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Reduced expression of apoptotic proteins in the ischemic rat brain following Sertoli cell transplantation

Nadia Khoshbaf Khiabanian[1], Mohammadreza Bigdeli[1,2]*, Sepideh Khaksar[3], Abbas Aliaghaei[4]

[1] Department of Physiology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran,
[2] Institute for Cognitive and Brain Science, Shahid Beheshti University, Tehran, Iran,
[3] Department of Plant Sciences, Faculty of Biological Sciences, Alzahra University, Tehran, Iran,
[4] Department of Anatomy and Cell Biology, School of Medicine, Shahid Beheshti University of Medical Science, Tehran, Iran,
** Email: bigdelimohammadreza@yahoo.com*

Sertoli cells (SCs) may be a new candidate to decrease ischemic damage due to their ability to secrete factors that actively protect neurons and inhibit uncontrollable immune responses. Pre-treatment with these cells was considered in the current study. SCs were injected into the right striatum in rats using the stereotaxic technique. Ten days after injection, middle cerebral artery occlusion surgery was performed. Following these procedures, neurological deficit scores, brain edema, blood-brain barrier integrity, infarct volume, and the expression of apoptotic factors in the cortex, striatum, and piriform cortex-amygdala were evaluated. Analysis showed that behavioral deficits, infarct volume, blood-brain barrier permeability, and edema in the striatal area in the allograft group demonstrated a significant decrease compared to the control group. Additionally, analysis of the expression of caspase-3 and Bcl-2 proteins in the striatum indicated a remarkable reduction and increase, respectively, in the allograft group compared to the control group. According to the obtained results, one possible mechanism for the neuroprotection induced by SCs in an ischemic brain is the reduction of apoptotic factors.

Key words: Sertoli cells transplantation, cerebral ischemia, infarction, blood-brain barrier integrity, edema, Bcl-2, caspase-3

INTRODUCTION

Among the neurovascular diseases, the second highest cause of death is attributed to stroke. The most prevalent type of stroke is ischemic stroke, which is caused by an interuption of the blood supply (Burrows et al., 2015). The increasing incidence of this disease, and its high morbidity and mortality rates, have prompted researchers to search for more effective and safer treatments (such as cell therapy), especially for patients who have problems with the current therapeutic methods (Lakhan et al., 2013).

Sertoli cells (SCs) are non-germ cells located within the testes that promote the development of antigenic germ cells within the seminiferous epithelium by creating a barrier (Sanberg et al., 1996). In addition, SCs stimulate neovascularization (Dufour et al., 2003), angiogenesis (Golat and Cameron, 2008), and secrete various anti-inflammatory cytokines, chemokines, growth factors, and nutrient factors (Griswold, 1988; Willing et al., 1998). SCs also have immunosuppressive characteristics in grafted tissues, can induce neuronal differentiation, and can increase cell proliferation (Hemendinger et al., 2005; Shamekh et al., 2006, 2008). Moreover, these cells are effective in animal models of diabetes (Fallarino et al., 2009), Huntington's disease (Emerich, 2004), Parkinson's disease (Sanberg et al., 1997; Willing et al., 1999), acute liver failure (Zheng

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et al., 2009), and major depressive disorders (Loftis, 2011). Due to their trophic and immunological properties, SCs may be an acceptable intervention for the treatment of cerebral ischemia.

Ischemic stroke causes a cascade of pathophysiological reactions, including excitotoxicity, microvascular injury, edema, breakdown of the blood-brain barrier (BBB), and intracerebral cellular death (Lakhan et al., 2009). Generally, neuronal death in cerebral ischemia consists of both necrotic and apoptotic cell death in the core and penumbra zones, respectively (Broughton et al., 2009). During ischemic stroke, the penumbra zone is metabolically active but functionally inactive, and can be saved if cerebral blood flow is reestablished quickly (Li et al., 1995). Apoptosis is regulated by the Bcl-2 family and caspases. DNA damage, cell cycle disruption, unusual proliferation or loss of trophic factors can initiate the intrinsic pathways of apoptosis within the dying cell *via* various signals. These signals interfere with the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins. Their complex combinations affect mitochondrial outer membrane permeability (Czabotar et al., 2014). The cytotoxic accumulation of intracellular Ca^{2+} activates the calpain enzymes, which, in turn, results in the activation of the Bcl-2 interacting domain (tBid) (Oberst et al., 2016). Subsequently, tBid targets the mitochondrial transition pores located in the outer membrane and changes the activities of other pro-apoptotic proteins, such as Bad, Bak, Bcl-xS and Bax. These pro-apoptotic proteins can interact with tBid and anti-apoptotic proteins, such as Bcl-2 or Bcl-xL (Kale et al., 2017). Release of cytochrome C from the intermembrane space into the cytosol occurs after the opening of the mitochondrial transition pores. Cytochrome C then joins to the cytosolic protein apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, forming an "apoptosome". The apoptosome, in turn, activates caspase-9, which leads to the activation caspase-3. Caspase-3 is known as an important mediator of apoptosis (Broughton et al., 2009). Caspase activation occurs up to 9 h after middle cerebral artery occlusion (MCAO) (Broughton et al., 2009). Therefore, inhibiting the activation of the caspases might reduce ischemic damage. Furthermore, the extrinsic mechanisms of apoptosis activitate caspase-3 (Liu et al., 2009). Cerebral ischemia is also associated with the activation of microglia and neuroinflammation (Jin et al., 2010). Previous studies have demonstrated that the activation of caspase-8 and caspase-3 regulates the activation of microglia *via* autocrine signaling by tumor necrosis factor alpha (TNF-α). Accordingly, the inhibition of caspase has neuroprotective effects against intracerebral cellular death in animal models of neurodegenerative diseases that are induced by the microglial activation, such as brain trauma, Parkinson's disease, and ischemic stroke (Depino et al., 2003; Block et al., 2007; Burguillos et al., 2011).

In the current study, the ability of SCs to reduce ischemia injuries by balancing the expression of Bcl-2 and caspase-3 is examined.

METHODS

Animals

For this study, male Wistar rats (9-13 weeks of age; weighing 230-330 g) were utilized. The animals were housed at a constant temperature (22 ± 2°C) and humidity under a 12 h light/dark cycle. Animal handling and experimental procedures were performed in accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals (NIH Publications; revised in 2011). The Ethics Committee at Shahid Beheshti University (NO#940.807) approved the research protocols. Every effort was made to decrease the number of animals used to a minimum.

