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Original Paper

Mesenchymal Stem Cell Treatment Prevents Post-Stroke Dysregulation of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

Bharath Chelluboina[a] Koteswara Rao Nalamolu[a] Gustavo G. Mendez[a]
Jeffrey D. Klopfenstein[a,b,e] David M. Pinson[c] David Z. Wang[d,e]
Krishna Kumar Veeravalli[a,b,d]

Departments of Cancer Biology and Pharmacology[a], Neurosurgery[b], Pathology[c], and Neurology[d], University of Illinois College of Medicine at Peoria, Peoria, IL, [e]Comprehensive Stroke Center, Illinois Neurological Institute, OSF HealthCare System, Saint Francis Medical Center, Peoria, USA

Key Words
Stem cells • Ischemia • Reperfusion • MMPs • TIMPs • Induction

Abstract
Background/Aims: Stem cell treatment is one of the potential treatment options for ischemic stroke. We recently demonstrated a protective effect of human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) in a rat model of ischemic stroke. The treatment attenuated apoptosis and prevented DNA damage. A collection of published studies, including several from our laboratory, indicated the induction and detrimental role for several matrix metalloproteinases (MMPs) in post-stroke brain injury. We hypothesized that the HUCB-MSCs treatment after focal cerebral ischemia prevents the dysregulation of MMPs and induces the expression of endogenous tissue inhibitors of metalloproteinases (TIMPs) to neutralize the elevated activity of MMPs. ***Methods:*** To test our hypothesis, we administered HUCB-MSCs (0.25 million cells/animal and 1 million cells/animal) intravenously via tail vein to male Sprague-Dawley rats that were subjected to a transient (two-hour) right middle cerebral artery occlusion (MCAO) and one-day reperfusion. Ischemic brain tissues obtained from various groups of rats seven days after reperfusion were subjected to real-time PCR, immunoblot, and immunofluorescence analysis. ***Results:*** HUCB-MSCs treatment prevented the induction of MMPs, which were upregulated in ischemia-induced rats that received no treatment. HUCB-MSCs treatment also prevented the induction of TIMPs expression. The extent of prevention of MMPs and TIMPs induction by HUCB-MSCs treatment is similar at both the doses tested. ***Conclusion:*** Prevention of stroke-induced MMPs upregulation after HUCB-MSCs treatment is not mediated through TIMPs upregulation.

B. Chelluboina and K. R. Nalamolu contributed equally to this work.

Dr. Krishna Kumar Veeravalli
Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, One Illini Dr., Peoria, IL 61605 (USA)
Tel. +1 (309) 671-8442, Fax +1 (309) 671-8403, E-Mail krishnav@uic.edu

Introduction

Stroke is currently the fifth leading cause of death in the United States of America. Moreover, it is the biggest reason for serious long-term disability. Several studies suggests that elevated matrix metalloproteinases (MMPs) play a detrimental role in various types of CNS pathology, including ischemic stroke [1-3]. Our recent studies demonstrated the predominant upregulation of MMP-12 and its pathological role in acute brain damage after ischemic stroke [4, 5]. The temporal expression profile of various MMPs in ischemic rat brains after focal cerebral ischemia and reperfusion revealed that MMP-9 and MMP-12 were upregulated (16-35 fold in case of MMP-9 and 47-265 fold in case of MMP-12 through days 1 to 7 after reperfusion) several fold higher than any other MMPs tested [4]. Evidence also suggests the detrimental role of MMP-9 and MMP-12 on post-stroke brain damage [4-8]. In the recent past, we demonstrated the prevention of MMPs induction in human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) treated spinal cord injured rats [9].

