Received: 21 June 2020 | Accepted: 24 August 2020

DOI: 10.1002/sctm.20-0268

FETAL AND NEONATAL STEM CELLS

STEM CELLS TRANSLATIONAL MEDICINE

Neuroprotective effects of human amniotic fluid stem cells-derived secretome in an ischemia/reperfusion model

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Abstract

Stem cells offer the basis for the promotion of robust new therapeutic approaches for a variety of human disorders. There are still many limitations to be overcome before clinical therapeutic application, including a better understanding of the mechanism by which stem cell therapies may lead to enhanced recovery. In vitro investigations are necessary to dissect the mechanisms involved and to support the potential development in stem cell-based therapies. In spite of growing interest in human amniotic fluid stem cells, not much is known about the characteristics of their secretome and regarding the potential neuroprotective mechanism in different pathologies, including stroke. To get more insight on amniotic fluid cells therapeutic potential, signal transduction pathways activated by human amniotic fluid stem cells (hAFSCs)-derived secretome in a stroke in vitro model (ischemia/reperfusion [I/R] model) were investigated by Western blot. Moreover, miRNA expression in the exosomal fraction of the conditioned medium was analyzed. hAFSCs-derived secretome was able to activate pro-survival and anti-apoptotic pathways. MicroRNA analysis in the exosomal component revealed a panel of 16 overexpressed miRNAs involved in the regulation of coherent signaling pathways. In particular, the pathways of relevance in ischemia/reperfusion, such as neurotrophin signaling, and those related to neuroprotection and neuronal cell death, were analyzed. The results obtained strongly point toward the neuroprotective effects of the hAFSCs-conditioned medium in the in vitro stroke model here analyzed. This can be achieved by the modulation and activation of pro-survival processes, at least in part, due to the activity of secreted miRNAs.

KEYWORDS

amniotic fluid, brain-derived neurotrophic factor, cerebral ischemia, conditioned medium, exosome, ischemia-reperfusion, miRNA, oxygen-glucose deprivation, stem cells, stroke

Vanessa Castelli and Ivana Antonucci equally contributed.

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1 | INTRODUCTION

Stroke is one of the primary causes of death and long-term disability in the world and represents a major public health problem. Stroke can be categorized into two major types, specifically ischemic and hemorrhagic stroke. Ischemic stroke is due to a block within a blood vessel distributing blood to the brain, while hemorrhagic stroke is due by the rupture of a blood vessel or an irregular vascular construct.[1,2] According to statistics, most stroke patients are affected by ischemic stroke.[3] Cerebral ischemia leads to different detrimental effects, including oxidative stress, ionic imbalance, apoptosis, and inflammation, which cause neuronal impairment and death.[4] This pathology lacks successful cures, due to both the limited time window for intervention before the development of deleterious consequences, and the absence of specificity of the treatments established so far. Furthermore, the inhibition of some molecular targets demonstrates to be ineffective due to secondary effects. To date, tissue plasminogen activator is the only therapeutic approach used in stroke patients.[4-6] Reperfusion injury occurring in consequence of ischemic stroke is an intricate process involving several processes. Also, thrombolytic therapy itself can lead to brain injuries, named cerebral ischemia/reperfusion (I/R) injury.[7,8] I/R injury is one of the major reasons for disability, high morbidity, and mortality worldwide. Due to the lack of effective neuroprotective treatments, the care for I/R injury continues to be a main medical concern and needs to be thoroughly investigated.

Due to its characteristics, the oxygen and glucose deprivation (OGD) challenge of neurons is a useful model of cerebral ischemia and provides the analysis of molecular pathways underlying stroke.[9-11] This challenge entails placing the cells in a glucose-free medium in an anaerobic condition; thus, merging the lack of these two components mimics the clinical situation in the brain during cerebral ischemia but in a simplified system.[11,12] Successively, to mimic in vitro the I/R injury, the OGD model is subjected to reperfusion (OGD/R or I/R) in normoxia condition.[13]

Differentiated SH-SY5Y are widely accepted for in vitro experiments requiring neuronal-like cells. Differentiated SH-SY5Y are low cost to culture, and the ethical concerns related with primary human neuronal culture are avoided. Furthermore, since SH-SY5Y cells are human-derived, they express several human-specific proteins and isoforms that would not be inherently present in rodent primary cultures.[14,15] Particularly, this cell line is used to develop the in vitro OGD/IR model.[15-21]

Current findings in regenerative medicine have strengthened the search for new stem cell sources with beneficial properties; in particular, amniotic fluid has been identified as a valid resource of stem cells in this field. Adult stem cells, even after reprogramming, could preserve epigenetic alterations, thus representing a limitation in their application.[22] On the other hand, fetal stem cells may overcome this limitation, and furthermore, ethical issues related to its isolation are minimal, in particular regarding amniotic fluid stem cells, as they are collected during routine amniocentesis, third-trimester amnioreduction or cesarean section.[23]

Significance statement

This article focuses on the therapeutic potential of human amniotic fluid stem cells (hAFSCs)-derived secretome in an ischemia/reperfusion in vitro model. Interestingly, in the presented experimental conditions, hAFSCs-derived secretome was able to activate pro-survival and anti-apoptotic pathways. Furthermore, microRNA analysis in the exosomal component revealed overexpressed miRNAs involved in neurotrophin signaling and those related to neuroprotection and neuronal cell death. In light of the data obtained, the use of conditioned medium and, in particular, exosomes, may constitute a potential approach to stimulate neuronal plasticity, ameliorate cognitive loss and neural replacement, and may represent a suitable treatment for ischemia/reperfusion injury.

The amniotic fluid is a protective liquid for fetus growth and offers mechanical support as well as essential nutrients during embryogenesis.[24] It is constituted primarily of water, cells, and chemical elements.[25] These cells are heterogeneous in morphology, in vitro, and in vivo characteristics.[26] They are mainly of fetal derivation (respiratory, epithelial, urinary, and intestinal tract), but also amniotic membranes and connective tissues. Furthermore, the amniotic fluid presents various cellular subcategories (amniotic, fibroblastic, and epithelioid), which differ in percentage depending on gestational period.[27] Amniotic fluid mesenchymal stem cells (AFMSCs) are really attractive due to their potential therapeutic purposes, and numerous procedures of expansion and isolation have been reported. AFMSCs show a wide differentiation potential toward mesenchymal lineages and have the capability to differentiate toward chondrogenic, osteogenic, and adipogenic, thus representing a suitable cell source for regenerative and therapeutic approaches.[28]

However, two processes exerting a crucial part in regenerative processes are reported. One process concerns the differentiation of the engrafted stem cells toward the specific cytotype of the injured area. This mechanism has been depicted by different authors,[29-32] but the small amount of exogenous cells constantly and directly engrafted into the regenerated tissue do not entirely clarify the achieved regenerative effect. The incomplete integration may be due to the heterogenic level of pre-differentiation produced in vitro before the transplant. In fact, the entire cell population stimulated might not undertake the differentiation process. This subpopulation could preserve low immunogenic characteristics but, notably, can influence the immune system and promote the recruitment of resident progenitor cells to regenerate injured tissue. Numerous investigations corroborated this hypothesis, indicating the protective activity of the stem cell-released molecules alone (conditioned medium) in the regeneration of the damaged area where, typically, a chronic inflammation condition is present.[33-36]

Founding on these theories, in the last decade, more interest has been focused on stem cells secretome and, consequently, on the paracrine effect.[37-39] Conditioned medium (CM) consists of extracellular vesicles (EV) and soluble factors. Recently, besides exerting role as biomarkers, secreted EV has been included among the players promoting mesenchymal stem cells (MSCs) regenerative potential[40-43] although, so far, a complete molecular description of their "cargo" is lacking.

