Stem Cells from Human Exfoliated Deciduous Teeth Alleviate High-Altitude Cerebral Oedema by Shifting Microglial M1/M2 Polarisation

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Objective: *To explore the high-efficiency and low-risk prevention and treatment strategies for stem cells from human exfoliated deciduous teeth (SHED) for high-altitude cerebral oedema.*
Methods: *A low-pressure and low-oxygen tank mimicking high-altitude conditions was used to establish the high-altitude cerebral oedema animal model. The preventive effects of SHED for cerebral oedema were then evaluated by haematoxylin and eosin (H&E) and histological staining. In vitro, SHED was co-cultured with BV-2 to analyse the effects of SHED by western blot and immunofluorescence staining.*
Results: *SHED can prevent and treat cerebral oedema in a high altitude rat animal model. Mechanistically, SHED treatment can protect brain cells from apoptosis induced by high altitude condition. Moreover, SHED treatment can inhibit M1-type polarisation and promote M2-type polarisation of microglia cells via the suppression of hypoxia inducible factor (HIF)-1α-mediated extracellular signal-regulated kinase (ERK) signalling activated in high altitude condition.*
Conclusion: *SHED treatment can relieve high-altitude cerebral oedema via inhibiting HIF-1α-mediated ERK signalling, which indicates that SHED is a promising alternative strategy to prevent and treat high-altitude cerebral oedema.*
Key words: *cerebral oedema, microglia, polarisation, SHED, stem cells*
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In the case of high altitude, low air pressure, thin air and low oxygen content, a series of physiological and pathological changes occur, known as acute mountain sickness (AMS). Cerebral oedema usually occurs in high-altitude areas above 4,000 metres and is the final stage of acute high-altitude reaction[1]. Within 24 hours, the patient may fall into a coma or die due to a cerebral haemorrhage[2]. The underlying mechanisms that cause high-altitude cerebral oedema remain unclear. Cerebral hypoxia is one of its main initiating factors[3]. This condition can cause brain swelling and thus increase brain size in high-altitude areas; cerebral blood flow also increases, which leads to increased intracranial pressure and more severe swelling of brain tissue[4,5]. Several prevention and treatment options are available for AMS and cerebral oedema, including acclimatisation, slow ascent[6], oxygen uptake, nutritional support and drug therapy[7]. Drugs, such as carbonic anhydrase inhibitors, medroxyprogesterone and glucocorticoids, are used to treat cerebral oedema by improving oxygenation and reducing the inflammatory response[6]; however, carbonic anhydrase inhibitors, medroxyprogesterone and glucocorticoids show variable side effects. As such, it is urgent to explore the high-efficiency and low-risk treatment strategies for cerebral oedema.

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Stem cells from human exfoliated deciduous teeth (SHED) are derived from the pulp of naturally replaced primary teeth; they are readily available, non-invasive and less subject to ethical controversy[8]. SHED have shown profound proliferation and multi-lineage differentiation capacity. They also exhibit therapeutic effects on multiple diseases, such as Alzheimer's disease, ischemic brain injury, Parkinson's disease, osteoarthritis and amyotrophic lateral sclerosis[9-12]. SHED have been found to demonstrate profound effects on neuronal diseases[13-15], which may be due to their embryonic neural crest origin and neurotrophic properties[16]. SHED may be a promising strategy for preventing and treating cerebral oedema that needs to be illustrated.

A variety of immune cells in brain tissue participate in the regulation and repair of brain tissue homeostasis, among which microglia, the macrophages in brain tissue, play an important role[17,18]. Macrophages can be activated by different stimulus factors into two different forms based on polarisation: M1 and M2 types[19]. M1-type macrophages can induce the production of a variety of inflammatory factors, such as inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-18 and CD86, which have proinflammatory effects[20]. M2-type macrophages can exert regulatory or inhibitory effects against proinflammatory and cellular immune responses. The polarisation of macrophages is reversible and plays an important role in disease development, reversal and treatment. Injury-related factors, such as ischemia and hypoxia, can induce M1-type polarisation of microglia and enhance the phagocytic ability of microglia and thus cause endothelial injury[21-23]. Thus, how to regulate the polarisation of macrophages to inhibit inflammation and promote tissue healing has become an important topic in the treatment of diseases[24-27]. Whether SHED treatment can be used to manipulate the polarisation of microglia remains unknown.

In the present study, we found that SHED showed preventive and therapeutic effects on cerebral oedema by controlling the polarisation of microglia, which is mediated by hypoxia-inducible factor (HIF)-1α/extracellular signal-regulated kinase (ERK) signalling.

Materials and methods

The study was approved by the Animal Use and Care Committee of Peking University (LA2020-124). All institutional and national guidelines for the care and use of laboratory animals were followed.

Animal model

Male Sprague-Dawley rats (8 to 10 weeks old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). The rats were randomly assigned to three groups: the control group, normoxia; the high-altitude group, placed in a low-pressure oxygen tank (FENGLEI, Shanghai, China) for 1 week; and the SHED infusion group, with SHED (5×10^6) suspended in 500 µl, then the cells were injected into the tail vein over the course of 1 minute. One week after the stem cell injection, the rats in the SHED infusion and high-altitude groups were placed in the low-pressure oxygen tank for 1 week. The environment mimicking high altitude was set at an altitude of 5,000 metres, a velocity of 10m/s, humidity of 60% to 70%, and a temperature of $25^0C \pm 2^0C$ with a 12-hour light-dark cycle and ad libitum access to feed and water.

Microglia culture

The microglia BV-2 were purchased from Procell Life Science&Technology (Wuhan, China) and cultured in BV-2 Cell Culture Medium (CM-0493, Procell) supplemented with 10% (vol/vol) foetal bovine serum (FBS, Procell), and 1% (vol/vol) penicillin/streptomycin solution (P/S solution, Procell) at 37^0C in a humidified atmosphere of 5% CO_2/95% air.