Stem cell therapy is emerging as therapeutic tool for ischemic stroke. It is not only effective for prevention of the ongoing damage but also beneficial in facilitating the functional recovery. Our recent studies demonstrated the effect of HUCB-MSCs treatment to attenuate the ischemic brain damage and prevent the induction of apoptosis [10, 11]. Evidence also suggests that the HUCB-MSCs treatment after cerebral ischemia mitigates of ischemic brain damage and facilitates neurological recovery [10, 12-14]. Based on the literature and the preliminary results from our laboratory, we hypothesized that the HUCB-MSCs treatment after focal cerebral ischemia prevents the dysregulation of MMPs. Tissue inhibitors of metalloproteinases (TIMPs) are a group of endogenous proteins that regulate the activity of MMPs by binding to the active and alternative sites of activated MMPs [15]. We also hypothesized that the HUCB-MSCs treatment induces the expression of TIMPs to neutralize the stroke-induced increase in MMPs activity. To test our hypothesis, we administered HUCB-MSCs to rats that were subjected to a two-hour focal cerebral ischemia and one-day reperfusion. To our knowledge, this is the first study that investigates the potential of HUCB-MSCs treatment on the expression of various MMPs and TIMPs in the context of ischemic stroke.

Materials and Methods

Ethics statement

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois College of Medicine Peoria approved the study design, surgical manipulations, post-operative care and humane endpoints.

Animals

Healthy, young adult, male Sprague-Dawley rats were used in this study. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the approved IACUC protocol. Animals weighing 230–250 g were procured from Envigo (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 various groups. Group I animals served as sham controls. Animals of groups 2, 3 and 4 were subjected to the middle cerebral artery occlusion (MCAO) procedure. Group 2 animals did not receive any further treatments after the MCAO procedure. Group 3 and Group 4 animals were intravenously injected with $0.25x10^6$ and $1x10^6$ HUCB-MSCs, respectively, suspended in 0.5 ml sterile saline via tail vein 24 hours post-MCAO procedure. All animals from the four groups were euthanized seven days post-MCAO procedure.

Experimental MCAO model

After the animals reached a weight of 260±5 g, they were subjected to right MCAO procedure by using a silicone rubber coated monofilament suture (Doccol Corporation, California) as described earlier by

our group [16]. 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 (ECA) was permanently ligated rostral with one ligature. Another loose ligature was made to the ECA near the bifurcation. Two microaneurysm clips were applied each to the common carotid artery (CCA) and internal carotid artery (ICA). A small puncture opening was made to the ECA between the two ligatures. The monofilament was inserted through the opening on ECA, and advanced into ICA. The microaneurysm clip was removed from ICA, and the monofilament was gently further advanced into ICA up to 19 to 20 mm distance that is pre-marked on the monofilament. The other loose ligature was tightened around the ECA containing the monofilament. Microaneurysm clip on CCA was removed, and skin on the neck incision was closed with surgical wound clips. To restore the blood flow 2 hours after MCA occlusion, the surgical site was re-opened by removing the wound clips. The microaneurysm clip was again applied to CCA, the knot was loosened, the monofilament was withdrawn, and the knot was re-tied to stop bleeding. Microaneurysm clip on CCA was removed and skin was sutured to close the neck incision.

Stem cell treatment

Cryo-preserved HUCB-MSCs obtained from Vitro Biopharma (Golden, CO) were used to establish cultures in MSC-GRO low serum complete MSC medium according to the manufacturer's 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 PBS, counted and suspended in sterile saline prior to intravenous administration. HUCB-MSCs obtained from subcultures of less than eight passages were administered to appropriate groups of animals.

Group 3 and 4 animals were intravenously injected with $0.25x10^6$ and $1x10^6$ HUCB-MSCs via tail vein, respectively, 24 hours post-reperfusion. The doses of stem cells utilized in this study were demonstrated to attenuate post-stroke brain damage in the same rodent model of focal cerebral ischemia and reperfusion [16]. MCAO subjected rats from all the groups that did not show any neurological/behavioral symptoms were excluded from the study. All animals subjected to the MCAO procedure were treated with an appropriate dose of analgesic (buprenorphine) and antibiotic (cefazolin), as mentioned in the approved IACUC protocol. On the seventh day post-MCAO procedure, all animals were euthanized. The ischemic brain tissues obtained from these animals were utilized for histology, gene and protein expression profiles.

