Suppression of inflammatory damage to the brain after global cerebral ischemia by transplanted mesenchymal stem cells via secretion of TSG-6

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Abstract

Objective: Numerous studies have shown that bone marrow-derived mesenchymal stem cells (MSCs) enhance neurological recovery after cerebral ischemia. However, the mechanisms are still not clear. The present study aimed to investigate the beneficial effects of MSCs on global cerebral ischemia induced by cardiac arrest (CA) and the underlying mechanisms.

Methods: Rats subjected to asphyxial CA were injected intravenously with MSCs (5×10^6) at 2 hours after resuscitation. Whole brain histopathologic damage scores (HDS) were assessed by histopathology at 3 and 7 days after resuscitation. The distribution of donor MSCs in the brain was evaluated. The expression of tumor necrosis factor-α-induced protein 6 (TSG-6) and pro-inflammatory cytokines in cerebral cortex was assayed. After intravenous infusion of TSG-6 siRNA-MSCs, HDS and pro-inflammatory cytokines were reevaluated at 7 days after resuscitation.

Results: Intravenously administered MSCs significantly reduced whole brain HDS after global cerebral ischemia. Immunofluorescence microscopy revealed that donor MSCs were primarily found in cerebral cortex and expressed TSG-6. MSCs treatment significantly increased the expression of TSG-6 and reduced the expression of pro-inflammatory cytokines in cerebral cortex. In addition, intravenous infusion of TSG-6 siRNA-MSCs failed to attenuate brain inflammation.

Conclusion: Systemically administered MSCs reduced inflammatory damage to brain in rats with global cerebral ischemia via secretion of TSG-6.

INTRODUCTION

Cardiac arrest (CA) is the critically ill condition, and the mortality rate of those who gain return of spontaneous circulation (ROSC) remains high. Despite advances in cardiopulmonary resuscitation (CPR), the overall survival rate for out-of-hospital CA is reported to be as low as 5%. Therefore, even small incremental improvements in outcomes would be likely to translate into thousands of lives saved every year. Brain damage caused by CA and CPR is initially triggered by complete temporary global cerebral ischemia and then exacerbated by reperfusion. Reperfusion can trigger a cascade of inflammation which can lead to dysfunction of the blood brain barrier, cerebral edema, and neuronal cell death. A number of cytokines related to inflammation such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) have been reported to exacerbate brain damage. Taken together, anti-inflammation therapy may benefit the neurological outcome after CA.

Mesenchymal stem cells (MSCs) have been shown to display a significant capacity of decreasing inflammation and protecting tissue from injuries, mostly by paracrine mechanisms. Increasing evidence suggests that administration of MSCs can produce beneficial therapeutic effects in experimental models of cerebral ischemia. Our previous study demonstrated that intravenous delivery of MSCs improved neurological function after global cerebral ischemia induced by CA.
in rats. However, the mechanisms of action of MSCs require further investigation. TNF-α-induced protein 6 (TSG-6), a 35 kDa secreted protein produced by MSCs in response to signals from injured tissues, can inhibit the amplification of the pro-inflammatory signals to limit lesions of tissues. Recent research finds that administration of TSG-6 decreases the lesion size in models of traumatic brain injury at 2 weeks.

This was a rat model study of global cerebral ischemia to provide the evidence in support of the hypothesis that administration of MSCs reduced post-ischemic inflammation in the brain via secretion of TSG-6. To test this hypothesis, we have assessed whole brain histopathologic alterations, the changes in pro-inflammatory cytokines levels, and the changes of TSG-6 expression in global ischemic rats following intravenously injected MSCs or TSG-6 siRNA-MSCs.

METHODS

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Five-week-old male healthy Sprague-Dawley (SD) rats (100-150 g body weight) and ten-week-old male healthy SD rats (300-400 g body weight) were obtained from Laboratory Animal Center of Sun Yat-sen University.

