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ORIGINAL PAPER

Stem Cell Treatment After Cerebral Ischemia Regulates the Gene Expression of Apoptotic Molecules

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Abstract Evidence suggests that apoptosis contributes significantly to cell death after cerebral ischemia. Our recent studies that utilized human umbilical cord blood-derived mesenchymal stem cells (hUCBSCs) demonstrated the potential of hUCBSCs to inhibit neuronal apoptosis in a rat model of CNS injury. Therefore, we hypothesize that intravenous administration of hUCBSCs after focal cerebral ischemia would reduce brain damage by inhibiting apoptosis and downregulating the upregulated apoptotic pathway molecules. Male Sprague–Dawley rats were obtained and randomly assigned to various groups. After the animals reached a desired weight, they were subjected to a 2 h middle cerebral artery occlusion (MCAO) procedure followed by 7 days of reperfusion. The hUCBSCs were obtained, cultured, and intravenously injected (0.25×10^6 cells or 1×10^6 cells) via the tail vein to separate groups of animals 24 h post-MCAO procedure. We performed various techniques including PCR microarray, hematoxylin and eosin, and TUNEL staining in addition to immunoblot and immunofluorescence analysis in order to investigate the effect of our treatment on regulation of apoptosis after focal cerebral ischemia. Most of the apoptotic pathway molecules which were upregulated after focal cerebral ischemia were downregulated after hUCBSCs treatment. Further, the staining techniques revealed a prominent reduction in brain damage and the extent of apoptosis at even the lowest dose of hUCBSCs tested in the present study. In conclusion, our treatment with hUCBSCs after cerebral ischemia in the rodent reduces brain damage by inhibiting apoptosis and downregulating the apoptotic pathway molecules.

Keywords Ischemia · Apoptosis · Stem cells · Stroke · Downregulation

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Introduction

Despite advances in understanding the mechanisms of acute ischemic stroke, the only FDA approved treatment is still intravenous recombinant tissue plasminogen activator. There are no other effective options due to perhaps multifaceted mechanisms involved in neuronal damage after brain ischemia [1]. Globally, fifteen million people suffer from a stroke each year, and five million stroke patients die with another five million left permanently disabled [2]. Recently, stem cell-based approaches have received much hope as potential methods for therapeutic use. Clinical trials that utilized stem cells in stroke thus far predominantly have targeted at subacute and chronic phases of stroke [3]. The findings reported from preclinical and

clinical studies demonstrate that stem cell-based therapies have the potential to improve the clinical outcome in stroke patients [4–6]. Stem cell transplantation offers a promising new therapeutic strategy for stroke not only to prevent the ongoing damage, which has been the focus of conventional therapy, but also to actually repair the injured brain.

In addition to ethical and moral concerns, limited availability of embryonic, fetal, and adult brain-derived neural stem cells have prompted the search for other stem cell sources. Bone marrow- and human umbilical cord blood-derived stem cells have emerged as alternative stem cell sources. Of these, human umbilical cord blood-derived mesenchymal stem cells (hUCBSCs) possess several advantages over other types of stem cells including those derived from bone marrow [7]. To date, very few authors have reported the use of human umbilical cord blood cells in animal models of acute cerebral ischemia. A few have reported that these cells survive, migrate and offer dose-dependent behavioral improvement and neuronal sparing after stoke [8–13]. However, none of these studies that utilized hUCBSCs have thus far clearly delineated the molecular mechanisms underlying the neuroprotection after focal cerebral ischemia.

It is rather clear that apoptosis contributes significantly to cell death subsequent to cerebral ischemia. Recently, we demonstrated the inhibition of neuronal apoptosis in a rat model of spinal cord injury after intraspinal implantation of hUCBSCs [14]. We hypothesize that "intravenous administration of hUCBSCs after focal cerebral ischemia would reduce brain damage by inhibiting apoptosis and downregulating the upregulated apoptotic pathway molecules." Considering the potential use of hUCBSCs in acute brain ischemia, the molecular mechanisms responsible for their neuroprotection and effective dose at which these cells offer neuroprotection need to be further elucidated prior to their clinical application. Therefore, in the present study, we aimed to investigate the effective dose of hUCBSCs that can reduce the brain damage and the effect of hUCBSCs treatment on the modulation of gene expression of apoptotic pathway molecules in a rat model of transient acute focal cerebral ischemia. To our knowledge, this is the first study that has been designed and conducted to explore the underlying molecular mechanisms of hUCBSCs-mediated neuroprotection after ischemic stroke.

Materials and Methods

Ethics Statement

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois College of Medicine at Peoria approved all surgical interventions and post-operative animal care.

Antibodies

Anti-Fas, anti-TNFα, anti-XIAP and anti-Smac antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-apoptosis-inducing factor (AIF) and anti-cleaved caspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Novus Biologicals (Littleton, CO).

Animals

In this study, we used male Sprague–Dawley rats. All animal experiments were conducted in accordance with the IACUC guidelines. Adult male Sprague–Dawley rats weighing 230–250 g were procured from Harlan Laboratories (USA). Animals were housed in a 12 h light/dark cycle with a controlled temperature and humidity and free access to food and water. After the animals reached a weight of 260 ± 5 g, they were randomly assigned to four groups, and each group consisted of at least 12 animals. Group 1 was used as sham controls. Groups 2, 3 and 4 were subjected to the middle cerebral artery occlusion (MCAO) procedure. Group 2 animals were subjected to the MCAO procedure without any further treatments. Group 3 and Group 4 animals were intravenously injected with 0.25×10^6 and 1×10^6 hUCBSCs, respectively, suspended in 0.5 mL sterile saline via tail vein 24 h post-MCAO procedure. All animals from the four groups were sacrificed 7 days post-MCAO procedure. All procedures that were performed on the animals were in compliance with the approved IACUC protocol.

