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ROLE OF HYPOXIA PRECONDITIONING IN THERAPEUTIC POTENTIAL OF MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES

Therapeutic potential of hypoxic MSC-derived EV

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Abstract

The use of mesenchymal stem cells (MSC) in cell therapy has received considerable attention, because of their properties. They include high expansion and differentiation *in vitro*, low immunogenicity and modulation of biological processes such as inflammation, angiogenesis and hematopoiesis. Curiously, the regenerative effect of MSC is partly due to their paracrine activity. This has prompted numerous studies, to investigate the therapeutic potential of their secretome, and mainly extracellular vesicles (EV). These contain proteins, lipids, nucleic acids and other metabolites, which can cause physiological changes when released into recipient cells. Interestingly, contents of EV can be modulated by preconditioning MSC under different culture conditions. Among them, exposure to hypoxia stands out. Thus, cells respond by activating hypoxia-inducible factor (HIF) at low O_2 concentrations. HIF has direct and indirect pleiotropic effects, modulating expression of hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, angiogenesis, metabolism and cell apoptosis. Expression of these genes is reflected in the contents of secreted EV. Interestingly, numerous studies show that MSC-derived EV conditioned under hypoxia have a higher regenerative capacity than those obtained under normoxia. In this review, we show the implications of hypoxia responses in relation to tissue regeneration. In addition, hypoxia preconditioning of MSC is being evaluated as a very attractive strategy for isolation of EV with a high potential for clinical use in regenerative medicine, applied to different pathologies.

Key Words: Cell priming; Extracellular vesicles; Hypoxia; Hypoxia inducible factor; Mesenchymal stem cells; Regenerative medicine

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Core Tip: MSC-derived EV have a high therapeutic interest. EV composition depends on the state of source cells, generating physiological changes in recipient cells. MSC culture preconditioning affects EV cargos. Thus, hypoxia exposition leads to HIF activation and modulation of hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, angiogenesis, metabolism and apoptosis. This affects the contents of secreted EV. Accordingly, numerous studies have shown that EV from MSC under hypoxia have a higher regenerative capacity than those obtained under normoxia. Therefore, the former have a high clinical potential in different pathologies.

INTRODUCTION

Mesenchymal Stem-Cells or Mesenchymal Stromal-Cells (MSC) derived from adult tissues are characterized by their low immunogenicity, high proliferation capacity, differentiation and modulation of physiological processes such as inflammation, hematopoiesis and angiogenesis[1–3]. Besides, MSC can be isolated from different tissues for their culture and expansion *in vitro*. Therefore, they are currently considered an important therapeutic tool in the field of regenerative medicine[4,5]. However, one of the main limitations of their use is the need to obtain and expand MSC *in vitro*, which cannot always be from the same patient to be treated. Besides, MSC manipulations may cause cell functionality loss and genetic instability, when performed outside their natural niches[6]. Moreover, one risk of the application of cell therapy in regenerative medicine is that MSC may remain undifferentiated and produce tumors[7].

Recently, numerous studies have shown that the regenerative capacity of MSC mainly depends on their paracrine functions. Therefore, an alternative or complement to cell therapy in regenerative medicine is the use of media enriched in factors and cytokines secreted by *in vitro* cultures of progenitor cells[8]. The MSC secretome is composed by soluble factors and extracellular vesicles (EV)[9]. The main function of EV are cell communications and interactions. Their contents depend on their cellular origin and

physiological conditions in which they are produced[10]. Therefore, preconditioning MSC under conditions that increase their regenerative power, like hypoxia, may induce production of EV with enhanced regenerative potential. In the presence of damage, tissues normally undergo ischemic processes. These reduce the supply of O_2 and nutrients to damaged areas. This causes cellular responses that induce the release of factors, promoting vessel formation and tissue regeneration[11]. Therefore, MSC preconditioning in hypoxia can induce this response, leading to production of EV rich in angiogenic factors and inducers of tissue regeneration.

In this context, the main aim of this review is to describe the effects that preconditioning in hypoxia can have on MSC, mainly in the contents of their EV, and how this strategy has a great potential, being therefore used in regenerative medicine.

MSC AND REGENERATIVE MEDICINE

MSC are multipotent cells, first discovered by Friedenstein *et al* in 1970[12]. They have fibroblast–like morphology, behaving as colony-forming units-fibroblasts (CFU-F). These cells originate in the mesoderm, having the ability to differentiate into different cell types including osteoblasts, adipocytes and chondrocytes[13]. The minimum characteristics that a cell must have to be considered MSC, according to the International Society for Cellular Therapy (ISCT), are: i) adhere to plastic under standard culture conditions; ii) exhibit several Clusters of Differentiation (CD): CD-73, CD-90 and CD-105, lacking CD-11b, CD-14, CD-19, CD-34, CD-45, CD-79a and Human Leukocyte Antigen – D-Related (DR) isotype (HLA-DR); and iii) differentiation potency into osteoblasts, adipocytes or chondrocytes *in vitro*[14]. Isolated MSC may have different origins, such as adipose tissue, placenta, umbilical cord blood or Wharton's jelly, synovium, periodontal ligament, menstrual blood and bone marrow, being the latter one of the essential sources of these cells, for research and clinical applications[15–17].