Brain tissue fixation and sectioning

On the seventh day post-MCAO procedure, untreated, ischemia-induced animals 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.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) from the ipsilateral brains of rats from various groups euthanized on the seventh day post-MCAO procedure as described earlier [10]. 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 RT2 Profiler PCR array.

Real-time PCR analysis

The forward and reverse primer sequences of rat MMPs and TIMPs used in this study have reported earlier by our group [9]. Reaction set-up for each cDNA sample was assembled using the FastStart SYBR Green Master (Roche, IN) as per the manufacturer's instructions. Samples were subjected to forty cycles at 95°C for fifteen seconds and 60°C for one min in iCycler IQ (Multi Color Real-Time PCR Detection System, Bio-Rad Laboratories, CA). Data were collected and recorded using the iCycler IQ software (Bio-Rad Laboratories, CA) and expressed as a function of the threshold cycle (Ct), which represents the number of cycles at which the fluorescent intensity of the SYBR Green dye is significantly above than that of the background fluorescence. The housekeeping gene, βactin was used for normalization of MMPs and TIMPs

expression. Average Ct values were normalized with average Ct values of βactin. After normalization of the Ct values, fold differences were calculated by using the formula 2^-(ΔCt of Test)/2^-(ΔCt of controls).

Immunofluorescence analysis

Immunofluorescence analysis was used to identify the changes in the expression of MMP-9 protein in untreated, ischemia-induced animals euthanized on the seventh day after reperfusion. Paraffin-embedded brain sections of various groups of animals were de-paraffinized, subjected to antigen retrieval, permeabilized, processed with anti-MMP-9 primary antibody followed by Alexa Fluor® 594 (goat anti-mouse IgG, red) fluorescent-labeled secondary antibody, counterstained with DAPI, cover slipped, and observed using a confocal microscope (Olympus Fluoview).

Immunoblot analysis

To study the expression of various proteins in tissue lysates of untreated and HUCB-MSCs-treated ischemic brains and the respective brain tissues of sham animals, immunoblot analysis was performed as described earlier [16]. 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

Statistical comparisons were performed using Graph Pad Prism software (version 3.02). Protein expression pattern of various molecules obtained from immunoblots was quantified by densitometry analysis (using Image J analysis software, NIH), and 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

Post-stroke induction of MMP-9 and MMP-12

Based on the data we recently published, we prepared a Venn diagram depicting various MMPs that were upregulated more than 5 fold at various reperfusion time points [4]. MMP-9 and MMP-12 are the only MMPs that were upregulated at all the reperfusion time points tested (Fig. 1). Their upregulation at mRNA level is more than 15 fold at any of the reperfusion time points [4]. Immunofluorescence analysis depicting MMP-12 protein upregulation in the ischemic brain of rats subjected to a two-hour ischemia followed by seven days reperfusion was recently reported by our group [4]. In this study, we performed immunofluorescence analysis of MMP-9 on the coronal brain sections of rats subjected to a two-hour ischemia followed by seven days reperfusion. As expected, MMP-9 protein was predominantly expressed in the ipsilateral brain hemisphere as compared to the contralateral hemisphere (Fig. 2). MMP-9 protein expression was negligible or absent in the contralateral brain hemispheres of ischemia- and reperfusion-induced rats.

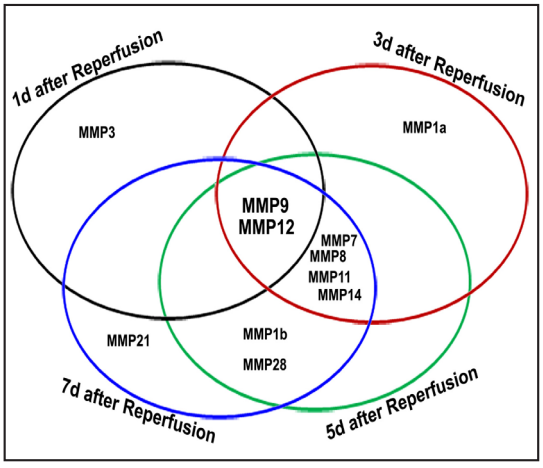

Fig. 1. Induction of MMPs after ischemic stroke. Venn diagram depicting a list of MMPs, whose mRNA expression in ischemic brain was upregulated >5 fold during reperfusion [4]. Of all the MMPs, MMP-9 and MMP-12 mRNA expression is increased more than 15 fold at all the reperfusion time points tested.