Group assignment

Ninety-six rats were divided randomly into four main groups (control, sham, intact, and transplantation of allograft SCs). The control group underwent stereotaxic/ischemia operations without receiving cell transplantation, the sham group received the media solution of SCs, the intact group received the transplantation of SCs without ischemic surgery, and the allograft group received the transplantation of SCs along with ischemic surgery. The rats in the sham group underwent the MCAO surgery procedure but a filament was not introduced (Khaksar and Bigdeli, 2017). This group was divided into 4 subgroups in which the following factors were assessed: the permeability of the BBB (n=7), brain edema (n=7), and the expression levels of Bcl-2 (n=5) and caspase-3 (n=5). In the intact group (n=10), only the effect of transplantation of SCs on Bcl-2 and caspase-3 was evaluated. The ischemia-operated groups (the control and allograft groups) were divided into subgroups for the separate evaluation of infarct volume (striatum, piriform cortex-amygdala, and cortex; n=7), brain edema (striatum, piriform cortex-amygdala, and cortex; n=7), BBB permeability (striatum, piriform cortex-amygdala, and cortex; n=7), and Bcl-2 and caspase-3 expression (striatum, piriform cortex-amygdala, and cortex; n=5).

Ten days after stereotaxic surgery for the transplantation of SCs, the ischemia-operated groups underwent

the MCAO surgery (the middle cerebral artery [MCA] was blocked for 60 min). Twenty-four h later, neurological deficit scoring and measurement of edema, BBB integrity, and infarct volume were carried out separately. The neurological deficit scoring was carried out in all animals (Khaksar and Bigdeli, 2017).

Cell isolation and cell culture

First, three male rats (20-30 days old) were put down according to the protocols of the Animal Care Committee of Shahid Beheshti University of Medical Sciences and their testes were removed. Next, the tunica albuginea (the lining around the testicles) was removed and the testicular tissues were cut into smaller sections. The tissues were then transferred to tubes and exposed to 0.25% trypsin for 15 min (Gibco, USA). After the seminiferous tubules were separated, the trypsin was removed from the tubes and the tissue samples were exposed to a 0.1% collagenase solution for 15 min. Fetal bovine serum (FBS; Gibco, USA) was then added to the samples, followed by centrifugation and transfer of the sediments to cell culture flasks. The flasks contained antibiotics, DMEM/F12 (Gibco, USA), and FBS (10%). Forty-eight h later, the culture media was changed in order to discard the debris and red blood cells. The cells were then passaged with 0.25% trypsin (Liu et al., 1999; Milanizadeh et al., 2018). SCs at passages 3 were used for transplantation.

Immunocytochemistry

The SCs were transferred to plates. After reaching stable conditions and an appropriate cell density, the cell culture media was removed. After being washed with phosphate buffered saline (PBS), the cells were fixed with a 4% paraformaldehyde-buffered solution (Merck, Germany) (Milanizadeh et al., 2018). A 0.3% Triton X-100 solution (Sigma Aldrich, USA) was then used to permeabilize the cells. Next, the cells were placed into a blocking solution and then incubated overnight with a primary antibody (anti-GATA4, Abcam, USA) at 4°C. After washing the samples with PBS, they were incubated with a secondary antibody (goat anti-rabbit conjugated with FITC; Abcam, USA) (Liu et al., 1999), stained with a fluorescent Hoechst stain, and imaged with a fluorescent microscope to visualize the nuclei. Immunocytochemistry is a common technique used for the identification of SCs since there are no other validated methods. Immunocytochemistry confirms the presence of SCs since GATA4 is known as a specific marker of SCs.

Stereotaxic surgery

Briefly, rats were anesthetized and fixed in a stereotaxic apparatus (Stoelting Instruments, USA). The surgical area was shaved, a midline injection of lidocaine/epinephrine was administered to the scalp, and a cut parallel to the sagittal groove was created on the scalp. After retraction of the skin, a hole was created in the skull above the right striatum according to the following stereotaxic coordinates relative to bregma: + 0.5 mm AP, ± 2.6 mm ML, – 5 mm DV relative to the skull. (Paxinos et al., 1980). Prior to injection, living SCs with an appropriate density were cultured and maintained in a suspension of 2 µl DMEM. The cells were counted using trypan blue and half a million cells labeled with DiI and Hoechst staining (Sigma Aldrich, USA) were prepared for injection at the coordinates outlined above (Fig. 3). During the procedure, the SCs were stored on ice and a 5 µL Hamilton microsyringe was used for the injection. For maximum graft survival, the cells were placed in the medioposterior part of the right striatum at two points that are devoid of an extracellular matrix. Following the injection, the skin was sutured. After recovery, the animals were returned to the holding room and housed separately for 10 days.

Tracing the transplanted Sertoli cells

The SCs were labelled with DiI and Hoechst staining prior to the injection. To label the cells, they were incubated with 4 µg/ml Hoechst for 1 h. To ascertain the survival of the transplanted cells and their distribution, a fluorescence microscope was used to detect the SCs following brain collection.

Focal cerebral ischemia

To prepare the animals for surgery, common protocols for anesthesia, eye lubrication (applied twice), shaving and preparation of the surgical site were followed, and the rats were placed on heated surgical pads (Pritchett-Corning et al., 2011). Ten days following SC cell injection, the animals were anesthetized by an intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (8 mg/kg) (Ramírez-Sánchez et al., 2019). Briefly, the MCA was blocked using the intraluminal filament technique suggested by Longa et al. (1989). An incision was made in the right common carotid and a suture was used to ligate the common and external carotids. A filament was made by rounding (by heating) and coating (by poly-L-lysine, Sigma, USA) a 3-0 nylon suture. The filament was then inserted into the

internal carotid artery until it hit an obstacle and did not move (20 mm). This procedure occluded the blood flow to the MCA. After 60 min of ischemia, reperfusion occurred by removing the filament. Rectal temperature was monitored throughout the surgery (Citizen-513w) and the temperature was maintained at 37°C (Khaksar and Bigdeli, 2017).

