Intravenous Administration of Hair Follicle Mesenchymal Stem Cells and Insulin Growth Factor-1 Improves the Neurological Performances of Rats Following Focal Cerebral Ischemia

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Research

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Abstract

Background: Focal cerebral ischemia is a common cerebrovascular disease with limited treatment options, and new treatments are therefore urgently needed. Hair follicle mesenchymal stem cells (HF-MSCs) are considered ideal cells for the treatment of neurological disorders. Insulin growth factor-1 (IGF-1) is an effective neuroprotective compound.

Methods: In the present study, we used middle *cerebral artery* occlusion (MCAO) *model* to evaluate the therapeutic effects of HF-MSCs and IGF-1 in focal cerebral ischemia. After middle cerebral artery occlusion (MCAO), rats were randomly divided into six groups. HF-MSCs and IGF-1 were transplanted into rat models by tail vein injection. The fate of transplanted HF-MSCs in the rat brain was assessed using immunofluorescence, immunohistochemistry, Western blot analysis, and reverse transcription polymerase chain reaction (RT-PCR). Beam balance tests and neurological severity scores were used to assess neurological recovery.

Results: HF-MSCs labeled with the green fluorescent dye PKH67 were found to colocalize with 4',6-diamidino-2-phenylindole (DAPI) and neural-specific markers. Rats in the HF-MSCs, IGF-1 and HF-MSCs + IGF-1 groups exhibited neural differentiation marker expression, with those in the HF-MSCs + IGF-1 group exhibiting the highest levels.

Conclusions: These results suggest that the combined treatment of HF-MSCs and IGF-1 can enhance neurological recovery, representing a new therapeutic strategy for cerebral ischemia.

Introduction

Stroke is one of the leading causes of high disability and is associated with high mortality rates worldwide [1]. Ischemic stroke is secondary to occlusion of the arteries that supply blood to the brain and accounts for more than 80% of all strokes [2]. The majority of stroke patients have some degree of sequelae, and the disability rate is as high as 3 out of 4, which seriously affects the patients' quality of life [3]. Currently, stroke treatments are limited to anticoagulation [4], thrombolysis [5], antiplatelet [6], and intravascular therapies [7]. An increasing number of scientists and clinicians are focusing on stem cell therapy because of the ability of stem cells to differentiate and proliferate. To elucidate the role of stem cells in regenerative medicine, scientists have isolated and cultured stem cells from different tissues or organs, and a nonsurgical approach for the treatment of neurological diseases, including nerve defects, has been proposed [8]. Transplanted stem cells may secrete some neuroprotective or neuroreparative neurotrophic factors [9], however many problems must be resolved before these cells can be used in the clinic. As a regenerative organ, hair follicles have the characteristics of periodic growth and continuous self-renewal [10]. Hair follicle mesenchymal stem cells (HF-MSCs) can be harvested from hair follicles [11]. Due to the abundance of hair follicle tissue, HF-MSCs are easily obtainable, and the feasibility of autotransplantation and avoidance of immune rejection have been demonstrated [12]. Studies have shown that transplanted HF-MSCs participate in not only skin reconstruction but also angiogenesis and

nerve repair [13]. In addition, HF-MSCs have great potential for myoblast differentiation and can provide functional smooth muscle for tissue regeneration and cell therapy [14]. Since the neural potential of HF-MSCs was first reported in 2004 [15], several studies have shown that transplanted HF-MSCs can differentiate in peripheral nerve injuries both *in vitro* and *in vivo* [16] including in spinal nerve injury [17–19] and Alzheimer's disease animal models [20]. Moreover, HF-MSCs can induce a rich population of glial and neuronal lineages after incubation with exogenous growth factors [21].

The polypeptide, insulin-like growth factor 1 (IGF-1) has neuroprotective effects in the central nervous system on the migration, growth and proliferation of cells. IGF-1 has been shown to be an effective neuroprotective compound in rat ischemic stroke models [22–24]. Studies have shown that injection of IGF-1 can reduce infarct volume, increase cell survival, and improve sensorimotor function [25] via the inhibition of the mRNA expression of iNOS and interleukin (IL)-1β in the ischemic hemisphere. It has been suggested that IGF-1 can induce neuroprotective effects by regulating neuroinflammation [26].

In the present study, we investigated whether intravenous administration of HF-MSCs can improve neurological function in a rat model of MCAO, and whether HF-MSCs and IGF-1 in combination are more effective than either is alone in improving neurological function in MCAO rats.

Materials And Methods

Experimental design

Thirty rats were randomly divided into 6 groups: healthy control group (n = 5), sham operation group (n = 5), MCAO group (n = 5), HF-MSCs group (n = 5), IGF-1 group (n = 5), and HF-MSCs + IGF-1 group (n = 5). Animals in the healthy control group were not treated, and those in the sham operation and MCAO groups were treated with 1 ml of saline. After the MCAO model was established (Fig. 1), the rats in the HF-MSCs transplantation group were injected with 1 ml of HF-MSCs (1×10□) through the tail vein, and those in the IGF-1 transplantation group were injected with 1 ml of IGF-1 (R&D Systems, MI, USA, 50 μg/ml) via the tail vein. Both HF-MSCs (1×10□) and IGF-1 (50 μg/ml) were injected into the tail veins of rats in the HF-MSCs + IGF-1 group.

On the 1st, 7th, 14th, 21st and 28th days after model establishment, the rats were assigned modified neurological severity scores (mNSS) and subjected to the beam-walking test. The samples were assayed for immunofluorescence on the 21st day, and immunohistochemistry analysis, western blot analysis and rt-PCR assays were performed on the 28th day, followed by statistical analysis (Fig. 1).

Isolation, identification, and in vitro **fluorescence labeling of HF-MSCs**

The skin of the whiskers of 1-week-old male Sprague-Dawley (SD) rats was excised after being disinfected with alcohol and digested with 3 mg/ml collagenase type I. The hair follicle bulge area was then separated under an anatomical microscope (Olympus, Japan), and rat HF-MSCs were cultured by tissue block attachment in F12 DMEM (HyClone) containing 10% foetal bovine serum (FBS, ScienCell

Research Laboratories). Upon reaching 80% confluence, the cells were digested, subcultured at 37℃ and 5% CO_2 and purified based on their adhesion to type I collagen, and cell density was adjusted to 1×10⁶ cells/ml. The expression levels of stem cell surface markers, including FITC-CD29, PE-CD90, PerCP-CD45 and APC-CD31, were measured by a flow cytometry (BD Biosciences) [11, 12, 27], and HF-MSCs were labeled with the green fluorescent dye PKH67 (SigmaAldrich, St. Louis, MO, USA). The labeled cells were observed under a fluorescence microscope (Olympus, Japan). Cell isotype control experiments were performed simultaneously to identify false positives.

Induced differentiation of HF-MSCs

To test the multidirectional differentiation function of the HF-MSCs, passage 3 cells were seeded in a 6-well culture plate. A lipogenic differentiation medium kit (Cyagen, RASMX-90031) and an osteogenic differentiation medium kit (Cyagen, RASMX-90021) for SD rat bone marrow mesenchymal stem cells (BMSCs) were used to induce osteogenesis and adipogenesis, respectively. After induction for 2 weeks to 1 month, the HF-MSCs were stained with Alizarin Red (Sigma-Aldrich) and Oil Red O (Sigma-Aldrich), and images were acquired by microscope (Olympus, Japan). Currently, there are no osteogenic or adipogenic media for inducing differentiation of HF-MSCs commercially available. As HF-MSCs and BMSCs are both mesenchymal stem cells, BMSC differentiation media were needed in this study.