Isolation and culture of bone marrow-derived MSCs

Of a total of 16 five-week-old rats, 12 rats were sacrificed to harvest MSCs for in vivo cell transplantation experiments and 4 rats for in vitro cell experiments. Rat MSCs were isolated and cultured from bone marrow as previously described. In brief, the femur and tibia of rats were excised and bone marrow was obtained by washing the cavity with Dulbecco’s Modified Eagle Medium-F12 (DMEM-F12; Gibco, USA). After collecting cell suspension, the cells were concentrated by centrifugation at 1500 rpm for 5 min. The cells were then resuspended in complete culture medium (CCM) containing DMEM-F12, 10% fetal bovine serum (FBS; Hyclone, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin, plated in culture flasks, and incubated at 37 °C with 5% CO2 for 3 days. The medium was discarded after 3 days and the adherent cells were cultured for 4 to 6 days until approximately 90% confluent. These cells subcultivated at the ratio of 1:2 by trypsinization (0.25% trypsin-EDTA; Gibco, USA). All experiments were performed with passage three MSCs. Flow cytometric analysis of MSCs was performed as previously described. MSCs were labeled with 4', 6'-diamidino-2-phenylindole (DAPI; Roche, Germany) before administration, according to previously described methods. Prior to transplantation, the cells were resuspended in 0.5 mL phosphate buffer solution (PBS) at 1×10⁷ cells/mL.

Incubation of MSCs with TNF-α

Rat MSCs were plated at 5×10⁴ cells/well in CCM in 6-well plates and incubated for 18 hours. The medium was discarded but without washing to retain some FBS on the cells and replaced with serum-free CCM containing 10 ng/mL recombinant human TNF-α (R&D Systems, USA). After incubation for 24 hours, total RNA was extracted (Trizol, invitrogen) and assayed for TSG-6 mRNA by real-time RT-PCR.

Transfection of MSCs with TSG-6 small interfering RNA (siRNA)

MSCs were plated at 5×10⁴ cells/well in Opti-MEM (only for transfection; Jinuo Biotech, China) in 6-well plates. One day after incubation, cells were incubated with only lipofectamine (no siRNA) or transfected with siRNA for TSG-6 (siB11111110529; Ribo Biotech, China) or RNAi negative control (Ribo Biotech, China) using a commercial kit (Lipofectamine RNAiMAX reagent; Invitrogen). Six hours later, the medium was replaced with 2 mL per well of Opti-MEM and MSCs were incubated for 18 hours. Total RNA was extracted from aliquots of the cells 24 hours (Trizol, Invitrogen) after transfection and assayed for TSG-6 by real-time RT-PCR to confirm TSG-6 knock-down efficiency. For in vivo experiments, TSG-6 siRNA-MSCs or negative siRNA-MSCs were injected into rats at 24 hours after transfection.

Global cerebral ischemia model

Global cerebral ischemia was induced by using a previously described method of asphyxial CA. Male rats weighing 300-400 g were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg). The tracheal was orally intubated with a 14 gauge cannula (Abbocath-T, USA). A 23 gauge polyethylene 50 (PE-50) catheter (Abbocath-T, USA) was inserted in the right atrium via the
jugular vein for infusion of MSCs. A PE-50 catheter was advanced from the left femoral artery into the thoracic aorta for withdrawal of blood for blood gas analysis and measurement of mean arterial blood pressure (MAP) with a pressure transducer (BD, Germany). Another PE-50 catheter was advanced from the left femoral vein into the inferior vena cava for intravenous access. Electrocardiogram lead II was continuously recorded. An incandescent heating lamp was used to maintain rectal temperature at 36.5±0.5 °C.

The animals were mechanically ventilated with 21% O₂ at a tidal volume of 0.65 mL/100 g body weight and 100 breaths/min. Hemodynamic data were recorded in a six channel recorder (Windaq acquisition system, USA).

Asphyxia was induced by intravenous injection of vecuronium (1 mg/kg) and discontinuation of ventilation. CA was determined by loss of aortic pulsation, defined as MAP ≤20 mmHg. At 6 min after onset of CA, precordial compression was initiated with an electrically driven mechanical chest compressor and mechanical ventilation (100% O₂) was restarted. Compression at a rate of 200 min⁻¹ was synchronized to provide a compression/ventilation ratio of 2:1. The depth of compression was adjusted to maintain an arterial diastolic pressure at 25±5 mmHg. ROSC was defined as return of a supraventricular rhythm with a MAP ≥60 mmHg for ≥5 min. Resuscitation was stopped if ROSC was not achieved within 4 min. Ventilation was continued for two additional hours after ROSC. The O₂ concentration was brought to 50% at 0.5-1 hours after ROSC and 30% at 1-2 hours. Sham-operated rats were subjected to the same surgery but without inducing CA.