MSC are involved in tissue regeneration, being necessary for maintenance of vital functions, delaying aging. The application of MSC in regenerative therapies is gaining great interest, due to their advantages. Thus, these cells can be isolated and cultured *in*

vitro, have the capacity to undergo multilineage differentiation, and also possess anti-inflammatory and immunosuppressive properties[5]. Indeed, such cells have great potential to treat various pathologies including those of the nervous system, bone, skin, myocardium and liver, among others[4,18–20]. In this regard, multiple clinical trials related to these pathologies have demonstrated the potential of MSC in human clinical practice[21–23]. Nevertheless, despite the potential and good results obtained in cell therapy, the risks involved when using cells in regenerative medicine should be considered, as indicated above. Therefore, in the last few years, cell-free therapies have gained attention, becoming the preferred options in many instances.

MSC-DERIVED EXTRACELLULAR VESICLES AS A NOVEL APPROACH TO CELL-FREE THERAPIES

It is well known that MSC-based cell therapy has beneficial effects in different pathologies. Nevertheless, some studies suggest that these benefits may not be due to the cells themselves, but to their paracrine effects; for instance, at the site of injury[13,24,25]. Recent research suggests that these therapeutic effects are mainly linked to their paracrine effectors, being the EV secreted by these cells key players[26]. Thus, the use of MSC-derived EV has been found to be beneficial to improve cartilage repair and regeneration, cardiac repair after myocardial infarction, wound healing and lung repair, among other applications[27–30].

According to their size, biogenesis, release pathways, function and content, EV have been classified into microvesicles, exosomes and apoptotic bodies. Microvesicles range between 100 to 1,000 nm in diameter, being formed through outward outgrowth. Exosomes are vesicles generating after fusion of multivesicular bodies with plasma membranes, ranging between 40 to 100 nm[10,31]. They should not be confused with RNA-degrading complexes with the same name, found in both archaea and eukaryotes. On the other hand, apoptotic bodies are released during early apoptosis, being larger than 1,000 nm[32,33]. However, there is a lack of consensus about classification and biochemical markers characterizing the different EV types. Therefore, the International

Society for Extracellular Vesicles stated the following in the "Minimal Information for Studies of Extracellular Vesicles 2018" (MISEV2018), in relation to the EV nomenclature: "EV is the preferred generic term for the subject of our investigations, and subtypes should be defined by physical and biochemical characteristics and/or conditions/sources. When other terms are used, careful definition is required"[34].

EV may contain proteins, nucleic acids (including coding and non-coding RNA), lipids and other metabolites. Normally, the content is rich in cytoskeletal proteins (such as TSG10 or CD63 tetraspanins), integrins and Major histocompatibility complex (MCH) molecules[35]. Depending on cell types and microenvironments in which they are secreted, contents of EV may change. Thus, EV reflect physiological states of cells generating them. For this reason, MSC growth under different conditions as hypoxia, presence of trophic and physical factors, or chemical and pharmacological agents, may stimulate secretion of EV enriched in certain cytokines, growth factors or non-coding RNA like microRNA (miRNA)[36].

Regarding the function of EV, at first it was thought that they were a mechanism for cells to get rid of unwanted material. It was later demonstrated that EV play a fundamental role in cellular homeostasis, being key elements in cell-to-cell communications[33,37]. Thus, these vesicles regulate different physiological processes such as cell proliferation, differentiation and migration[38].

EV can be isolated from various sources, including blood, urine, breast milk, amniotic fluid and synovial fluid, among others, as well as supernatants from cell cultures such as endothelial, epithelial, cancer, MSC, etc [39]. There are different purification approaches such as differential and density gradient ultracentrifugation, ultrafiltration, size exclusion chromatography, precipitation, immunoaffinity and microfluidic-based methods [33]. Likewise, isolated EV can be characterize by different techniques like electron microscopy, flow cytometry, Nanoparticle Tracking Analysis (NTA), Dynamic Light Scattering (DLS), Tunable Resistive Pulse Sensing (TRPS) and Atomic Force Microscopy (AFM), among others[38,40].

Using EV in regenerative medicine has some advantages, in comparison with whole-cell therapy[41], including: i) can be easily stored, being immediately available for clinical applications; ii) production of large quantities of cells is not required; iii) can be evaluated for safety, dosage and activity, in a manner similar to conventional pharmaceutical agents; iv) are stable, exhibiting a long half-life. Indeed, the lipid bilayers of their membranes protect their contents from degradation *in vivo*; v) can be more easily applied for clinical purposes than proliferative cells. For example, they can be intravenously injected, circulate through the smallest capillaries and cross the blood-brain barrier; vi) risks of immune rejection, cellular dedifferentiation or tumor formation are lower than in whole-cell therapies; and vii) it is important to note that EV can be manipulated for more precise effects as therapeutic agents[10,41]. Therefore, the use of EV in therapy has become a great tool for regenerative medicine in recent years.