SHED and microglia co-culture

For the SHED co-culture group, SHED were co-cultured with microglia by transwell assay. In the transwell plates, 2×10^5 BV-2 cells were plated in the lower chamber and 1×10^5 SHED were plated in the upper chamber, and BV2 specific medium was used as the co-culture medium.

Brain water content analysis

Brains were collected after anaesthesia with 1% sodium pentobarbital, and the wet weight was immediately determined using a precision electronic balance. The samples were then dried at 105^0C to a constant weight. The brain water content (BWC) was calculated according to the following formula: BWC(%) = ((wet weight−dry weight)/wet weight) × 100%.

Haematoxylin and eosin staining

The brain samples were fixed in 4% paraformaldehyde for 24 hours at 4^0C, dehydrated through a graded alcohol series and embedded in paraffin. Sections with a thick-

ness of 6 μm were obtained and stained with haematoxylin and eosin (H&E) according to the manufacturer's instructions to observe the morphology of the cortex and medulla. Photomicrographs were captured using a digital camera attached to a light microscope (Olympus, Tokyo, Japan).

Immunofluorescence

The cells were cultured on chamber slides, fixed in 4% paraformaldehyde for 24 hours at 4^0C, then washed three times with phosphate-buffered saline (PBS, pH 7.4), immersed in 5% bovine serum albumin solution for 30 minutes and incubated with primary antibodies diluted in PBS overnight at 4^0C. Next, the sections were washed with PBS and incubated with secondary antibodies for 60 minutes in the dark. The nucleus was stained with DAPI mounting medium and the slides were observed using an upright fluorescence microscope (Olympus).

Deparaffinised sections were treated with 5% bovine serum albumin (BSA) for 30 minutes, then the sections were incubated with BAX (50599-2-lg, Proteintech, Rosemont, IL, USA) and IBA-1 (1022-5, Santa Cruz Biotechnology, CA, USA) primary antibodies overnight at 4°C, respectively. The samples were then incubated with secondary antibodies for 60 minutes in the dark. The nucleus was stained with DAPI mounting medium and the slides were observed using a fluorescence microscope.

Western blot

Proteins were extracted from cells by using RIPA lysis and extraction buffer. Protein concentration was determined using the bicinchoninic acid (BCA) method. 20 μg protein was separated by the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method, then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in tris-buffered saline with 0.1% Tween 20 detergent (TBST) buffer (150 mM NaCl, 50 mM Tris, pH 7.5, with 0.1% Tween 20) containing 5% BSA (v/v) at room temperature for 1 hour. Next, the membranes were incubated with primary antibodies (rabbit polyclonal anti-iNOS, ARG-1, HIF-1α, p-ERK and β-actin; 1:1,000) overnight at 4^0C, then then the membranes were incubated with the HRP-conjugated secondary antibody for 1 hour at room temperature. The protein band was visualised using chemiluminescence with a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). β-actin was used as a control, and the density of protein bands was semi-quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction

The total RNA of cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was synthesised using a reverse transcription system (Toyobo, Osaka, Japan) and quantitative polymerase chain reaction (qPCR) was carried out using SYBR Green PCR master mix (Applied Biosystems, Waltham, MA, USA) on an ABI 7900 fast real-time PCR system (Applied Biosystems). The gene expression levels were normalised to the internal controls (β-actin) and the relative expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method. The primers for iNOS, TNF-α, ARG-1 and IL-10 are listed in Table S1 (provided on request).

Immunohistochemistry staining

For immunohistochemistry staining analysis, deparaffinised sections were incubated with 3% H_2O_2 for 15 minutes and then treated with 5% BSA for 10 minutes. Next, the sections were incubated with iNOS (18985-1-AP, Proteintech), ARG-1 (D4E3M, Cell Signaling Technology, Danvers, MA, USA), HIF-1α (20960-1-AP, Proteintech), cleaved CASPASE-3 (9664S, Cell Signaling) and ERK (4695, Cell Signaling) primary antibodies overnight at 4°C, respectively. Then, the samples were incubated with HRP-conjugated secondary antibodies and visualised with DAB substrate kit staining (ZLI-9017, ZSGB-Bio, Beijing, China). The nucleus was stained with hematoxylin and the images were photographed by microscope (Olympus).

Determination of apoptosis

Apoptosis in brain tissues was detected by terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end labelling (TUNEL) staining. Paraffin-embedded sections were deparaffinised and digested with 20 μg/mL proteinase K for 30 minutes at room temperature. After washing with PBS, the slides were incubated with the TUNEL reaction overnight at 4°C and visualised with DAB substrate kit staining. TUNEL+ cells were observed and counted in at least three different fields per section.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY, USA). Independent unpaired two-tailed Student *t* tests were used to analyse

the comparisons between two groups. The differences among more than two groups were assesses with a one-way analysis of variance (ANOVA) followed by a Fisher least significant difference post hoc test. The level of statistical significance was set at $P < 0.05$.

Results

SHED infusion alleviated cerebral oedema

To analyse whether SHED infusion can attenuate cerebral oedema, we injected 1×10^6 SHED into rats placed in a low-pressure oxygen tank setting at a 5,000-metre altitude environment to mimic high-altitude conditions (Fig 1a). The BWC of rats in the high-altitude group was significantly higher than that of rats in the control group, and SHED infusion decreased the BWC elevated by hypoxia stimulation (Fig 1b).

We then analysed the rats' blood and the results showed that the red blood cell specific volume (haematocrit, HCT), haemoglobin (HGB) and red blood cell (RBC) count in the high altitude and SHED infusion groups were significantly higher than those in the control group (Figs 1c to 1e). These results indicated that the number and density of erythrocytes in the blood increased under the high-altitude environment, and the oxygen uptake ability enhanced to adapt to it.

Next, the HE staining results showed that karyopyknotic, cell oedema and vacuolar degeneration of brain stromal cells were detected in the high-altitude group. The number of blood vessels in the medulla and the circumference of blood vessels increased in the high-altitude group. After SHED infusion, cell oedema reduced significantly (Fig 1f). These results indicated that SHED infusion can alleviate cerebral oedema at high altitude and reduce BWC.