Stem Cells

Cryo-preserved hUCBSCs obtained from Vitro Biopharma (Golden, CO) were used to establish cultures in MSC-GRO low serum complete MSC medium according to the provided instructions. Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 with a change of culture medium twice a week. When the cell cultures were about 80 % to 90 % confluent, cells were split and subcultured. Cells were detached, washed twice with sterile phosphate buffered saline (PBS), counted and suspended in sterile saline prior to intravenous administration.

Experimental MCAO Model

After the animals reached a weight of 260 ± 5 g, they were subjected to right MCAO procedure as described

earlier [15]. Briefly, a ventral midline incision (~25 mm) was made in the neck and the right common carotid, internal carotid, and external carotid arteries were surgically exposed. The external carotid artery was permanently ligated rostral with one ligature. A microaneurysm clip was applied to the external carotid artery near its bifurcation with the internal carotid artery. A small puncture opening was made in the external carotid artery. Monofilament was inserted through the opening, and the other loose ligature was tightened around the lumen containing the monofilament. The microaneurysm clip was removed from the external carotid artery, and the monofilament was then gently advanced from the lumen of the external carotid artery into the internal carotid artery for a distance of ~19–20 mm beyond the bifurcation of the common carotid artery. Skin on the neck incision was closed with surgical wound clips. To restore the blood flow 2 h after MCA occlusion, the surgical site was re-opened by removing the wound clips. The microaneurysm clip was removed, the knot was loosened, the monofilament was withdrawn and the knot was re-tied to stop bleeding. The skin was sutured to close the neck incision.

Group 3 and 4 animals were intravenously injected with 0.25×10^6 and 1×10^6 hUCBSCs respectively, via tail vein, 24 h post-MCAO procedure. All animals subjected to the MCAO procedure were treated with appropriate doses of analgesics and antibiotics as mentioned in the approved IACUC protocol. On the seventh day post-MCAO procedure, all animals were sacrificed. The brain tissues obtained from these animals were utilized for various experimental procedures.

Brain Tissue Fixation and Sectioning

On the seventh day post-MCAO procedure, animals from all four groups were placed under deep anesthesia with pentobarbital and perfused through the left ventricle with 70–100 mL of PBS, followed by 100–150 mL of 10 % buffered formalin (Fisher Scientific, NJ). The brains of the animals from various treatment groups were then removed, fixed in 10 % buffered formalin, and embedded in paraffin. Serial coronal brain sections were cut at a thickness of 5–6 µm with a microtome.

Terminal Deoxy Nucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay

The extent of apoptosis in the ischemic core and the penumbra regions of paraffin-embedded coronal brain sections of animals from all the groups were analyzed by TUNEL assay using an In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Briefly, the paraffin-embedded tissue sections were de-paraffinized, re-hydrated, treated with proteinase K working solution, and permeabilized. Permeabilized tissue sections were incubated with the TUNEL reaction mixture in a humidified atmosphere for 60 min at 37 °C in the dark. Sections were counterstained for nuclei with DAPI (Dako, Carpinteria, CA), cover slipped using fluorescent mounting medium (Dako), and observed under a fluorescence microscope (Olympus IX71).

Hematoxylin and Eosin Staining

Paraffin-embedded brain sections of various groups of animals including sham controls were de-paraffinized, rehydrated and then subjected to hematoxylin and eosin (H&E) staining according to a standard protocol. H&E stained sections were cover slipped and observed under a light microscope.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) from the ischemic ipsilateral brain regions of rats sacrificed on the seventh day post-MCAO procedure. Briefly, total RNA was extracted with TRIzol and precipitated with isopropyl alcohol, washed in ethanol, and resuspended in RNase-free water. One µg of total RNA was reverse transcribed into first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. The cDNAs obtained from this process were used for rat apoptosis RT^2 Profiler PCR array.

Apoptosis PCR Array

We used the RT^2 Profiler Rat Apoptosis PCR Array (SA Biosciences). Each array contains a panel of 96 primer sets of 84 relevant apoptosis pathway focused genes, plus five housekeeping genes and three RNA and PCR quality controls. The cDNAs obtained from the brain sections of hUCBSCs-treated rats sacrificed on day seven post-MCAO procedure were loaded onto microarray plates along with Fast Start SYBR Green (Roche, Indianapolis, IN) according to the manufacturer's instructions. The sample-loaded microarray plate was subjected to real-time PCR analysis in iCycler IQ (Multi Color Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA). Real-time PCR was carried out under the following conditions: one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. We performed the PCR microarray analysis on the samples obtained from three different animals per group.

Immunofluorescence Analysis

Immunofluorescence analysis was used to identify the changes in the expression of cleaved-caspase-3 protein upon treatment with various doses of hUCBSCs. Paraffin-embedded brain sections of various groups of animals were deparaffinized, subjected to antigen retrieval, permeabilized, processed with anti-cleaved-caspase-3 primary antibody followed by Alexa Fluor® 488 (goat anti-rabbit IgG, green) fluorescent-labeled secondary antibody, counterstained with DAPI, cover slipped, and observed using a confocal microscope (Olympus Fluoview). Negative controls (without primary antibody or using isotype specific IgG) were maintained for all the samples. The results were quantified by counting the number of cleaved-caspase-3-positive cells in at least five different ischemic zones of ipsilateral brain regions of the tissue sections obtained from at least three animals per group.