Exosomes represent a subpopulation of microvesicles, ranging from 40-100 nm, which, initially, were identified as artifacts under the electron microscope. Recently, different investigations were focused on exosomes for their cell-to-cell communication, storage of biological information, the use as biomarkers, and, in particular, for the potential application in regeneration and neural protection.[44-46] Exosomes have cup-shaped morphology and derive from the endocytic pathway after the fusion of multivesicular bodies with the plasma membrane and then release into the extracellular environment.[44]

Exosomes contain several proteins, miRNA, DNA, saccharides, and lipids, indicated with the term "cargo."[47,48] Because of their nanosize and non-complexed structure, exosomes efficiently cross the blood-brain barrier, consequently, represent innovative approaches to design therapeutic strategies for different cerebral diseases. Thus, exosomes offer an alternative therapeutic approach as a substitute for cell transplantation.[49]

On these bases, in the present work, the signal transduction pathways activated by hAFSCs-secretome on a stroke in vitro model (I/R) and the main miRNA involved in the modulation of the observed effects were investigated, shedding light on some of the possible mechanisms of hAFSCs-induced neuroprotection.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatments

The human neuroblastoma cell line SH-SY5Y has comparable electrophysiological, morphological, and neurochemical characteristics of neurons and was bought from the European Collection of Authenticated Cell Cultures.Cells were maintained in DMEM high-glucose medium complemented with 10% FBS and 1% antibiotics (Corning) and were maintained in a humidified condition with 5% carbon dioxide at 37°C. Cells were seeded at 10^4 cells/cm^2; after 24 hours, to induce differentiation, medium was replaced with DMEM 1% FBS, and N2 supplement (Gibco) for 7 days, as previously reported,[50] and then subjected to OGD. Differentiated SH-SY5Y are widely accepted for in vitro experiments requiring neuronal-like cells.[17]

2.1.1 | OGD model

In brief, cells were gently rinsed with phosphate-buffered saline, and the medium was substituted with EBSS containing L-glutamine (Sigma-Aldrich) that had been deoxygenated with an anaerobic gas mixture (0.1% O_2) for 30 minutes before use. Cells were then incubated in a hypoxic condition incubator, 0.1% O_2 at 37°C at different time points. To set the experimental conditions, we measured cell viability after different OGD timepoints. In particular, 3 hours OGD insult showed a 50% reduction in cell viability and was chosen for the following experiments.

2.1.2 | I/R model

We set 3 hours OGD as timepoint, then to perform reperfusion conditions, medium was substituted with DMEM (for I/R control) or conditioned medium (I/R + CM), and cells were incubated for 24 hours in 95% air/5% CO_2 in a humidified incubator (reperfusion). Control cells were exposed to the identical experimental processes with vehicle only and with no exposure to anoxia and glucose-free medium (Normoxia). About 24 hours after OGD/Reperfusion we assayed again the cell viability to understand if conditioned media could exert positive effects.

2.1.3 | CM preparation

Cells used to collect the conditioned medium were approved by the ethic committee for biomedical research of the G. D'Annunzio University of Chieti-Pescara, Italy. Two milliliters of amniotic fluid were collected after informed written consent and centrifuged at 1200 rpm for 5 minutes and the pellet was utilized to establish the cell line.[51]

hAFS cells were cultivated in Iscove's Modified Dulbecco's medium (Corning), complemented with 10% FBS (Corning), 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mM l-glutamine (Corning), and 5 ng/mL basic FGF 2 (Peprotech, United Kingdom), and cultured at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced every 2 days.

To collect conditioned medium, when the culture reached the confluence of 70% the medium was replaced with IDMEM supplemented with exosome-free FBS (depleted using a kit by Norgen Biotek, Canada) and collected after 72 hours.

2.1.4 | Exosome preparation

To isolate and purify the conditioned media exosomes different ultracentrifuge procedures were performed. Briefly, the conditioned media were subjected to centrifuge for 5 minutes at 200*g*, then 10 minutes at 200*g* in order to remove cell debris. Then, the supernatant was collected and centrifuged for 30 minutes at 16 500*g* (+4°C); the supernatant was again collected and centrifuged at 120 000 g for 90 minutes (+4°C). The pellet obtained contains the exosomes and was washed with Ultrapure water (90 minutes, 120 000*g* at 4°C).

2.2 | MTS assay

Cell viability at different time points and conditions were determined, using Cell Proliferation Assay (Promega) a colorimetric assay established on the amount of formazan produced, as a function of viability. The assay was read at 490 nm using a plate reader, Infinite F200 (Tecan, Swiss), and analyzed in triplicate.

2.3 | Immunofluorescence

Treated and untreated cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at RT and permeabilized in cold methanol for 15 minutes. Unspecific sites were blocked using 4% BSA for 20 minutes. As primary antibody, rabbit anti-NHF (1:200, Invitrogen by Thermo Fisher Scientific) diluted in bovine serum albumin was used. Cells were then washed with PBS thoroughly and then incubated with the secondary antibody, goat anti-rabbit conjugated with Alexa Fluor 633 (1:2000; Life Technologies) for 40 minutes. Coverslips were thoroughly washed, Vectashield mounting medium with DAPI (Vector Laboratories) was used, and then observed at fluorescence microscopy AXIOPHOT (Zeiss microscope, Germany).

2.4 | Neurite analyses

Neuronal cells were photographed from 8 to 10 random fields per coverslip from three independent experiments using an immunofluorescence microscope (AXIOPHOT, Zeiss microscope). Cell clusters were excluded from morphometric analyses. Neurite length was defined as the distance from the soma to the tip of the longest primary neurite. Cells were traced using NeuronJ (plugin of Fiji software). The neurite length was then divided on soma diameter (cell body). All the detailed points can be found in the article by Pemberton et al, 2018.[52]

2.5 | Protein assay

Protein amount was assayed using Pierce BCA Protein Assay (Pierce) and the absorbance was read at 550 nm.

2.6 | Western blotting

Treated and untreated cell cultures were collected and lysated in ice-cold RIPA buffer, as previously reported.[53,54] Protein lysates (30-40 μg) were run on 9-13% SDS-polyacrylamide gel and blotted on polyvinyldifluoride membrane (PVDF; Sigma-Aldrich). Regarding the extraction of proteins from CM and EXO components, RIPA buffer was used as the above described, and then 50 μg of protein content was run on gradient SDS gel (Thermo). Unspecific sites were blocked in 5% lyophilized blocking buffer Blotto (Santa Cruz) diluted in Tris-buffered saline with Tween for 1 hour at RT. Membranes were then incubated overnight at 4°C with the below primary antibodies, prepared in blocking buffer: rabbit anti-HIF-1α 1:200 (Santa Cruz); rabbit anti-BDNF 1:1000 (Abcam, United Kingdom); rabbit anti-p-TrkB and TrKB 1:500 (Abcam, United Kingdom); rabbit anti-p-ERK5 and ERK5 1:1000 (Cell Signaling), rabbit anti PSD95 1:1000 (Cell Signaling); rabbit anti-p-CREB and CREB 1:1000 (Cell Signaling), rabbit anti P75 1:1000 (Abcam, United Kingdom); mouse anti-RhoA 1:500 (Santa Cruz); rabbit anti-p-JNK and JNK 1:500 (Santa Cruz); mouse anti-ProBDNF 1:500 (Invitrogen). After extensive washings, membranes were incubated with peroxidase-conjugated anti-rabbit or mouse IgG (1:10 000; Vector Laboratories). To visualize, the immunoreactive bands were incubated with luminol (Bio-Rad Laboratories), according to the producer's directions. The bands were revealed using Uvitec (United Kingdom) machine and digital images were collected. To reprobe the same membrane, ReBlot Plus Strong Antibody Stripping Solution was used following manufacturer's protocol (Sigma). Relative densities were analyzed using Fiji software and to normalize and check the loading, HRP-conjugated actin was used (Cell signaling). Phosphorylated proteins are normalized upon their respective total protein. Values were reported as relative units.

2.7 | BDNF ELISA kit

mBDNF ELISA kit was purchased from Aviscera Bioscience, United States. Both the exosome component and liquid component were separated by ultracentrifugation. Then we assayed the kit following the manufacturer's protocols and finally, we read the optical density, using a microplate reader set to 450 nm. Data were expressed as mBDNF (pg/mL).

2.8 | RNA extraction and miRNAs analysis

Total RNA was extracted from CM exosomes, obtained as above described, and exosome-free control medium using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek, Canada) according to the manufacturer's instructions.

