and Express Neural Markers in a Rat Model of Cerebral Ischaemia/Reperfusion

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Abstract

Background: Ischaemic stroke has become the main cause of death and severe neurological disorders, for which effective restorative treatments are currently limited. While stem cell transplantation offers therapeutic potential through neural regeneration, this approach is associated with challenges of limited applicable sources. Hair follicle stem cells (HFSCs) are multipotential cells which can differentiate into ectodermal and mesodermal lineages and proliferate for long periods. The therapeutic potentials of HFSCs have not been investigated in ischaemic stroke models and, therefore, in this study, we aimed to determine whether they can survive and migrate to ischaemic areas after stroke attack.

Methods: A rat model of middle cerebral artery ischaemia/reperfusion was established and intravenously administrated HFSCs. The potential of HFSCs to migrate and differentiate into neuron-like cells, as well as their ability to reduce the infarct size, was evaluated. Rat brain tissue samples were collected 2 weeks after cell transplantation and analysed via TTC staining, immunofluorescence, and immunohistochemistry methods. The data were statistically analysed and are presented as the means ± standard deviations (SDs).

Results: Intravenously administrated rat HFSCs migrated to the penumbra where they expressed neuron-specific markers, reduced the infarct volume, and promoted neurological recovery.

Conclusion*:* HFSC transplantation has therapeutic potential for ischaemic stroke, which implies that HFSC treatment might be useful for stroke patients

in the future and is, therefore, worthy of further investigation toward possible clinical development.

Keywords: Hair follicle stem cells**,** ischaemia/reperfusion**,** cell transplantation, homing, differentiation

Background

Ischaemic stroke has become the main cause of disability and death worldwide [1]. The number of patients who can undergo recanalisation therapy is restricted because of the strict eligibility criteria and narrow time window [2, 3]. The central nervous system (CNS) can hardly regenerate under pathological conditions, which leads to irreversible neurological disabilities. However, regenerative medicine brings new hope for functional organ reconstruction.

For CNS diseases, stem cell transplantation seems to be the most practical regenerative strategy; however, it is associated with inevitable challenges. For example, neural or embryonic stem cells are the most suitable stem cell sources for CNS diseases; however, they are very sparse and extremely difficult to acquire. In contrast, bone marrow mesenchymal or hemopoietic stem cells, which have been used in many diseases, originate from a different embryo layer than the neural system, and thus, they have limited potential to differentiate into neurons [4, 5]. Moreover, the identified stem cell sources cannot meet the substantial clinical requirements in a short time.

Hair follicle stem cells (HFSCs) are a highly promising source of multipotent cells as they are abundant, accessible, and active in adult

mammals. Throughout the whole lifetime, mammals' hair undergoes a unique regenerative phenomenon consisting of cyclic periods, growth, regression, and rest. The stem cells contained in hair follicle bulge region have been verified based on several criteria [6]. HFSCs continuously produce new cells to restore follicles and cutaneous appendages during the anagen phase [7] and can differentiate into most mesodermal and ectodermal derivatives [8]. Despite of skin diseases, these characteristics make HFSCs a promising cell source for the treatment of nervous system disorders [9].

Stem cells isolated from the rat vibrissa were demonstrated to differentiate into ectodermal lineages such as neural cells, astrocytes, oligodendrocytes, and Schwann cells when cultured *in vitro* [10], as well as mesodermal derivatives including myocytes [11], chondrocytes [12] and osteocytes [13]. Interestingly, Robert M. Hoffman proved that after 7 days of culture in RPMI 1640 medium containing 10% FBS, 48 ± 8% and 30 ± 8% of the population of HFSCs differentiated into neurons and glial cells, respectively [14].Moreover, HFSCs have been transplanted into skin injury [15, 16], spinal cord injury [17], peripheral nerve injury [6], and Alzheimer's disease [18] animal models with results suggesting their potential to localise to the injury site and promote angiogenesis or neurological functional recovery.