Immunoblot Analysis

In order to study the expression of various proteins in tissue lysates of untreated and hUCBSCs-treated ischemic brains and the brain tissues of sham controls, immunoblot analysis was performed as described earlier [15]. Briefly, appropriate portions of the brain tissues of sacrificed rats were suspended in 0.2 mL of homogenization buffer, homogenized using a Tissue Tearor (Biospec Products, Inc.), and followed by sonication. Tissue homogenate was centrifuged at 15,000$\times g$ for 30 min at 4 °C and the protein levels in the supernatant were determined using the BCA assay (Pierce, Rockford, IL). Samples [equal amount (30–50 μg) of total protein/well] were subjected to 12–14 % SDS-PAGE based on the specifications of the protein, and the protein bands on the gel were transferred onto nitrocellulose membranes. The membranes were processed with primary antibodies followed by appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using chemiluminescence ECL Western blotting detection reagents on Hyperfilm-MP autoradiography film (Amersham, Piscataway, NJ). Immunoblots were re-probed and processed with GAPDH antibody to verify that similar amounts of protein were loaded in all lanes.

Statistical Analysis

Apoptotic gene analysis data obtained in the form of Ct values was analyzed by using web-based analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Changes in gene expression were shown as a fold increased or decreased. The cut-off induction determining expression was ±2.0 fold-changes, and the genes fitting these criteria were considered to be upregulated or downregulated. Statistical comparisons for cleaved-caspase-3 immunofluorescence were performed using Graph Pad Prism software (version 3.02). These data were evaluated for statistical significance using one-way ANOVA. Bonferroni's post hoc test (multiple comparison tests) was used to compare any statistical significance among the groups. Differences in the values were considered significant at $p < 0.05$.

Results

Stem Cell Treatment Reduces Brain Damage After Acute Focal Cerebral Ischemia

Recently, we demonstrated that ischemia-induced apoptotic cell death results in significant brain injury in the ipsilateral hemisphere of rats after MCAO procedure followed by reperfusion [15]. In the present study, TUNEL analysis of the coronal brain sections of rats subjected to a 2 h MCAO procedure followed by 7 days of reperfusion demonstrated a prominent TUNEL staining in both the ischemic core and the penumbra regions of cortex and striatum (Fig. 1). Intravenous administration of hUCBSCs 24 h post-MCAO procedure prominently reduced the intensity of TUNEL staining and the number of TUNEL-positive cells in both the ischemic and the penumbra regions (Fig. 1). The reduction of apoptosis as evidenced by TUNEL staining after hUCBSCs treatment was not dose-dependent, i.e., the lowest dose of hUCBSCs administered (0.25×10^6 cells) produced a similar response to that offered by the highest dose of hUCBSCs (1×10^6 cells) utilized in the present study. TUNEL-positive cells were absent in the contralateral brain regions of rats subjected to various treatment conditions. Cells in the cortex and striatal regions of ipsilateral hemisphere of hUCBSCs-treated animals showed cleaved caspase-3 protein expression (Fig. 2a). The percent of cleaved caspase-3 positive cells in the ipsilateral brain regions of hUCBSCs-treated animals was significantly ($p < 0.05$) reduced compared to untreated animals (Fig. 2b). Similar to what we noticed in TUNEL staining, there was no significant difference in the percent of cleaved caspase-3 positive cells between the two doses of hUCBSCs tested in the present study.

In addition, H&E staining performed on the paraffin-embedded coronal brain sections from all groups of rats revealed a prominent reduction in the structural brain damage in the ipsilateral regions of hUCBSCs-treated animals compared to untreated animals (Fig. 2c). Magnified images of the cortex and striatal regions of the ischemic core showed absence of interstitial edema and a very few pyknotic nuclei in hUCBSCs-treated animals compared to untreated animals. The extent of interstitial edema