Neurobehavioral evaluation

Twenty-four h after the MCAO surgery, the rats performed five tests to measure neurological deficits. The total neurological score (the sum of the partial scores) could range from a minimum of 0 points in normal rats to a maximum of 18 points in ischemic rats. Furthermore, neurological deficits were evaluated using a chart that assesses the rat sensorimotor abilities and coordination (Zhou et al., 2013). For instance, the motor ability of rats was was measured by examining the symmetry of muscle tone, climbing, and gait disturbance tests. To examine sensorimotor incorporation, limb placing is an acceptable test (Khaksar and Bigdeli, 2017).

Brain water content measurement

After decapitation, the pons, olfactory bulb, and cerebellum were separated from the rat brain. The evaluation of cerebral edema was done separately in the piriform cortex-amygdala, cortex, and striatal areas (Khaksar and Bigdeli, 2017). The wet weights (WW) of the right and left parts of the brains were measured separately. After 24 h, the dry weights (DW) were computed at 120°C. The formula [(WW–DW)/WW]×100 was used to calculate cerebral edema (Bigdeli et al., 2007).

Infarct volume assessment

Twenty-four hours after the MCAO surgery, the brains of the sacrificed rats were removed and kept in cool saline at 4°C for 15 min. The brains were then cut into sections in the coronal plane at a thickness of 2 mm (Brain Matrix, Iran). Next, the slices were exposed to a 2% 2,3,5-triphenyltetrazolium chloride solution (TTC) (Merck, Germany) and were placed in a water bath at 37°C for 15 min. A digital camera (Canon EOS 500D) was used to capture images of the slices, which were then transferred to a computer. Image analysis software (ImageJ; version: 1.46r) was utilized to calculate the infarct volume. The unstained areas were considered as infarction regions. Measurement of the infarct volume in each slice was carried out by multiplying the infarcted area of the slice by its thickness (2 mm). The sum of the infarct volumes of seven brain slices was considered as the total infarct volume for each brain. The contribution of the edema to the infarct volume was corrected using the following formula:
corrected infarct volume=the left hemisphere volume – (the right hemisphere volume – the calculated infarction volume) (Swanson et al., 1990). The infarct volumes in the piriform cortex-amygdala, cortex, and striatum areas were separately evaluated (Fig. 4) (Khaksar and Bigdeli, 2017).

The blood-brain barrier integrity

BBB permeability was measured using Evans blue dye (EBD, Sigma Chemicals, USA) extravasation. Thirty min after the MCAO surgery, 4 ml/kg of a 2% EBD solution was injected intravenously. 24 h after ischemic induction, the rats were anesthetized and transcardially perfused to remove the intravascular EBD and to replace it with 250 ml saline. Next, the rats were decapitated and the brains were removed. The hemispheres were quickly separated and weighed. To homogenize each hemisphere, 2.5 ml of PBS was used to extract the EBD. After mixing the samples with 2.5 ml of 60% trichloroacetic acid, the proteins were allowed to precipitate. The samples were then kept at 4°C for 30 min, centrifuged for 30 min at 1000 rpm, and the supernatants were separated. Using a spectrophotometer (Perkin-Elmer, Illinois, USA), the EBD absorbance in the supernatants was measured at 610 nm. Using a standard curve, the EBD concentrations were calculated and are presented as µg/g of brain tissue (Bigdeli et al., 2007). BBB permeability was separately examined in the piriform cortex-amygdala, cortex, and striatal areas (Khaksar and Bigdeli, 2017).

Western blot analysis

The expression of the Bcl-2 and caspase-3 proteins were evaluated by the western blot technique. The expression levels of Bcl-2 and caspase-3 were separately measured in the piriform cortex-amygdala, cortex, and striatal areas. For homogenization, the tissue samples were placed in a lysis buffer at 4°C for 1 min. The tissues were then centrifuged at 12,000 rpm at 4°C for 20 min. The tissue extracts were obtained from the supernatants. The Bradford assay was utilized to determine the protein concentration in each tissue extract. The samples (the tissue extracts) were then exposed to a sample buffer. After heating the extracts at 100°C,

separation gels (10%) for SDS-PAGE were prepared to separate the proteins. Semi-dry blotting (Biorad) was done. 2% non-fat dry milk in Tris-buffered saline in 0.1% Tween 20 was prepared and the blots were blocked with this solution at 4°C for 75 min. Next, the blots were separately incubated for 18 h with polyclonal rabbit antibodies for caspase-3 (1:500, H-277, Santa Cruz Biotechnology, USA), Bcl-2 (1:500, sc-492, Santa Cruz Biotechnology, USA), and GAPDH (1:1000, sc-166574, Santa Cruz Biotechnology, USA). Next, a secondary anti-rabbit antibody (1:500, sc-2004, Santa Cruz Biotechnology, USA) was used and the blots were incubated for 90 min. Detection of caspase-3 and Bcl-2 immunoreactive proteins was accomplished by advanced chemiluminescence (Enhanced Chemiluminescence, Amersham Biosciences). The films were then developed and ImageJ software (v. 1.46r) was used to analyze the intensity of the blots (Khaksar and Bigdeli, 2017).

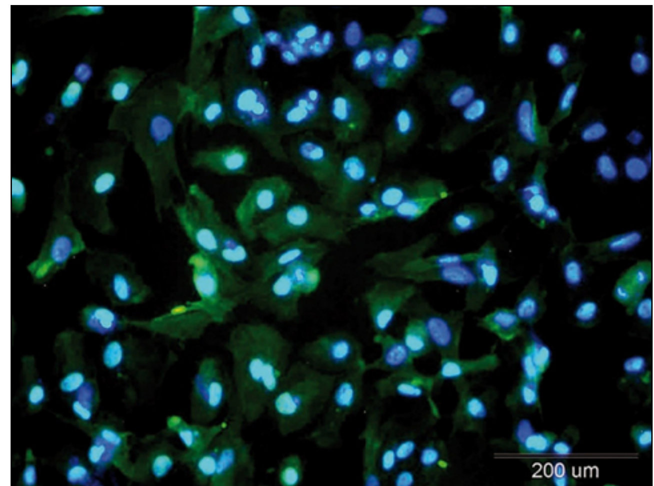

Fig. 1. Immunostained Sertoli cells with anti-GATA-4 exhibiting a green color and Hoechst staining (cell nuclei exhibiting a blue color). Immunocytochemistry shows that Sertoli cells were immunopositive for GATA4.