**Post-resuscitation treatment**

Two hours after ROSC, animals were randomized to receive the following treatment: in PBS-treated group (n=20) or MSCs-treated group (n=20), animals received 0.5 mL PBS alone or 5×10⁶ MSCs labeled with DAPI in 0.5 mL PBS injected into the right atrium; in negative siRNA-MSCs treated group (n=10) or TSG-6 siRNA-MSCs treated group (n=10), animals received 5×10⁶ MSCs treated with negative siRNA or TSG-6 siRNA in 0.5 mL PBS. There were 10 rats in different time points per group. After treatment, the animals were extubated and returned to their cages when upper airway reflexes were active. At 3 or 7 days after CPR, rats were deeply reanaesthetised and the brains were removed. The cerebral cortex of 5 rats in each group was isolated by dissection and stored at -80 °C for RNA isolation or ELISA analysis. The whole brain from another 5 rats was used for histopathology.

**Histopathologic evaluation**

The brains were fixed with 4% paraformaldehyde for 24 hours, dehydrated in a graded sucrose series, and cut into 5 to 7 coronal sections. Each coronal section was embedded in paraffin, sliced at 5 μm and stained with hematoxylin and eosin. A single slide for each animal was examined by the same investigator blind to the experiment according to the histopathologic damage scoring system established previously. In brief, nine brain regions including frontal cortex, parieto-occipital cortex, temporal cortex, hippocampus, basal ganglia, thalamus, midbrain, cerebellum and caudate putamen were examined separately under a light microscope for edema, ischemic neuronal changes and microinfarcts. The severity score of lesions was evaluated on a scale of 4 (minimal 1, moderate 2, severe 3 and maximal 4). The severity score was then multiplied by a weighing factor based on the type of damage (edema × 1, ischemic neuronal changes × 2 and microinfarcts × 4). Total scores for each animal were the sum of scores of 9 bilateral regions.

**Immunofluorescent examination**

Continuous coronal cryostat slides (10 μm) from the region between 3.5 and 3.6 mm posterior to Bregma were processed for immunofluorescence examination. Slides were pre-incubated with normal rabbit serum to block nonspecific binding, treated first with a goat polyclonal antibody against TSG-6 (1:100, Santa Cruz Biotech, USA), and then with the anti-goat antibody conjugated to Cy3. The identical preparations were performed for negative control slides, except that the first antibody was omitted.

**Real-time RT-PCR analysis**

Total RNA was extracted from cerebral cortex or cell cultures using Trizol reagent (Invitrogen, USA). Double-stranded cDNA was synthesized by reverse transcription (PrimeScript RT Master Mix; TaKaRa, China). The cDNA amplification was performed using GoTaq qPCR Master Mix (Promega, USA). Real-time PCR reactions were incubated at 95 °C for 2 min, and then 40 cycles at 95 °C for 3 seconds followed by 60 °C for 30 seconds. The relative mRNA levels of the target gene were calculated based on the
2-ΔΔCts methods normalized to mRNA levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific primers used in this study were listed as follows: TSG-6 forward, 5'-AAGCAGCCAGAAAGATTGGA-3'; reverse, 5'-TTGGGTTAGCAATAGGC3'; IL-1β forward, 5'-TCCTCTGTGACTCGTGGGAT-3'; reverse, 5'-TCAAGACAGACAGGGCATT-3'; IL-6 forward, 5'-AGAGACTTCCAGCAGTTCG-3'; reverse, 5'-AGGGCTTCCGACCTGCTG-3'; TNF-α forward, 5'-TCGTCTACTCCTCAGAGCCC-3'; reverse, 5'-ACTCTCGTCTCCTGTT-3'; GAPDH forward, 5'-CAAGGTCATCCATGACAACTTTG-3'; reverse, 5'-GTCCACCACCCTGTTGCTG3'.