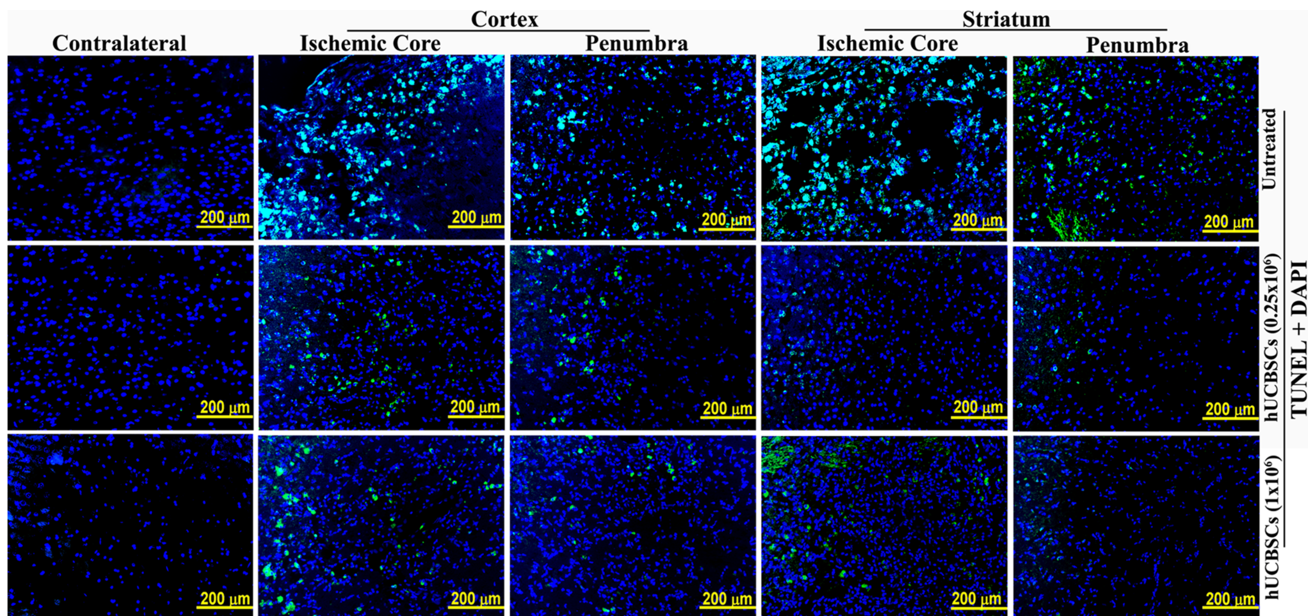


Fig. 1 Apoptosis after MCAO procedure in rats subjected to stem cell treatment. TUNEL assay on the paraffin-embedded coronal brain sections of rats from various groups sacrificed on the seventh day post-MCAO procedure. Green fluorescence in the ischemic core and the penumbra regions of the cortex and striatum of ipsilateral rat brains indicates TUNEL-positive cells. Blue fluorescence indicates DAPI staining of the nuclei. n $\geq$ 3. Representative images shown are the merged images of TUNEL staining and respective DAPI staining. *Scale bar* 200 µm

and the number of pyknotic nuclei were prominently reduced in hUCBSCs-treated animals at both the dose levels tested in the present study.

Regulation of Apoptotic Molecules After Stem Cell Treatment

Our recent study clearly demonstrated the upregulation of several apoptotic pathway molecules after focal cerebral ischemia in rats [15]. In the present study, hUCBSCs treatment 24 h post-MCAO procedure to rats reverted the mRNA expression profile of a majority of the apoptotic molecules to basal levels (Fig. 3). Even the minimum dose of hUCBSCs utilized in the present study (0.25×10^6 cells/animal) was sufficient to revert the upregulated apoptotic pathway molecules in response to focal cerebral ischemia to basal levels. The mRNA expression profile of various apoptotic pathway molecules in animals after hUCBSCs treatment at both of the dose levels tested in the present study was comparable to sham-operated animals (Fig. 3). Several fold downregulation of various genes involved in the induction or inhibition of apoptosis in animals after hUCBSCs treatment compared to untreated MCAO-subjected animals is listed in Tables 1 and 2, respectively. Similarly, the fold downregulation of genes that can either induce or inhibit apoptosis upon hUCBSCs treatment is listed in Table 2. Almost all of the apoptotic pathway molecules that were affected after focal cerebral ischemia were corrected with the minimum dose of hUCBSCs tested in the present study (Tables 1, 2).

Immunoblot analysis of the key apoptotic molecules such as Fas, TNFα, AIF and Smac and the anti-apoptotic molecule XIAP revealed a prominent reduction in protein expression after hUCBSCs treatment (Fig. 4). The protein expression of all these molecules, except Smac, was prominently reduced at the minimum dose of hUCBSCs tested in the present study. Smac protein expression was reduced at the highest dose of hUCBSCs (1×10^6 cells/animal) tested in the study. The reduction in the expression profile of these apoptotic pathway molecules at both mRNA and protein levels was in agreement with the reduction of the extent of apoptosis upon hUCBSCs treatment described in the earlier section.

Discussion

Compelling evidence suggests that regulating apoptosis in both the ischemic core and the penumbra after cerebral ischemia can provide an opportunity to reverse the cell death and neurovascular integrity [16–18]. TUNEL staining performed in this study clearly demonstrated the reduction of apoptotic cell death after intravenous hUCBSCs treatment in both the ischemic core and the penumbra. These results are in agreement with the earlier reported data where authors demonstrated a significant