Statistical analysis

The non-parametric Kruskal-Wallis analysis of variance (ANOVA) followed by the Dunn's test (SPSS v. 22.0) were used to compare the neurological deficit scores. Analysis of infarct volume data was done using the non-parametric Mann-Whitney U test. Brain edema and EBD extravasation data were analyzed by two-way ANOVA, and the data from the Bcl-2 and caspase-3 assays were compared using one-way ANOVA and *post-hoc* LSD tests (SPSS v22.0). Mean ± SEM was used to display the data and p-values lower than 0.05 were considered statistically significant (Khaksar and Bigdeli, 2017).

RESULTS

Confirmation of the presence of Sertoli cells in the testis-extracted samples

Immunocytochemical staining showed that the testis-extracted cells were SCs since GATA4 is specifically expressed in these cells. Using anti-GATA4, the immunostained SCs appeared green in color, while their nuclei appeared blue due to Hoechst staining (Fig. 1).

Confirmation of survival of the transplanted cells in the striatum

In this study, we randomly selected a number of rats and examined cell tracing in their brains. Ten days after transplantation, the rats were sacrificed and SCs cells labeled with DiI and Hoechst staining (prior to injection) were considered by fluorescent microscopy. The SCs displayed a fibroblast-like morphology – especially in the long processes – indicating that these cells were active and prolific (Fig. 2). Labeling SCs with DiI and Hoechst demonstrated that the transplanted cells (SCs) were alive in the striatum after 10 days (Fig. 3).

The effects of transplanting Sertoli cells on neurological deficit scores

Evaluating the effects of neuroprotective factors on motor and sensory functions is very important in stroke studies. In the current research, the transplantation of SCs caused significant differences in the

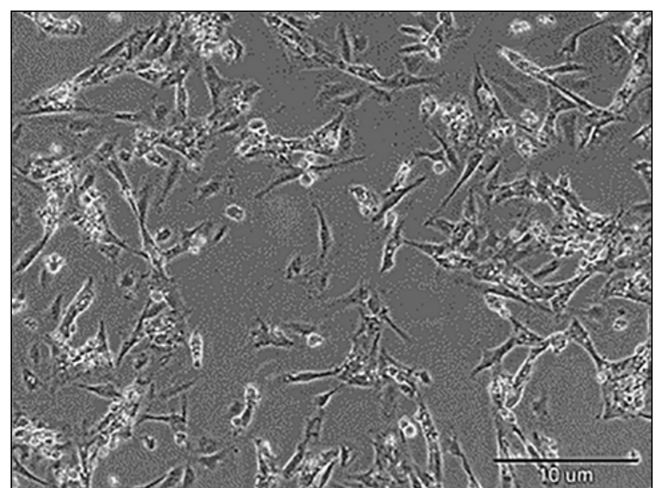

Fig. 2. Phase contrast microscopy showing that Sertoli cells exhibit a fibroblast morphology after 10 days.

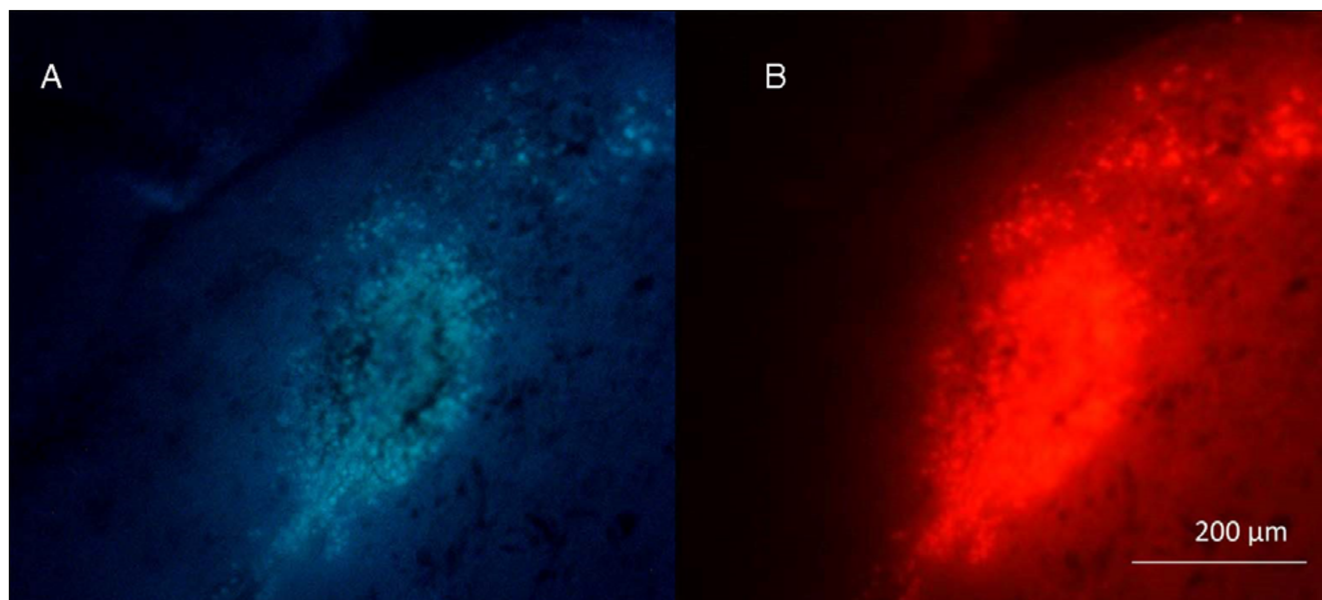

Fig. 3. (A) Hoechst-stained Sertoli cells (*20) and (B) DiI-labeled Sertoli cells (*20) traced in the brain. Fluorescent microscopy confirmed the survival of labeled transplanted Sertoli cells in the striatum.

transplant recipients compared to the control group, where transplanted animals showed an improvement in the total neurological deficit scores (P<0.001). As shown in Table 1, the allograft and control groups significantly differed in all the conducted behavioral tests (P=0.001). Furthermore, a significant difference was observed in the sham group compared to the control group (P<0.001), showing a disruption in neurological behaviors after MCA occlusion (Table 1).