ELISA analysis of pro-inflammatory factors in the brain

The cortex was minced into small pieces and placed in cell lysate. The samples were homogenized on ice with a Dounce homogenizer (Wheaton Science Products, Millville, USA). The homogenate was centrifuged at 12000 rpm at 4°C for 10 min, and the supernatant was collected and assayed by ELISA for IL-1β, IL-6 and TNF-α (rat ELISA kits; CUSABIO, USA) following the manufacturer's directions.

Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables are presented as means ± SD. Comparisons between two groups were made by unpaired Student's t test. Multiple comparisons were made by one-way ANOVA, followed by Bonferroni’ post hoc test. Differences were considered statistically significant if P <0.05.

RESULTS

Baseline physiologic parameters

The baseline body weight, rectal temperature, MAP, arterial blood gases, and lactic acid were similar among the groups (Table 1).

Characteristics of passage three rat MSCs

Passage three rat MSCs showed a plastic adherent, spindle shaped and flatten morphology in culture. Flow-cytometric analysis confirmed that rat MSCs had a CD11b+, CD45−, CD29+, and CD44+ marker which is typical for rat MSCs (Figure 1).

Intravenous rat MSCs reduced brain damage in rats

Histopathology in the brain after CPR from CA was primarily marked by ischemic neuronal changes scattered throughout most brain regions. These were most pronounced in the cortices and hippocampus (Figure 2A, 2B). MSCs-treated group demonstrated significantly lower whole brain HDS than PBS-treated group at 3 and 7 days after CPR, indicating less brain damage (Figure 2C, 2D).

Intravenous rat MSCs suppressed brain inflammation in rats

MSCs treatment significantly decreased the production of pro-inflammatory cytokines in cerebral cortex as measured by real-time RT-PCR and ELISA, compared with PBS-treated group (Figure 3A-F). In addition, the expression

| Table 1: Baseline physiologic parameters among the five groups before cardiac arrest |
|--------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Parameters                          | Sham (n=10)     | PBS (n=20)      | MSCs (n=20)     | Negative siRNA-MSCs (n=10) | TSG-6 siRNA-MSCs (n=10) |
| Body weight (g)                     | 350±27          | 340±35          | 336±23          | 333±21          | 343±33          |
| Rectal temperature (°C)             | 36.3±0.1        | 36.4±0.2        | 36.8±0.6        | 36.6±0.3        | 36.4±0.2        |
| MAP (mmHg)                          | 130±2.9         | 133±7.9         | 135±10.2        | 134±5.3         | 136±10.8        |
| PH                                  | 7.378±0.045     | 7.391±0.031     | 7.369±0.038     | 7.358±0.047     | 7.401±0.039     |
| PaO₂ (mmHg)                         | 82±4            | 84±4            | 81±3            | 82±2            | 85±4            |
| PaCO₂ (mmHg)                        | 36.3±2.6        | 35.8±3.6        | 36.0±3.3        | 36.2±2.9        | 35.9±1.1        |
| Lactic acid (mmol/L)                | 0.6±0.03        | 0.7±0.06        | 0.7±0.04        | 0.4±0.05        | 0.3±0.04        |

PBS, phosphate buffer solution; MSCs, mesenchymal stem cells; MAP, mean arterial pressure; siRNA, small interfering RNA; TSG-6, tumor necrosis factor-α-induced protein 6.
Figure 1. Flow cytometric analysis of surface antigen expression on the three-passage rat MSCs. CD29 and CD44 markers represented more than 95% of MSCs, however, CD11b and CD45 markers represented less than 5% of MSCs.

Figure 2. Intravenous injection of MSCs reduced brain damage at 3 (A, C) and 7 (B, D) days after CPR. (A, B) Representative photographs demonstrated ischemic neuronal changes in the frontal cortex and CA1 region of the hippocampus (arrow). n=5 per time point per group. Magnification: 200×. (C, D) Whole brain histopathologic damage scores (HDS). Intravenous injection of MSCs decreased whole brain HDS. n=5 per time point per group. CPR, cardiopulmonary resuscitation.
of TSG-6 in cerebral cortex was significantly increased in MSCs-treated group as measured by real-time RT-PCR (Figure 3G).