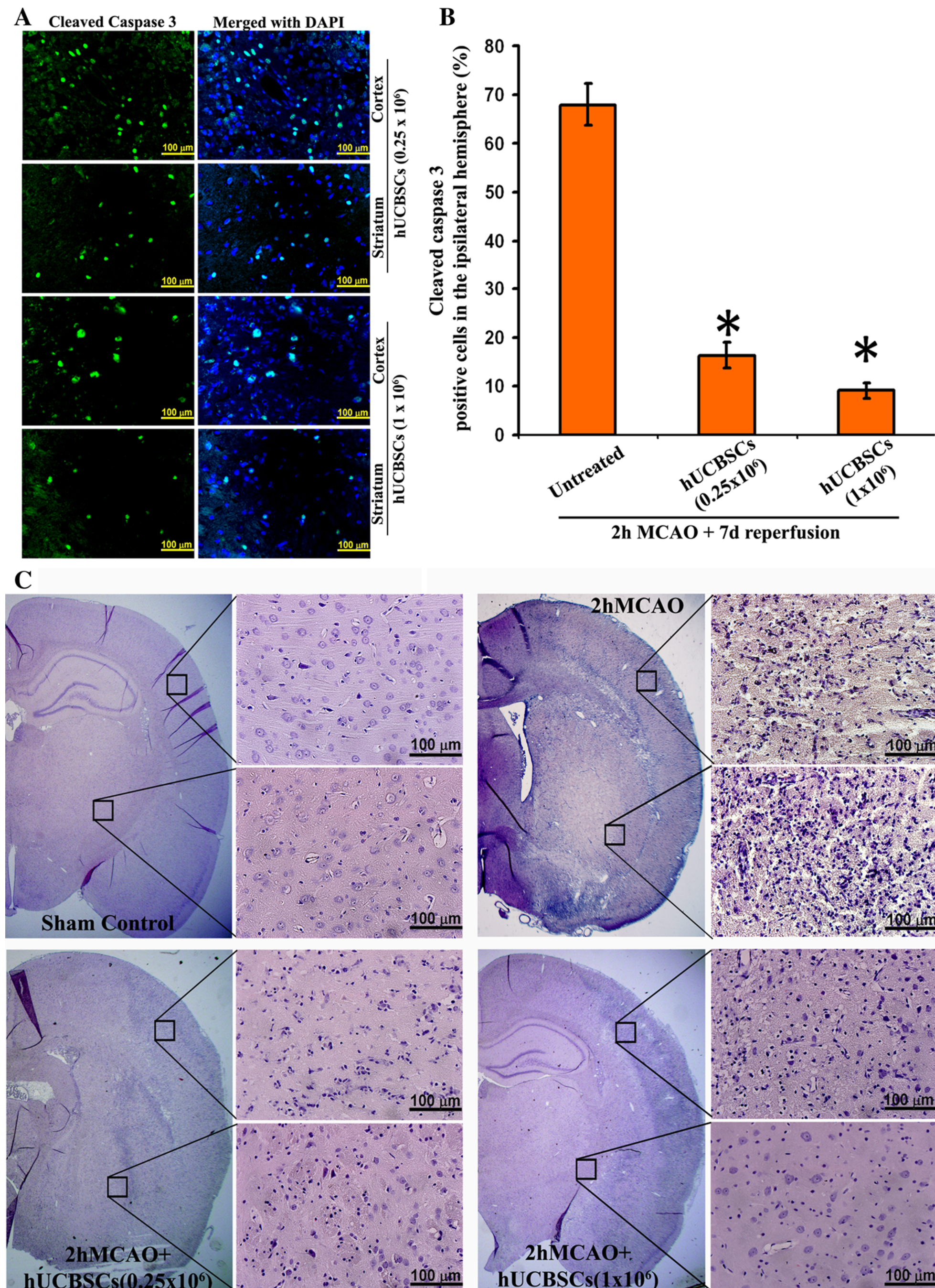
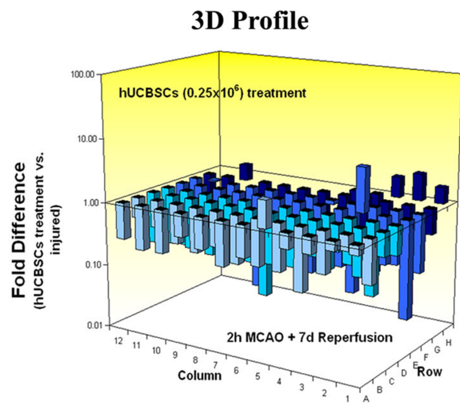


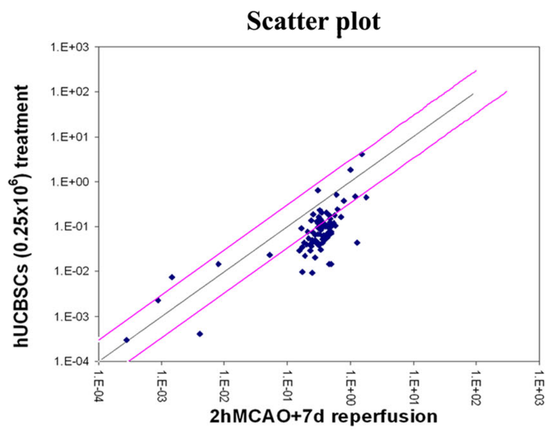
Fig. 2 Stem cell treatment after MCAO procedure reduces caspase-dependent apoptosis and brain damage. **a** Green fluorescence indicates cleaved caspase 3 protein expression. Representative cleaved caspase-3 images were merged with respective DAPI images. *Scale bar* 100 µm. **b** Quantification of cleaved caspase-3 protein expression in the ipsilateral hemisphere of untreated [15] and hUCBSCs-treated animals. $n \geq 3$. Values are expressed as mean ± SEM; $*p < 0.05$ compared to untreated MCAO subjected animals. **c** Representative hematoxylin and eosin stained paraffin-embedded tissue sections from rat brains. Higher magnification images from the ischemic cortex and striatal regions of MCAO-subjected and untreated animals show interstitial edema and damaged neurons that have a condensed, irregular shaped and darkly stained nuclei which are absent or less frequent in control/hUCBSCs-treated brain sections. Each group consisted of a minimum of three animals. *Scale bar* value for the magnified images = 100 µm

reduction in the numbers of TUNEL-positive cells 28 days after intraventricular transplantation of hUCBSCs in MCAO-subjected rats [13, 17]. Another group of researchers recently reported that the intravenous administration of human umbilical cord blood cells did not reduce caspase-dependent cell death in premorbid rats [11, 17]. These authors injected 8×10^6 cells/kg body weight to spontaneously hypertensive rats 24 h after permanent thermo-occlusion of the right middle cerebral artery. Conversely, in the present study, 24 h after reperfusion of