MiRNAs analysis in exosomal component was investigated by quantitative Real-Time PCR (qRT-PCR) using TaqMan Advanced miRNA Human Serum/Plasma Card (Applied Biosystem). This method allows evaluating the expression of 188 miRNAs, each of them in duplicate, with high sensitivity and specificity starting from a very low amount of total RNA input.

The analysis was performed by examining 2 replicates, by using RNAs extracted by two distinct CM-exosome preparations compared with control medium. Comparative expression analysis was performed by QuantStudio Software v 1.3 and Expression Suite software v 1.3 (Applied Biosystems). Ath-miR159a was used as exogenous control for normalization of data, and global normalization analysis was conducted as well. ΔΔCt method was applied to determine the relative miRNAs expression levels. Furthermore, manual analysis focused on

PCR amplification plots profiles was performed, and miRNAs showing mean Ct less than 30 and relative quantification (RQ) values more than twofold (linear scale) were considered.

2.9 | In silico target genes and pathways analysis

Pathway-based analysis and target gene prediction of miRNAs identified were performed using DIANA miRPath v.3 software (http://snf-515788.vm.okeanos.grnet.gr/).[55] We used Tarbase analysis,[56] which provide data about experimentally supported target genes and pathways and KEGG database annotation with standard statistics and genes union settings for exploratory functional analysis. Graphs of miRNAs/target genes interactions were obtained by R software (www.r-project.org).

2.10 | Statistical analyses

Data were reported as means ± SE (n = 3). Statistical analysis was performed through Graphpad software, and data were evaluated using one-way analysis of variance. The level of significance was set at $P < .05$.

3 | RESULTS

3.1 | Phenotypic characterization of the hAFSCs

The hAFSC line used for this study was assessed for various intracellular and surface markers to determine that the cells are in an intermediary state between pluripotent stem cells and lineage-limited adult progenitor cells. As previously described,[51] the hAFSCs do not express hematopoietic surface markers (ie, CD34, CD14, and CD45), while showing different mesenchymal markers (ie, CD90, CD73, and CD105), numerous related surface adhesion molecules (ie, CD146, CD44), and stemness markers, such as Sox-2, SSEA-4, and Oct3/4 (Data already published[51]).

3.2 | I/R model

To establish the I/R model, differentiated SH-SY5Y cells (using N2 supplement, as explained in the method section) were subjected to OGD for 1 hour, 3 hours, 6 hours, or 12 hours. MTS assay revealed that cell viability gradually diminished with increasing OGD time (Figure 1A). Data confirmed also by contrast phase microscopy analyses (Figure 1B). About 3 hours OGD insult showed a 50% reduction in cell viability and was chosen for the following experiments. To further validate the model, the hypoxia inducible factor (HIF), a transcriptional activator that directs evolutionarily conserved adaptive reactions to hypoxia,[12,57] was assayed by Western blotting. HIF is induced in cerebral ischemia and the heterodimer HIF1α is regulated by oxygen-dependent degradation. Indeed, in our experimental condition, it is possible to appreciate that the protein level is significantly increased in the OGD condition compared to normoxia (Figure 1C).

Regarding the reperfusion, 24 hours time point was chosen, and different percentages of hAFSCs-conditioned medium were tested. In Figure 1D, it is possible to appreciate that the conditioned medium significantly increased cell viability respect to the control reperfusion. To confirm these data and show a similarity in hAFSCs-derived CM, its therapeutic effect in primary culture was conducted, in particular preliminary experiments on rat cortical neurons were performed as reported in File S1. Once we confirmed the protective effect and the similarity with our I/R model, to better understand the "protective" mechanism, the pathways involved in ischemia-reperfusion injury in differentiated SH-SY5Y I/R model were dissected.

3.3 | Protective mechanism analyses

To investigate the potential mechanisms underlying the conditioned media protective effects, the BDNF pathway upon both normoxic and I/R conditions, by Western blot analysis was examined.

Evidence demonstrated that BDNF resulted in increased neuronal survival via anti-apoptotic effect.[53,58-60] Accordingly, in Figure 2A, it is possible to appreciate that the conditional medium can increase mature BDNF protein level respect to the reperfusion with normal media.

BDNF exerts multiple biological actions through TrkB (tropomyosin receptor kinase) receptors, it binds TrkB triggering autophosphorylation of the tyrosine residue in its intracellular domain, inducing ligand-induced dimerization in each receptor, which triggers numerous intracellular signaling cascades with many roles; three enzymes represent the key regulators: mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), and phospholipase C γ (PLCγ).[53,58]

In our experimental condition, also TrkB protein levels were significantly increased upon conditioned medium treatment, as well as the active forms of CREB (cAMP response element-binding protein) and ERK5 (Extracellular signal Regulated kinase 5), all of which are involved in neuronal survival pathways (Figure 2A).

Therefore, it has been examined whether the conditioned media-driven increase of BDNF and TrkB translated into variations in PSD95 (postsynaptic density protein 95) levels, a marker of post-synaptic integrity. It is possible to observe a significative increase in the PSD95 protein level upon CM reperfusion (Figure 2A). PI3K/Akt signaling pathway was also analyzed and, as it is shown in the same figure (Figure 2A), the conditioned medium was able to restore stroke-induced decrease.

Further, it has been examined whether the observed neuroprotective effect of the BDNF-TrkB signaling was related to alterations in the proBDNF-p75NTR signaling cascade. WB analysis of these pathways confirmed that compared to control reperfusion, CM treatment promoted a strong decrease in p75NTR levels as well as in

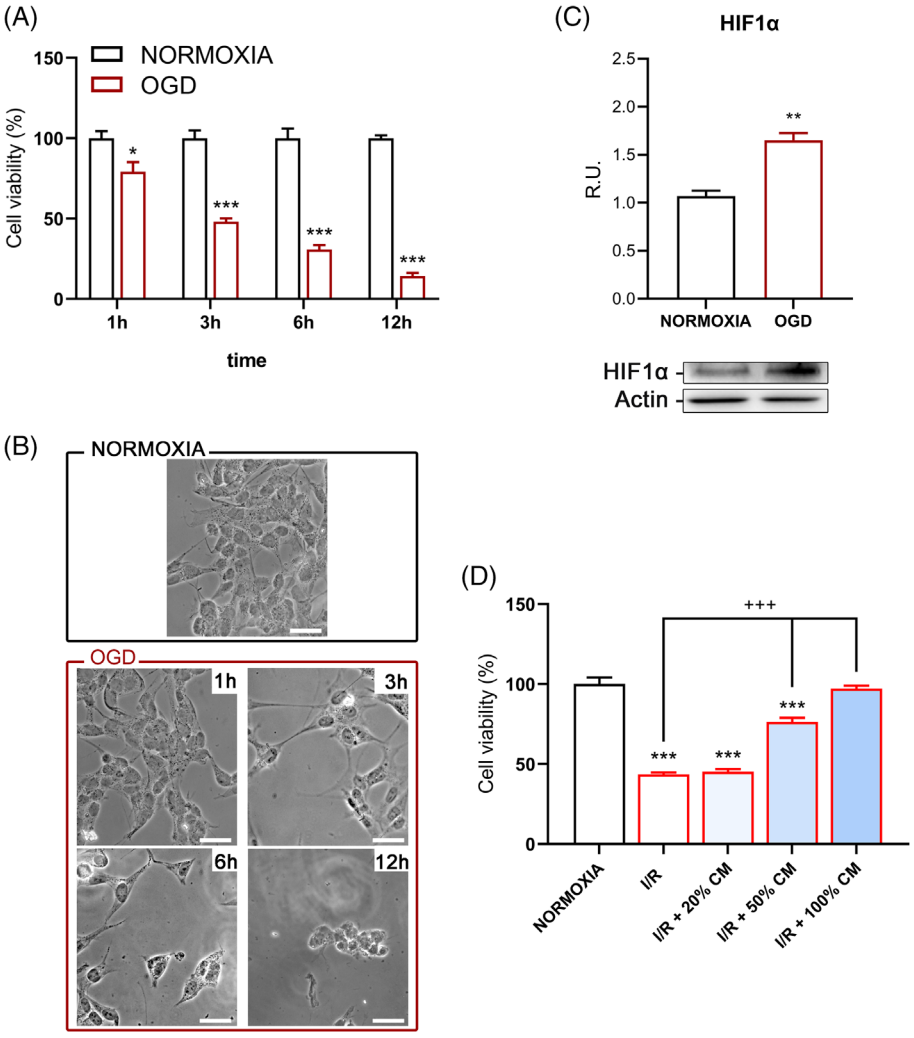

FIGURE 1 A, Viability test upon different OGD timepoints. B, Contrast phase representative pictures of differentiated SH-SY5Y upon normoxia and OGD conditions. Bar = 20 μm. C, Western blotting and relative densitometric analysis for HIF-1α. D, Viability test upon different concentration of CM. Results are mean ± SE of 3 experiments (n = 3). **P* < .05, ***P* < .005, ****P* < .0005 vs Normoxia; +++*P* < .0005 vs I/R. Representative WB images are shown

proBDNF levels, paralleled with low levels of phosphorylated (active form) of JNK (p-JNK) (Figure 2B).