Middle cerebral artery occlusion model

Adult male clean-grade SD rats weighing 240–260 g were obtained from the Animal Experiment Center of the Second Affiliated Hospital of Harbin Medical University. All relevant procedures were approved by the Ethics Committee of Harbin Medical University and were in compliance with National Institutes of Health (NIH) guidelines for the use and care of laboratory animals. After 24 hours of fasting and 2 hours of water deprivation, the rats were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) and placed in the supine position for surgery, with their limbs and heads being fixed. The proximal common carotid artery (CCA) and the external carotid artery (ECA) of each rat were ligated, and the internal carotid artery (ICA) was clamped with arteriolar forceps. A silicon-coated monofilament was slowly inserted into the ICA through the CCA. After loosening the arterial forceps, the monofilament finally reached the middle cerebral artery [28], and insertion of the monofilament was halted as soon as resistance was felt. During the surgery, the body temperature of the rat was maintained at 37 ± 1℃ with an electric blanket. After one hour, part of the monofilament was removed, but it was retained in the ICA. Finally, the incision was sutured, and the exposed portion of the monofilament was cut to prevent the rat from removing it on its own. Rats in the sham-operated group underwent only vascular dissection. The occurrence of hemiplegia was indicative of successful model establishment.

2,3,5-triphenyltetrazolium chloride (TTC) staining

Ischemic brain injury was assessed by TTC staining at 24 hours after model establishment. The brain tissue was quickly removed from ice, flash frozen at -80℃ for 1 minute, sliced into an average of five

consecutive coronal sections and incubated in 2% TTC (St Louis, Missouri). A digital camera was used to image the brain tissue sections.

Hematoxylin-eosin (HE) staining

HE staining can be used to distinguish normal cells from necrotic cells. Brain tissues were paraffinized and then cut into successive coronal sections. According to a standard protocol, the specimens were stained with HE, and observed under a light microscope.

Beam-walking test and modified neurological severity scores (mNSS)

Motor coordination in rats can be evaluated by the beam-walking test and mNSS. For the beam-walking test, rats were placed on a high, narrow beam and allowed to walk, and hemiplegia and even falling were observed. The mNSS test was used to examine the behaviors of the model rats, which received scores ranging between 0 and 18 [29, 30]. Rats with mild or severe symptoms and even those that died were excluded from the analysis. On days 1, 7, 14, 21 and 28 after the operation, the beam-walking test and mNSS test were performed to evaluate the improvement of motor function.

Immunofluorescence

After heart perfusion, the brain tissue was fixed in 4% paraformaldehyde (pH 7.4) at 4℃ for 24 hours and then dehydrated in 20% and 30% sucrose solutions until it sank to the bottom of the container. The brain tissue was sectioned into coronal frozen sections (8 μm) and immediately immobilized in acetone for 10 minutes. After overnight incubation at 4℃ with anti-neuronal nuclei protein (anti-NeuN) (Abcam, ab177487, 1:100), anti-neurofilament heavy polypeptide (anti-NF-H) (Abcam, ab207176, 1:200), anti-doublecortin (anti-DCX) (Abcam, ab207175, 1:100) and anti-glial fibrillary acidic protein (anti-GFAP) (Abcam, ab33922, 1:100) antibodies, frozen brain tissue sections were incubated with normal goat serum for 20 minutes at room temperature, washed with PBS 3 times, and incubated with rhodamine-conjugated goat anti-mouse IgG (ZSGB, ZF-0313) and anti-rabbit IgG (ZSGB, ZF-0316) for 1.5 hours. The brain sections were counterstained with the DAPI (Sigma-Aldrich) at 37℃ for 10 minutes. The HF-MSCs were observed under a laser scanning confocal microscope, and the positive cells' average number was calculated from counts of five randomly selected regions.

Immunohistochemistry

After heart perfusion, the brain tissue was embedded in paraffin and sectioned (10 μm) before being dewaxed and hydrated in xylene and ethanol solutions. The sections were heated at 95℃ for 10 minutes, incubated overnight with anti-NeuN (1:400), anti-NF-H (1:200), anti-DCX (1:50), and anti-GFAP (1:400) antibodies at 4℃, and then reacted with the biotinylated secondary antibodies for 20 minutes at room temperature. After the sections were incubated in 0.05% diaminobenzidine (DAB) and PBS, the product of this reaction turned brown, at which point the sections were counterstained with hematoxylin. After dehydration in an ethanol solution, the sections were sealed on glass slides and analyzed under an

optical microscope, and semiquantitative analysis was conducted with ImageJ (National Institutes of Health, Bethesda, USA).

Western blot

Fresh brain tissue was taken, and the corresponding volume of lysate was added according to the quality and volume of each sample and then centrifuged at 12000 rpm and 4℃ for 10 minutes. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. The electrophoretic device was assembled, and the electrophoretic protein sample (every 40 μg) was transferred to a polyvinylidene fluoride (PVDF) membrane, which was incubated with TBST buffer and 5% m/V skimmed milk powder under gentle shaking and then incubated overnight at 4℃ with anti-NeuN (1:500), anti-NF-H (1:1000), anti-DCX (1:500) and anti-GFAP (1:500) antibodies overnight. After the membrane was incubated with the secondary antibody (1:5000) coupled to horseradish peroxidase at 37℃ for 45 minutes, the substrates were detected with an ECL chemiluminescence detection kit. The film was scanned, and the optical density of the target band was analyzed.

Real-time polymerase chain reaction (rt-PCR)

Total RNA was harvested from frozen brain tissue with the TRIzol (Invitrogen, Carlsbad, CA, USA) extraction kit in accordance following the manufacturer's instructions, and the RNA concentrations in each sample were determined using a NANO 2000 photometer. The RNA samples were reverse-transcribed by PCR to obtain the corresponding cDNA, and β-actin served as an internal reference. Fluorescence quantitative analysis was performed on an ExicyclerTM 96 fluorescence quantizer made by Bioneer (Korea) under the following conditions: pre-denaturation at 94 for 5 minutes, followed by 40 cycles of 94℃ for 10 seconds, and an annealing step at 60℃ for 20 seconds. The gene expression levels of neural-specific markers in each group were compared using the $2^{-\Delta\Delta CT}$ method. All PCRs were repeated three times, and the existence of a single peak was verified by the melting curve. The primers are shown in Table I.