**MSCs engrafted into damaged brain and expressed TSG-6**

Three or seven days after DAPI-labeled MSCs had been intravenously injected into CA rats, DAPI-labeled (blue) cells were primarily found in cerebral cortex (Figure 4A-B). On immunofluorescent staining of TSG-6, positive cells had a red cytoplasm (Figure 4C-D). Some DAPI+ cells in cerebral cortex of MSCs-treated group colocalized with TSG-6, indicating that donor MSCs engrafted into recipient brain expressing TSG-6 (Figure 4E-F).

**MSCs were activated in vitro to secrete TSG-6 in response to TNF-α**

To test the hypothesis that the pro-inflammatory cytokine TNF-α would activate MSCs to express TSG-6, the expression of TSG-6 mRNA in MSCs incubated with 10 ng/mL TNF-α for 24 hours was assayed by real-time RT-PCR. The results demonstrated that the expression of TSG-6 mRNA was increased about 4.6-fold in MSCs incubated with TNF-α compared to MSCs cultured alone (Figure 5).

**MSCs with TSG-6 siRNA knockdown did not reduce brain inflammation**

To further investigate the role of TSG-6 in the action of MSCs, the expression of TSG-6 in MSCs was knocked down by transient transfection with a TSG-6 siRNA. Real-time RT-PCR analysis revealed that the knockdown efficiency was about 85% (Figure 6B). MSCs with a siRNA knockdown of the TSG-6 gene did not significantly decrease whole brain HDS and pro-inflammatory cytokines levels in rats 7 days after CPR (Figure 6A, C-F).
However, whole brain HDS and pro-inflammatory cytokines levels were significantly decreased in rats receiving MSCs with negative siRNA controls (Figure 6A, C-F).

**DISCUSSION**

This study demonstrated that systemic delivery of MSCs significantly reduced brain damage and suppressed inflammatory response to global cerebral ischemia. We also found that some MSCs engrafted into damaged brain and the up-regulation of TSG-6 in the brain was observed after MSCs treatment. In vitro experiments demonstrated that the pro-inflammatory factor TNF-α significantly up-regulated the expression of TSG-6 in rat MSCs. Therefore, the TSG-6 may be a factor in reducing brain inflammatory response after treatment of global cerebral ischemia with MSCs. In addition, in the rat model of global ischemia, knockdown of TSG-6 expression in MSCs was not able to reduce brain inflammation. Collectively, these results indicated that MSCs treatment reduced inflammatory damage to brain in rats with global cerebral ischemia by secretion of TSG-6. In accordance with this, Wang *et al.* showed that intravenously injected MSCs attenuated peritoneal adhesion by secretion of TSG-6 in a rat model of acute peritoneal adhesion.21,22

It has been shown that inflammation is one of the most important pathophysiological mechanisms involved in cerebral ischemia.23 Inflammation mediated by pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α occurs after cerebral ischemia and can exacerbate brain damage.6-8 In vivo experiments have demonstrated that exogenous IL-1β administered into the brain can lead to exacerbation of ischemic brain damage.24,25 IL-6 as a powerful predictor of early neurological deterioration is up-regulated in the brain following cerebral ischemia.26 TNF-α also plays an important role in the execution of inflammation cascade after cerebral ischemia.27 Application of recombinant TNF-α after cerebral ischemia exacerbates brain damage.28 In our study, global cerebral ischemia induced by CA resulted in apparent ischemic neuronal changes in the cortex and hippocampus at 3 or 7 days after CPR. Consistent with pathological changes,
Figure 6. Intravenous injection of MSCs with TSG-6 siRNA knockdown did not reduce brain inflammation 7 days after CPR. (A) Representative photographs demonstrated ischemic neuronal changes (arrow) in the frontal cortex and CA1 region of the hippocampus 7 days after CPR. n=5/group. Magnification: 200×. (B) Real-time RT-PCR assays TSG-6 of control rat MSCs (Control), MSCs treated with transfection reagents only (no siRNA), MSCs transfected with a negative siRNA (Negative siRNA), or MSCs transfected with TSG-6 siRNA (TSG-6 siRNA). The knockdown efficiency of TSG-6 in rat MSCs was about 85%. n=3. (C) Whole brain histopathologic damage scores (HDS). Intravenous injection of MSCs with TSG-6 knockdown did not decrease whole brain HDS, however, MSCs with negative siRNA significantly decreased HDS. n=5/group. *P<0.01 vs PBS-treated group. (D-F) The levels of pro-inflammatory cytokines were not decreased by MSCs with siRNA knockdown of TSG-6, however, MSCs with negative siRNA significantly decreased pro-inflammatory cytokines levels in cerebral cortex. n=5/group. *P<0.05 vs PBS-treated group. CPR, cardiopulmonary resuscitation.