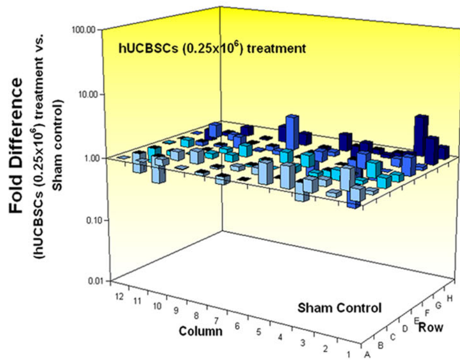
Fig. 3 Stem cell treatment after cerebral ischemia alters the gene expression profile of apoptotic molecules. PCR microarray analysis of rat apoptotic genes from the cDNAs obtained from the ischemic region of hUCBSCs-treated, MCAO-subjected rats. 3D profile graphs show the fold difference in the expression of each gene in MCAO-subjected and hUCBSCs-treated ischemic rat brain samples versus untreated MCAO subjected/sham controls [15]. Columns pointing up (with z-axis values >1) indicate upregulation of gene expression, and columns pointing down (with z-axis values <1) indicate downregulation of gene expression. Corresponding scatter plots show the validity of the experiment and the expression level of each gene in hUCBSCs-treated versus untreated/sham control samples. $n \geq 3$

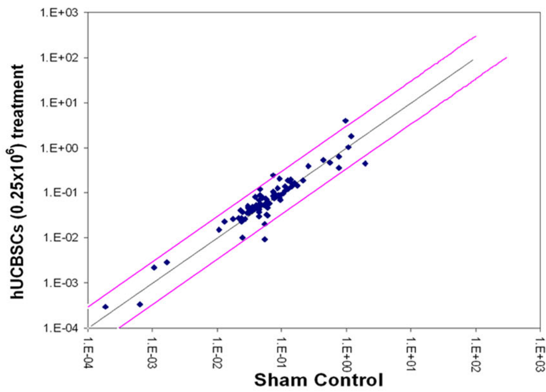
3D Profile **Scatter plot**



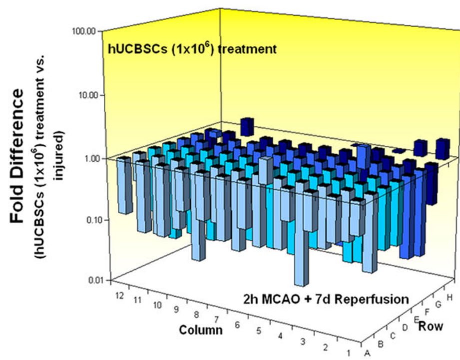


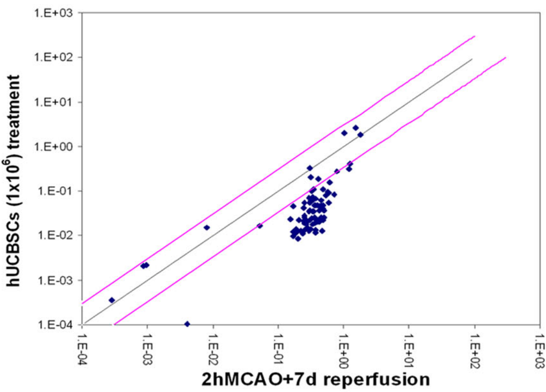
hUCBSCs (0.25x10⁶) treatment vs. 2hMCAO+7d reperfusion



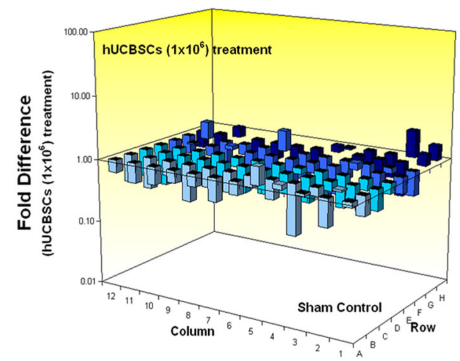


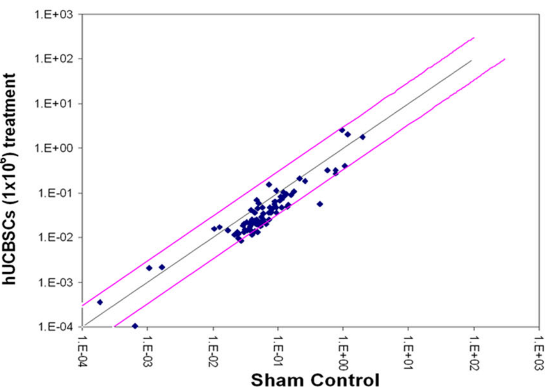
hUCBSCs (0.25x10⁶) treatment vs. Sham control





hUCBSCs (1x10⁶) treatment vs. 2hMCAO+7d reperfusion





hUCBSCs (1x10⁶) treatment vs. Sham control

Table 1 Effect of hUCBSCs treatment on the expression of genes involved in the positive regulation of apoptosis