Table I. rt-PCR primer sequences

Gene of interest	Direction	Primer sequence (5′-3′)
NeuN	Forward	GGTGCTGAGATTTATGGAGG
NeuN	Reverse	CGATGGTATGATGGTAGGGA
GFAP	Forward	TGACTATCGCCGCCAACTGC
GFAP	Reverse	CTGGTAACTCGCCGACTCCC
NF-H	Forward	AGTGAACACGGATGCTATGC
NF-H	Reverse	CAGTGACTCCTTGGTGCTTT
DCX	Forward	AGAGGGTCACGGATGAATGG
DCX	Reverse	GAGGCAGGTTGATGTTGTCG
β-actin	Forward	GGAGATTACTGCCCTGGCTCCTAGC
β-actin	Reverse	GGCCGGACTCATCGTACTCCTGCTT

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) from three repeated experiments and were analyzed by Student's t-test, Bonferroni's multiple comparison test, a two-factor factorial design and ANOVA in a fully randomized manner. All statistical analyses were performed with SPSS 24.0 (SPSS Inc, Chicago, IL, USA) and Prism 8.0 (GraphPad), and differences between the means were considered statistically significant for $P < 0.05$.

Results

Evaluation of the MCAO rat model

An experimental model was established with MCAO. Focal cerebral infarction in the temporal, parietal, frontal, occipital and striatum regions was induced by this model. TTC staining showed white ischemic brain tissue in the MCAO group (Fig. 2B), and red in the healthy control group (Fig. 2A). HE staining showed that noninfarcted brain tissue contained normal nerve cells with round or oval shapes (Fig. 2C), whereas a large number of nerve cells disappeared in the infarcted area, and the remaining neurons were sparsely distributed. In addition, we observed a substantial amount of inflammatory cell infiltration (Fig. 2D, E). Under normal conditions, the rats showed significantly different mNSS and beam-walking test performances, and rats of the acute cerebral ischemia model showed obvious signs of neurologic deficits.

Characteristics of HF-MSCs

After reaching approximately 80% confluence, HF-MSCs were passaged, and they began to exhibit a paving-stone shape after the third passage (Fig. 3A, B). Calcium precipitation was observed in HF-MSCs cultured in osteoblast differentiation medium for 1 month (Fig. 3D). After HF-MSCs were cultured in adipogenic differentiation medium for two weeks, lipid droplets were formed in HF-MSCs stained with Oil Red O (Fig. 3C). Fluorescence-activated cell sorting (FACS) analyses revealed that CD29 (Fig. 3E, I, J) and CD90 (Fig. 3F, I) were highly expressed in HF-MSCs, while the expression of endothelial cell surface marker CD31 (Fig. 3H, J, L) and hematopoietic cell surface markers CD45 (Fig. 3G, K, L) was lower.

Migration of transplanted HF-MSCs and expression of neural-specific markers

The expression of four neural-specific markers was examined, including NeuN, NF-H, DCX and GFAP, was examined to evaluate the therapeutic effect of HF-MSCs and IGF-1. The expression levels of the neural-specific markers in each group were analyzed and compared.

Immunofluorescence analysis revealed that the transplanted HF-MSCs exhibited green fluorescence in the cell membrane (Fig. 4B, F, J and N), red fluorescence in the cytoplasm (Fig. 4E, I and M) and blue fluorescence in the nucleus (Fig. 4C, G, K and O) after proliferation and differentiation as determined by laser scanning confocal microscopy. After cosynthesis, HF-MSCs exhibited a yellow-green fluorescence, and the nucleus was blue (Fig. 4H, L and P). Since NeuN is a nuclear protein (Fig. 4A), the nucleus showed both blue and red (Fig. 4D). We can clearly see the color of the cell membrane, cytoplasm and nucleus from the enlarged picture (Fig. 4d, h, l and p). These findings indicated that HF-MSCs had homing abilities, allowing their migration to ischemic brain tissue areas and their differentiation into related nerve cells to promote the expression of neural-specific proteins (Fig. 4). In addition, our data suggest that PKH67, as an ideal marker of living cells, can help track the implantation, proliferation and differentiation of transplanted HF-MSCs in brain tissue.

Immunohistochemistry analysis showed that the percentage of positive cells of NeuN, NF-H, DCX and GFAP of the infarct zone in the HF-MSCs, IGF-1 and HF-MSCs + IGF-1 groups was higher than that in the MCAO group. In addition, the percentage of positive cells in the HF-MSCs + IGF-1 group was significantly higher than that in the HF-MSCs and IGF-1 groups (Fig. 5). The expression of NeuN, DCX and NF-H in Western blot (Fig. 6) and rt-PCR (Fig. 7) analyses confirmed the above results at the protein and mRNA levels respectively. While GFAP expression was markedly different among the groups, as the highest GFAP expression was observed in the MCAO group, while the healthy control and sham operation groups exhibited the lowest GFAP expression.

Treatment enhances functional recovery

The beam-walking test and mNSS were used to evaluate the neural function of the rat MCAO model on days 1, 7, 14, 21 and 28 after surgery. After the surgery, the rats showed symptoms of neurological deficits on the first day. The treated animals showed improved neurological function in the beam-walking

test (Fig. 8B) and higher mNSSs (Fig. 8A) than rats in the MCAO group at 1 week. Rats in the HF-MSCs + IGF-1 group exhibited the greatest improvement in neural function. These data indicate that intravenous injection of HF-MSCs and IGF-1 significantly improves the neural function of rats after MCAO.

Disscussion

Acute cerebral ischemia is a kind of focal ischemic necrosis caused by disturbance of blood circulation in the brain, which can lead to different degrees of neurological impairment and sequelae. Stem cells have homing ability and can migrate to the border area of infarcted tissue and colonize, proliferate and differentiate. In the past, our team has studied the therapeutic effects of transplanted BMSCs [31] and dental pulp stem cells [32] on ischemic stroke models. However, the methods of obtaining stem cells are harmful to the individual, and HF-MSCs can improve this shortcoming, as they are easy to not only obtain but also expand, culture and cryopreserve. Moreover, no controversies are known in regards to immune rejection and mortality for HF-MSCs, and they could thus be considered an ideal source of stem cells for transplantation in neurological disorders. IGF-1 is involved in many central nervous system processes, including cell growth, proliferation, neurogenesis and migration, and has neuroprotective effects. Treatment of the acute cerebral blood model with IGF-1 is associated with decreased expression of inflammatory factors in hemispheres. IGF-1 could induce microglial changes and reduce neuroinflammation [26]. Experimental studies show that transplanted HF-MSCs can migrate to the area around the infarcted tissue. In addition, HF-MSCs and IGF-1 transfuse intravenously, which is not only nonsurgical but also safe and effective.

The MCAO model was established, and TTC and HE staining showed the ischemic regions in the brain tissues of the model rats. In addition, successful rat models were characterized by neurological dysfunction, and rats in the sham operation and healthy control groups showed no differences. HF-MSCs is a type of MSC. CD29 and CD90 are mesenchymal stem cells indicating antigens. The two antigens were positive in flow cytometry, confirming that HF-MSCs belongs to MSC, which are cells with multipotential differentiation potential. Moreover, it has been proved that HF-MSCs can differentiate into osteogenic and adipogenic cells.