The effects of transplanting Sertoli cells on infarct volume

The impact of transplanting allograft SCs on the infarction size caused by ischemia was examined in the piriform-cortex amygdala, cortex, and striatum. Analysis showed that the transplantation of SCs 10 days before MCAO surgery resulted in a significant decrease of infarct volume in the whole ischemic hemisphere in the allograft group (166.82 ± 8.7 mm^3, P=0.03) compared to the control group (221.33 ± 8.7 mm^3). In addition, the infarct volume in the striatal area in the allograft group (21.82 ± 9.34 mm^3, P=0.01) was significantly different from the control group (59.4 ± 20.5 mm^3). This means that the infarction volume in the striatal area of the SC recipients was significantly decreased compared to that of the control group. Moreover, no change in the infarct size in the cortex and piriform cortex-amygdala in the allograft group was detected compared with that of the control group (Fig. 4).

The effects of transplanting Sertoli cells on brain edema

As the main indicator of brain edema, brain water content was also considered in the MCA-occluded rats 24 h after ischemia. It was found that there was a significant increase in the amount of cerebral edema in the piriform cortex-amygdala, cortex, and striatal areas of the control group (P=0.01, P=0.01, and P<0.01, respectively). Thus, the occurrence of cerebral edema was confirmed in the control group after the MCAO surgery. Furthermore, a significant decrease of cerebral edema was observed in the striatal area (72.54 ± 0.35, P<0.001)

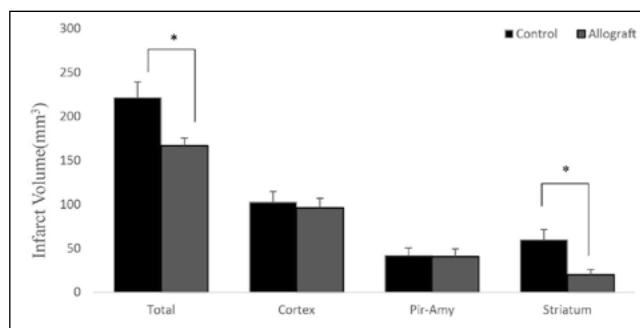

Fig. 4. The graph shows the comparison of middle cerebral artery occlusion (MCAO)-induced total infarct size in the piriform cortex-amygdala (Pir-Amy), cortex, and striatum between the control and allograft groups. Total infarction volume decreased significantly. In the striatum, the decreasing effect of SC transplantation was also detected. Each column represents the mean ± SEM of the infarct volume (n=7). P<0.05 considered as significant. (*P<0.05, **P<0.01, ***P<0.001; Mann-Whitney U test).

Table 1. The partial neurological deficits scores in different experimental groups. This table indicates partial scores in 10 rats. Total neurological deficit scores and behavioral tests were analyzed and reported separately. RT: raise the tail, MF: motor function, SF: sensory function, BT: beam test, RA: reflex action. *1: Sham group; *2: Control group; *3: Allograft group. (Nonparametric Kruskal-Wallis analysis).

Groups	Rat	Raise the tail	Motor function	Sensory Function	Balance perfor-mance	Reflex action	Sum	Mean	Mode	Statistical results
Sham	1	1	1	2	1	1	6	5	4.5	–
	2	1	0	1	2	0	4			
	3	1	1	2	2	1	7			
	4	0	2	0	3	0	5			
	5	1	1	0	2	0	4			
	6	2	0	1	3	1	7			
	7	0	2	1	1	0	4			
	8	1	1	2	0	0	4			
	9	0	1	2	1	0	4			
	10	1	1	0	2	1	5			
Control	1	2	2	3	4	2	13	13.5	13	Significant change in total neurological scores compared to the sham group (P=0.000), RT: *1-*2 (P=0.001), MF: 1-2 (P=0.000), SF: 1-2 (P=0.004), BT: 1-2 (P=0.003), RF: 1-2 (P=0.001)
	2	2	3	3	6	2	16			
	3	1	3	3	4	2	13			
	4	3	3	3	2	3	14			
	5	2	3	0	5	2	12			
	6	2	3	3	2	3	13			
	7	2	3	1	5	3	14			
	8	2	2	3	4	2	13			
	9	2	2	3	2	3	12			
	10	2	3	3	5	2	15			
Allograft	1	1	1	2	0	0	4	6.4	5	Significant change in total neurological scores compared to the control group (P=0.000), RT: 2-*3 (P=0.002), MF: 2-3 (P=0.039), BT: 2-3 (P=0.039), RA: 2-3 (P=0.001)
	2	1	0	0	0	0	1			
	3	2	1	3	6	2	14			
	4	1	2	0	0	0	3			
	5	2	2	2	4	0	10			
	6	0	1	3	6	3	13			
	7	1	3	0	0	1	5			
	8	1	1	3	0	0	5			
	9	1	2	3	0	0	6			
	10	1	1	1	0	w0	3			

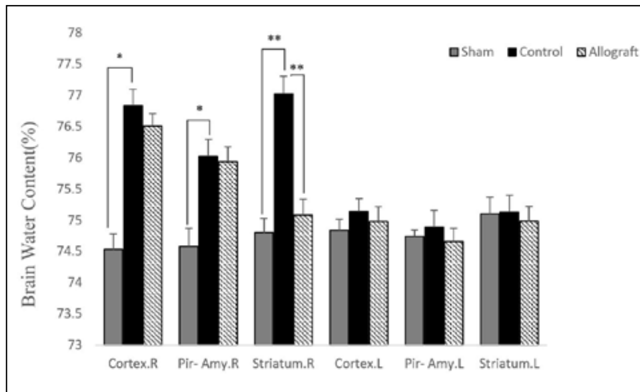

Fig. 5. Brain water content in different experimental groups in the piriform cortex-amygdala (Pir-Amy; right and left), cortex (right and left), and striatum (right and left) in the control, sham-operated, and allograft groups. Values are expressed as the mean ± SEM (n=7). *P*<0.05 is considered as significant. (**P*<0.05, ***P*<0.01, ****P*<0.001; two-way ANOVA).

in the allograft group compared to the control group (77.01 ± 0.28). In addition, no significant differences in the piriform cortex-amygdala and cortex were detected between the allograft and control groups (Fig. 5).