Gene	Fold up- or down-regulation	
	hUCBSCs (0.25×10^6) treatment versus ischemic injury	hUCBSCs (1×10^6) treatment versus ischemic injury
Bad	**−3.15**	**−5.53**
Bak1	**−4.83**	**−13.43**
Bax	**−4.95**	**−13.40**
Bcl2l11 (Bim)	**−9.75**	**−38.69**
Bcl10	**−3.76**	**−7.70**
Bid	**−5.36**	**−13.21**
Bik	**−5.67**	**−18.05**
Bok	**−2.28**	**−8.38**
Caspase 1 (Ice)	**−2.56**	**−7.23**
Caspase 12	**−3.52**	**−12.88**
Caspase 14	**−6.01**	**−19.04**
Caspase 2	**−6.29**	**−17.76**
Caspase 3	**−6.67**	**−15.93**
Caspase 4	**−3.50**	**−9.70**
Caspase 6	**−6.89**	**−21.52**
Caspase 7	**−6.24**	**−19.75**
Caspase 8	**−5.85**	**−27.55**
Caspase 8ap2	**−6.47**	**−11.16**
Cd40	**−13.40**	**−13.49**
Cidea	**−4.20**	**−13.31**
Cideb	**−4.05**	**−17.60**
Dapk1	**−6.15**	**−17.93**
Dffa	**−8.42**	**−16.19**
Dffb (Cad)	**−7.86**	**−20.88**
Diablo (Smac)	**−4.12**	**−9.47**
Fadd	**−33.93**	**−19.17**
Fasl (CD95-L)	**−8.03**	**−19.26**
Gadd45a	**−3.21**	**−5.93**
Hrk	**−3.91**	**−8.48**
Lta (Tnfb)	**−30.87**	**−19.26**
Ltbr	**−1.98**	**−5.45**
Mapk1 (Erk2)	**−3.98**	**−5.30**
Pycard (Tms1/Asc)	**−2.52**	**−3.94**
Ripk2	**−6.25**	**−10.01**
Tnfrsf11b	**−6.24**	**−12.33**
Tnfrsf1a (Tnfr1)	**−2.52**	**−4.26**
Tnfrsf1b (Tnfr2)	**−6.97**	**−31.37**
Tnfsf10 (Trail)	**−6.59**	**−19.26**
Tnfsf12	**−4.47**	**−8.32**
Tp53 (p53)	**−2.24**	**−3.16**
Tp63	**−6.43**	**−22.90**
Tradd	**−7.28**	**−13.62**
Traf3	**−7.40**	**−20.98**

Fold up- or down-regulation values which are greater or <2 are indicated in bold

Table 2 Effect of hUCBSCs treatment on the expression of genes involved in the inhibition of apoptosis and those involved in either induction or inhibition of apoptosis

Gene	Fold up- or down-regulation	
	hUCBSCs (0.25×10^6) treatment versus ischemic injury	hUCBSCs (1×10^6) treatment versus ischemic injury
Involved in the inhibition of apoptosis		
Annexin A5 (Anxa5)	−1.74	**−3.10**
Api5	**−3.16**	**−5.49**
Aven	**−4.97**	**−9.76**
Bcl2	**−4.75**	**−14.06**
Bcl2a1d	−1.74	**−5.81**
Bcl2l1 (Bcl-x)	**−4.45**	**−8.78**
Bcl2l2	**−5.60**	**−12.27**
Birc2 (c-IAP2)	**−3.59**	**−7.62**
Birc3 (c-IAP1)	**−5.08**	**−36.19**
Birc4 (XIAP)	**−5.20**	**−6.52**
Birc5 (Survivin)	**−4.24**	**−13.06**
Bnip2	**−2.66**	**−18.64**
Card10	**−5.36**	**−15.28**
Cd40lg (Tnfsf5)	**−5.08**	**−23.54**
Faim	**−5.60**	**−6.44**
Il10	**−8.25**	**−14.23**
Mcl1	**−3.83**	**−9.81**
Naip2	**−8.06**	**−22.33**
Nol3	**−11.12**	**−9.85**
Polb	**−27.06**	**−8.74**
Prlr	**−17.28**	**−17.97**
Sphk2	**−4.41**	**−6.53**
Involved in either induction or inhibition of apoptosis		
Akt1	−1.79	**−3.75**
Cflar (Casper) (Flip)	**−7.08**	**−11.53**
NF-kB (Nfkb1) (p50)	**−7.02**	**−9.79**
Tnf	**−6.04**	**−20.08**
Tp73	**−5.89**	**−18.95**

Fold up- or down-regulation values which are greater or <2 are indicated in bold

transient (2 h) right MCAO, intravenous administration of 0.25×10^6 hUCBSCs/animal to normal Sprague–Dawley rats significantly reduced the number of cleaved caspase-3 positive cells in the ipsilateral hemisphere (Fig. 2b). The variation noticed in these results could be attributed to the differences in animal strain, occlusion method, occlusion time, reperfusion time and/or the type of cells utilized. Intra-arterial administration of hUCBSCs (1×10^6 cells/animal) to adult beagle dogs 1 day after occlusion of the middle cerebral artery by injecting thrombus emboli reduced the infarct volume [10]. Another group of

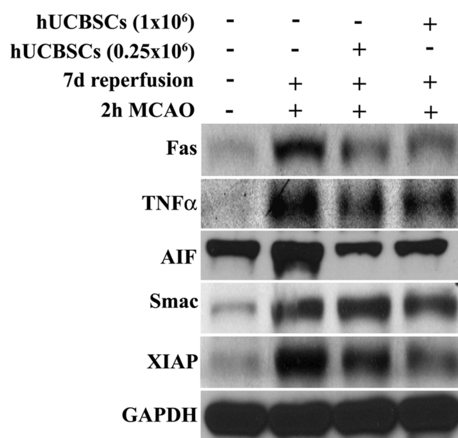


Fig. 4 Immunoblots depict the protein expression profile of apoptotic and anti-apoptotic molecules. Focal cerebral ischemia increased protein expression of all these molecules. Stem cell treatment reduced the increased expression of these molecules. $n \geq 3$. GAPDH was used as a loading control

researchers also reported that intravenous administration of human umbilical cord blood cells to rats 24 h after transient (2 h) MCAO improved functional recovery and neuronal sparing [8, 9]. All of these authors reported that the administered cells entered the brain, survived and migrated and trans differentiated to various neuronal cells. We could attribute a significant part of the neuroprotection offered by stem cells in these studies and in the present study to the inhibition of apoptosis. Other possible mechanisms of neuroprotection offered by hUCBSCs which were reported earlier by our research team include trans differentiation to neurons and oligodendrocyte, increased expression of myelin basic protein, remyelination of the demyelinated/hypomyelinated axons, altered mRNA and protein expression of several matrix metalloproteinases and other apoptotic pathway molecules, etc. [7, 19–21].