pro-inflammatory cytokines overexpression (IL-1β, IL-6 and TNF-α) were also observed in the PBS group at 3 or 7 days after CPR. These results show that inflammatory response following global cerebral ischemia has a pivotal role in brain damage.

MSCs are multipotent cells with proven efficacy in central nervous system injury including cerebral ischemia in preclinical studies.\(^{10-12,16}\) However, the mechanisms of the therapeutic benefits following MSCs transplantation are still unclear. More recently, the anti-inflammatory effects of MSCs have attracted a great deal of interest. Several studies have demonstrated that infusion of MSCs significantly decreases the levels of pro-inflammatory cytokines in animal models of injury.\(^{29-31}\) In agreement with these studies, our results showed that MSCs treatment reduced the expression levels of IL-1β, IL-6 and TNF-α after global cerebral ischemia, which were accompanied by reduced brain damage. Next, the key question is how administration of MSCs attenuates the inflammatory response after global cerebral ischemia. Currently it is of interest that an anti-inflammatory molecule TSG-6 plays a crucial role in mitigating pro-inflammatory networks.

TSG-6 is a multifunctional protein associated with inflammation and up-regulated in many pathological conditions.\(^{32}\) The protein has strong anti-inflammatory effects in many models of inflammation. Administration of recombinant human TSG-6 dramatically improved the arthritis,\(^{33}\) reduced inflammatory damage to the cornea following injury,\(^{14}\) suppressed the excessive inflammatory response after myocardial infarction,\(^{34}\) and global cerebral ischemia.\(^{13}\) The anti-inflammatory activity of TSG-6 was mainly attributed to its ability to down-regulate the inflammatory network of proteases, to regulate the expression of various factors that controlled inflammatory response, and to suppress neutrophil migration into sites of inflammation.\(^{35,36}\) More recently, it has been shown that TSG-6 is also secreted by MSCs in response to stimulation with
TNF-α and other pro-inflammatory cytokines. In vitro, the results here demonstrated that MSCs incubated with TNF-α dramatically up-regulated the expression of TSG-6. In several murine models of inflammation including corneal injury, lung injury, myocardial infarction, and peritonitis, transplanted MSCs secreted TSG-6 in vivo in response to inflammatory signals and reduced inflammatory response. Consistent with these researches, our results demonstrated that systemic delivery of MSCs suppressed inflammatory response and reduced brain damage produced by global cerebral ischemia through secretion of TSG-6.

There are a number of weaknesses in this study. Firstly, the distribution of donor MSCs in the damaged brain is not quantified. Several animal models demonstrated that most of intravenously infused MSCs were trapped in lung, suggesting that only small numbers of MSCs engrafted into the injured tissues. For detecting small numbers of the cells in the injured tissues, animals must be transplanted with xenologous MSCs such as human MSCs. Then real-time PCR was performed to assay human-specific Alu sequences in the injured tissues. The number of MSCs in the injured tissues could be calculated by standard curves. The technique was not presently available to us. Another limitation is that the study does not further explore the fate of MSCs engrafted into the damaged brain. The results here demonstrated that the prelabeled transplanted MSC were detected in the brain of the recipient animals until 7 days. However, what is the fate of MSCs in the brain after 7 days are unknown. One possibility is that some transplanted MSCs can differentiate into cells of neural lineage and interact with host brain parenchymal cells. Another possibility is that they restore brain function by the release of growth factors, antiapoptotic factors and anti-inflammatory cytokines.

In conclusion, systemic delivery of MSCs significantly reduced inflammatory damage to brain after global cerebral ischemia in rats via secretion of TSG-6.

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DISCLOSURE
Conflicts of interest: None

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