SHED infusion reduced apoptosis of brain cells

Low oxygen levels may lead to cell apoptosis[28]. TUNEL staining showed that the ratio of TUNEL positive cells increased significantly in the high-altitude group compared with the control group, and decreased in the SHED infusion group (Figs 2a to c). Caspase-3 is one of the key enzymes of apoptosis. Immunohistochemical results of brain tissue paraffin sections showed that the expression of cleaved Caspase-3 increased significantly in the high-altitude group and decreased after SHED infusion (Figs 2a to c). The immunofluorescence co-staining of microglia marker IBA-1 and apoptotic markers BAX in brain tissue sections showed that the positive sites of IBA-1 overlapped with the positive sites of BAX in the high-altitude group, suggesting that apoptosis occurred in microglia cells (Fig 2d). Microglia cells were cultured in a low oxygen incubator to mimic a high-altitude low oxygen environment, and the results showed that the ratio of apoptotic cells increased significantly in the high-altitude low oxygen group. Then, the ratio of cell apoptosis induced by low oxygen increased after SHED co-culturing (Fig 2e). These results indicate that SHED can prevent cell apoptosis caused by high-altitude conditions in vivo and in vitro.

SHED treatment suppressed M1-type polarisation of microglia in an environment mimicking high altitude and low oxygen

Microglia are a kind of macrophage in the nervous system and are involved in the regulation of nervous system homeostasis[17,18]. Regulation of microglial polarisation may be involved in the pathology of and therapy for neurological diseases[24-27]. First, we analysed the expression of iNOS, which is a representative marker of M1-type polarisation. The results showed that the ratio of iNOS positive cells increased significantly in the high-altitude group compared with the control group and decreased significantly in the SHED infusion group (Fig 3a). Next, we cultured microglia in a low-oxygen incubator, which mimicked a high-altitude, low-oxygen environment, and the qPCR results revealed that the mRNA expression levels of M1-type polarisation markers iNOS and TNF-α in the high-altitude group were significantly higher than those in the control group. On the other hand, the expression levels of these genes decreased in the SHED co-culture group (Figs 3b and c). Moreover, the expression of iNOS in the high-altitude group was significantly higher than that in the control group, but the iNOS expression in the SHED co-culture group was lower than that in the high-altitude group, as assessed by Western blot and immunofluorescence staining (Figs 3d and e). These results indicate that hypoxia stimulation can promote the transformation of microglial cells to M1-type, which was attenuated by SHED treatment.

SHED treatment promoted M2-type polarisation of microglia in an environment mimicking high altitude and low oxygen

The proportion of arginase-1 (ARG-1) positive cells increased significantly after SHED infusion compared with the control and high-altitude groups (Fig 4a). We then analysed the effects of SHED on M2-type polarisation in microglia using a co-culture system, and found

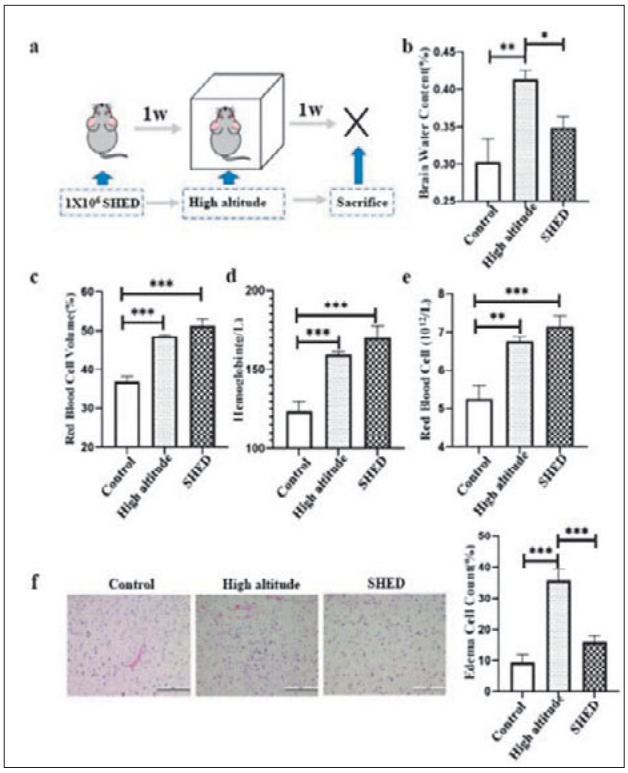

Fig 1 SHED infusion showed therapeutic effects on hypoxic high altitude cerebral oedema in vivo. **(a)** The schema of high-altitude and SHED therapy rat models (n = 5). **(b)** Quantitative analysis of BWC in the control, high-altitude and SHED infusion groups. **(c)** Red blood cell volume in the control, high-altitude and SHED infusion groups. **(d)** Haemoglobin in the control, high-altitude and SHED infusion groups. **(e)** Red blood cells in the control, high-altitude and SHED infusion groups. **(f)** Representative images of HE staining of brain tissue in the control, high-altitude and SHED infusion groups. Scale bars 200 μm. Data are presented as mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001).