ROLE OF HYPOXIA-INDUCIBLE FACTOR IN ADAPTATION TO HYPOXIA AND TISSUE REGENERATION

When oxygen concentrations decrease to less than 5% in tissues, cells have to adapt their metabolism and functions to such hypoxic conditions. Moderate (<5 - >2% O_2), severe (≤2 - ≥0,1% O_2) and anoxia (<0.1% O_2) are hypoxia levels below 5% oxygen concentration. Depending on O_2 concentration and hypoxia time, cells show different responses, as observed in human embryonic stem cell-derived MSC[42]. That occurs mainly through activation of Hypoxia-Inducible Factor (HIF). This is a transcription factor consisting in a heterodimer of two basic helix-loop-helix proteins, alpha (HIFA) and beta (HIFB)[43,44]. While expression of alpha subunits is induced by hypoxia, HIFB, also known as aryl hydrocarbon-receptor nuclear translocator (ARNT), is constitutively expressed[45]. There are three alpha subunits (HIF1A, HIF2A and HIF3A), being well-know the first two. HIF1A and HIF2A have 48% amino acid sequence identity, and similar protein structures. Although they share functions, they can regulate expression of different genes[46]. Thus, HIF2A, which is also known as Endothelial PAS domain protein-1 (EPAS1), was originally associated with endothelial development and

regulation. Its encoding gene exhibits a more restricted expression relative to the one of HIF1A[47]. Furthermore, whereas HIF1A requires very low O_2 concentrations for stabilization, H2FA can be activated at less severe levels of hypoxia (~5%). Therefore, HIF1A would act in the initial response, whereas HIF2A would regulate the response to long periods of hypoxia[48,49]. On the other hand, HIF3A has three isoforms [HIF3A, Neonatal and Embryonic PAS (NEPAS), and Inhibitory PAS protein (IPAS)]. They inhibit the transcriptional activity of HIF1A and HIF2A, by preventing their heterodimerization with HIF1B[50,51].

Under normoxia, HIF1A protein in the cytoplasm is continuously degraded, through the proteasome pathway[52]. However, when the O_2 concentration decreases, HIF1A is not degraded; it is accumulated and translocated into the nucleus (Figure 1). Regulation of HIF1A levels depends on presence of an Oxygen-Dependent Degradation Domain (ODDD) in the protein. This domain is constituted by Fe^{2+} and two prolyl residues (Pro402 and Pro564). Such residues undergo hydroxylation through Prolyl HyDroxylases (PHD1, PHD2 and PHD3) in the presence of oxygen and α-ketoglutarate, allowing HIF1A to be recognized by von Hippel-Lindau tumor suppressor protein (pVHL), a component of the E3 ubiquitin-ligase complex. That way, it is degraded by the ubiquitin-proteosome pathway[53,54] (Figure 1). In addition to PHD, another enzyme called Factor Inhibiting HIF1 (FIH) can inhibit the transcriptional activity of HIF1A. In this case, FIH hydroxylases residues within the C-terminal transactivation domain of HIF1A, preventing its binding to coactivators to initiate transcription in the nucleus[55].

Under hypoxic conditions, prolyl hydroxylation is inhibited, and thus the degradation of HIF1A. It accumulates and translocates into the nucleus, where it forms heterodimers with HIF1B. That way, it can induce gene transcription through binding to pentanucleotide sequences (A/GCGTG) called Hypoxic-Response Elements (HRE) in the promoters of target genes. For transcription of target genes to occur, coactivators are recruited; mainly p300/CBP[56] (Figure 1).

It has been described that more than 1,000 genes can be directly or indirectly regulated by HIF. These genes are involved in adaptation of cells to hypoxic conditions. They

affect different physiological processes including metabolism, angiogenesis, inflammatory response, cell differentiation, migration and apoptosis[57]. In order to present an overview of the various functions of genes regulated by HIF1α and HIF2α, we have analyzed the information contained in Qiagen Ingenuity Pathway Analysis (Qiagen IPA) web-based software application <https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa>[58]. This platform allows querying information gathered from databases and findings described in the literature for a given gene. Information from 493 references related to genes regulated by HIF1A and 215 for HIF2A were integrated in the description of functions of human HIF, at the time of writing. From the information of these references, the application shows 191 genes regulated by HIF1α and 111 by HIF2α. Among them, 72 are common to both. Functional analyses of HIF1A- and HIF2A-regulated genes with IPA show categories and functional annotations in which they are involved. Table 1 and 2 show these data, together with *p* values and the number of genes identified for each of the categories, obtained from such application. Regarding the functional annotations, a maximum of the five most significant annotations in each category are shown. The list of genes for HIF1A and HIF2A obtained from IPA, as well as genes corresponding to each of the categories presented in Tables 1 and 2, are shown in supplementary material (Tables 1S to 3S). Among the functions of genes regulated by HIF1A and HIF2A are those related to glucose metabolism, vessel formation, inflammatory response, cell proliferation, cell migration and apoptosis. Interestingly, they play relevant roles in tissue regeneration[57]. The importance of HIF1A in tissue regeneration has been further demonstrated using Murphy Roths Large (MRL) mouse model. These animals are characterized by high basal expression of *HIF1A* gene. That has been associated with the ability of these animals to regenerate significant ear lesions, without the appearance of fibrotic areas[59]. Indeed, HIF1A induction upregulates genes such as Vascular Endothelial Growth Factor (*VEGF*), Stromal cell-Derived Factor-1 Alpha protein (*SDF-1A*), Transforming Growth Factor Beta 1 (*TGFB1*), Platelet-Derived Growth Factor (*PDGF*) and Matrix