As a promising source of stem cells for ischaemic stroke treatment, HFSCs have three important advantages making them prominent among all stem cells. First, they are abundant and accessible. Second, they are pluripotent, holding the capability to differentiate into both ectodermal and mesodermal lineages. Third, they are active and proliferate for long periods, making it possible for most patients to accept auto-transplantation without ethical or

logistic problems. However, HFSCs have not been evaluated in ischaemic stroke models to date, and whether they can survive and migrate to ischaemic areas after stroke attack is still unclear. Therefore, in present study, we aimed to investigate this phenomenon using a middle cerebral artery (MCA) ischaemia/reperfusion (I/R) rat model based on immunohistochemical, immunofluorescence, and TTC staining assays.

Materials and Methods

Experiment design

A total of 48 rats were divided into four groups at random: (i) control, (ii) sham, (iii) I/R + saline, and (iv) I/R + HFSCs. Rats in the control group were heathy and not subjected to an operation, whereas the I/R model was established via surgical MCA occlusion (MCAO). In the sham group, rats received a similar operation but without MCA occlusion. The I/R + HFSCs group underwent HFSC transplantation (1×10^6 cells dispersed in 1 mL saline) via tail vein injection 24 h after reperfusion, whereas animals in the I/R + saline group and sham group were similarly administered 1 mL saline.

Animals were maintained for 2 weeks after transplantation, and then, relevant tissue samples from half of the animals were analysed using TTC (Amresco, OH, USA) and the other half were used for histological staining (n = 6 each, Fig. 1). The 2-week survival point was chosen because it might be the shortest time required for the transplanted cells to migrate to the penumbra and exert putative protective effects on stroke recovery.

Neurological scores (Table 1) were recorded daily after cell transplantation [19]. The score of each rat was estimated three times for consistency. A full score represents a normal neurological status, whereas lower scores are indicative of behavioural deficits.

Animals

Male Sprague-Dawley (S-D) rats weighing 280 ± 10 g were purchased from the animal centre of the Second Affiliated Hospital of Harbin Medical University. The animals were housed at 22 ± 2 °C with a humidity of 40 ± 5%, under a 12-h light/dark cycle, and fed a standard diet and water ad libitum. The rats were forbidden to eat 12 h before the experiments but were allowed free access to drinking water. All study design and experimental procedures were conducted in accordance with institutional guides for animal experiments approved by the Experimental Center of the Second Affiliated Hospital of Harbin Medical University.

Isolation, culture, labelling, and transplantation of rat-derived HFSCs

We harvested hair follicles via enzyme digestion following mechanical dissection [20]. The upper lip containing the vibrissa pad of 4-week-old male S-D rats was cut and digested with 0.1% collagenase in DMEM (both from Gibco, BRL, Gaithersburg, MD, USA). Then, the vibrissa follicles were gently plucked from the pad under a binocular microscope, placed in 24-well tissue-culture dishes (Corning, NY, USA) pre-treated with IV collagen, and cultured

in DMEM containing 10% FBS (ScienCell, Santiago, CA) and 1% penicillin and streptomycin (Gibco, BRL, Gaithersburg, MD, USA) at 37°C in an atmosphere of 95% air–5% CO_2. All surgical procedures were conducted in accordance with aseptic principles, and the entire culture medium was first changed 12 h later. The non-adherent cells were discarded with the waste culture medium, and the culture medium was replaced every 48 h. When the culture grew to about 80% confluency (approximately 10 days), the new adherent cells were passaged using the same culture method. The prepared HFSCs were used at passage 3 in the experiments. In order to monitor the grafted cells in the brain, the HFSCs were pre-labelled by non-invasive membrane-labelling green fluorescent dye PKH 67. Two weeks after HFSC transplantation, the TTC and histological staining were performed.

Induction of focal cerebral I/R

The focal cerebral I/R model was induced by right MCA occlusion (MCAO) for 1 h following reperfusion, as reported previously [21, 22]. Briefly, after intraperitoneal anesthetisation with 10% chloral hydrate (0.3 mL/100 g), a midline ventral incision was made on the neck to expose the vessels. The right external carotid artery (ECA) was isolated and the branches were cauterised. The right MCA was occluded by gently inserting a monofilament nylon suture with a rounding tip through the right ECA. After a 60-min occlusion, the suture was slowly pulled back to achieve reperfusion. The rats in the sham group received the similar procedure except that the right MCA was not occluded. The body temperature was maintained at 37 ± 5 °C using a

thermostat-controlled heating pad from the start of the operation until the animals recovered from anaesthesia.