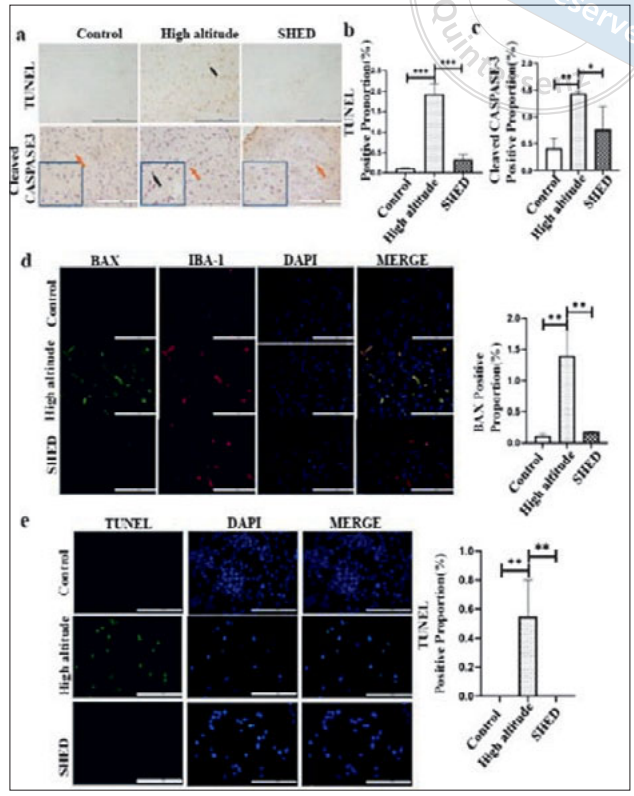

Fig 2 SHED infusion reduced brain cell apoptosis. **(a)** Representative images and quantification of TUNEL (upper panel) and cleaved Caspase-3 (lower panel) staining of brain tissue in the control, high-altitude and SHED infusion groups (scale bars 200 μm). **(b and c)** Quantitative analysis of TUNEL **(b)** and cleaved Caspase-3 **(c)** level in the control, high-altitude and SHED infusion groups. **(d)** Expression of BAX and IBA-1 in the control, high-altitude and SHED infusion groups, analysed by immunofluorescence (scale bars 200 μm). **(e)** Representative images and quantification of TUNEL staining in BV-2 microglia in the control, high-altitude and SHED infusion groups (scale bars 200 μm). Data are presented as mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001).

that the mRNA expressions of M2-type polarisation markers ARG-1 and IL-10 in the SHED co-culture group were significantly higher than those in the control and high-altitude groups (Figs 4b and c). Moreover, the protein expression of ARG-1 in the SHED co-culture group was significantly higher than that in the control and high-altitude groups, as assessed by Western blot and immunofluorescence staining (Figs 4d and e).

SHED treatment reduced HIF-1α expression in an environment mimicking high altitude and low oxygen

Next, we analysed the expression of HIF-1α in an environment mimicking high altitude and low oxygen and found that the expression of HIF-1α in the high-altitude group increased significantly, attenuated by SHED treatment analysed by qPCR and immunofluorescence staining (Figs 5a to c). YC-1, an inhibitor of HIF-1α protein, was used to treat microglia to verify the role of HIF-1α. The results showed that the elevated gene expressions of M1-type polarisation markers iNOS and TNF-α in the high-altitude group decreased significantly after YC-1 treatment (Fig 5d). Immunofluorescence staining results also revealed that the expression of iNOS, which was elevated in the high-altitude group, was decreased after HIF inhibitor YC-1 treatment (Figs 5e and f). Meanwhile, the expressions of ARG-1 and IL-10 related with M2-type polarisation increased significantly in the YC-1 treatment group compared with the high-altitude group

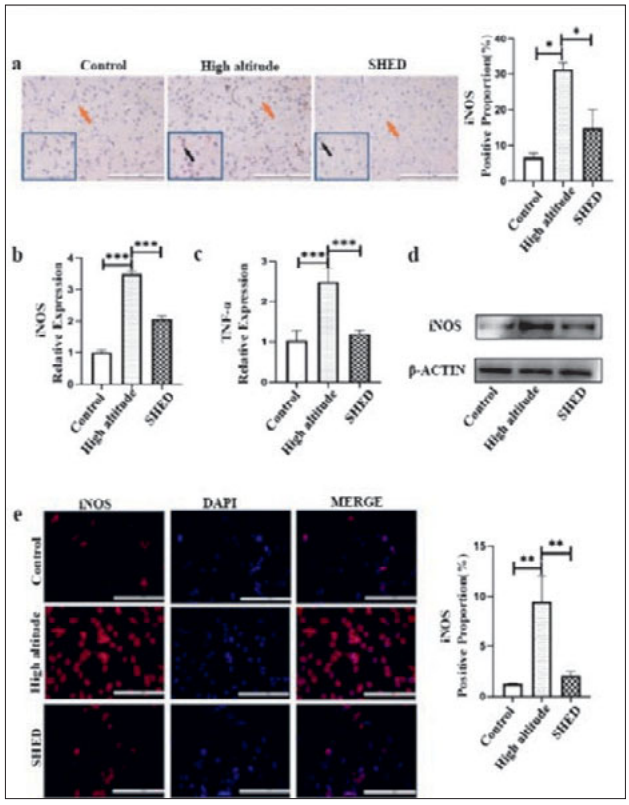

Fig 3 SHED treatment dampened the M1-type polarisation phenotype of BV-2 microglia in an environment mimicking high altitude and low oxygen. **(a)** Expression of iNOS in the control, high-altitude and SHED co-culture groups as assessed by immunohistochemical staining (scale bars 200 μm). **(b and c)** Expression of iNOS and TNF-α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, assessed by qPCR. **(d)** Expression of iNOS in BV-2 microglia in the control, high-altitude and SHED co-culture groups was assessed by Western blot. **(e)** Expression of iNOS in BV-2 microglia in the control, high-altitude and SHED co-culture groups as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