The effects of transplanting Sertoli cells on the integrity of the blood-brain barrier

As one of the most commonly used markers for determining the BBB integrity, the EBD absorption rate was analyzed in the piriform-cortex amygdala, cortex, and striatal areas 24 h after ischemia. The data showed significant differences between the sham and control groups in the piriform-cortex amygdala, cortex, and striatal areas (*P*<0.001, *P*<0.01, and *P*<0.001, respectively). Moreover, there was a significant decrease (*P*=0.01) in BBB permeability in the striatum of the ischemic hemisphere in the allograft group (ipsilateral hemisphere=2.90 ± 0.58; the right hemisphere=7.01 ± 0.27) compared to that of the control group (ipsilateral hemisphere=3.0 ± 0.22; the right hemisphere=10.31 ± 0.44; Fig. 6).

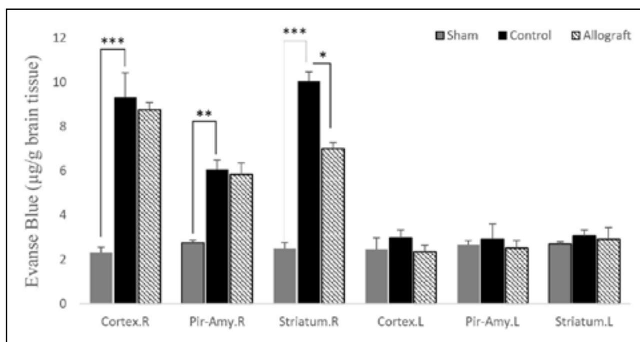

Fig. 6. Evans blue extravasation in the piriform cortex-amygdala (Pir-Amy; right and left), cortex (right and left), and striatum (right and left) in the control, sham-operated, and allograft groups. Values are expressed as the mean ± SEM (n=7). *P<0.05* is considered significant. (**P*<0.05, ***P*<0.01, ****P*<0.001; two-way ANOVA).

The effects of transplanting Sertoli cells on the expression of caspase-3

The expression of caspase-3 protein in the piriform cortex-amygdala, cortex, and striatum was detected using the Western blot technique (Fig. 7). Regarding the

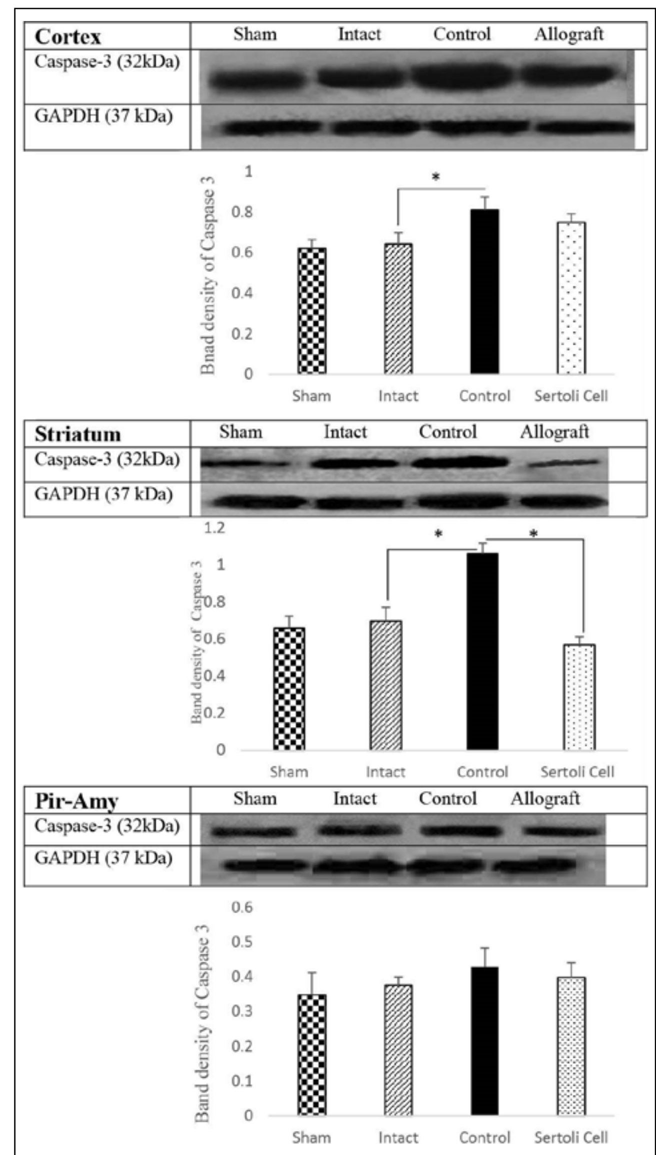

Fig. 7. The evolution of caspase-3 protein expression in the cortex (a), striatum (b), and piriform cortex-amygdala (Pir-Amy; c) in the sham, intact, control and allograft groups. After normalization of caspase-3 protein bands with GAPDH (as loading control), analysis was done. Values are expressed as the mean ± SEM (n=5; one-way ANOVA).

expression of caspase-3 protein, an increase was observed in the cortex and striatum of the control group compared to those of the intact group (*P*=0.04, *P*=0.02, respectively) (Fig. 7A and B). The expression of caspase-3 protein decreased in the striatum of the allograft group compared to that of the control group (*P*=0.01) (Fig. 7B). There was no significant difference in the piriform cortex-amygdala areas of the experimental groups (Fig. 7C).

The effects of transplanting Sertoli cells on the expression of Bcl-2

Bcl-2 protein was expressed in the piriform cortex-amygdala, cortex, and striatal areas (Fig. 8). The analysis of the control and allograft groups showed a significant increase of Bcl-2 expression in the striatal area of the allograft group compared to that of the control group (*P*=0.03) (Fig. 8B). No significant differences were observed between the control and allograft groups in the cortex and piriform cortex-amygdala areas (Fig. 8A, C). Moreover, there were significant decreases in the cortex and striatal areas of the control group compared to the intact group (*P*<0.01, respectively) (Fig. 8A, B). It should also be noted that there were no significant differences in the piriform cortex-amygdala area across the experimental groups (Fig. 8C).