DCX plays an important role in the final localization of new neurons, which are biomarkers of immature neurons [31, 33, 34]. NeuN is a common marker of mature peripheral and central nervous system neurons and is widely expressed in rats. Moreover, the NeuN protein was expressed only in nerve tissue and mainly in the nucleus [35]. NF-H is a mature neuronal marker [36]. GFAP is a hallmark intermediate filament protein mainly expressed in astrocytes[37, 38]. GFAP increases dramatically in the periinfarct area after infarction, which may be associated with swelling of the neuroglia, increased exposure to postdisintegrating sites, and reactive astrocyte. Furthermore, the GFAP of infarct area was reduced due to necrosis[39–42]. This is the reason for the highest GFAP expression in the model group. The reason that GFAP expression trends differed among the three treatment groups may be due to a reduction in GFAP expression by intravenous injection of cells and factors that reduce swelling of the neuroglia and proliferation of reactive glial cells in the periinfarct area, the ability of stem cells to differentiate increases

the expression of GFAP by increasing the neuroglia of the infarct zone. In WB and PCR our brain tissue samples included both infarct and periinfarct regions. So it was precisely because of the dissynchronization of GFAP expression in the infarcted area and periinfarct regions that there was no significant difference in GFAP expression between the HF-MSCs + IGF-1 group and the IGF-1 group. The HF-MSCs group mainly decreased the expression of GFAP in the periinfarct regions, but had little effect on increasing the expression in the infarct area, So its GFAP expression was minimal compared to the other two treatment groups. Although the amount of GFAP expression does not account for the efficacy of combination therapy or monotherapy, it does at least account for the efficacy of either therapy in comparison with the model group.

In this study, HF-MSCs labeled with green PKH67 dye were tracked *in vivo* and colocalized with neural-specific markers and DAPI. The transplanted HF-MSCs expressed the neural-specific markers NF-H, DCX, NeuN and GFAP, which showed that they could differentiate into neuron-like cells. Semi- and quantitative data analysis revealed that the positive expression levels of NeuN, NF-H and DCX in the IGF-1, HF-MSCs and HF-MSCs + IGF-1 groups were much higher than those in the MCAO group. In addition, the expression levels of NeuN, NF-H and DCX were highest in the HF-MSCs + IGF-1 group. The finding suggest that IGF-1 can improve the migration and differentiation of HF-MSCs after transplantation, thus enhancing the neuroprotective effect. Simultaneous transplantation of IGF-1 and HF-MSCs has a synergistic protective effect in improving ischemic stroke, thus representing an effective method to restore neural function.

However, the limitation of this study was the short-term follow-up of HF-MSCs that survived *in vivo* transplantation. Therefore, further studies are needed to elucidate the molecular mechanism underlying the effects of HF-MSCs in combination with IGF-1 in the treatment of acute cerebral ischemia.

In summary, this study is the first to demonstrate that intravenously transplanted HF-MSCs and IGF-1 can effectively improve the neurological function of an animal model of focal cerebral ischemia without significant side effects, despite lacking mechanisms to establish the therapeutic effect of stem cells. These results indicate that intravenous injection of HF-MSCs and IGF-1 may be a promising treatment for focal cerebral ischemia.

Abbreviations

BBB	blood-brain barrier
BCA	bicinchoninic acid
BMSCs	bone marrow mesenchymal stem cells
CCA	common carotid artery
DAB	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCX	doublecortin
ECA	external carotid artery
FACS	Fluorescence-activated cell sorting
GFAP	glial fibrillary acidic protein
HE	Hematoxylin-eosin
HF-MSCs	hair follicle mesenchymal stem cells
ICA	internal carotid artery
IGF-1	insulin growth factor-1
MCAO	middle cerebral artery occlusion model
mNSS	modified neurological severity scores
NeuN	neuronal nuclei protein
NF-H	neurofilament heavy polypeptide
NIH	National Institutes of Health
PVDF	polyvinylidene fluoride
real-time PCR	real-time polymerase chain reaction
SD	Sprague-Dawley
TTC	2,3,5-triphenyltetrazolium chloride

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Experiment Ethic Committee of Second Affiliated Hospital of Harbin Medical University (SYDW 2019-176).

Consent for publication

Not applicable.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Contributions

J. Fu designed the experiment and provided guidance; X. Zhang and W. Li implemented the experiment and were major contributors in writing the manuscript; X. Zhang and W. Li contributed equally; Y. Zhou, Y. Ouyang,D. Wang, H. Yue, Z. Jiao and Y. Wang participated in the animal experiments; X. Wang, Y. Shi, Y. Xu, Z. He and J. Fu supervised the project and edited the manuscript; J. Fu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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Figures

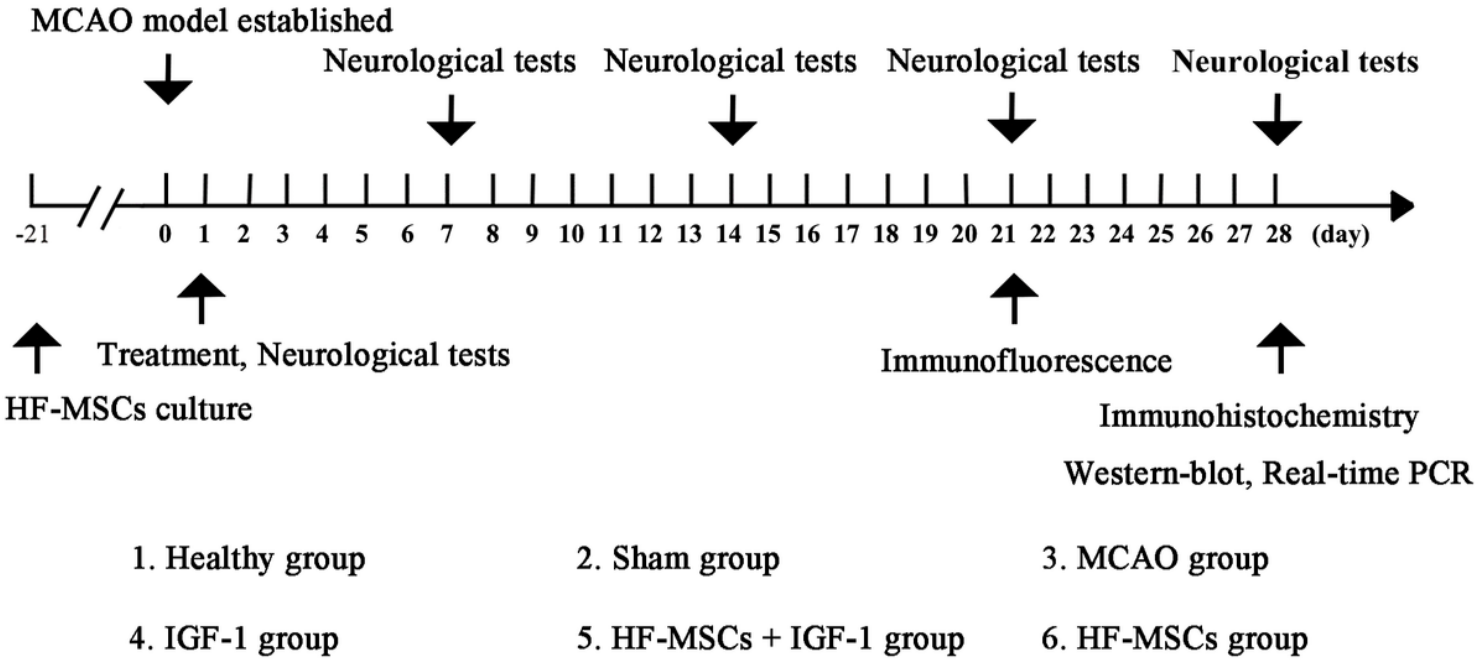

Figure 1

Experimental flow chart. HF-MSCs were isolated and cultured for about 21 days before the MCAO model was established. Rats were divided into six groups randomly. On the 1st, 7th, 14th and 21st days after the model established, the rats were given behavioral tests. The samples were assayed for immunofluorescence on the 21st day, and the immunohistochemistry, western blot and rt-PCR assays were performed on the 28th day prior to statistical analysis.