TTC staining

To measure the infarct volume, TTC staining was carried out as described previously [23]. Briefly, brain tissue was cut into five coronal slices (2 mm thick) and incubated in 1% TTC dissolved in PBS for 15 min at 37 °C. The non-infarcted tissue was stained red, whereas the infarcted tissue area remained white. The infarct volume was calculated as follows: [(left hemisphere area − right non-infarcted area) / (left hemisphere area × 2)] to avoid the influence of oedema [24].

Nissl staining

Following anaesthesia, rats were trans-cardially perfused with 0.9% saline until no blood flowed out, followed by 4% paraformaldehyde in PBS (pH 7.4). Brain tissue was removed and kept in 4% paraformaldehyde for 48 h, cryoprotected in 30% sucrose in PBS for another 48 h at 4 °C, and then embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). The brain specimens were cut into coronal slices at 10 μm thickness between the optic chiasma and the cerebral caudal end using a frozen section machine (Thermo Scientific Microm HM560, Waltham, MA, USA). The sections were then air dried and processed for pathological experiments, including Nissl staining. Nissl bodies were stained using Cresyl violet acetate (Sigma-Aldrich,

St Louis, MO, USA). Briefly, the brain tissue slices were immersed in the cresyl violet acetate solution for 2 h at 37 °C, successively dehydrated and hyalinised, and observed under an optical microscope [21].

Immunofluorescence

Frozen sections were blocked with 5% goat serum (Absin, Shanghai, China) in PBS for 30 min and incubated with primary rabbit anti-doublecortin (DCX, Abcam, Cambridge, UK), mouse anti-neuron-specific nuclear protein (NeuN, Millipore Corp, Billerica, MA, USA), and rabbit anti-glial fibrillary acidic protein (GFAP, Abcam, Cambridge, UK) antibodies overnight at 4 °C. After rinsing with PBS, the sections were incubated with rhodamine-conjugated anti-mouse/rabbit IgG/IgM (1:500, Cell Signaling Technology, VT, USA) for 90 min at 25 °C. The nuclei were stained with DAPI. PKH 67 (green), neuron-specific markers (red), and DAPI (blue) were observed using laser scanning confocal microscopy (Zeiss LSM800; Carl Zeiss, Jena, Germany) at wavelengths of 594 nm (red), 488 nm (green), and 405 nm (blue), respectively.

Immunohistochemistry

The brain sections were incubated with 0.3% H_2O_2 to eliminate endogenous peroxidase, blocked with 10% goat serum, and treated with 0.1% triton X-100 in PBS for 30 min at 25 °C. Then, the sections were incubated with rabbit anti-DCX, mouse anti-NeuN, and rabbit GFAP antibodies overnight at 4 °C. After

washing with PBS, the sections were incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (1:500, Cell Signaling Technology, VT, USA) for 60 min at 25 °C, and stained with DAB (Cell Signaling Technology, VT, USA). The sections were rinsed with PBS, counterstained with haematoxylin, then dehydrated and observed using an optical microscope.

Statistical analysis

The statistical analysis was performed by GraphPad Prism 6.0 (GraphPad Prism Software, San Diego, CA, USA) using one way-ANOVA followed by a Tukey test for multiple comparisons. Values for $P < 0.05$ were considered statistically significant. All data are expressed as means ± standard deviations (SDs).

Results

Characteristics of HFSCs

The self-renewal HFSCs exhibited colony formation, plastic adherence and a paving stone-like morphology (Fig. 2a-c). The capacity of multipotent differentiation was verified by osteogenesis and adipogenesis experiments (Fig. 2d-e). The image of PKH-67 pre-labelled HFSCs under a fluorescence microscope is presented in Figure 2f. The expression of mesenchymal stem cell surface markers was detected by FACS (BD FACS Canto II, NJ, USA)

analyses (Fig. 3). In conclusion, the cells derived from S-D rat hair follicle vibrissa appeared to be largely HFSCs.