MetalloPeptidase 9 (*MMP9*), among others. All of them have important functions in the healing process. Therefore, the activation of HIF1A can accelerate wound healing. This has been observed in HyperBaric Oxygen Therapy (HBOT) treatments of diabetic skin ulcers. Interestingly, HBOT treatments increased HIF1A levels[60], probably due to high Reactive Oxygen Species (ROS) concentrations, produced by increased O_2 in the tissue, which may inhibit PHD and FIH, thus stabilizing HIF1A. In fact, HIF1A activity is decreased in diabetics, being associated with wound healing difficulty in these patients[61].

However, if hypoxia is maintained, wounds may become chronic, and fibrotic processes may appear. This is because, among the genes regulated by the HIF pathway, there are some that encode pro-fibrotic enzymes, producing an excess of extracellular matrix. Some of these genes are *TGFB*, related to collagen biosynthesis like COLlagen type –IV, V, IX and XVIII– Alpha –1 and 2– chains (*COL4A1*, *COL4A2*, *COL5A1*, *COL9A1* and *COL18A1*, accordingly), and the ones encoding enzymes that produce modifications in collagen, such as procollagen prolyl hydroxylases and lysyl hydroxylases[62].

Inflammation is the first phase activated by injury, and hypoxia is related to inflammatory response. Several protein-encoding genes of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) complex, such as ReticuloEndotheLiosis (REL)-Associated (RELA) proto-oncogene (transcription factor p65, also known as nuclear factor NF-kappa-B p65 subunit p65, involved in NF-κB heterodimer formation, nuclear translocation and activation) and NF-κB subunit (RELA) are induced by HIF1A[63]. NF-κB is a family of transcription factors whose activation regulates different physiological processes. They include inflammatory response, as well as cell differentiation, proliferation and survival[64]. Among the genes that NF-κB regulates is HIF1A, thus producing a reciprocal regulation[65]. HIF1A also induces expression of genes encoding proteins belonging to the Toll-Like Receptor (TLR) family. Thus, it enhances the activation of NF-κB[66]. This is because TLR have the capacity to recognize pathogen-associated molecules, inducing immune responses through activation of transcription factors, such as NF-κB[67].

Upon injury, the resulting hypoxia intervenes in macrophage recruitment, through regulation of Sphingosine 1-Phosphate (S1P) levels. It acts as a signal for recruitment, activation, differentiation and polarization of macrophages[68]. This may be mediated by induction of expression of genes such as SPHingosine Kinase 1 (*SPHK1*). Such gene is involved in the last step of S1P synthesis. It has been described that HIF1A and HIF2A act on M1 and M2 macrophages, through different pathways. While HIF1A induces inducible nitric-oxidase synthase, HIF2A acts through arginase-1, maintaining nitric oxide homeostasis during inflammation. In the case of HIF1A, its overexpression induces glycolysis metabolism, resulting in macrophage polarization to M1 (proinflammatory)[69]. However, although HIF2A has also been associated with the M1 phenotype, other studies have shown that it may promote anti-inflammatory and pro-resolving/regenerative M2 macrophages[70]. HIF1A may also produce immunosuppression, through induction of Programmed Death-Ligand 1 (PD-L1) encoding gene (*CD274*). Binding of PD-L1 to its PD-1 receptor on activated T cells inhibits immunity, by counteracting T cell-activating signals[71].

Adaptation to hypoxia also requires metabolic changes. Cells must reduce mitochondrial oxygen consumption. In this sense, glycolysis is activated, as the only way to produce Adenosine TriPhosphate (ATP) under such hypoxic conditions. Not surprisingly, HIF1A upregulates genes related to glucose metabolism. Among them is SoLute Carrier family 2 member A1 (*SLC2A1*), encoding glucose transporter-1, necessary for glucose uptake by cells[72]. Also, the genes encoding PhosphoGlycerate Kinase 1 (*PGK1*) and Pyruvate Kinase M1/2 (*PKM*) are transcriptionally upregulated by HIF1A[73,74]. Additionally, in the adaptation to hypoxia, the TriCarboxylic Acid or Krebs cycle (TCA) must be suppressed, to prevent accumulation of ROS in mitochondria. For this purpose, HIF1A induces the gene encoding Pyruvate Dehydrogenase Kinase 1 (*PDK1*). This inactivates Pyruvate DeHydrogenase (PDH), which is responsible for converting pyruvate to acetyl-CoA in the TCA[75].