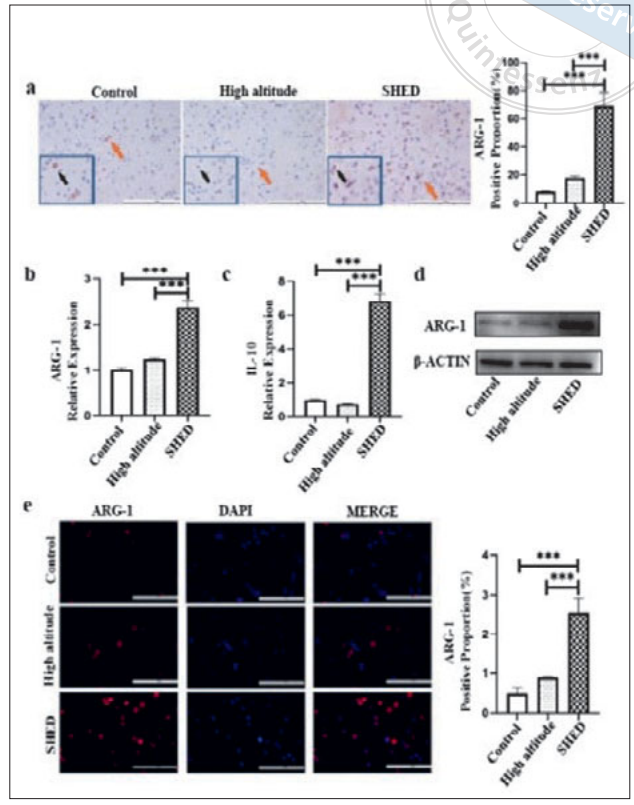

Fig 4 Stem cell therapy promoted the M2-type polarisation phenotype of BV-2 microglia in an environment mimicking high altitude. **(a)** Expression of ARG-1 in the control, high-altitude and SHED co-culture groups, as assessed by immunohistochemical staining (scale bars 200 μm). **(b and c)** Expression of ARG-1 and IL-10 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by qPCR. **(d)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by Western blot. **(e)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and SHED co-culture groups, as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

(Fig 5g), and immunofluorescence staining results showed the expression of ARG-1 in the YC-1 treatment group was significantly higher than that in the high-altitude group (Figs 5h and i). These results indicated that hypoxia induced HIF-1α to regulate microglia polarisation, which can be restored by SHED treatment.

SHED treatment alleviated the activation of ERK signalling pathway in an environment mimicking high altitude and low oxygen

HIF-1α activation interacts with multiple signalling pathways, such as the ERK signalling pathway[29,30]. In this study, the expression of phosphorylated ERK (p-ERK) increased significantly in the high-altitude group but decreased after co-culture with SHED, as assessed by immunofluorescence staining and Western blot (Figs 6a to c). To verify the role of the ERK signalling pathway, we used U0126, an inhibitor of this pathway, to treat microglia in an environment mimicking high altitude and low oxygen, and the results showed the decreased gene expression of M1-type polarisation markers iNOS and TNF-α (Fig 6d). The elevated expression of iNOS in the high-altitude group was inhibited after U0126 treatment, as assessed by immunofluorescence staining (Fig 6e). Meanwhile, the gene expression level of M2-type polarisation markers ARG-1 and IL-10 increased significantly after U0126 treatment (Fig 6f). The expression of ARG-1

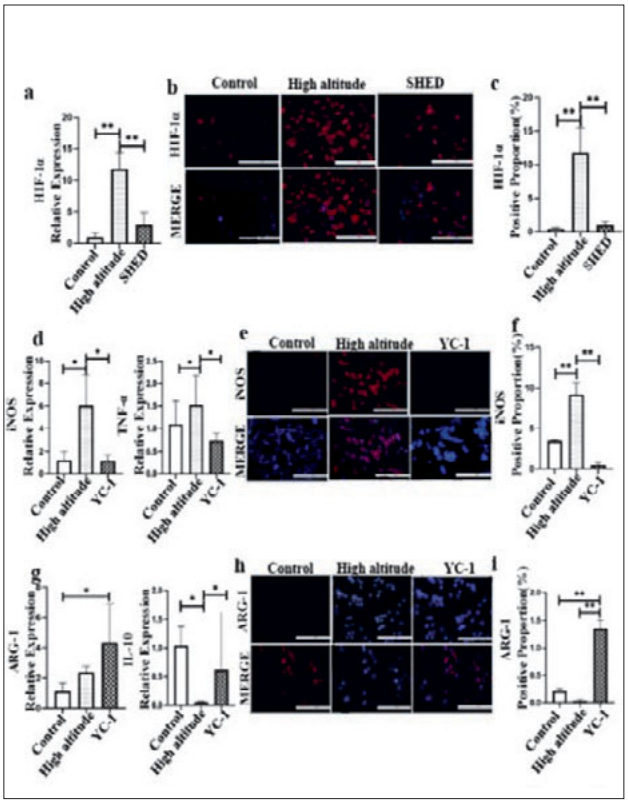

Fig 5 SHED treatment reduced the expression of HIF-1α. **(a)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, as assessed by qPCR. **(b and c)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and SHED co-culture groups, analysed by immunofluorescence staining (scale bars 200 μm). **(d)** Expression of iNOS and TNF-α of BV-2 microglia in the control, high-altitude and YC-1 groups analysed by qPCR. **(e and f)** Expression of iNOS in BV-2 microglia in the control, high-altitude and YC-1 groups (scale bars 200 μm). **(g)** Expression of ARG-1 and IL-10 expression in BV-2 microglia in the control, high-altitude and YC-1 groups, analysed by qPCR. **(h and i)** Expression of ARG-1 in BV-2 microglia in the control, high-altitude and YC-1 groups, as assessed by immunohistochemical staining (scale bars 200 μm). Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