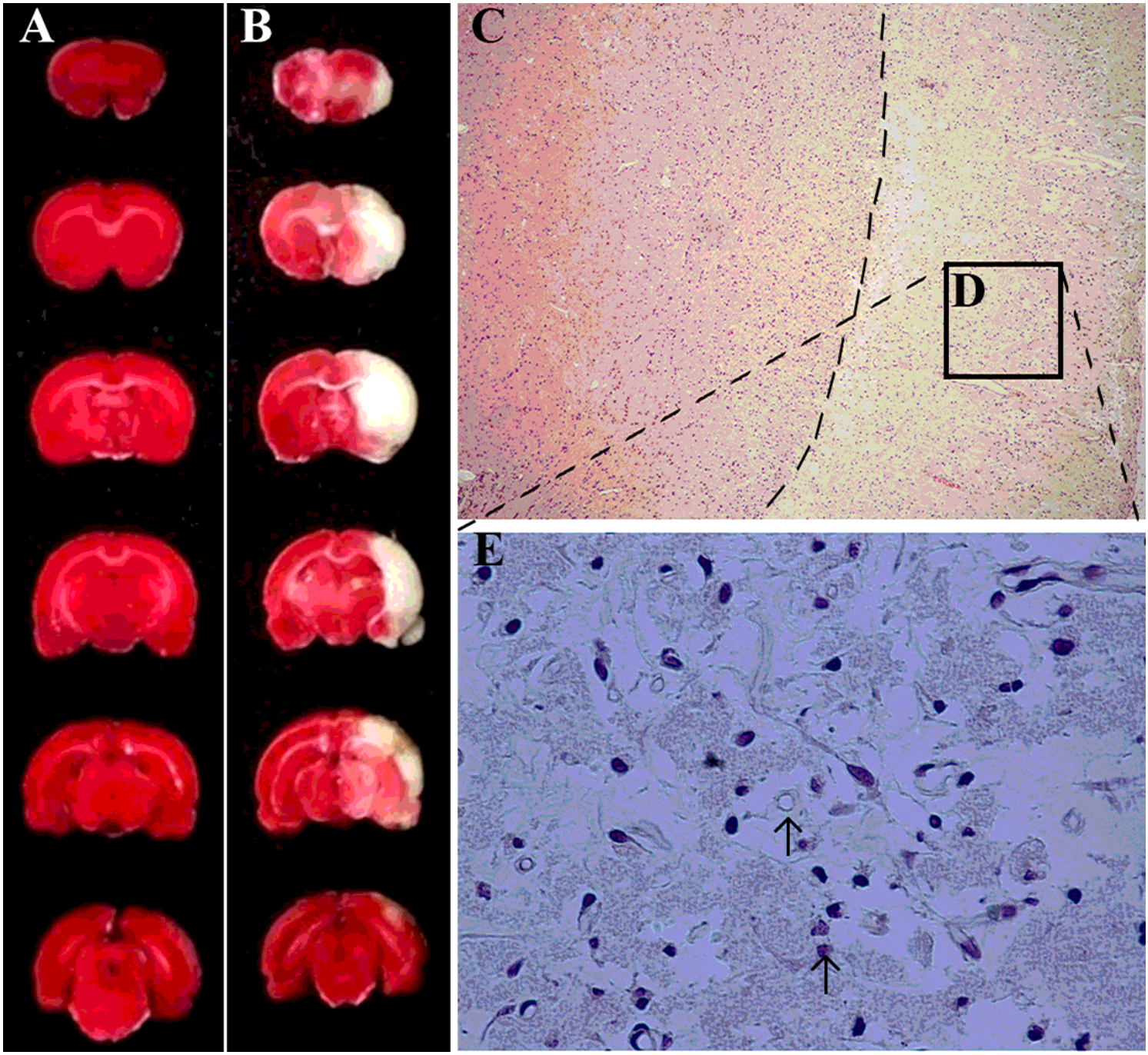

Figure 2

Model evaluation. A): Serial coronary sections of the rats in the healthy control group. B): serial coronal sections of rats in the MCAO group. TTC staining showed the healthy area to be red and the ischemic area to be white. C): HE staining section of MCAO group rats showed obvious normal and pathological areas of brain tissue under low power microscope. D): HE staining section of infarcted brain tissue in the MCAO group. E): At high magnification, the infarcted area of HE stained sections in the MCAO group showed a decrease in the number and structural disorganization of neurons.