In response to hypoxia caused by tissue damage, cells produce angiogenic factors to induce generation of vessels, to restore oxygen levels and nutrient delivery. HIF1A and

HIF2A induce expression of genes encoding these factors. Among them, stand out *VEGF*, *SDF-1A*, C-X-C chemokine-Receptor type 4 (*CXCR4*), ANGiopoietin-2 (*ANG-2*), Platelet-Derived Growth Factor (*PDGF*) and *TGFB* [76]. These factors favor endothelial-cell proliferation, differentiation and migration for vessel formation. That involves also mobilization and recruitment of Endothelial Progenitor Cells (EPC) from bone marrow[61]. Mobilization of EPC is mediated by production of SDF-1 in hypoxic tissues. It acts as a chemoattractant of EPC, expressing its receptor CXCR4[77]. On the other hand, regulation of EPC migration to ischemic tissues through CXCR4/SDF1 axis is specific to HIF2A[78].

Tissue regeneration also induces cell proliferation and migration processes. HIF activation can affect cell-cycle progression, due to regulation of genes such as CyCliN D1 (*CCND1*) and cellular MYeloCytomatosis (*c-MYC*; *MYC*)[79,80]. Interestingly, While HIF1A downregulates *c-MYC* expression and results in cell-cycle arrest[79], HIF2A upregulates *c-MYC* expression, promoting cell-cycle progression and proliferation[81]. Regarding cell migration, HIF regulates genes encoding InTeGrin –Alpha and Beta– 1, 3 and 5 (*ITGA1*, *ITGA*5, *ITGAV*, *ITGB3* and *ITGB5*, accordingly) and Matrix MetalloProteinases 2, 7 and 9 (*MMP2*, *MMP7* and *MMP9*, respectively), which are important in such process[82]. The induction of cell migration by hypoxia is essential under physiological conditions, for tissue regeneration after injury. This favors recruitment and homing of inflammatory and precursor cells, eliminating pathogens and cellular debris, further regenerating damaged tissues[68].

Hypoxia causes important changes in cellular microenvironments that might condition cell viability. Therefore, another set of important genes regulated by hypoxia are related to cell survival and death. Thus, HIF1A regulates genes activators of apoptosis such as the ones encoding Tumor Protein p53 (*TP53*) and B-Cell Lymphoma 2 (BCL2)/adeNovirus E1B 19 kDa protein-Interacting Protein 3 (*BNIP3*)[83], as well as anti-apoptotic genes, such as Baculoviral Inhibitor of Apoptosis Protein (IAP) Repeat Containing 2 (*BIRC2*) and *BCL2*[83,84]. The balance of expression of these genes, and thus cell survival, will depend on the adaptation of cells to hypoxic conditions. Thus, cell

survival may predominate under mild hypoxia, but apoptosis is preferentially activated under severe hypoxia[85].

HYPOXIA AND MESENCHYMAL STEM-CELLS

MSC reside in areas of 3-9% of oxygen tension, allowing this hypoxic niche its self-renewal, proliferation, migration and ultimately, their differentiation[86,87]. Based on this, MSC in culture have been grown at low levels of oxygen to condition or acclimate them before their therapeutic use[88]. These cells exposed to hypoxic conditions activate Protein Kinase B (PKB; also known as Akt, name derived from Ak mouse strain, with thymoma transforming tumors) or AKT signaling pathway mediated by HIF-1 activation, to improve their survival and proliferation[89]. However, different modes, severity and duration of hypoxic exposure could provoke different responses on MSC. Indeed, cells can become stressed and even undergo apoptosis under extreme (<1.5%) oxygen levels [87]. Furthermore, if hypoxic exposures are maintained, internal energy reserves of glucose are rapidly consumed. That is due to glycolysis characteristic of MSC, causing poor survival after implantation[90]. Ischemic conditions could be solved by providing glucose supplementation to hypoxic MSC. That allows them to retain their proliferative capacity and differentiation potency[91]. Therefore, survival of MSC could be improved by preconditioning them at 1-4% O_2 for 24 to 48 h, prior to implantation[88]. Hypoxia could also reduce cell viability and proliferation of MSC. Nevertheless, reoxygenation processes might promote recovery of cells, enhancing expression of pro-survival genes, as well as various trophic factors[92], further promoting multipotency of MSC[93,94]. Therefore, maintenance of MSC cultures in hypoxia may influence processes such as proliferation[87,94,95], migration[87], differentiation[93,95], metabolism[87] and apoptosis[88,96], which may affect their regenerative capacity. Interestingly, Cyclic Hypoxic (CH) exposure, defined as periodic exposure to hypoxia, interrupted by normoxic exposure, or lower levels of hypoxia[97], could have positive effects on proliferation and migration abilities of MSC[98].