Focal cerebral I/R rat model

TTC staining and Nissl staining were used to confirm and analyse the successful establishment of the focal cerebral I/R model along with neurological system scores. A white zone within the brain tissue slices represents the infarcted area caused by I/R injury, whereas the red zone represents normal brain tissue (Fig. 4A and B). Nissl staining (Fig. 4C and D) showed significant damage to neurons in the infarcted area. Neurons in the healthy area exhibited normal morphological features, whereas diverse neuronal damage was observed in the infarcted area such as cell loss, nuclear karyorrhexis, and pyknosis.

HFSCs migrate to ischaemic penumbra

The spatial distribution of HFSCs within the brain was monitored and analysed after transplantation into the cerebral I/R model. The green fluorescent dye pre-labelled HFSCs were easily identified using a fluorescent microscope (Fig. 5). Figure 5a and c show the regions of the non-infarcted hemisphere, whereas images of the I/R-insulted tissue are presented in Figure 5b and d. PKH 67-labelled HFSCs were visibly localised to the penumbra area, but rarely found in the normal hemisphere.

Grafted HFSCs express neuron-specific markers

The co-localisation of PKH 67 pre-labelled HFSCs and neuron-specific markers was performed by laser scanning confocal microscopy. Cell tracker PKH 67 emitted green fluorescent signals both *in vitro* and *in vivo*. Immunolabelling of neural-specific markers, which conjugated with red fluorescent indicator, was used to detect the differentiation activity of HFSCs in the I/R model. As shown in Figure 6, PKH 67 labelled cells expressed the neuron-specific markers DCX, NeuN, and GFAP, which appeared yellow in the cytoplasm, whereas DAPI stained the nuclei blue.

Neuron-specific marker expression in penumbra

Immunohistochemistry staining was conducted to analyse the number of neurons in the penumbra regions. Figure 7 shows the number of neuron-specific marker-positive cells around the ischaemic core. The number of positive cells decreased in the I/R + saline group compared with the sham group, whereas these decreases were significantly inhibited by HFSC treatment ($P < 0.05$).

HFSCs reduce infarct volume and improve neurological scores

To investigate whether intravenously transplanted HFSCs could alleviate damage in the cerebral I/R model, TTC staining was conducted to measure the infarct volume 2 weeks after cell transplantation (Fig. 8). The TTC staining photographs of the I/R groups are exhibited in Figure 8a, where the red and white colours indicate non-infarcted and infarcted tissue, respectively. Compared to that in the I/R + saline group, the I/R + HFSCs group showed a significant reduction in I/R-induced cerebral infarction.

Quantitation of the cerebral infarct volume, illustrated in Figure 8b, shows that the proportion was 44.20 ± 2.16% in the I/R + saline group, whereas in the I/R + HFSCs group, the white zone was significantly smaller at 29.80 ± 1.86% ($P < 0.05$). The neurological score presented in Figure 8c indicates that the rats in the I/R + saline group underwent slow recovery from neurological deficits induced by I/R injury, whereas HFSC transplantation obviously improved neurological functional recovery ($P < 0.05$).

Discussion

In this study, we demonstrated that trans-venously grafted rat HFSCs could survive in a cerebral I/R rat and migrate to the ischaemic boundary zone. Moreover, most PKH67-labelled HFSCs expressed the neuron-specific cell markers DCX, NeuN, and GFAP in the penumbra regions. Furthermore, HFSCs exerted a protective effect on I/R-induced brain injury by reducing the infarct volume and enhancing neurological functional recovery. These phenomena suggest that HFSCs could be a novel alternative source for ischaemic stroke regenerative treatment with remarkably appealing potential.

Stem cell transplantation, as a promising therapeutic approach in stroke diseases, has been investigated for many years in numerous studies [25]. However, obtaining an optimal cell source has been challenging. HFSCs are considered an outstanding candidate among different stem cells because of their appealing characteristics such as abundance, easy accessibility, low

invasiveness, multipotency, and auto-transplantability with no ethical or logistical problems.