Rho family GTPases received substantial appreciation as regulators of actin cytoskeletal organization. In addition, Rac and/or Rho GTPase dysregulation has been described in different neuronal injuries and neurodegenerative disorders, including I/R. Normally, Rac and its downstream effectors stimulate neuronal survival, while Rho and its downstream effectors can induce neuronal apoptosis.[61-63] Indeed, in our experimental conditions, a strong decrease in RhoA protein levels upon conditioned media reperfusion was observed, while both Rac and Cdc42 are significantly increased, as it is shown in Figure 2B, thus, suggesting a protective role exerted by the CM.

In Figure 3, immunofluorescence analyses for NFH (Neurofilament Heavy) is reported. Interestingly, upon CM reperfusion, a restore of neurite branches was observed, and this is even more evident in the graph, in which the length of the neurites was compared with the diameter of the soma. In fact, in control I/R, a significant neurites length reduction was observed, while, upon CM reperfusion, the length of the neurites was sharply increased, thus, suggesting that the CM exerted neuroprotective activities.

Afterward, to compare to the whole conditioned media with the exosomal fraction, the viability assay on the same model, treating the cells with the soluble component (CM w/o EXO) and the exosome extract (EXO) was performed. It is possible to notice in Figure 4A that, compared to I/R control, both the components increased cell viability, but the exosomal fraction (EXO) more significantly than the soluble component (CM w/o EXO).

Further, mature BDNF levels through ELISA kit in whole CM, exosomal component, and CM without exosomes were analyzed. Interestingly, elevated levels of mBDNF in CM and exosomal components (EXO) compared to the control medium were observed (Figure 4B); these data were also confirmed by Western blot analysis in proteins extracted from exosomes compared to CM w/o EXO (Figure 4C).

miRNAs expression profile in conditioned media-derived exosomes was examined to investigate a possible role of miRNAs as an exosomal component involved in the neuroprotective effects.

Among the miRNAs analyzed, we identified 16 hyper-expressed miRNAs in CM hAFSC-derived exosomes; these included let-7a-5p, let-7f-5p, let-7g-5p, miR-146a-5p, miR-154-5p, miR-155-5p, miR-221-3p, miR-222-3p, miR-22-3p, miR-23a-3p, miR-24-3p, miR-27a-3p, miR-28-5p, miR-29a-3p, miR-31-5p, miR-34a-5p (Figure 4D).

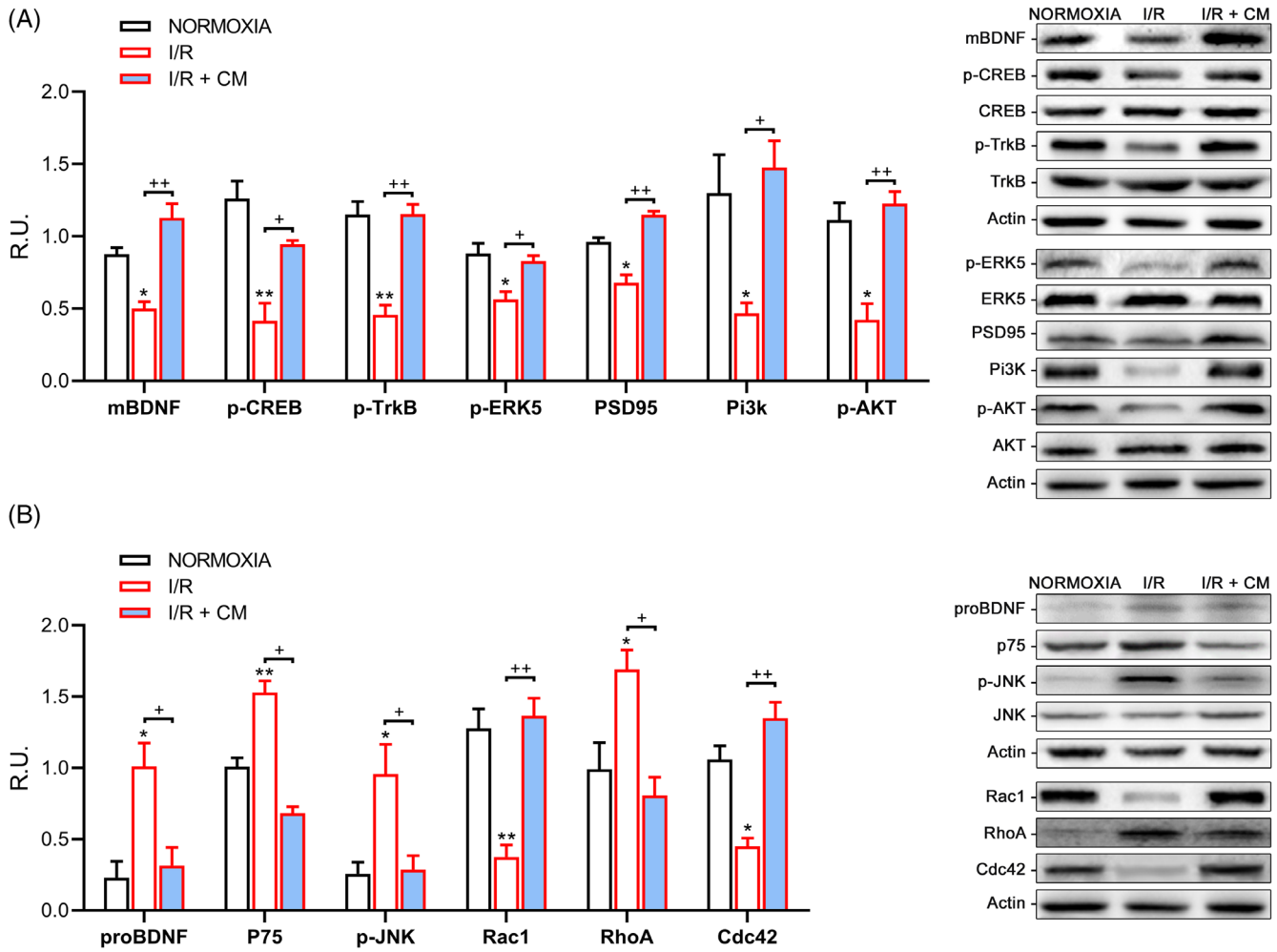

FIGURE 2 A, Western blotting and relative densitometric analysis for the neuroprotective pathway. Results are mean ± SE of 3 experiments (n = 3). *P < .05, **P < .005 vs Normoxia; +P < .05, ++P < .005 vs I/R. Representative WB images are reported. B, Western blotting and relative densitometric analysis for the neuronal death pathway and for Rho-family GTPases. Results are mean ± SE of 3 experiments (n = 3). *P < .05, **P < .005 vs Normoxia; +P < .05, ++P < .005 vs I/R. Representative WB images are reported. Phosphorylated proteins are normalized upon their respective total protein

In some of the miRNA assays, wide error bars can be detected, arguably due to the nature of samples subjected to analysis, obtained from heterogeneous, and most probably variable, secreted exosomal components.