THERAPEUTIC POTENTIAL OF EV DERIVED FROM MSC PRECONDITIONED IN HYPOXIA

Microenvironments in which MSC are cultivated are extremely important for their proliferation, differentiation and therapeutic potential. Factors such as time in culture, oxygen levels, medium composition or cell-material interactions should be considered[99]. As indicated in previous sections, many factors induced by hypoxia are involved in processes related to tissue regeneration such as inflammation, angiogenesis, cell proliferation and migration[100]. Thus, priming of MSC in hypoxia favors generation of EV enriched in hypoxia-induced factors. Their functions include alterations of microenvironments for tissue adaptations to low O_2 concentrations[101–103]. Production and isolation of these EV for use in regenerative medicine is of great interest, from a clinical point of view. Therefore, numerous studies have evaluated potential therapeutic applications. In this scenario, time exposure and degree of hypoxia may represent relevant factors influencing contents and therapeutic properties of EV (Table 3).

Hypoxia is an important inducer of angiogenesis, which plays a key role in tissue regeneration. Therefore, numerous studies have evaluated whether MSC-derived EV exposed to low levels of O_2 are enriched in angiogenic factors. Likewise, whether this has an impact on their ability to induce vessel formation. One of these studies showed that MSC cultivated for 72 h under hypoxic conditions (1% O_2) produced exosomes with proangiogenic effects, through overexpression of Urokinase receptor [also known as urokinase Plasminogen-Activator surface Receptor (*uPAR*)], Angiogenin (*ANG*), *VEGF*, Insulin-like Growth Factor (*IGF*), angiopoietin receptor Tyrosine kinase with immunoglobulin-like and Epidermal Growth Factor (EGF)-like domains 2 (*Tie-2*) [also known as Tyrosine Endothelial Kinase (*TEK*)] and InterLeukin 6 (*IL-6*)[104]. Also, umbilical cord MSC-derived EV have the ability to enhance endothelial-cell angiogenesis *in vitro*, and in a rat hindlimb ischemia model, being able to restore blood flow[103]. Preconditioning adipose-derived MSC in moderate hypoxia (5% O_2) also produced EV with capacity to increase formation of tubular structures in Human Umbilical-Vein Endothelial Cells (HUVEC), with respect to EV obtained in normoxia.

On the other hand, effects of EV were greater than those produced by media obtained after isolation of microvesicles. This indicates that EV, rather than soluble factors in the media, are responsible for angiogenic induction[32]. *In vivo* studies have also shown the potential of EV derived from MSC grown under hypoxia on angiogenesis. For example, in a mouse model of fat grafting, co-transplantation of exosomes in subcutaneous fat grafting enhanced angiogenesis, neovascularization and graft survival[105]. A significant rise in protein synthesis of EGF, Fibroblast Growth-Factors (FGF), VEGF/VEGF Receptors (VEGF-R), Angiopoietin-1 (Ang-1) and angiopoietin receptor Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1) were shown in grafted animals, 30 days after transplantation[106]. Inclusion in hydrogels that allow local release of EV with high angiogenic capacity has also been used for treatment of spinal-cord injuries[107]. One of the proteins that has been found to be over synthesized in MSC-derived EV under hypoxia, with respect to those obtained in normoxia, is Jagged-1 (JAG1). This is one of the Notch ligands. Notch pathway modulates process as angiogenesis, embryonic development and Hematopoietic Stem-Cell (HSC) biology[108,109]. Hematopoietic stem-cells from umbilical-cord blood were *in vitro* treated with EV from MSC preconditioned in 1% O_2 for 48 h. As expected, their expansion capacity, self-renewal, and clonogenic potential, was increased through Jagged-1/Notch pathway regulation[110].

Treatment of endothelial cells with hypoxia-conditioned MSC-derived EV modulates angiogenesis-related signaling pathways. For instance, it has recently been described that EV obtained from MSC maintained in 5% O_2 for six days induced angiogenesis in HUVEC. That was accomplished through increased synthesis of High-Mobility Group Box 1 (HMGB1). It activates c-Jun N-terminal kinases (JNK) pathway (name derived from viral homolog v-jun, discovered in avian sarcoma virus 17 and named ju-nana, the Japanese word for 17) and consequently upregulated *HIF1A*/*VEGF* expression[111]. The angiogenic effects of MSC-derived EV exposed to hypoxia are mediated, in part, by their cargos; and specifically by certain miRNA. One of them is miR-612, which inhibits

translation of TP53 mRNA, favoring the activity of HIF-1A-VEGF signaling, and consequently angiogenesis[112].