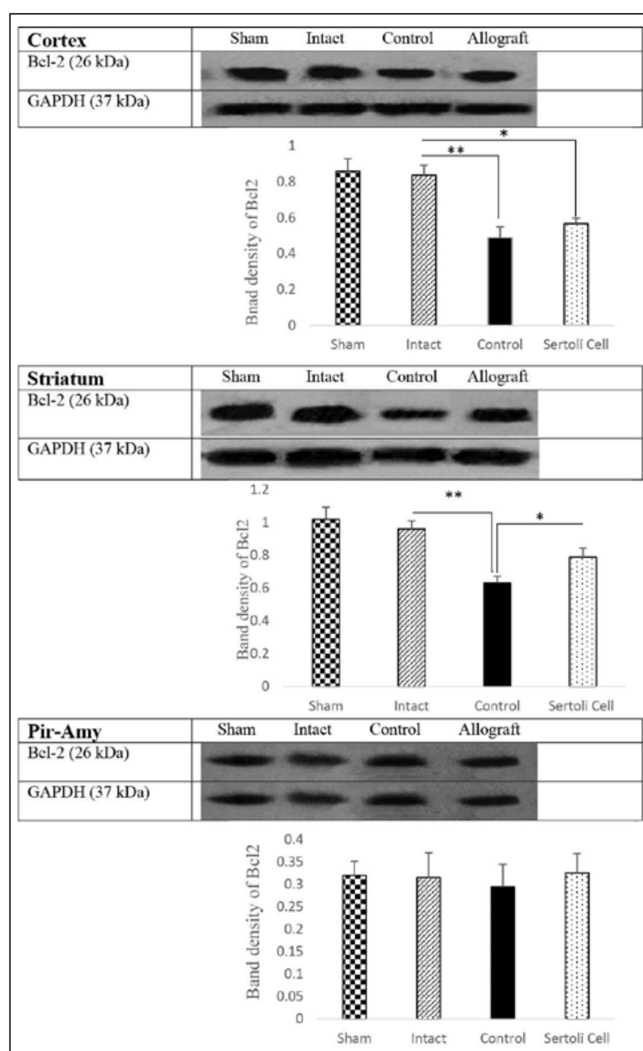

Fig. 8. Effect of Sertoli cell transplantation on Bcl-2 protein expression in the cortex (a), striatum (b), and piriform cortex-amygdala (Pir-Amy; c). After normalization of Bcl-2 protein bands with GAPDH (as loading control), analysis was done. Values are expressed as the mean ± SEM (n=5; One-way ANOVA).

DISCUSSION

The effects of Sertoli cells on neurological deficit scores and infarct volume

The present research shows that the transplantation of SCs ameliorated the neurological deficits induced by MCA occlusion. Patients with acute ischemic stroke typically lose more than 100 million neurons. Therefore, it is obvious that most stroke patients will suffer from motor and sensory disabilities due to the destruction of related neurons (Lakhan et al., 2009). In order to advance therapeutic interventions for ischemic stroke, it is necessary to know the pathological events that happen during the acute phase of ischemia in the brain (Burrows et al., 2015).

Previous experimental stroke studies have shown that an adult brain is able to promote neurogenesis after cerebral damage. Neurogenesis can lead to a certain level of functional recovery and brain repair by enhancing the activity in preexisting networks (Calautti and Baron, 2003; Yamashita et al., 2006). This phenomenon is known as 'brain plasticity' and can lead to improvements by inducing endogenous neurogenesis (Jin et al., 2010). The ability of an ischemic brain is not sufficient for neurogenesis since only a small number of the dead neurons may be replaced by newborn neurons and because the trophic support is poor (Yamashita and Abe, 2012). Knowing that SCs can induce and support neuronal differentiation (Sanberg et al., 1997; Willing et al., 1999), it can be anticipated that they may have a similar effect on adult neural progenitor cells and induce functional recovery.

It has been shown that inflammation increases infarct volume and worsens the neurological deficits following cerebral ischemia. After free radicals and pro-inflammatory mediators are increased, microglia cells are activated and the circulating inflammatory cells infiltrate the brain tissue. These are known as the characteristics of post-ischemic brain damage. The in-

farct volume is the main result of the post-stroke malfunction of the inflammatory cells (Hug et al., 2009). The current findings show a significant decrease in infarct volume in the striatal area following SC transplantation. Accordingly, it is assumed that SCs might reduce the infarct volume by secreting trophic and neuroprotective factors. The migration rate of the injected cells in the striatum tissue is low, due to the lack of space for cell migration in brain tissue and the absence of connective tissue in the parenchyma. On the other hand, due to the relatively short time frame, this study did not examine the migration of injected cells over the long-term. However, visualization of the injected cells indicated that the cells had accumulated near the injection site and that the migration rate of these cells from the injected area was low.

It should also be stated that SCs are known to express many trophic factors, including transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF-I), desert hedgehog (Dhh), and sulfated glycoproteins-1 and -2 (SGP-1 and -2). In addition to their therapeutic potential, SCs improve the endurance of the surrounding neurons (Sanberg et al., 1997; Luca et al., 2016), survive, and cause no harm to the central nervous system (Senut et al., 1996). In addition, it has been shown that SCs increase the engraftment of allogeneic and xenogeneic cells (Dufour et al., 2004; Kaneko et al., 2013).

There is evidence that the neuroprotective effects of TGF-β are mediated *via* a suppression of microglial activation and inflammatory responses, an upregulation of the extracellular matrix, an increase in cytoprotection, and an inhibition of excitotoxicity (Pratt and McPherson, 1997). Furthermore, previous work has suggested that the effects of TGF-β on glutamate excitotoxicity are due to reducing the concentration of Ca^{2+} and enhancing expression of the Bcl-2 protein (Krupinski et al., 1996). Additionally, in rats, TGF-β increases neurogenesis indirectly (Mathieu et al., 2010) and inhibits p38 mitogen-activated protein kinase (an oxidative stress-associated enzyme) (Loftis, 2011). Accordingly, the reduced infarct volume in this study may partially be due to the expression of TGF-β. It has been demonstrated that insulin-like growth factor-1 (IGF-1) decreases ischemic damage by reducing infarct volume and ameliorates behavioral neurological deficits (Liu et al., 2001). IGF-1 also promotes the survival of neurons and glial cells and regulates cell proliferation (Mitsiades et al., 2002). Additionally, IGF-1 inhibits apoptosis and protects neurons against oxidative stress and excitotoxicity (Kooijman et al., 2009; Liu et al., 2001). Moreover, an *in vitro* experiment has demonstrated that IGF-1 induces the proliferation and differentiation of neuronal progenitors. Hence, it regulates brain plasticity and improves sensory and motor functions (Kooijman et al., 2009). Studies have also emphasized the roles of the Hedgehog (Hh) signaling pathway in the regulation of cellular proliferation and differentiation, tissue repair, and regeneration (Han et al., 2009). In particular, the Hh signaling pathway can regulate the expression of Bcl-2 (Han et al., 2009). Based on these findings, it is assumed that, in the current study, SC-derived Dhh may have decreased the infarcted area *via* the regulation of Bcl-2 and cell survival.