Pathway-based analysis, predicting the putative biological functions of the 16 miRNAs, suggested their involvement in a high number of KEGG signaling pathways (File S2). Among them, we focused on pathways relevant in I/R model, such as neurotrophin signaling and other pathways related to neuroprotection and neuronal cell death (Table 1).

Further, we generated the list of experimentally supported target genes (unique n = 582) involved in the pathways mentioned above (File S3). Among them, several genes are known to play a role in inducing cell proliferation and survival (eg, PI3K, AKT, RAS, ABL, SHC, BCL2, NF-kB), on the contrary, others are involved in negative regulation of the signals mentioned above (eg, BAX, MAPK8, CDC42, RAC1, GSK3B, JUN, TP53). Consequently, depending on the specific role in such networks of genes overall targeted by miRNAs here considered, both pro-survival/proliferation and apoptotic or inflammatory signals inhibition would be induced but contextually, fine-tuned too.

With this regard, we focused on the analysis of four of the most interesting pathways listed in Table 1: HIF-1 signaling, apoptosis, and neurotrophin signaling pathway and PI3K signaling. In Figures 5 and 6, pathways representations and miRNA/target genes interactions are reported, while relative target gene lists in File S4 are reported. Of note, most of the genes involved in the HIF-1 signaling pathway were targeted by the 16 miRNAs here identified, with consequent extensive putative down-regulation possibly driving to a global pathway switching off (Figure 5A).

Effects leading to apoptosis and inflammation may be deduced by observing the apoptosis pathway map, where a relevant number of target genes (Figure 5, complete list in File S4) with physiological pro-apoptotic and pro-inflammatory functions, here supposed to be downregulated, are shown. Interactions among the target mentioned

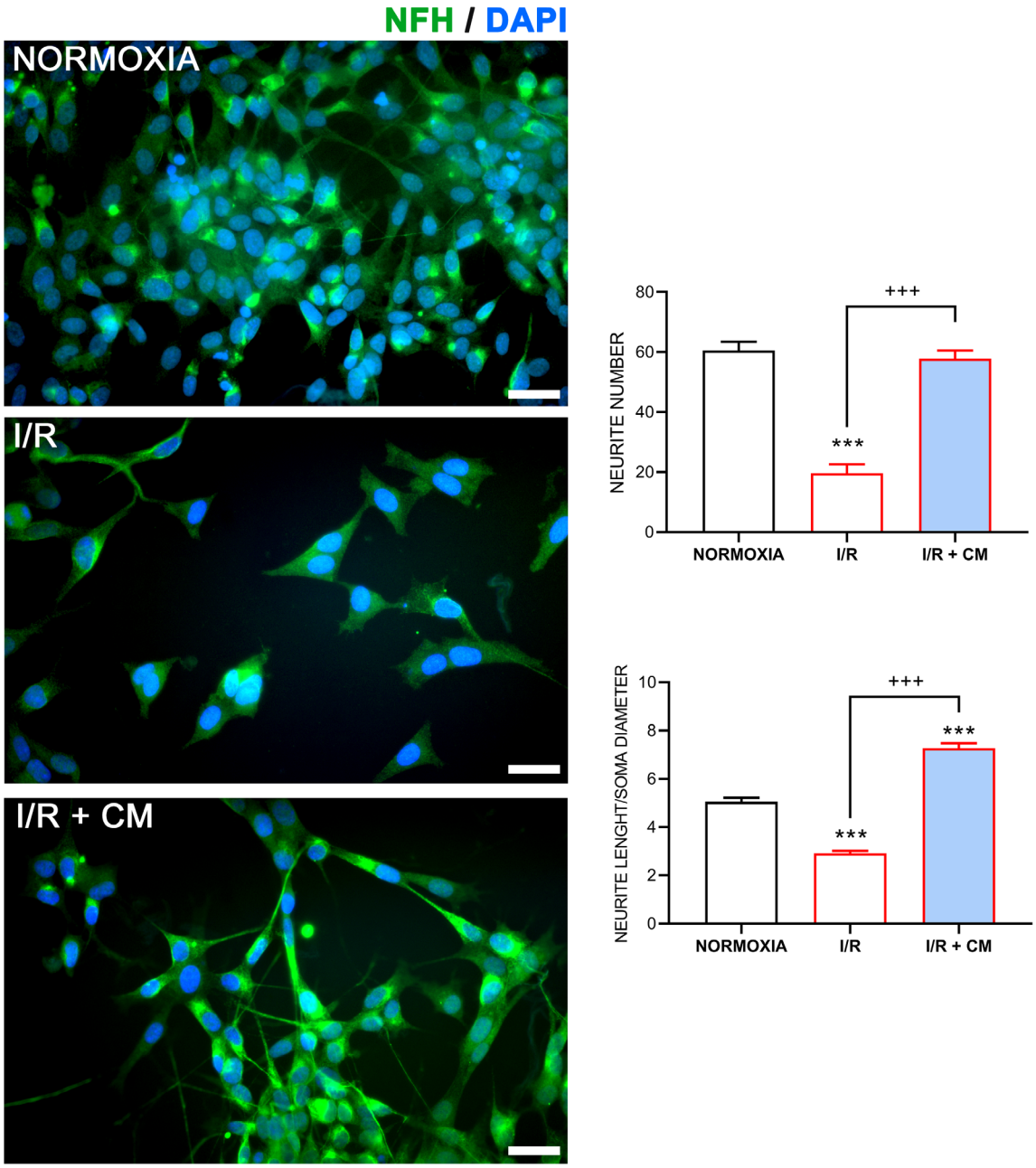

FIGURE 3 Immunofluorescence for NFH and neurite analyses graphs. Bar = 10 μm. Results are mean ± SE of 3 experiments (n = 3). ***P < .0005 vs Normoxia; +++P < .0005 vs I/R. Representative IF images are shown

above genes (red circles) and related miRNAs (light-blue circles) are displayed as well in a graph (Figure 5B, right).

Similarly, many genes known to be implicated in neuronal death, here presumed with decreased levels, are included and highlighted in the neurotrophin signaling pathway (complete list in File S4), in particular downstream p75NTR (Figure 6, left).

Of note, among the target genes, there is RhoA, down-regulation of which was also described by Western blotting in I/R + CM conditions (Figure 2B). Also, in this case, interactions among target genes playing a role in neuronal death (red circles) and related miRNAs (light-blue circles) are shown in the graph (Figure 6, right).

Conversely, especially in the latter two pathways, several genes, physiologically involved in promoting cell survival and proliferation, were included in the list of miRNAs target genes as well. For this reason, we hypothesize that even if supposed downregulated in our model, with consequent inhibition of such pathways, the complex microenvironment, where they play their function, may influence the effective downregulation of these players. This phenomenon, however, can be expected, being miRNAs fine regulators at the epigenetic level, and thus contributing to the balance of different functions within a broad multitude of cell process networks. Furthermore, due to the complexity of such networks, even though we refer to an