According to the properties of MSC-derived EV under hypoxia, their applications may be useful in multiple disease treatments (Figure 2). Among them is Alzheimer's disease. That is characterized by neuronal and synaptic loss, caused by deposition of beta-amyloid peptides[113], due to erroneous protein folding. Experiments have been carried out with an Alzheimer's transgenic mouse model, overexpressing mutated forms of human Amyloid-Precursor Protein (APP) and PreSenilin 1 (PS1) (APP/PS1). Interestingly, they improved learning and memory functions after treatment with exosomes from MSC preconditioned for 12 h under hypoxia. These improvements could be due to reduced β-amyloid accumulation, through increase of miR-21 in the brain, synthesis of synaptic proteins and decrease of inflammatory factors[114]. Also, related with the nervous tissue, the effects of exosomes released during 48 h under hypoxia (1% O_2), on repair traumatic injuries of spinal cords, have been studied. An enrichment of miR-216a-5p in exosomes was observed, involving Toll-Like Receptor 4 (TLR4)/NF-κB/PhosphoInositide 3-Kinase (PI3K)/AKT signaling cascades. These miR-216a-5p-enriched exosomes promote functional behavioral recovery using both *in vitro* and *in vivo* models. That is carried out by shifting microglial polarization, from classically-activated macrophage (M1) to alternatively-activated macrophage (M2) phenotype, effectively switching from pro-inflammatory to non-inflammatory states[115]. Also, in a mouse model of cerebral ischemia, application of EV derived from bone marrow MSC preconditioned in hypoxia (1% O_2), through their effect on angiogenesis, reduced neuronal degeneration, brain atrophy and improved neurological recovery[116].

Additionally, EV may be used in bone-fracture healing. Thus, exosomes generated by MSC obtained from human umbilical cord were exposed to 1% O_2 during 48 h. They promoted bone fracture healing in an animal model. These exosomes are enriched in miR-126 by the action of HIF1A, exerting proangiogenic effects by means of SPRouty-related, N-terminal Enabled (Ena)/VASodilator-stimulated Phosphoprotein (VASP) Homology-1 (EVH1) Domain-containing protein 1 (SPRED1)/Ras (name derived from

RAt sarcoma-virus protein)/Mitogen-Activated Protein Kinase MAPK (originally called Extracellular signal-regulated kinases or Erk) pathway activation[117]. Also, in a rat model of steroid-induced osteonecrosis of femoral head, treatment with EV released from MSC preconditioned in 2% of oxygen prevented bone loss, increasing blood-vessel formation[118]. In relation to the skeletal system, other studies have show that MSC-derived EV grown in hypoxia protect from intervertebral disc degeneration, through their content in mir-17-5p. That modulates proliferation of nucleus pulposus cells (NPC) matrix, *via* TLR4/PI3K/AKT pathway[119]. Furthermore, preconditioning in hypoxia also increased the capacity of MSC-derived EV in cartilage regeneration, by positively acting on chondrocytes. Thus, *in vivo* assays have shown that an injectable silk-fibroin hydrogel, containing articular chondrocytes and MSC-derived EV, in hypoxia promoted cartilage regeneration[120]. Several miRNAs are involved in this process, including miR-205-5p, miR-181c-5p, miR-18a-3p, miR-376a-5p and miR-337-5p[120,121].

Treatment with EV derived from MSC has also been proposed for kidney injury. Thus, EV from Adipose tissue-Derived Mesenchymal Stem Cells (ADMSC) cultured 72 h under hypoxia (1% O_2) or normoxia conditions were compared, in treatment of kidney injury induced by ischemia in a rat model. Both conditions reduced tissue damage, but renal regeneration was higher under hypoxia conditions, triggering antiapoptotic, angiogenetic, immunomodulatory and anti-oxidative stress responses. This could be due to differences in proteomic profiles of EV types[122].

On the other hand, EV derived from MSC cultured in hypoxia have been applied, using models of myocardial infarction, in several studies. Generally, protective effects of cardiac tissues from ischemic injury were observed. They were due, at least in part, to the ability of these EV to promote blood-vessel formation [123]. Additionally, exosomes from conditioned bone-marrow MSC cultured in hypoxia (24 h, 0.5% O_2) or normoxia were used. They were intramyocardially injected into infarcted hearts of C57 black 6 (C57BL/6) inbred mice strain. Treatment with hypoxia-derived exosomes produced interesting results: i) decrease in fibrotic tissue and apoptotic cardiomyocytes; and ii) increase in cardiac progenitor cells. These exosomes, compared to normoxia ones, had a

significant increase in expression of miR-210, which had positive effects on endothelial cells and cardiomyocytes[124]. Such miRNA were also abundant in EV secreted by rat bone-marrow MSC, cultured in 1% O_2 for 72 h. Their antiapoptotic effects in cardiomyocytes have also been demonstrated in a rat model of myocardium infarction[125]. Other EV derived from MSC cultured under hypoxia were also enriched in miRNA, showing antiapoptotic activity in cardiomyocytes. They include miR-125b-5p, which works through repression of *p53* and B-Cell Lymphoma 2 (BCL2) Antagonist/Killer 1 (*BAK1*)[126]. It has also been shown that EV obtained from MSC cultured in hypoxia were enriched in miRNA-26a, in relation to EV obtained in normoxia. The former is involved in upregulating Glycogen-Synthase Kinase 3 Beta (*GSK3B*) expression. That enhances beta-catenin pathway, reducing ischemia-reperfusion injury in a rat model[127].