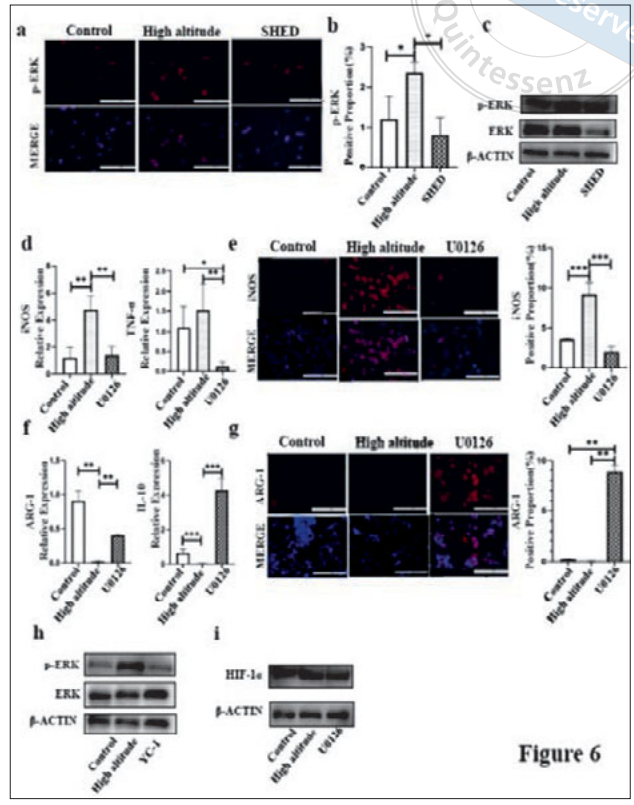

Fig 6 SHED treatment inhibited the ERK signalling pathway activated by an environment mimicking high altitude and low oxygen. **(a and b)** Expression and quantification of p-ERK in BV-2 microglia in the control, high-altitude and SHED co-culture groups, assessed by immunohistochemical staining (scale bars 200 μm). **(c)** Expression of p-ERK in BV-2 microglia in the control, high-altitude and SHED co-culture groups analysed by Western blot. **(d)** Expression of iNOS and TNF-α in BV-2 microglia in the control, high-altitude and U0126 groups analysed by qPCR (U0126 is the inhibitor of the ERK pathway). **(e)** Expression and quantification of iNOS in BV-2 microglia in BV-2 microglia in the control, high-altitude and U0126 groups (scale bars 200 μm). **(f)** Expression of ARG-1 and IL-10 in BV-2 microglia in the control, high-altitude and U0126 groups analysed by qPCR. **(g)** Expression and quantification of ARG-1 in BV-2 microglia in BV-2 microglia in the control, high-altitude and U0126 groups analysed by immunohistochemical staining (scale bars 200 μm). **(h)** Expression of p-ERK and ERK in BV-2 microglia in BV-2 microglia in the control, high-altitude and YC-1 groups analysed by Western blot. **(i)** Expression of HIF-1α in BV-2 microglia in the control, high-altitude and U0126 groups. Data are presented as mean ± standard deviation (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

increased significantly after U0126 treatment compared with that in the high-altitude group (Fig 6g).

Next, we analysed whether the ERK signalling pathway is regulated by HIF-1α. The findings showed that HIF-1α inhibitor YC-1 treatment inhibited the activated ERK signalling under high altitude (Fig 6h), whereas ERK inhibitor U0126 treatment failed to suppress the elevated level of HIF-1α induced by high altitude hypoxia stimulation (Fig 6i). Thus, SHED can attenuate high-altitude cerebral oedema by inhibiting the M1-type polarisation of microglia and promoting M2-type polarisation, which is mediated by the HIF-1α/ERK signalling pathway (Fig 7).

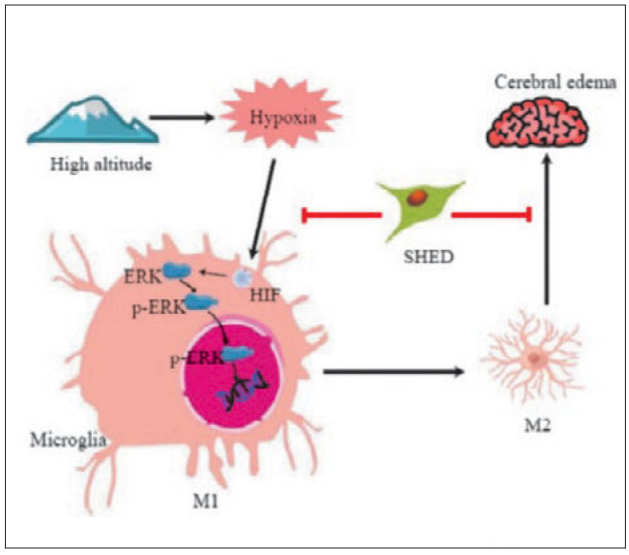

Fig 7 The schema showed that SHED treatment can relieve high-altitude cerebral oedema by inhibiting HIF-1α mediated ERK signalling activation in a hypoxic environment mimicking high altitude.

Discussion

The human body may undergo a series of physiological and pathological changes during sudden exposure to a high-altitude environment due to the thin air and low oxygen content[1,2]. The main symptoms of acute altitude sickness are headache, nausea and vomiting. If the acute altitude sickness is not treated in time, it may develop and lead to cerebral oedema or death, which is the terminal stage of acute altitude sickness. The aetiology of and treatment strategy for cerebral oedema remain to be studied. Mesenchymal stem cells have shown profound therapeutic effects on neuronal injury, such as rescuing injured neurons exposed to oxygen-glucose deprivation by inhibiting the inflammatory cytokine TNF-α[31]. In this study, we established a high-altitude cerebral oedema model to mimic a high-altitude, low-oxygen environment. The results showed that the BWC, brain oedema and cell apoptosis of brain tissues increased significantly under high altitude conditions, whereas SHED infusion can relieve cerebral oedema and reduce the proportion of apoptosis and oedema of brain cells to effectively reduce the occurrence of cell oedema. To the best of the present authors' knowledge, this study is the first report about the prevention and therapeutic effects of SHED on high-altitude cerebral oedema, suggesting that SHED is a promising strategy for preventing and treating cerebral oedema.

Mesenchymal stem cells can induce the polarisation of macrophages to a M2-type phenotype[32] and play a therapeutic role in the cardiovascular, autoimmune and central nervous systems and in the event of inflammatory disease[33,34]. Microglia, one of the main types of cells that regulate the inflammatory response after brain injury, play an important role in high-altitude sickness[35]. Microglia may differentiate into two forms: the pro-inflammatory M1 type and anti-inflammatory M2 type. Under hypoxic and ischemic conditions, microglia are prone to polarise into the M1 type, which releases pro-inflammatory cytokines, leading to secondary brain injury[36]. In the case of ischemia and hypoxia, M2-type polarisation of microglia can activate peroxisome proliferator–activated receptor gamma and promote microglia to release anti-inflammatory cytokines, which alleviates brain injury[37,38]. Regulation of microglial polarisation from M1 to M2 type may be the key to treating ischemic encephalopathy[39]. SHED have shown a profound capacity for proliferation and multi-lineage differentiation. Studies have found that SHED can affect the local microenvironment of bone regeneration, secrete anti-inflammatory factors and promote angiogenesis through the paracrine effect[40]. It has been reported that SHED and their conditioned media can be effective against neurodegeneration through multiple mechanisms, including cell replacement, paracrine effects, angiogenesis, synaptogenesis, immunomodulation and apoptosis inhibition[41]; however, the specific mechanism of SHED remains to be studied. Thus, we speculated that SHED could regulate the inflammatory environment of brain cells and relieve the symptoms of brain cell oedema. In our study, when microglia co-cultured with SHED, the mRNA and protein levels of M1-type polarisation marker molecules decreased compared with groups not co-cultured with SHED in the high-altitude conditions, whereas the levels of M2-type polarisation marker molecules increased significantly. Therefore, after hypoxia stimulation, SHED can inhibit M1-type polarisation of microglia and promote M2-type polarisation, which alleviates the occurrence of cerebral oedema.