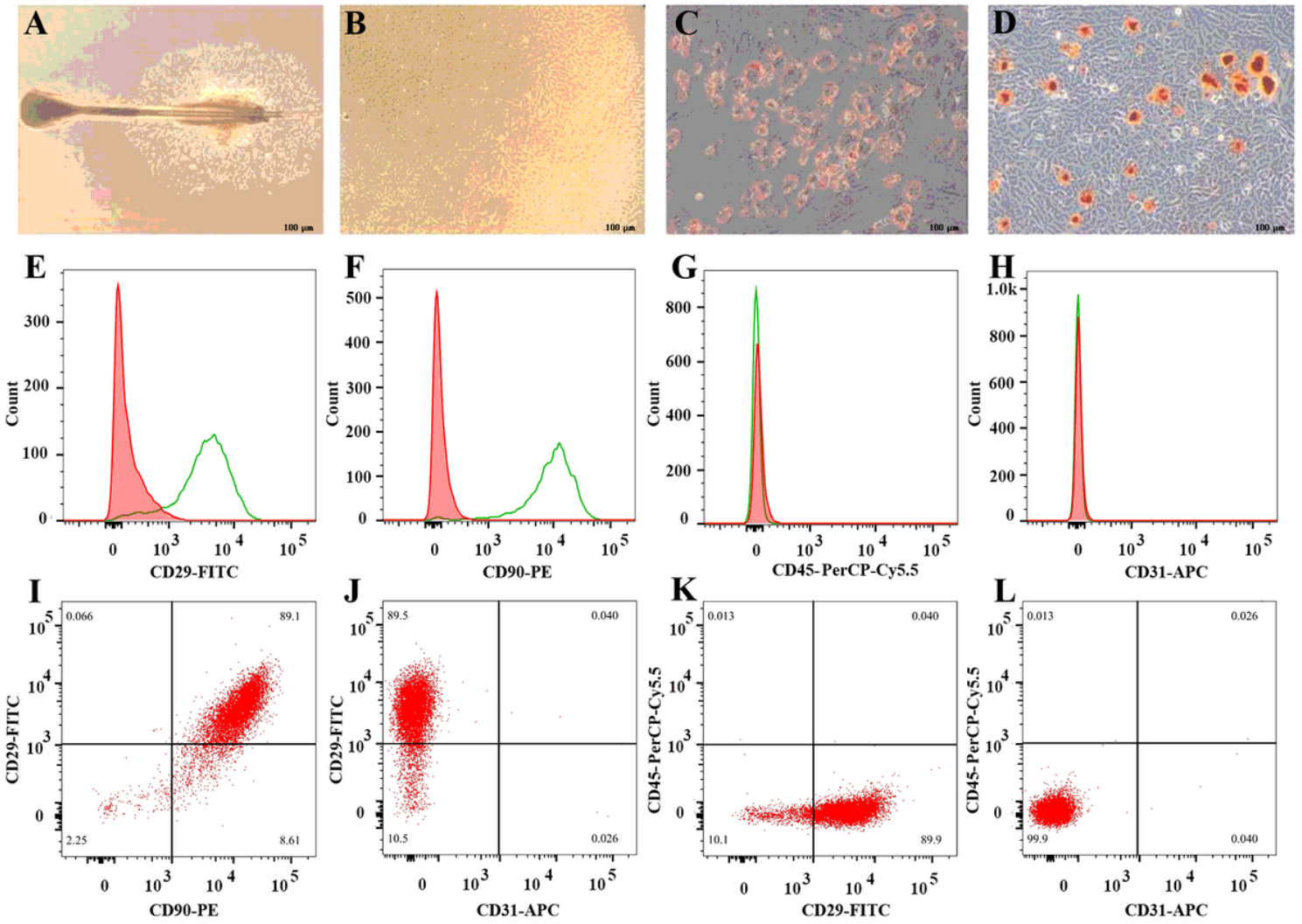

Figure 3

Characteristics of HF-MSCs. A): the primary culture of HF-MSCs showed plastic adhesion and colony formation. B): The third generation of HF-MSCs has a paving stone-like morphology. C, D): multilineage differentiation of HF-MSCs: mineralized nodules and fat droplets. E-L): Surface Markers of HF-MSCs: FITC-CD29 and PE-CD90 were overexpressed, while APC-CD31 and PerCP-CD45 were underexpressed.

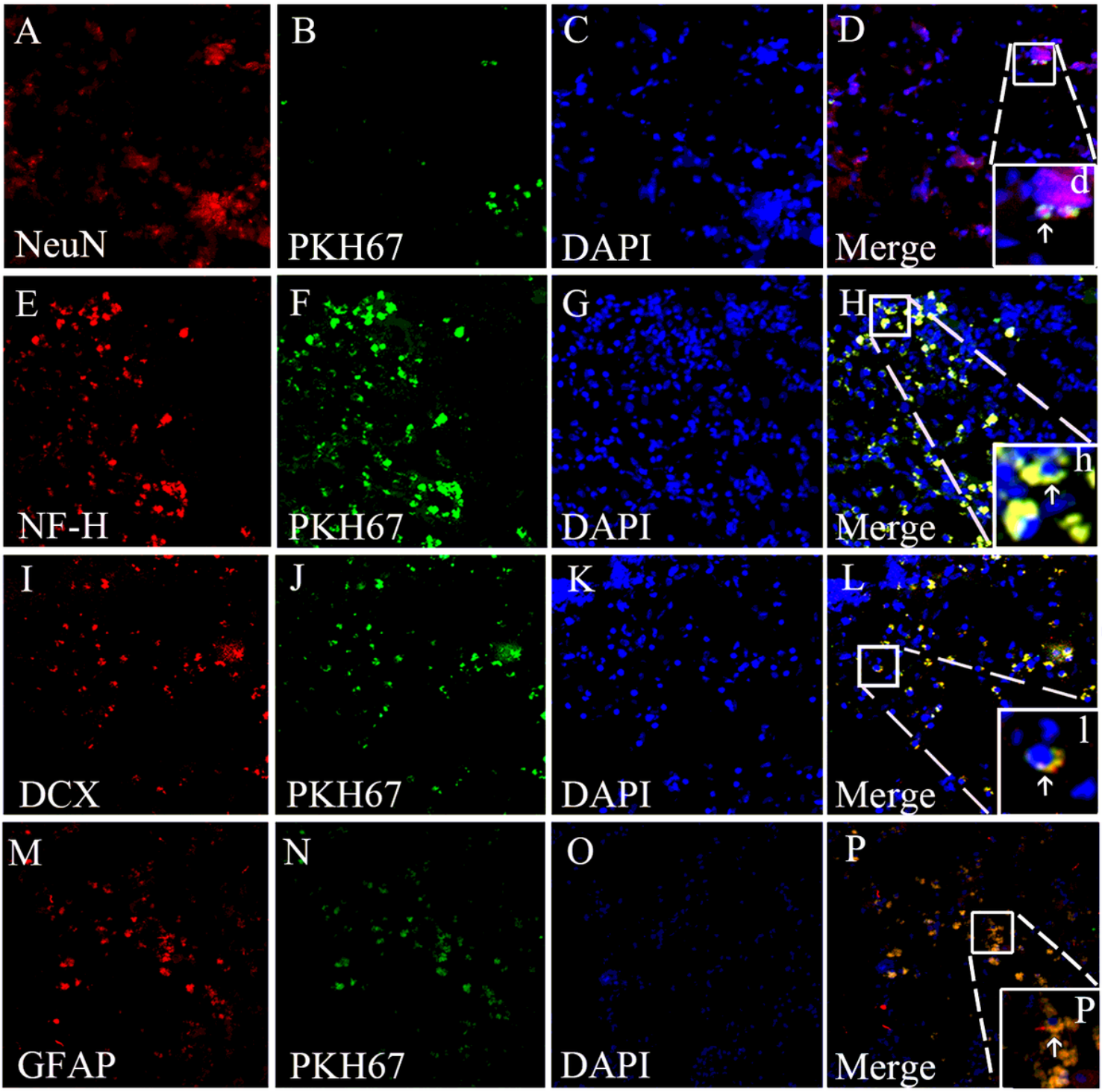

Figure 4

Immunofluorescence expression of transplanted HF-MSCs in the brain tissue. The HF-MSCs labeled with PKH67 were located in the infarcted area. The cells expressed neural-specific markers, including NeuN (A-D), NF-H (E-H), DCX (I-L) and GFAP (M-P). DAPI staining showed blue fluorescence at 405 nm. d, h, l, p) Co-localization of PKH67-labeled HF-MSCs with neural-specific markers and DAPI.