Since HFSCs have not been investigated in a cerebral I/R rat model, we prudently considered the administration route and transplantation time window. First, intravenous infusion, compared with other administration routes such as intraarterial or intraventricular routes, is more suitable for the administration of stem cells, with the least invasive injury or risk of thrombosis. In the present study intravenous administration proved to be a safe and effective approach and could be a practical method for the clinical application of these findings. Second, an optimal time point for transplanted stem cell survival after a stroke exists before the maximal activation of the microglia [26]. In a stroke rat model, the corresponding time window would be before days 2–3 after the insult, which is when the maximum accumulation of macrophages is observed [27]. For this reason, we chose to conduct HFSC transplantation 24 h after reperfusion. Third, the HFSCs differentiate into GFAP-positive astrocytes 7 days after culture in RPMI 1640 medium and differentiate into neurons after 2 weeks of culture. Moreover, 2 weeks after transplantation into nude mice, the HFSCs differentiated into neural class III β-tubulin-positive and CD31-negative neurons [14]. Thus, in the present study, the brain tissue was collected 2 weeks after cell transplantation. DCX, a valuable marker of immature neuron, has been used to detect neurogenesis [28]. NeuN is usually used to mark mature neurons [29], whereas GFAP is a specific marker of glial cells [30]. Our study demonstrates that 2 weeks after transplantation into the cerebral I/R rat model, HFSCs migrated to the

315 ischaemic penumbra without tumorigenesis and expressed neuron-specific
316 cell markers (Fig. 6), as expected.

317 We first verified the homing capability of HFSCs in a cerebral I/R rat model
318 after intra-venous administration. Studies have reported that stem cell
319 mobilisation mechanisms might involve chemokines, growth factors, and
320 adhesion molecules released from the injured area and hypoxic environment
321 [31]. Stromal cell-derived factor 1 (SDF-1) and its receptor C-X-C motif
322 chemokine receptor 4 (CXCR4) play vital roles as the drivers of stem cells
323 homing [32, 33]. A recent study suggests that platelets participate in
324 metastasis and homing of stem cells by spatial approach and direct contact,
325 which indicates a new main source of homing-related factors [34, 35]. In the
326 present study, however, we did not attempt to elucidate the molecular
327 mechanisms, but rather focused on the cell homing phenomenon by
328 performing pathological experiments. The cytokines and factors involved in
329 HFSC homing after transplantation into I/R rats thus require further study.

330 Transplanted mesenchymal stem cells possess the potential to differentiate
331 into neurons [36]. The results in Figure 6 shows that the grafted HFSCs in the
332 penumbra expressed neuron-specific markers, thereby indicating that they
333 might differentiate into neuron-like cells (Fig. 6). Stem cell differentiation might
334 partially account for the increase in neuron-specific marker-positive cells in
335 the HFSC group (Fig. 7). Additionally, many studies have reported that stem
336 cells contribute to neuroprotection via pro-angiogenesis [37, 38], pro-

neurogenesis [39], anti-inflammation, and anti-apoptosis [40] mechanisms in I/R injury, which could be concluded to represent paracrine effects. In the present study, the increase in neurons induced by HFSCs is most likely mediated via their paracrine ability, but further studies are needed to confirm this notion. What can be verified from the results is that HFSC transplantation reduced neuron loss and the infarct volume and improved neurological recovery (Fig. 8).

Conclusions

In conclusion, our results illustrate a homing phenomenon in which trans-venously grafted HFSCs localised around the ischaemic regions, without requiring a brain-blood barrier permeabiliser, and expressed neuron-specific cell markers. Moreover, HFSCs exhibited therapeutic effects by reducing the infarct volume and promoting neurological functional recovery, which require more fundamental studies to illuminate the underlying mechanisms before application in a clinical setting.

Abbreviations

HFSCs, hair follicle stem cells; I/R, ischaemia/reperfusion; MCAO, middle cerebral artery occlusion

Declarations

Ethics approval and consent to participate

All experimental procedures were conducted in accordance with the relevant ethical guidelines and regulations approved by the Experimental Center of the Second Affiliated Hospital of Harbin Medical University.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

All authors declare that they have no conflicts of interest regarding this study.

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Authors' contributions

XZ contributed to the conceptualisation and methodology. HT contributed to performing the experiments, statistical analyses, and writing of the manuscript. SM contributed to the background investigation. BL and YZ took photos and videos. HY was responsible for software analysis. DW took care of animals. YW validated the data. JF contributed to the project administration and staff management. All authors read and approved the final manuscript.