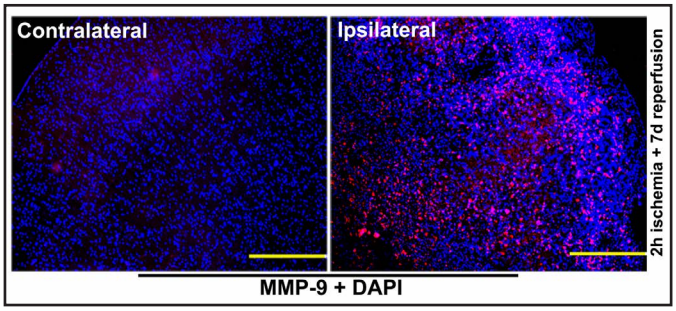

Fig. 2. Immunofluorescence analysis depicting MMP-9 expression in coronal brain sections of rats subjected to a two-hour ischemia followed by seven days reperfusion. Nuclei were stained with DAPI (blue). Each image is the representative of at least six confocal images obtained from six different animals. Scale bar = 200 µm.

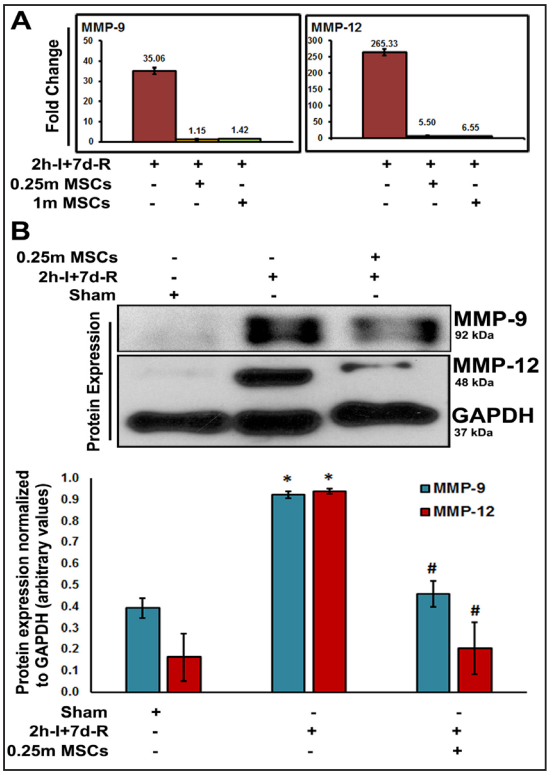

Fig. 3. Effect of stem cell treatment after ischemic stroke on MMP-9 and MMP-12 expression. (A) Bar graphs depicting the real-time PCR data (as fold change vs sham) of MMP-9 and MMP-12 in ischemic brains of untreated [4] and HUCB-MSCs-treated (intravenous administration of 0.25 million cells/animal or 1 million cells/animal, via tail vein one day after reperfusion), ischemia-induced rats. n=6. Error bars indicate SEM. I-ischemia; R-reperfusion. (B) Representative immunoblots depicting the protein expression of MMP-9 or MMP-12 in ischemic brains of sham and untreated or HUCB-MSCs-treated (intravenous administration of 0.25 million cells/animal via tail vein one day after reperfusion), ischemia-induced rats. GAPDH served as a loading control. Bar graph represents the densitometry analysis of immunoblots. n=6. Error bars indicate SEM. *p<0.001 vs. sham; #p<0.001 vs untreated 2h-I+7d-R.