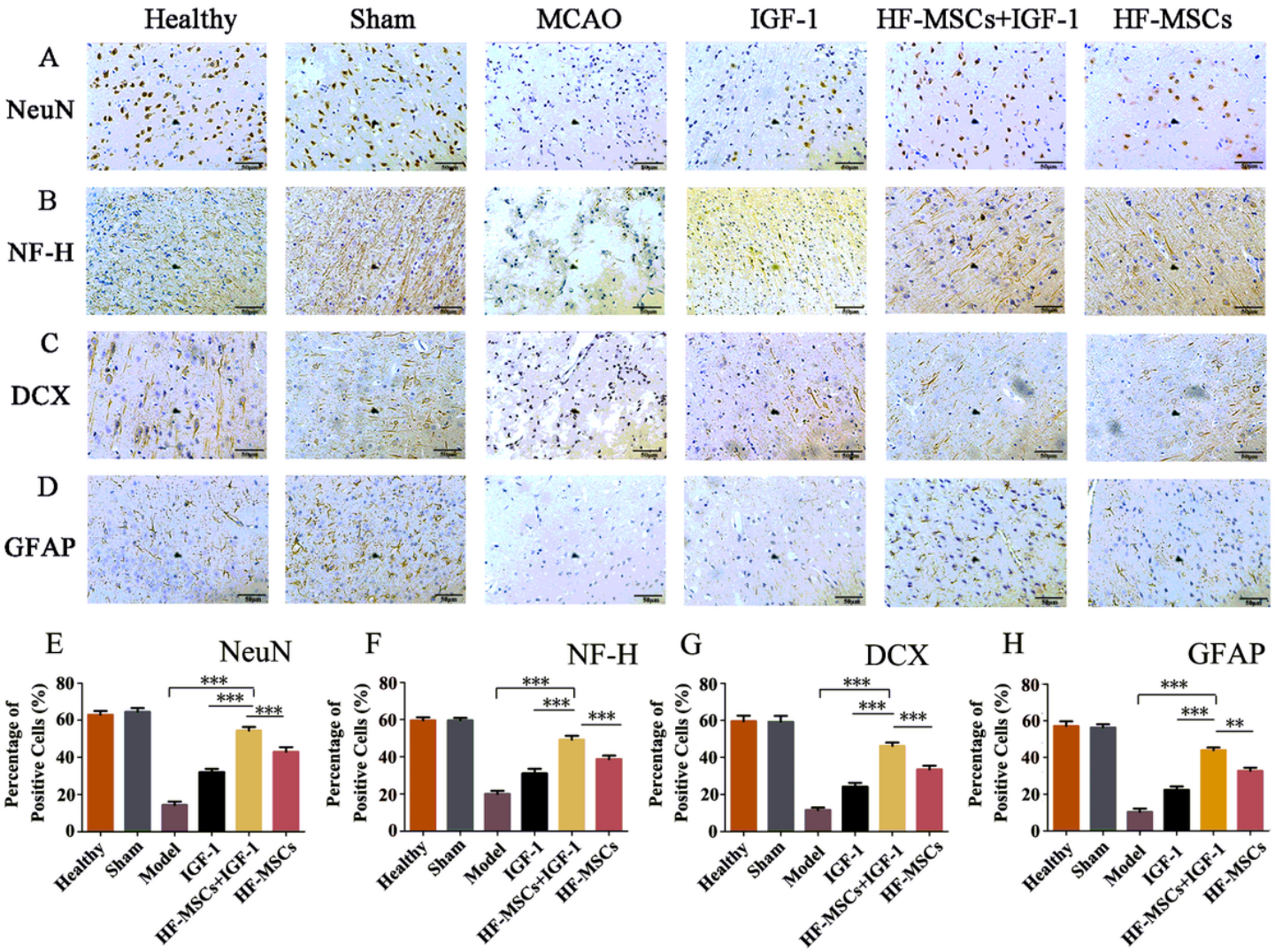

Figure 5

Immunohistochemical expression of transplanted HF-MSCs in the brain tissue. A-D) NeuN-, DCX-, GFAP-, and NF-H-immmunoreactive cells show neuronal morphology in the infarcted area. E-H) Semi-quantitative data. The percentage of positive cells in the IGF-1, HF-MSCs + IGF-1, and HF-MSCs groups was significantly higher than that in the MCAO groups. In addition, the percentage of positive cells in the HF-MSCs + IGF-1 group was significantly higher than that in the IGF-1 and HF-MSCs groups. (*P <0.05, **P 0.01, ***P 0.001).

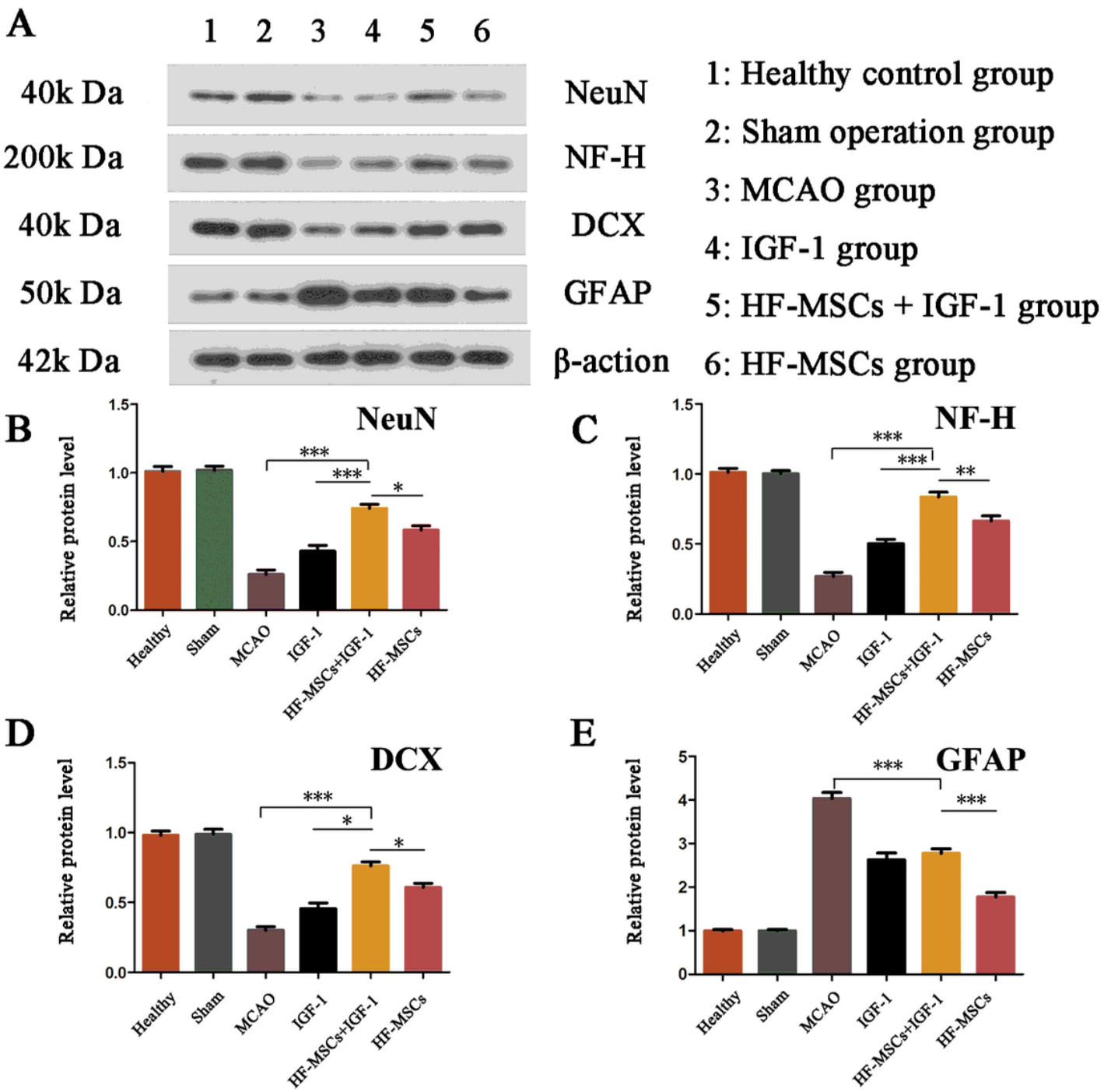

Figure 6

Protein level expression of HF-MSCs after transplantation. A) The immune response band. B-E) The protein level of NeuN, DCX, and NF-H in the HF-MSCs + IGF-1 group was significantly higher than that in the IGF-1 and HF-MSCs groups. The protein level of the HF-MSCs and IGF-1 groups was significantly higher than that in the MCAO group. The protein level of GFAP was the highest in the MCAO group (*P <0.05, **P□0.01, ***P□0.001).