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549 **Figure legends**

550 **Fig. 1** Experiment design and procedure. The rats in sham group and I/R +
551 saline group were treated with 1 mL saline, whereas rats in I/R + HFSCs
552 group were administrated with HFSCs (1×10^6 in 1 mL saline) transplantation,
553 24 h after reperfusion. Behavioral tests were conducted daily after reperfusion
554 until 14 d post-transplantation. TTC staining, immunofluorescence and
555 immunohistochemistry assays were conducted on day 15.

556 **Fig. 2** Identification of hair follicle stem cells. (**a).** P0: Primary HFSCs started
557 to form colonies 7-10 d and attached to the plate surface. (**b, c).** P1 and P3:
558 HFSCs displayed triangular and paving stone-like morphology. (**d, e).**
559 Multilineage differentiation of HFSC: mineralised nodules and fat droplets. (**f).**
560 PKH67 labelled HFSCs emitted green fluorescence under fluorescence
561 microscope. The nuclei were stained blue with DAPI. Scale bar = 50 μm.

562 **Fig. 3** Fluorescence-activated cell sorting (FACS) of HFSCs. CD29 and CD90
563 are surface markers of mesenchymal stem cells. CD31and CD45 are the
564 surface antigens of endothelial cells and leucocytes, respectively. The
565 cultured HFSCs mainly expressed CD29 and CD90, rarely expressed
566 CD31and CD45.

567 **Fig. 4** MCAO modeling assessment. (**a).** Consecutive coronal slices of sham
568 operated rats. (**b).** Serial coronal slices of MCAO rats. Typical photographs of
569 rat brain stained with 2,3,5-Triphenyltetrazolium chloride (TTC), wherein no

infarction tissue was stained red, whereas the infarction area remained white. (**c, d).** Nissl staining of MCAO models revealed lesions in the brain tissues, with diminished numbers of neurons and chaotic neuronal configuration. (**e, f).** Enlargement of healthy area and infarcted area. The black arrow indicates nissl-positive neurons. The double arrows indicate nuclear pyknosis with karyorrhexis. Scale bar = 100 μm.

Fig. 5 PKH67-labeled hair follicle stem cells (HFSCs) present in the penumbra. (**a, c).** Different regions of contralateral hemisphere of I/R. (**b, d).** Typical regions of the hemisphere with I/R injury. Majority of PKH67-labeled HFSCs gathered around the ischemic area, but rarely migrated to the contralateral hemisphere. Scale bar = 1 mm.

Fig. 6 HFSCs in the penumbra express neuron-specific markers including doublecortin (DCX), neuron-specific nuclear protein (NeuN), and glial fibrillary acidic protein (GFAP). (**a, f, k).** PKH-67 labelled HFSCs emitted green fluorescence in the penumbra. (**b, g, l).** The neuron-specific markers with Alexa Fluor® 594-conjugated secondary antibodies emitted red fluorescence. (**c**, **h**, **m).** DAPI staining emitted blue fluorescence at the wavelength of 405 nm. (**d**, **i**, **n)** PKH67-labeled HFSCs were overlayed with the panel of neuron-specific markers and DAPI. Scale bar = 50 μm. (**e**, **j**, **o).** The white arrows indicate the co-localised cells. Scale bar = 25 μm.

Fig. 7 The expression of neuron-specific markers DCX, NeuN and GFAP in the penumbra. (**a - l).** The representative photographs of neuron-specific markers expression in all groups. Scale bar = 25 μm. (**m - o).** The quantitation of the neuron specific markers positive cells. The number of positive cells was upregulated in HFSCs group compared with I/R + saline group by immunohistochemistry assay. Values are the mean ± SD. * $P < 0.05$ vs. sham group, $^{\#}$ $P < 0.05$ vs. I/R group, (n = 6).

Fig. 8 The effect of HFSCs on brain infarction and neurological scores. (**a).** Representative photographs of rat brain stained with TTC in different groups. (**b).** Quantitative analysis of infarct size in all groups, showing that infarct volume in saline group is about 44.20 ± 2.16%, whereas the HFSCs transplantation group is to 29.80 ± 1.86%. (**c).** Neurological scores of animals in different groups. Values are the mean ± SD. *$P < 0.05$ vs. sham group, $^{\#}$ $P < 0.05$ vs. I/R group, (n = 6).