Stem cell treatment prevented the upregulation of MMPs

Intravenous administration of HUCB-MSCs one day after reperfusion, almost completely prevented the upregulation of all MMPs (MMP-7, -8, -9, -11, -12, -14, -21, and -28), which have been found upregulated in the absence of any treatment (Figs. 3A and 4). The extent of effect noticed at the lowest dose (0.25 million cells/animal) of HUCB-MSCs tested was similar to the effect noticed at the highest dose (1 million cells/animal). Therefore, evaluation of the protein expression of MMP-9 and MMP-12 by immunoblot analysis was restricted to the lowest dose of HUCB-MSCs. Immunoblot analysis revealed a predominant upregulation of both MMP-9 and MMP-12 in ischemia- and reperfusion-induced ipsilateral rat brains compared to their expression in the respective brain tissue of sham rats (Fig. 3B). Further, the increase in MMP-9 and MMP-12 protein expression after focal cerebral ischemia followed by reperfusion compared to their expression in sham operated controls was significant ($p<0.001$ for both MMP-9 and MMP-12) as compared to their expression in sham rats. Treatment with HUCB-MSCs one day after reperfusion almost completely prevented the induction of MMP-9 and MMP-12 protein expression (Fig. 3B). The protein expression of MMP-9 and MMP-12 in HUCB-MSCs treated rats was not significantly different from the expression in sham rats. Next, we tested the effect of HUCB-MSCs treatment on the regulation of MMPs, which have

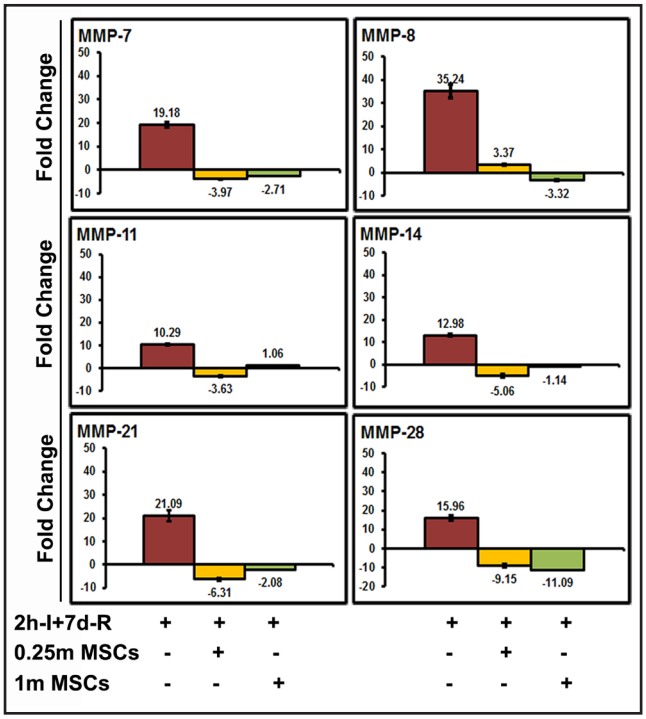

Fig. 4. Real-time PCR data (as fold change vs sham) of MMPs (that were upregulated more than 10 fold on the seventh day after reperfusion with no treatment [4]) in ischemic brains of rats subjected to HUCB-MSCs-treatment. HUCB-MSCs were administered one day after reperfusion intravenously via tail vein at a dose of 0.25 million cells/animal or 1 million cells/animal), to ischemia-induced rats. n=6. Error bars indicate SEM. I-ischemia; R-reperfusion.