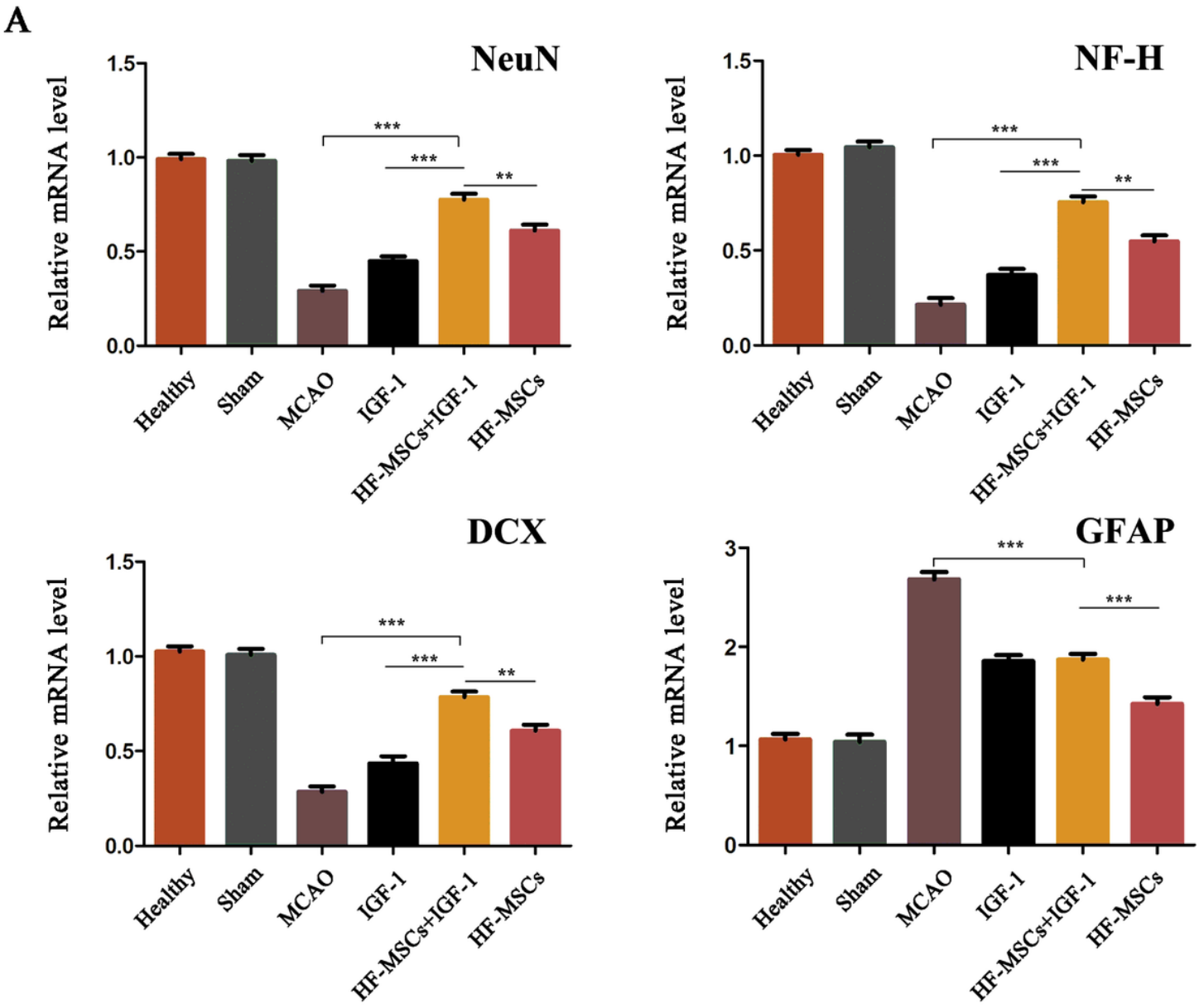

Figure 7

mRNA expression of transplanted HF-MSCs in the brain tissue. A) mRNA expression levels of neural-specific markers in each group. The mRNA expression level of NeuN, DCX, and NF-H in HF-MSCs + IGF-1 group was higher than that of in HF-MSCs and IGF-1 groups. The mRNA expression level of NeuN, DCX, and NF-H in HF-MSCs and IGF-1 groups was significantly higher than that of in MCAO group. The mRNA expression level of GFAP was the highest in the MCAO group (*P <0.05, **p□0.01, ***p□0.001).

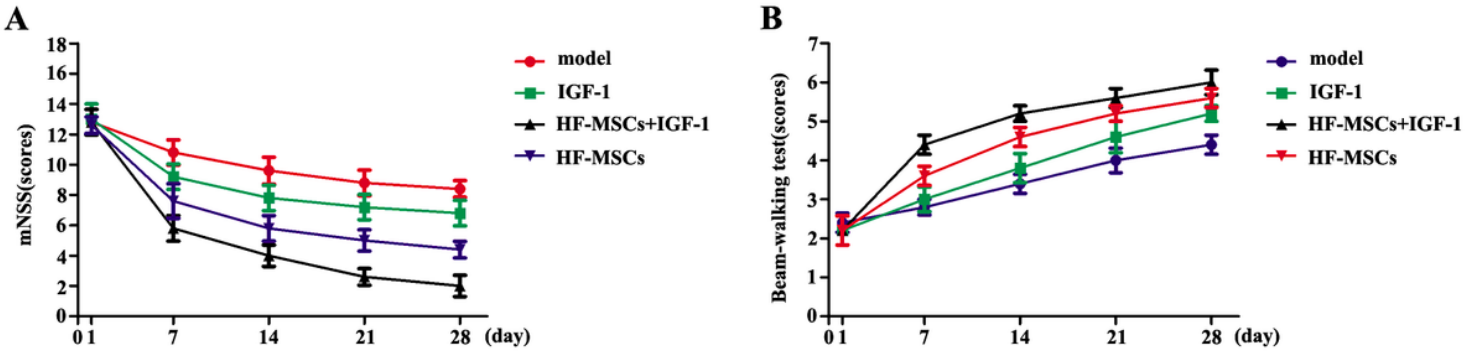

Figure 8

Modified Neurological Severity Score (mNSS) and the Beam-Walking test. Compared with Healthy group and Sham group, the other groups all have neurological deficit symptoms. The score of mNSS in MCAO group was the highest, with the score in HF-MSCs + IGF-1 group was lower than that of IGF-1 group and HF-MSCs Group. While the score of the beam-walking test was highest in HF-MSCs + IGF-1 group, with lowest in MCAO group. (*P <0.05, **P□0.01, ***P□0.001).