The effects of Sertoli cells on cerebral edema and blood-brain barrier integrity

The current results showed a significant reduction in EBD concentration following SC transplantation. Increased permeability of the BBB after cerebral ischemia results in vasogenic edema formation (Simard et al., 2007). Based on previous reports, TNF-α can also increase the expression of endothelial surface antigens (ICAM-I, VCAM-I, P-selectin, and E-selectin) and enhance leukocyte adherence, leading to BBB leakage and brain injuries (Dobbie et al., 1999; Shen et al., 1997).

As a potent mediator for cell growth, PDGF is produced in different cell types — including endothelial cells — and its effect is similar to that of IGF-1 (Ahn et al., 2012). PDGF is mainly expressed in the penumbra and has angiogenic and neuroprotective properties (Shih and Holland, 2006). It has been reported that PDGF, similar to IGF-1, regulates the expression of Bcl-2 and cell survival (Ahn et al., 2012). Consequently, the reduction of BBB integrity in the present study might partially be due to the effects of PDGF and IGF-1. Other trophic factors secreted from SCs are SGP-1 and -2 (Collard and Griswold, 2002). SGP-1 exerts an inhibitory effect on the activity of nuclear factor kappa (NF-kB) and the expression of TNF-α in a model of Alzheimer's disease. Hence, it reduces inflammation, oxidation, and apoptosis, and consequently, preserves the BBB integrity (May, 1993). SGP-2 has also exhibited a high protection against ischemia-induced damage to hippocampal neurons, suggesting its neurotrophic action. Moreover, it can facilitate cell survival *via* the upregulation of Bcl-xl (Wu et al., 2012). Based on the data outlined above, it can be suggested that SGP-1 and SGP-2 may promote BBB integrity and reduce the infarct size *via* inhibiting apoptosis. Another characteristic of SCs that might partially reduce BBB leakage following edema is the production of Fas ligand (FasL). SCs downregulate the immune responses induced by Fas-bearing T-cells. Hence, it is suggested that they

may decrease TNF-α levels and subsequently preserve the BBB integrity (Lakhan et al., 2013; Willing et al., 1998). Also, SCs produce transferrin and ceruloplasmin to deliver iron and copper to the germ cells, respectively. SCs also contribute to the synthesis of superoxide dismutase and cytochrome C oxidase (Willing et al., 1999), which likely has a protective effect by targeting oxidative stress pathways.

The effect of Sertoli cells on the expression of Bcl-2 and caspase-3 proteins

According to the current data, it is hypothesized for the first time that the improved outcomes after cerebral ischemia observed in the present study may be due to the transplantation of SCs, which reduces the expression of caspase-3 and leads to an upregulation of Bcl-2. As the increased expression of Bcl-2 inhibits the caspase cascade and the mitochondrial apoptosis pathway, it likely decreases the release of mitochondrial cytochrome C and prevents irreversible cell damage. SCs may be a powerful tool for delivering many different trophic and neuroprotective factors to increase neuronal survival and plasticity. It can be suggested that the beneficial effects of transplanting SCs may be due to the enhanced expression of the Bcl-2 protein.

Injuries induced by cerebral ischemia are not only limited to stroke, but also occur in patients with a history of aneurysms, sickle cell anemia, and heart attack. Therefore, in addition to the treatment of this disease, pre-treatment with neuroprotective factors to induce ischemic tolerance may be helpful for patients with a variety of conditions. The various studies that have investigated the effects of different stem cells on stroke patients (clinical trials) have demonstrated potential efficacy and safety, without the occurrence of any adverse or severely adverse events (Singh et al., 2020). There are many properties of SCs that distinguish them from other stem cells. For example, SCs inhibit the immune system, which prevents transplant rejection by creating tight connections and forming an immunological barrier around the nerve cells. In addition, these cells do not express the major histocompatibility complex surface antigens or Fas ligands, which attach to Fas molecules on the surface of lymphocytes and induce apoptosis. It is for these reasons that SCs were selected to be studied in the current research. SCs have also been shown to aid in local immunosuppression after transplantation without additional immunosuppression (Willing et al., 1998). Furthermore, SCs can secrete antioxidant proteins and cyto-protective factors, and act as nursing cells that support the function of other cells. Moreover, it has been shown that SCs can be used in the co-culture technique, through co-overexpression of various factors, in organ transplantation studies (Xu et al., 2019). Thus, it is suggested that SCs should be isolated from individuals and banked before an injury. The banked cells can then be expanded *ex vivo* and transplanted back into the patient. Banking SCs prior to ischemia would allow for the cells to be administered as off-the-shelf medications in the clinic (Kaneko et al., 2013). SCs taken from an individual prior to ischemic stroke can be transplanted into patients who are at high risk for stroke.

CONCLUSION

In sum, the administration of SCs may be a new therapeutic intervention for cerebral ischemia that is not toxic to the brain and will not be rejected due to their ability to restrict immune responses. Further experiments should be conducted to increase the likelihood of neuronal survival and neuroplasticity in the ischemic area following SC transplantation.

Implantation of Sertoli cells is a new effective candidate to decrease the damage induced by neurovascular diseases. This treatment showed neuroprotective effects in an animal model of cerebral ischemia by decreasing neurological deficits, reducing infarction volume, inhibiting brain edema, and maintaining blood-brain barrier integrity. The cerebro-protective effects of Sertoli cells likely relate to their anti-apoptotic properties, which are mediated by effects on the Bcl-2 and caspase-3 pathway.

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