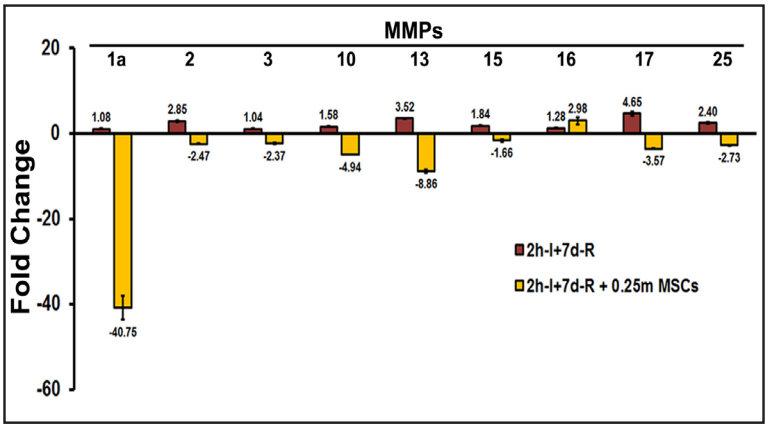

Fig. 5. Real-time PCR data of MMPs (that were unchanged or changed less than 5 fold on the seventh day after reperfusion with no treatment [4]) in ischemic brains of rats subjected to HUCB-MSCs-treatment. HUCB-MSCs were administered one day after reperfusion intravenously via tail vein at a dose of 0.25 million cells/animal), to ischemia-induced rats. n=6. Error bars indicate SEM.

been found unchanged (dysregulated <5 fold) in the absence of any treatment. Except MMP-1a (downregulated ~40 fold) and MMP-13 (downregulated ~9 fold), none of the MMPs (MMP-2, -3, -10, -15, -16, -17 and -25) that were unchanged without any treatment were changed with HUCB-MSCs treatment (Fig. 5).

Regulation of TIMPs with or without treatments after focal cerebral ischemia

TIMPs are the endogenous tissue inhibitors of MMPs. After demonstrating the upregulation of various MMPs after focal cerebral ischemia, we hypothesized a possible upregulation of endogenous TIMPs in the ischemic rat brains as a natural defense mechanism to inhibit the expression of elevated MMPs. As expected, all the TIMPs (TIMP-1, -2, -3, and -4) were upregulated in the ischemic brains of rats subjected to a two hour ischemia and seven days reperfusion as compared to their expression in the respective brain tissue of sham rats. TIMP-1, -2, -3 and -4 at mRNA level were upregulated approximately to 14, 5, 5, and 7 fold, respectively when compared to their expression in sham brains (Fig. 6A). Both the doses of HUCB-MSCs tested in the current study were able to prevent the upregulation of all the four TIMPs. The lowest dose of HUCB-MSCs (0.25 million cells/animal) tested was as effective as