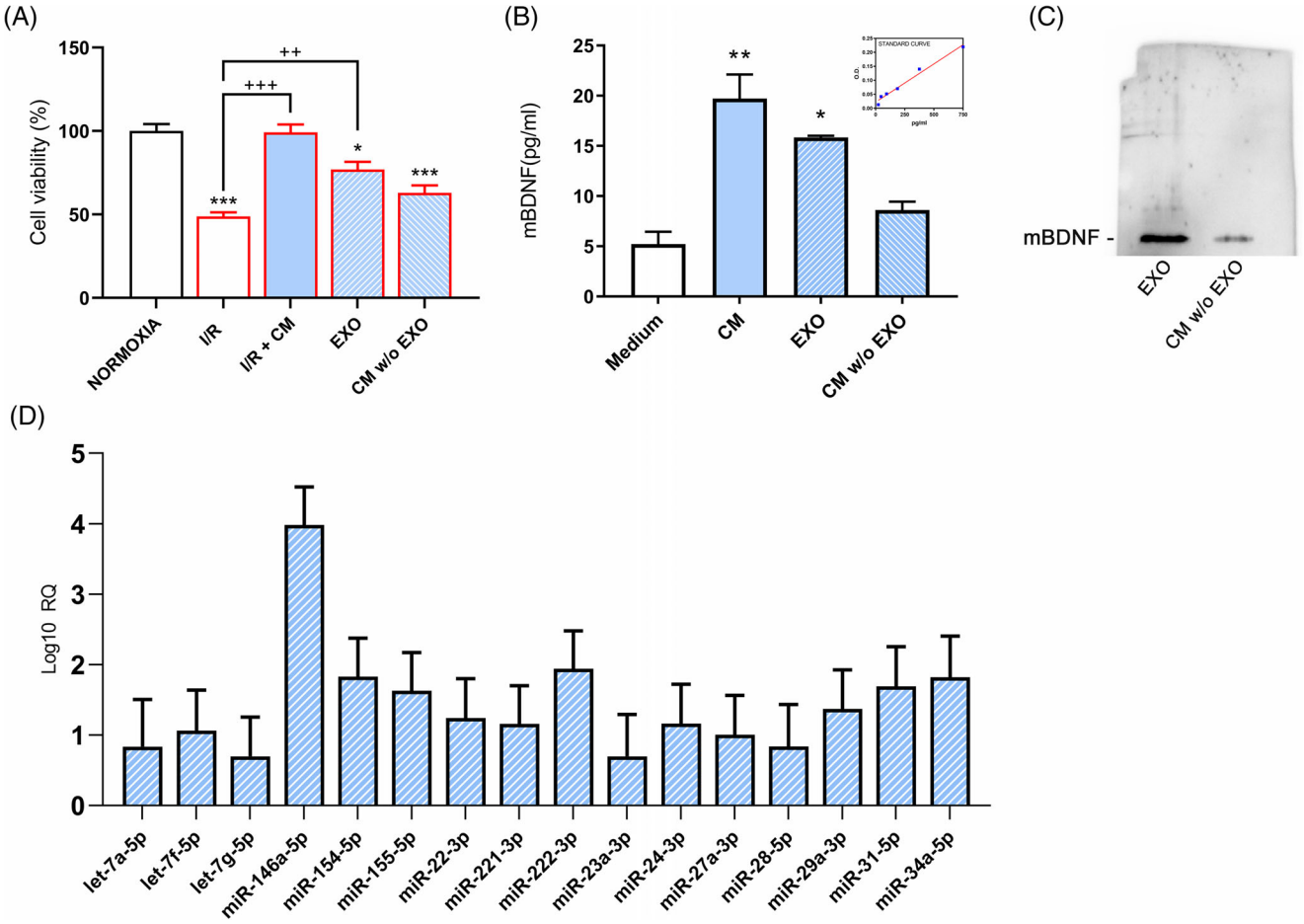

FIGURE 4 A, Viability test upon I/R, I/R+ CM, exosomal component (EXO) and soluble component (CM w/o EXO). B, mBDNF levels through ELISA kit and its relative standard curve. C, Western blotting analysis for mBDNF in the exosome protein lysates and CM w/o EXO. Results are mean ± SE of 3 experiments (n = 3). *P < .05, **P < .005, **P < .0005 vs Normoxia; ++P < .005, ++P < .0005 vs I/R. Representative WB image is reported. D, overexpressed miRNAs detected in hAFSC-CM derived exosomes. RQ ± SE values obtained are reported and shown in the logarithmic (log10) scale

TABLE 1 Pathway-based analysis of the 16 upregulated miRNAs in conditioned media exosomes

KEGG pathway	*P*-value	#genes	#miRNAs
Hippo signaling pathway (hsa04390)	7.36E-08	103	16
TNF signaling pathway (hsa04668)	3.76E-05	78	16
mTOR signaling pathway (hsa04150)	6.70E-05	48	16
Neurotrophin signaling pathway (hsa04722)	.000235281	85	15
HIF-1 signaling pathway (hsa04066)	.000639284	75	16
Apoptosis (hsa04210)	.003465202	62	15
PI3K-Akt signaling pathway (hsa04151)	.019922794	209	16
MAPK signaling pathway (hsa04010)	.043639368	152	15
Axon guidance (hsa04360)	.04536589	78	16

Note: Only terms considered as the most relevant among the significant ones are reported.

experimentally supported database for miRNA-target genes interactions in in silico analysis, it has to be considered that also additional regulatory mechanisms able to define the cell fate can play a role. The well-known PI3K-AKT survival pathway, already studied by Western blotting, was analyzed as well (Figure 6B). A relevant part of miRNA target genes promoting cell proliferation was present, but also others with pro-apoptotic and anti-proliferative functions (complete list in File S4), represented in the target genes/miRNA interaction graph (Figure 6, right). Interestingly, most of those factors here hypothesized to be downregulated as miRNAs targets, act downstream the major

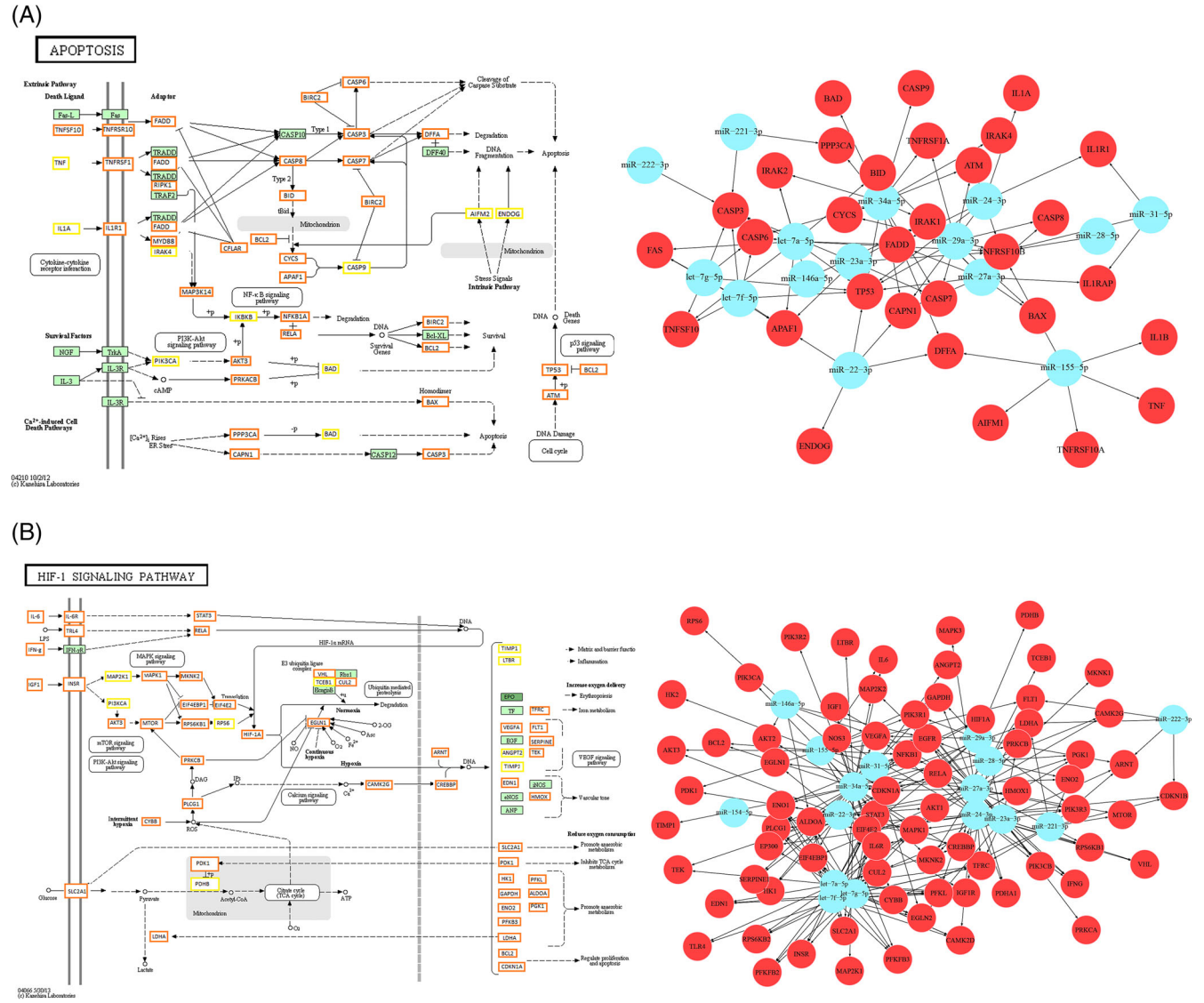

FIGURE 5 A, On the left, HIF-1 signaling pathway (modified from KEGG hsa04066), showing genes targeted by miRNAs (n = 16) involved in its modulation. Orange boxes, genes targeted by more than 1 miRNA; yellow boxes, genes targeted by 1 miRNA and in green, not targeted genes. The complete list of target genes is reported in File S3. On the right, the graph showing interactions between miRNAs and related target genes. B, On the left, the Apoptosis pathway (modified from KEGG hsa04201), showing genes targeted by miRNAs (n = 15) involved in its modulation. Orange boxes, genes targeted by more than 1 miRNA; yellow boxes, genes targeted by 1 miRNA and in green, not targeted genes. The complete list of target genes is reported in File S3. On the right, the graph showing interactions between miRNAs and related target genes with pro-apoptosis and pro-inflammatory functions

player of this pathway, possibly further enhancing pro-survival and proliferation processes.