Hypoxic conditions can activate the ERK signalling pathway and lead to a series of diseases[42]. The regulation of the ERK signalling pathway can play an important role in hypoxic disease treatment[43]. In our study, the expression of p-ERK significantly increased under hypoxia in vitro and decreased after co-culturing with SHED. The in vivo experiments indicated that the expression level of ERK increased significantly under hypoxia stimulus. These results suggest that the ERK signalling pathway regulates microglial polarisation to control high-attitude cerebral oedema. Whether other pathways participate in this process needs to be investigated further.

HIF-1α is an oxygen-sensitive transcriptional activator. Under hypoxia, HIF-1α induces regulation of angiogenesis, glucose metabolism, cell proliferation and apoptosis. Regulation of HIF-1α has become a therapeutic target for numerous diseases. HIF-1α knockdown reduces ischemic injury, which suggests that HIF-1 may contribute to tissue damage during cerebral ischemia[44]. In this study, the expression of HIF-1α increased significantly in the high-altitude group and decreased after co-culturing with SHED. Moreover, we observed that ERK signalling activation was regulated by HIF-1α. The M1-type polarisation induced by hypoxia stimulus was inhibited after ERK or HIF inhibitor treatment. These results indicate that ERK and HIF signalling may be potential targets for cerebral oedema treatment, but further investigations are still needed.

Conclusion

In this study, SHED displayed profound preventive and therapeutic effects on high-altitude cerebral oedema through regulating M1/M2 polarisation of microglia, which was controlled by HIF-1α/ERK signalling. It provides new evidence for the prevention and treatment of high-altitude cerebral oedema in the future.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Yi Ming WANG performed the experiments and drafted the manuscript; Drs Yi Kun ZHOU, Chun Shan HAN, Liu Jing CHEN and Zi Meng ZHUANG offered experimental advice; Drs Rui Li YANG and Wei Ran LI designed the study. All the authors approved the submission.

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References

1. Zhou Y, Huang X, Zhao T, et al. Hypoxia augments LPS-induced inflammation and triggers high altitude cerebral edema in mice. Brain Behav Immun 2017;64:266–275.
2. Basnyat B. High altitude cerebral and pulmonary edema. Travel Med Infect Dis 2005;3:199–211.
3. Zelmanovich R, Pierre K, Felisma P, Cole D, Goldman M, Lucke-Wold B. High altitude cerebral edema: Improving treatment options. Biologics (Basel) 2022;2:81–91.
4. Mehta SR, Chawla A, Kashyap AS. Acute mountain sickness, high altitude cerebral oedema, high altitude pulmonary oedema: The current concepts. Med J Armed Forces India 2008;64:149–153.
5. Hackett PH, Yarnell PR, Weiland DA, Reynard KB. Acute and evolving MRI of high-altitude cerebral edema: Microbleeds, edema, and pathophysiology. AJNR Am J Neuroradiol 2019;40:464–469.
6. Aksel G, Çorbacıoğlu ŞK, Özen C. High-altitude illness: Management approach. Turk J Emerg Med 2019;19:121–126.
7. Luks AM, Auerbach PS, Freer L, et al. Wilderness Medical Society clinical practice guidelines for the prevention and treatment of acute altitude illness: 2019 update. Wilderness Environ Med 2019;30:S3–S18.
8. Miura M, Gronthos S, Zhao M, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 2003;100:5807–5812.
9. Ueda T, Ito T, Inden M, Kurita H, Yamamoto A, Hozumi I. Stem cells from human exfoliated deciduous teeth-conditioned medium (SHED-CM) is a promising treatment for amyotrophic lateral sclerosis. Front Pharmacol 2022;13:805379.
10. Wakayama H, Hashimoto N, Matsushita Y, et al. Factors secreted from dental pulp stem cells show multifaceted benefits for treating acute lung injury in mice. Cytotherapy 2015;17:1119–1129.
11. Mita T, Furukawa-Hibi Y, Takeuchi H, et al. Conditioned medium from the stem cells of human dental pulp improves cognitive function in a mouse model of Alzheimer's disease. Behav Brain Res 2015;293:189–197.
12. Inoue T, Sugiyama M, Hattori H, Wakita H, Wakabayashi T, Ueda M. Stem cells from human exfoliated deciduous tooth-derived conditioned medium enhance recovery of focal cerebral ischemia in rats. Tissue Eng Part A 2013;19:24–29.
13. Kitase Y, Sato Y, Ueda K, et al. A novel treatment with stem cells from human exfoliated deciduous teeth for hypoxic-ischemic encephalopathy in neonatal rats. Stem Cells Dev 2020;29:63–74.
14. Bai X, Zhang X, Wang C, et al. Stem cells from human exfoliated deciduous teeth attenuate trigeminal neuralgia in rats. Stem Cells Int 2021;2021:8819884.
15. Zhu S, Min D, Zeng J, Ju Y, Liu Y, Chen X. Transplantation of stem cells from human exfoliated deciduous teeth decreases cognitive impairment from chronic cerebral ischemia by reducing neuronal apoptosis in rats. Stem Cells Int 2020;2020:6393075.
16. Gonmanee T, Thonabulsombat C, Vongsavan K, Sritanaudomchai H. Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells. Arch Oral Biol 2018;88:34–41.
17. Nayak D, Roth TL, McGavern DB. Microglia development and function. Annu Rev Immunol 2014;32:367–402.
18. Colonna M, Butovsky O. Microglia function in the central nervous system during health and neurodegeneration. Annu Rev Immunol 2017;35:441–468.
19. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. F1000Prime Rep 2014;6:13.
20. Hu X, Li P, Guo Y, et al. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 2012;43:3063–3070.
21. Wang X, Chen G, Wan B, et al. NRF1-mediated microglial activation triggers high-altitude cerebral edema. J Mol Cell Biol 2022;14:mjac036.