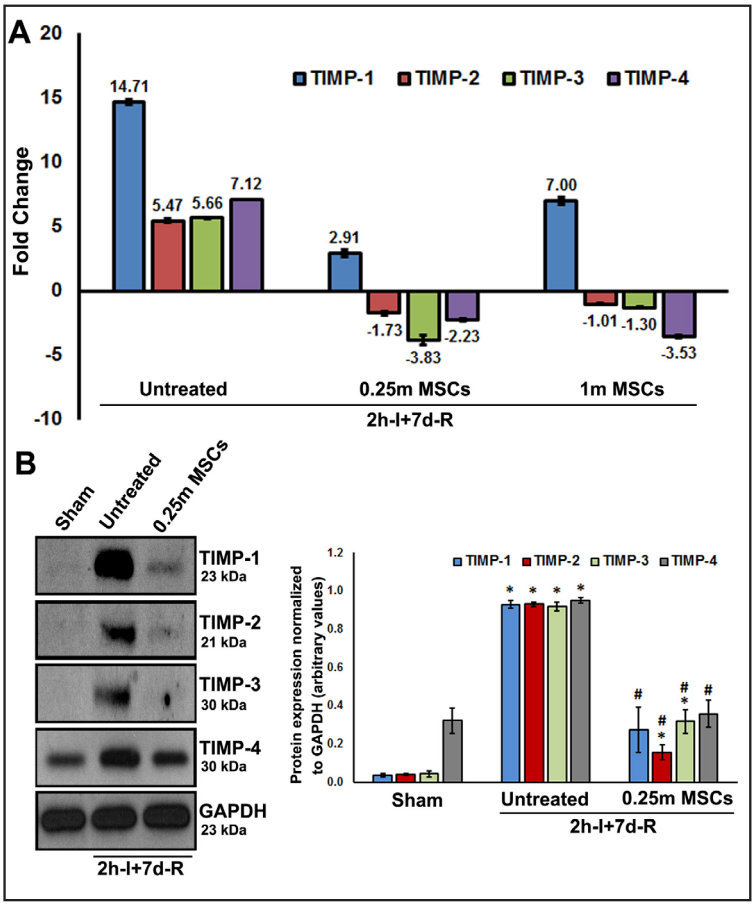

Fig. 6. Effect of stem cell treatment after ischemic stroke on regulation of TIMPs. (A) Bar graph depicting the real-time PCR data (as fold change vs sham) of TIMPs in ischemic brains of untreated and HUCB-MSCs-treated (intravenous administration of 0.25 million cells/animal or 1 million cells/animal, via tail vein one day after reperfusion), ischemia-induced rats. n=6. Error bars indicate SEM. I-ischemia; R-reperfusion. (B) Representative immunoblots depicting the protein expression of various TIMPs in ischemic brains of sham and untreated or HUCB-MSCs-treated (intravenous administration of 0.25 million cells/animal via tail vein one day after reperfusion), ischemia-induced rats. GAPDH served as a loading control. Bar graph represents the densitometry analysis of immunoblots. n=6. Error bars indicate SEM. *p<0.05 vs. sham; #p<0.001 vs untreated 2h-I+7d-R.

the highest dose (1 million cells/animal) tested in preventing the upregulation of TIMPs. In addition, immunoblot analysis revealed a prominent increase in the protein expression of all four TIMPs in ischemic brains of untreated rats subjected to ischemia and reperfusion as compared to their expression in the respective brain tissue of sham rats (Fig. 6B). Further, the increase in protein expression of these TIMPs was statistically significant ($p<0.001$ for all TIMPs). HUCB-MSCs treatment at a dose of 0.25 million cells/animal to ischemia- and reperfusion-induced rats completely prevented the induction of protein expression of TIMPs. There was no statistical difference in the expression of TIMPs between sham-operated rats and ischemia-reperfusion rats treated with HUCB-MSCs.

Discussion

In this study, we demonstrated in a rat model that the stem cell treatment-mediated prevention of post-stroke MMPs dysregulation is not mediated through TIMPs. We also showed the stem cell treatment-mediated prevention of the post-stroke induction of both MMPs and TIMPs.

Astrocytes participate in a variety of homeostatic functions and elicit repair responses against CNS injuries as balance mechanisms. One of the key homeostatic mechanisms of astrocytes in tissue repair is maintained through the production of TIMPs. To date, four homologous TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified [17]. Focal cerebral ischemia and reperfusion induced the expression of all four TIMPs in ischemic rat brains at both the mRNA and protein levels (Fig. 6). Our results are in agreement with the previously reported data wherein the authors reported the induction of TIMP-1 and TIMP-

2 in rat ischemic brain at various reperfusion time points [18]. TIMPs inhibit the function of MMPs, stabilize the extracellular matrix, and reduce BBB disruption and brain edema after cerebral ischemia [18]. Several research groups reported the induction of MMPs after cerebral ischemia [19-23]. In a recent systematic study, our group reported the temporal expression profile of several known MMPs after focal cerebral ischemia until seven days of reperfusion [4].

Increased MMP-9 expression and gelatinolytic activity that was accompanied by increased blood-brain barrier (BBB) disruption, neuronal apoptosis, and ischemic injury was reported in 30-minute focal cerebral ischemia-induced TIMP-1 knockout mice [24]. In agreement with these studies, our recent studies in a rat model of transient focal cerebral ischemia and reperfusion reported the post-ischemic induction of MMP-9 activity through gelatin zymography analysis [4, 5]. TIMP-1 upregulation after stroke in the human infarcted brain tissue was recently reported [25]. Correspondingly, BBB leakage was ameliorated and infarction volume was reduced in TIMP-1 overexpressed mice subjected to a two-hour transient focal cerebral ischemia [26]. TIMP-1 can inhibit a wide range of MMPs, even though it has been described as being particularly potent against MMP-9 [27]. BBB disruption after focal cerebral ischemia was also reported in TIMP-2 knockout mice without any further increase in MMP-9 expression or exacerbation of neuronal loss compared to their wild type mice [24]. A collection of published studies suggests that TIMP-2 inhibits majority of MMPs, especially MMP-2. Significant elevation of serum TIMP-3 and TIMP-4 levels were recently reported in stroke patients [28]. TIMP-3 and TIMP-4 were able to inhibit MMP-9, MMP-14, and TNFα activities and prevent neuronal damage [29, 30]. The roles of TIMP-3 and TIMP-4 in stroke and the TIMP that regulates the activity of MMP-12 are still unclear.