4 | DISCUSSION

Ischemic stroke is one of the leading causes of morbidity, mortality, and disability in aging patients.[64] Appropriate reperfusion of the ischemic region is the standard approach to preserve brain structure and function.[8] However, reperfusion approaches after ischemic stroke trigger further brain injuries, described as cerebral I/R injury. I/R injury decreases the clinical benefits of reperfusion approaches for patients with ischemic stroke.[65,66] Consequently, defining the molecular mechanisms underlying the I/R injury can improve the success of reperfusion treatment and enhance the clinical advantages for patients with stroke.[67] Numerous biological processes involving calcium excess, oxidative stress, inflammatory reactions, and endoplasmic reticulum stress are closely related to the brain I/R injury.[65,68]

Interestingly, each of these activities can be influenced by mitochondria. In reply to the brain I/R injury, mitochondria cannot generate sufficient energy and thus affect calcium reuptake by the endoplasmic reticulum, which induces calcium excess. Moreover, alterations in mitochondria can be due to excessive ROS, which causes oxidative stress.[69] Consequently, altered mitochondria can

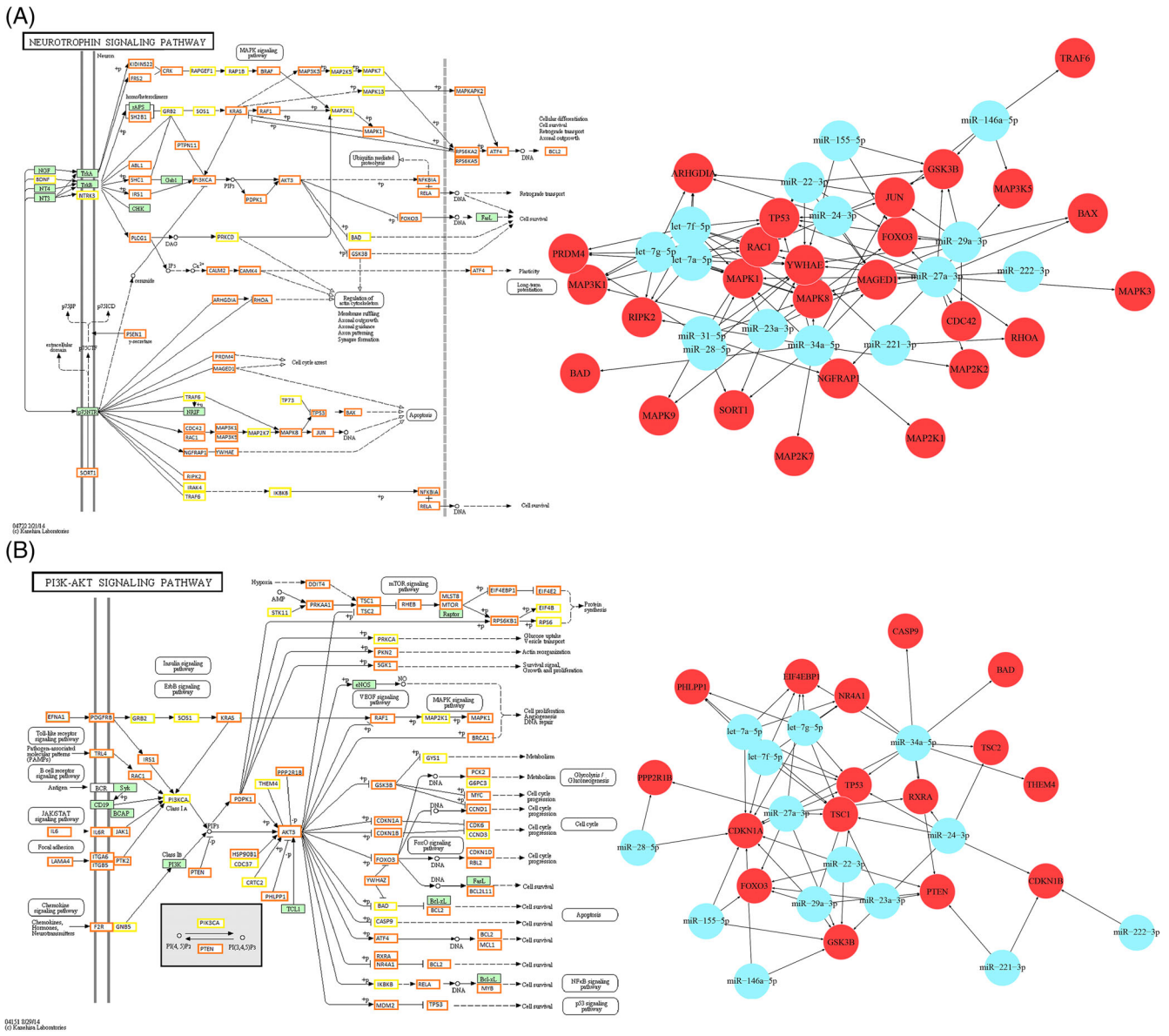

FIGURE 6 A, On the left, the Neurotrophin signaling pathway (modified from KEGG hsa04722), showing genes targeted by miRNAs (n = 15) involved in its modulation. Orange boxes, genes targeted by more than 1 miRNA; yellow boxes, genes targeted by 1 miRNA and in green, not targeted genes. The complete list of target genes is reported in File S3. On the right, the graph showing interactions between miRNAs and related target genes involved in neuronal death. B, On the left, the PI3K-Akt signaling pathway (modified from KEGG hsa04151), showing genes targeted by miRNAs (n = 16) involved in its modulation. Orange boxes, genes targeted by more than 1 miRNA; yellow boxes, genes targeted by 1 miRNA and in green, not targeted genes. The complete list of target genes is reported in File S3. On the right, the graph showing interactions between miRNAs and related target genes with anti-survival and anti-proliferative functions

stimulate pro-apoptotic factors in the cytoplasm or the nucleus to begin mitochondria-supported cellular apoptosis.[70]

In the present study, for the first time, the efficacy of hAFSCs-secretome in protecting cells by death signals in I/R in vitro model was studied.