22. Yang J, Zhao Y, Zhang L, et al. RIPK3/MLKL-mediated neuronal necroptosis modulates the M1/M2 polarization of microglia/macrophages in the ischemic cortex. Cereb Cortex 2018;28:2622–2635.
23. Lu Y, Chang P, Ding W, et al. Pharmacological inhibition of mitochondrial division attenuates simulated high-altitude exposure-induced cerebral edema in mice: Involvement of inhibition of the NF-κB signaling pathway in glial cells. Eur J Pharmacol 2022;929:175137.
24. Yang X, Xu S, Qian Y, Xiao Q. Resveratrol regulates microglia M1/M2 polarization via PGC-1α in conditions of neuroinflammatory injury. Brain Behav Immun 2017;64:162–172.
25. Du X, Xu Y, Chen S, Fang M. Inhibited CSF1R alleviates ischemia injury via inhibition of microglia M1 polarization and NLRP3 pathway. Neural Plast 2020;2020:8825954.
26. Fan H, Tang HB, Shan LQ, et al. Quercetin prevents necroptosis of oligodendrocytes by inhibiting macrophages/microglia polarization to M1 phenotype after spinal cord injury in rats. J Neuroinflammation 2019;16:206.
27. Cui C, Xu P, Li G, et al. Vitamin D receptor activation regulates microglia polarization and oxidative stress in spontaneously hypertensive rats and angiotensin II-exposed microglial cells: Role of renin-angiotensin system. Redox Biol 2019;26:101295.
28. Zhang L, Wei W, Ai X, et al. Extracellular vesicles from hypoxia-preconditioned microglia promote angiogenesis and repress apoptosis in stroke mice via the TGF-β/Smad2/3 pathway. Cell Death Dis 2021;12:1068.
29. Tsao CC, Baumann J, Huang SF, et al. Pericyte hypoxia-inducible factor-1 (HIF-1) drives blood-brain barrier disruption and impacts acute ischemic stroke outcome. Angiogenesis 2021;24:823–842.
30. Fan Y, Ou L, Fan J, et al. PLCε regulates metabolism and metastasis signaling via HIF-1α/MEK/ERK pathway in prostate cancer. J Cell Physiol 2020;235:8546–8557.
31. Huang P, Gebhart N, Richelson E, Brott TG, Meschia JF, Zubair AC. Mechanism of mesenchymal stem cell-induced neuron recovery and anti-inflammation. Cytotherapy 2014;16:1336–1344.
32. Liu F, Qiu H, Xue M, et al. MSC-secreted TGF-β regulates lipopolysaccharide-stimulated macrophage M2-like polarization via the Akt/FoxO1 pathway. Stem Cell Res Ther 2019;10:345.
33. Li R, Zhao K, Ruan Q, Meng C, Yin F. Bone marrow mesenchymal stem cell-derived exosomal microRNA-124-3p attenuates neurological damage in spinal cord ischemia-reperfusion injury by downregulating Ern1 and promoting M2 macrophage polarization. Arthritis Res Ther 2020;22:75.
34. Wang L, Pei S, Han L, et al. Mesenchymal stem cell-derived exosomes reduce A1 astrocytes via downregulation of phosphorylated NFκB P65 subunit in spinal cord injury. Cell Physiol Biochem 2018;50:1535–1559.
35. Prinz M, Jung S, Priller J. Microglia biology: One century of evolving concepts. Cell 2019;179:292–311.
36. Chen AQ, Fang Z, Chen XL, et al. Microglia-derived TNF-α mediates endothelial necroptosis aggravating blood brain-barrier disruption after ischemic stroke. Cell Death Dis 2019;10:487.
37. Zhou D, Ji L, Chen Y. TSPO modulates IL-4-induced microglia/macrophage M2 polarization via PPAR-γ pathway. J Mol Neurosci 2020;70:542–549.
38. Liu R, Diao J, He S, et al. XQ-1H protects against ischemic stroke by regulating microglia polarization through PPARγ pathway in mice. Int Immunopharmacol 2018;57:72–81.
39. Perego C, Fumagalli S, Zanier ER, et al. Macrophages are essential for maintaining a M2 protective response early after ischemic brain injury. Neurobiol Dis 2016;96:284–293.
40. Zhang N, Xu L, Song H, et al. Tracking of stem cells from human exfoliated deciduous teeth labeled with Molday ION rhodamine-B during periodontal bone regeneration in rats. Int J Stem Cells 2023;16:93–107.
41. Aydin S, Şahin F. Stem cells derived from dental tissues. Adv Exp Med Biol 2019;1144:123–132.
42. Cheng WH, Chen CL, Chen JY, Lin CH, Chen BC. Hypoxia-induced preadipocyte factor 1 expression in human lung fibroblasts through ERK/PEA3/c-Jun pathway. Mol Med 2021;27:69.
43. Geng YN, Zhao M, Yang JL, et al. GP-14 protects against severe hypoxia-induced neuronal injury through the AKT and ERK pathways and its induced transcriptome profiling alteration. Toxicol Appl Pharmacol 2022;448:116092.
44. Helton R, Cui J, Scheel JR, et al. Brain-specific knock-out of hypoxia-inducible factor-1alpha reduces rather than increases hypoxic-ischemic damage. J Neurosci. 2005;25:4099–4107.