The induction of TIMPs expression after ischemic stroke could be the body's defense mechanism to neutralize the elevated activity of stroke-induced MMPs. The extent of proteolysis in the ischemic brain that contributes to BBB disruption depends on the balance between the proteases and their inhibitors. The induced expression of TIMPs in the ischemic brain interferes with the activity of MMPs and thereby affect the extent and duration of proteolytic damage. After an ischemic stroke, the extent of MMPs-mediated brain injury including the BBB disruption despite the elevated TIMPs expression indicates that the extent of TIMPs upregulation is not sufficient to neutralize the induced MMPs. We initially thought that HUCB-MSCs treatment after ischemic stroke could induce the expression of TIMPs to neutralize the increased proteolytic activity of MMPs. In contrast, ischemia- and reperfusion-induced upregulation of TIMPs was prevented in HUCB-MSCs treated animals (Fig. 6).

Manipulation of either MMP-9 or MMP-12 by neutralizing antibodies or siRNA-/shRNA-mediated gene silencing attenuated the brain damage in rats after ischemic stroke [4-8]. HUCB-MSCs treatment in spinal cord injured rats prevented the dysregulation of various MMPs including MMP-9 and MMP-12 as well as improved their locomotor recovery [9, 31]. Recently, we reported that the treatment with HUCB-MSCs in rats after ischemic stroke prevented apoptosis by preventing the upregulation of apoptotic and DNA damage signaling molecules in ischemic rat brains [10, 11]. As expected, in the current study, HUCB-MSCs treatment after focal cerebral ischemia in rats prevented the dysregulation of various MMPs including MMP-9 and MMP-12 and did not affect the expression of MMPs (except MMP-1a and MMP-13), which were unchanged without any treatment. Prevention of MMPs dysregulation after ischemic stroke could be one of the underlying mechanisms for the reported HUCB-MSCs treatment-mediated attenuation of brain damage and improved neurological recovery [10, 12-14].

Our results clearly indicate the absence of TIMPs involvement in HUCB-MSCs treatment-mediated prevention of MMPs dysregulation after focal cerebral ischemia. The possible reason for the prevention of TIMPs induction after ischemic stroke in HUCB-MSCs treated rats could be the absence of body's defense mechanism due to the prevention of MMPs induction after HUCB-MSCs treatment. In summary, our results demonstrated the induction of TIMPs after focal cerebral ischemia as well as the HUCB-MSCs treatment-mediated prevention of the induction of both MMPs and TIMPs. Based on the results of this study, we hypothesize

that the prevention of the post-stroke induction of MMPs and TIMPs by HUCB-MSCs could facilitate the neurological/functional recovery of stroke-induced rats. Our future studies will address this hypothesis.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

KKV involved in the conception and hypotheses delineation, design and execution of the study, analysis and interpretation of results, and preparation of the manuscript. BC and KRN contributed equally to this work. BC handled animals, performed animal surgeries, conducted PCR, immunoblot, and immunofluorescence analysis, and assisted KKV in study design. KRN involved in animal handling and post-surgical care of few animals, and conduct of immunoblot, PCR, and immunofluorescence analysis, acquisition and quantification of the data and assisted KKV for data analysis and manuscript preparation. GGM assisted BC in the conduct of PCR analysis. All authors reviewed and read the draft and the final manuscripts.

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