As mentioned above, the OGD/R model is a widely studied model to mimic ischemia. Differentiated SH-SY5Y cells have been largely utilized to investigate neuronal activity since they have comparable morphology and biochemical characteristics to mature neurons.[71-73] Differentiation of SH-SY5Y cells with N2 supplement demonstrated upregulated neuron-specific markers, such as β-tubulin III, Neurofilament 200, and GAP43.[74]

Previous reports demonstrated that in ischemic conditions, different pathways were altered, including PI3K/AKT, AKT/GSK-3β/β-catenin, ERK1/2, apoptotic pathways, mTOR pathway as well as BDNF pathway.[60,75,76]

Mostly, MSCs or MSCs-CM administration showed structural and functional advantages: it can diminish apoptosis at the injured area, to modulate pro-inflammatory reaction, to offer a permissive environment for axonal expansion, to stimulate neurogenesis and enhance

neurological impairments.[36,77,78] The composition of the CM secretome "cargo" determines the therapeutic potential.[79] CM contains a variety of growth factors and tissue-regenerative agents, which were released by the stem cells. Indeed, proteomic studies showed the presence of various growth factors and other cytokines in the CM.[80,81] Besides, MSCs are the most efficient exosome-producing cells.[82]

Based on this evidence, and on the paracrine theory which establishes that the positive effects of stem cell therapeutic approach are due to stimuli of resident cells by the release of bioactive molecules and EV, the use of secretome could offer several advantages over MSCs such as a superior safety profile. Different reports demonstrated that secretome released from MSCs could decrease cognitive impairments related to numerous neurological disease models, comprising Traumatic Brain Injury, Parkinson's disease, Alzheimer's disease, and stroke.[28,83-86] Secretome and conditioned media showed the ability to interact and promote the release of neurotrophins, comprising vascular endothelial growth factor, BDNF. Nerve growth factor, improving neurite development, stimulating neurorestorative and neurological improvement.[78,87]

Exosome release is deemed an adaptation mechanism and its composition, biogenesis, and secretion are influenced by microenvironment with which cells interrelate.[78,87]

The high proliferative capacity, the low immunogenicity, and tumorgenicity and the anti-inflammatory activity support the hAFSCs use as safe and effective donor cells for stroke therapy.[88] Furthermore, human stem cell utilization is useful and helpful for clinical trial translation. In vitro systems have a partial capacity to reflect the complicated situation that occurs during stroke in vivo. Still, they represent a useful research tool to understand cellular and molecular mechanisms that are difficult to dissect in vivo.

Thus, the growing availability of human cells renders in vitro systems a solid low-cost base for the drug discovery pyramid and for strengthened the in vivo research, improving clinical translation.

In this study, we established an I/R model and analyzed the downstream responses to hAFSC-secretome exposure. Our findings indicated that the CM and, in particular, the exosomal fraction played a crucial role in promoting cell survival. This pro-survival effect was paralleled by the activation of the neurotrophic pathways BDNF/TrkB and by the suppression of the death pathways (p75/JNK). These effects were paralleled by the increase of the survival pathway PI3K/Akt and ERK5.

Exosomes encapsulate and transfer numerous functional molecules, including proteins, lipids, and regulatory RNAs, which can alter cell metabolism.[79] Notably, in the MSC-derived exosomes protein component, we identified a high level of mBDNF. Numerous studies showed that exosomes carry different neuronal proteins and that they can cross the blood-brain barrier.[89] However, till now, it was not clear whether BDNF was present in the exosomes and the soluble component of CM, and, interestingly, in our experimental condition, we found that both exosomal fractions and soluble CM contains high levels of mBDNF, with exosomes particularly endowed of this neurotrophin.

Further, within exosomal cargo, a broad variety of miRNAs has been detected, which can influence functions associated with neural remodeling and neurogenic processes. Several miRNAs, already identified as players in neuroprotective mechanisms (miR-146a-5p, miR-154-5p, miR-22-3p, miR-23a-3p, miR-27a-3p, miR-29a-3p, and miR-31-5p), were also identified in our hAFSC-derived exosomes. Interestingly, miR-146a was already described in the negative regulation of inflammation[90,91] and in promoting oligodendrogenesis[92] in the post-injury perinatal brain. MiR-154 was detected in astrocyte-derived extracellular vesicles, involved in regulating neurotrophic signaling in neurons.[93] Moreover, miR-154 is known to target DKK2 (Dickkopf-related protein 2) with consequent β-catenin up-regulation and classical Wnt signaling pathway activation,[94] crucial both for the maintenance of synaptic structures and neuronal survival.[95] MiR-22-3p, 23a, and 27a are involved in neuroprotection mechanisms through apoptosis inhibition[96-100] Furthermore, miR-22-3p can regulate neuronal morphology during migration by targeting the CoREST/REST complex,[101] and miR-27a is involved in controlling neuroinflammation.[99] Also, among the other miRNA identified, miR-29a was already described to protect against cell damage after ischemia-like stress and astrocyte ischemic injury[102,103]; miR-31-5p, also identified in our exosomal component, is known to reduce the inflammatory response and oxidative stress-induced neuronal damage[104] and, further, shows pro-angiogenic activity.[105]

On the other hand, regarding other miRNAs here identified, such as miR-221-3p, miR-222-3p, miR-24-3p, and miR-28-5p, limited or conflicting data about their function and implication in neuronal processes have been reported.[106-109]

Besides neurotrophin signaling, interestingly, the pathway-based analysis revealed that these miRNAs act on pathways known to be altered in ischemic conditions and involved in neuroprotection, such as TNF, Hippo, and PI3K.[110-112] Among the miRNAs targets here identified, supposed to be downregulated in our model, we highlighted several genes involved in apoptosis and inflammation processes.

Interestingly, based on the experimentally supported miRNAs/target genes, in HIF-1 pathway, a sort of global switch-off mechanism can be observed. In contrast, other pathways of interest, including apoptosis, neurotrophins, and PI3K-AKT, showed several groups of genes, all supposed to be downregulated in response to miRNA activity, physiologically playing a role in inducing or, conversely repressing different processes, such as proliferation, apoptosis, inflammation. This is, however, consistent with the pleiotropic and versatile activity and function of miRNAs, molecules specifically designated to regulate and fine tune, at the epigenetic level, expression of multiple target genes within complex molecular circuitries.[113] Further studies are needed to confirm the involvement and biological impact of miRNAs identified in this in silico study regulating neurogenic processes after stroke, as well as to shed light on molecular mechanisms underlying their possible neuroprotective role, even if the biochemical data obtained strongly support the involvement of these miRNAs in regulating several actors of the analyzed pathways, that is, pro-BDNF, PI3K-Akt, Rho/Rac.

The central nervous system includes various cells other than neurons, thus, undoubtedly it is important also to study the effect of the conditioned media derived from hAFSCs in vivo; future studies will be devoted to clarify the therapeutic effect of hAFSCs-CM in stroke in vivo models.

5 | CONCLUSIONS

These data indicate that miRNAs represent a relevant component of the exosomal cargo and that the effect of CM-derived exosomes could be in part mediated by secreted miRNAs. Specifically, our findings suggest that miRNAs may mediate neuroprotection mainly through anti-apoptotic and pro-survival pathways. However, it is conceivable that some effects may also be due to the soluble component of the CM, being this latter endowed by neurotrophic activity.

Taking this into consideration, the use of CM, and in particular, exosomes, may constitute a potential approach to stimulate neuronal plasticity, ameliorate cognitive loss, and neural replacement in stroke. Consequently, it is possible to indicate that the exosomal fraction of the hAFSC-CM may represent a suitable treatment for I/R injury.

ACKNOWLEDGEMENTS

The authors thank University of L'Aquila, RIA funding for their support.

CONFLICT OF INTEREST

C.B. disclosed employment/leadership position with University of South Florida, patent holder and stock ownership with Sanbio Inc, Athersys Inc, advisory role with KMPHC, Chiesi, research funding from NIH, Asterias, Astellas. The other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

V.C.: conception and design, experiments execution, collection and assembly of data, data analysis and interpretation, manuscript writing; I.A.: provision of study material, supervision of experiments, critical review of the manuscript; M.d.A.: experiments execution, data analysis and interpretation, figure preparation; A.T., V.Z.: miRNA experiments and analysis, critical review of the final manuscript; E.B., C.F.: critical review of the manuscript; G.D.: critical review of the manuscript, final approval of the manuscript. C.B.: critical review of the manuscript and experimental execution, final approval of the manuscript; L.S.: supervision, critical review of manuscript and experimental execution, final approval of the manuscript; A.C.: conception and design, supervision, data analysis and interpretation, manuscript writing, financial support.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available from the corresponding authors upon reasonable requests.

ETHICS STATEMENT

Amniotic fluid was used upon informed written consent.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Castelli V, Antonucci I, d'Angelo M, et al. Neuroprotective effects of human amniotic fluid stem cells-derived secretome in an ischemia/reperfusion model. *STEM CELLS Transl Med*. 2021;10:251–266. https://doi.org/10.1002/sctm.20-0268