Both extrinsic and intrinsic (mitochondrial pathway) apoptotic pathway molecules that were upregulated after transient right MCAO in rats were reverted to normal levels after hUCBSCs treatment (Fig. 3; Tables 1, 2). Bax, Bad, and Bid which cause the release of cytochrome *C*, the main trigger for mitochondrial-mediated apoptosis, were downregulated at the lowest dose of hUCBSCs tested in the present study (Table 1). As expected, caspase-3, the downstream of cytochrome *C*, mRNA levels also reverted to normal at the lowest dose of hUCBSCs tested. Similar to cytochrome *C*, the mRNA levels of Smac/Diablo, an apoptotic molecule released from the mitochondria, were also reduced after treatment with hUCBSCs. Smac inhibits XIAP, an anti-apoptotic molecule that inhibits caspase-3. Although the mRNA levels of Smac were reverted to normal at the lowest dose of hUCBSCs tested, the protein expression of Smac was prominently reduced only at the highest dose of hUCBSCs tested in the present study (Table 1; Fig. 4). The numbers of cleaved caspase-3 positive cells were also significantly reduced in the ipsilateral brain regions after hUCBSCs treatment (Fig. 2b). Interestingly, the highest dose of hUCBSCs tested in the present study further downregulated Bax, Bad, Bid, and caspase-3 mRNA levels to below normal levels (Table 1). In addition to caspase-3 mRNA levels, hUCBSCs treatment also downregulated other caspases including caspase-1, caspase-2, caspase-3, caspase-4, caspase-6, caspase-7, caspase-8, caspase-12, caspase-14, and caspase-8ap2, many of which were upstream to caspase-3. Earlier investigations that utilized caspase inhibitors such as zVADfmk, BAF, zDEVDfmk, IETDfmk, and YVADcmk have been demonstrated to reduce the number of apoptotic cells or to be neuroprotective in animal models of focal and global cerebral ischemia [22–27]. Similarly, recent investigations that utilized hUCBSCs in animal models of cerebral ischemia have been reported to reduce the infarct size and improve the neurological recovery [10, 12, 13]. Therefore, the present study that utilized hUCBSCs in a rat model of focal cerebral ischemia and demonstrated the inhibition of apoptosis and downregulation of various apoptotic pathway molecules further strengthens these earlier reports by providing possible mechanisms that would have been responsible for the reported neuroprotection offered by hUCBSCs. In addition to caspases downregulation, treatment with hUCBSCs prominently reduced the elevated protein expression of AIF, the key molecule involved the non-caspase-mediated induction of apoptosis by intrinsic apoptotic pathway (Fig. 4).

In addition to affecting the intrinsic apoptotic pathway molecules, hUCBSCs treatment in the present study also regulated the Fas/TNF family death receptor-mediated extrinsic apoptotic pathway molecules such as Fasl, Fas, FADD, TNFα, TNFR1, TRADD, etc. All the extrinsic apoptotic pathway molecules in addition to those listed earlier were downregulated after treatment with hUCBSCs (Table 1). The protein expressions of Fas and TNFα were also prominently reduced after hUCBSCs treatment (Fig. 4). The death domains of FADD and TRADD proteins interact with the death effector domains of procaspase-8, cleaving it, and in turn, activating downstream effector caspase and apoptosis [28, 29]. As mentioned earlier, stem cell treatment downregulated many of the elevated caspase and reduced the cleaved-caspase-3 protein expression irrespective of the involvement of either extrinsic or intrinsic apoptotic pathway. The lowest dose of hUCBSCs utilized in the present study was sufficient to revert these molecules to normal levels from their elevated levels after focal cerebral ischemia [15]. Contrary to our expectation, hUCBSCs treatment downregulated the gene expression levels of various anti-

apoptotic molecules including Annexin A5, Bcl2, cIAP1, cIAP2, XIAP, Naip2, and survivin, among others, which were upregulated in the ipsilateral brain regions after focal cerebral ischemia (Table 2). The protein expression of XIAP was also prominently reduced after hUCBSCs treatment (Fig. 4). Downregulation of anti-apoptotic molecules after hUCBSCs treatment in the present study could be due to the effect of endogenous mechanisms in response to reduced gene and protein expression of apoptotic molecules. Because of the apoptotic cross-talk among several pathways, inhibition of one apoptotic pathway may augment an alternative one. Interestingly, our treatment strategy that utilized hUCBSCs inhibited apoptosis by regulating the expression of several molecules which could have been contributed to apoptosis after cerebral ischemia.

In summary, the present study provided an in-depth understanding of the molecular mechanisms underlying the neuroprotective effects of mesenchymal stem cells derived from human umbilical cord blood in a rat model of transient focal cerebral ischemia. Our study clearly demonstrated the potential of hUCBSCs to regulate various molecules responsible for cell death after transient focal cerebral ischemia followed by reperfusion. Based on these results, it appears that administration of hUCBSCs after ischemic stroke could offer a therapeutic advantage over other treatment strategies that target one or two molecules/mechanisms. The survival and transdifferentiation of these hUCBSCs to various neuronal cells in the ischemic brain regions after their administration to animals has already been reported. However, we still need to investigate the beneficial and toxic effects of single and multiple doses of hUCBSCs by intravenous route administered at various time points after focal cerebral ischemia on the long-term neurological outcome.

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