Other miRNA enriched in EV derived from adipose and bone-marrow mesenchymal stem-cells preconditioned in hypoxia were miR224-5p and miR-24. The former decreased expression of thioredoxin-interacting protein (TXNIP), which facilitates degradation of HIF1A. EV enriched in miR224-5p favored adaptation of cardiomyocytes to hypoxia, therefore protecting them against myocardial infarction[128]. On the other hand, miR-24 decreased in infarcted myocardium of rats. Thus, the application of EV containing this miRNA protected cardiomyocytes from apoptosis, reducing infarct size and improving cardiac function[129].

In addition to miRNA, other RNA types have been identified with cardioprotective effects in EV generated by MSC, under hypoxia conditions. This is the case of long non-coding RNA of Urothelial Carcinoma-Associated 1 (lncRNA-UCA1), which is related with the anti-apoptotic miR-873-5p/X-linked Inhibitor of Apoptosis Protein (XIAP)/phosphorylated AMP-activated protein Kinase (pAMPK) pathway[130].

Exosomes derived from MSC grown under hypoxia may be also useful for treatments of chronic skin-ulcers. They are associated with pathologies such as diabetes. Their healing is difficult, being a serious problem for patients and public health systems[131]. Recently, a study has evaluated the potential application of EV obtained from adipose-tissue stem

cells maintained at 1% O_2 for 24 h. *In vitro* assays showed that they promoted fibroblast proliferation and migration. That was accomplished by activating PI3K/AKT pathway, in a more effective way than when EV obtained under normoxia were used. Differential expression analyses of miRNA contents between both types of EV showed upregulated miR-21-3p, miR-126-5p and miR-31-5pand downregulated miR-99b and miR-146-a. They may be involved in signaling pathways, related to fibroblast proliferation and migration, modulating immune responses. Thus, they confirmed in a diabetic nude mice model of wound healing that treatment with hypoxia-derived EV improved healing. That was carried out downregulating *IL-6*, upregulating *VEGF* and modulating extracellular matrices[132]. Additionally, EV derived from umbilical cord MSC exposed to 1% O_2 for 3 to 6 h were used in a full-thickness skin-injury mouse model, improving wound healing, with respect to EV obtained in normoxia. In this case, it was demonstrated that EV in hypoxia had anti-apoptotic effects on endothelial cells. That was due to miR-125b, which suppressed expression of Tumor Protein p53-Inducible Nuclear Protein 1 (*TP53INP1*)[133].

CONCLUSION

In recent years, the therapeutic potential of using MSC-derived EV has become apparent. This is because the regenerative effect of MSC is partly due to their paracrine activity. The contents of EV can be modulated through preconditioning of MSC under different culture conditions. Among they, exposure to hypoxia stands out. HIF activation affects hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, metabolism and cell apoptosis. That is related to the contents of secreted EV, and thus their therapeutic potential, being higher than the one of EV obtained under normoxic conditions. Therefore, hypoxia preconditioning of MSC is a very attractive strategy for isolation of therapeutic EV. They have a high potential for use in regenerative medicine, applied to different pathologies. However, studies published to date show a great variability. That includes sources of MSC, culture media, O_2 concentrations and exposure times to hypoxia, as well as methods of

EV isolation. Such factors may influence the degree of induction of *HIF1A* and *HIF2A*, and therefore MSC responses and EV cargos. Thus, it would be necessary in the future to perform studies to optimize and standardize conditions for obtaining EV, according to their therapeutic applications. Also, *in vivo* studies carried out so far have been performed mainly in animal models. Only two active MSC-derived EV clinical trials in recruitment phase, in which hypoxia is being evaluated, are shown in ClinicalTrials <https://clinicaltrials.gov>: "Treatment of Severe COVID-19 Patients Using Secretome of Hypoxia-Mesenchymal Stem Cells in Indonesia" (ID: NCT04753476) and "Regeneration of Posterior Cruciate Ligament Injury Using Hypoxic Conditioned Allogenic Adipose Mesenchymal Stem Cell and Condition Medium" (ID: NCT04889963). Therefore, in order to ascertain the greater potential effectiveness of EV obtained from MSC preconditioned in hypoxia, it would be necessary to carry out a greater number of properly designed clinical trials.

Using EV in regenerative medicine is very promising, as shown above. Yet, possible adverse effects associated with the use of the ones derived from MSC in human clinical practice must be taken into account. One of them is that the contents of EV may enhance tumor-cell activity[134]. In any case, that should be significantly lower –if ever exists– than using whole stem-cells. Therefore, these risks should be properly evaluated in animal models, and potential clinical trials. In this regard, there are several challenges, for the use of MSC-derived EV in regenerative medicine, that must be properly addressed beforehand. These include: i) identification of the most suitable MSC sources for each pathology; ii) optimization and consensus of culture methods and conditions to obtain EV with greater regenerative capacity; iii) scaling up of production for clinical use; iv) control of variability and stability of produced EV; v) increase in clinical trials to make them statistically significant; and vi) a better understanding of pharmacokinetics and biodistribution of applied EV[135].

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