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ORIGINAL ARTICLE

The effects of fibroblast growth factor-2 and pluripotent astrocytic stem cells on cognitive function in a rat model of neonatal hypoxic-ischemic brain injury

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Abstract

Objective: This study aimed to determine the effect of pluripotent astrocytic stem cells (PASCs) and fibroblast growth factor-2 (FGF-2) on cognitive function in neonatal rats with hypoxic-ischemic brain injury (HIBI).

Methods: The study was performed on 7-d-old rats that were randomly divided into four groups. All rats, except those in the sham group, were kept in a hypoxic chamber containing 8% oxygen for 2 h after the ligation of the right carotid artery. Next, 5 d after HIBI was induced, PASCs were administered to the motor cortex, and FGF-2 was administered intraperitoneally to group AF; PASCs were administered to the motor cortex, and salt solution buffered with phosphate was administered intraperitoneally to group A; and fresh cell culture solution (medium) was administered to group M. Immunofluorescence was used to localize the administered PASCs in the brains of rats from groups A and AF. The Morris water maze tank (MWM) test was performed to assess the rats' cognitive functions at week 12. The rats that were administered PASCs were observed for the development of neoplasms and autopsies were performed after 30 months.

Results: PASCs migrated to damaged brain regions surrounding the hippocampus in groups A and AF. The mean platform finding time (PFT) significantly decreased over time in each group on day 1–4 of MWM testing ($p < 0.001$). On day 2–4, the mean PFT was shortest in group S followed by group AF. In group A, the PFT was significantly longer than in group S on day 3–4 ($p = 0.01$ and 0.007, respectively). On day 5 of the MWM test, the time spent in the eastern quadrant (which previously contained the platform) was longest in group S followed by groups AF, A, and M; however, the differences between groups were not significant ($p = 0.51$). After 30 months, none of the rats in groups A or AF had benign or malignant neoplasms.

Conclusions: Following the administration of PASCs in rats with experimentally induced HIBI, PASCs migrated to the injured brain regions; however, treatment with PASCs did not have a positive effect on cognitive function. The administration of FGF-2 together with PASCs resulted in positive cognitive results, although not at the level of significance.

Keywords

Fibroblast growth factor-2, hypoxic-ischemic brain injury, stem cell

History

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Introduction

Perinatal hypoxic-ischemic encephalopathy (HIE) is one of the leading factors associated with neonatal disability and mortality worldwide [1–4]. Approximately 20–30% of newborns with HIE die during the neonatal period, and 33–50% of survivors are left with permanent neurodevelopmental disabilities such as cerebral palsy and mental retardation [5]. Cognitive impairment, with or without neuromotor impairment, is a significant problem after HIE [6]. Perinatal HIBI models suggest that learning impairment and memory and behavioral problems persist among the survivors of HIE [7]. In a current study investigating the cognitive functions of school-age children with HIE, more than 30% of patients required special educational services and nearly 35% had intelligence quotients (IQs) lower than 84 [6].

In recent years, the incidences of mortality and disability have been reduced as a result of hypothermia therapy [8–15]. However, serious disabilities continue to develop despite hypothermia treatment, particularly in cases with severe HIE.

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Therefore, more effective HIE treatments are required [16–18]. Experimental studies on HIE and stem cell administration have been conducted, and animal studies have reported that hypoxic-ischemic brain injury (HIBI) can be ameliorated through the administration of stem cells [16,17,19].

Fibroblast growth factor-2 (FGF-2) is present throughout the central nervous system (CNS) and is important for the survival, reproduction, and differentiation of cells [20,21]. The administration of FGF-2 in rats with experimentally induced ischemic brain injury increases cellular reproduction in the brain and stimulates neuronal, astrocytic, and oligodendrocytic differentiation [21]. In one study, neurons collected from the cortices of 18-d-old rat embryos were cultured and then exposed to hypoxic conditions, after which FGF-2 production increased 2.5-fold [22]. Furthermore, the external administration of FGF-2 mRNA reduced the rate of neuronal death due to hypoxia [22]. Based on these findings, FGF-2 is hypothesized to protect neurons against hypoxic stress.

The present study aimed to determine the effect of the administration of pluripotent astrocytic stem cells (PASCs) together with FGF-2 on learning and memory in neonatal rats with experimentally induced HIBI.

Methods

This study was performed on male neonatal Wistar rats aged 7 d ($n = 78$) that were randomly assigned to the following four groups:

Group S (sham group) ($n = 15$): The right carotid artery was located via neck dissection following anesthesia, without ligation or hypoxia, and needles were introduced into the motor cortex 5 d after neck dissection;

Group M (medium group) ($n = 17$): Fresh cell culture medium was administered to the motor cortex 5 d after inducing HIBI;

Group A (astrocyte group) ($n = 22$): PASCs labelled with BrdU (5-Bromo-2′-deoxyuridine) were administered to the motor cortex and phosphate buffered solution (PBS) was administered intraperitoneally 5 d after inducing HIBI;

Group AF (astrocyte + FGF-2 group) ($n = 24$): PASCs labelled with BrdU were administered to the motor cortex and FGF-2 was administered intraperitoneally 5 d after inducing HIBI.

During the study, halothane inhalation anesthesia was administered to the neonatal rats for <5 min after which HIBI was induced according to the Levine-Rice model [23]. A midline neck incision was performed on the rats, and the right carotid artery was located via microscopy. The right carotid arteries of all rats except those in group S were ligated using 6–0 silk sutures. Next, all rats except those in group S were placed in a hypoxic chamber and exposed to a continuous flow of 8% oxygen and 92% nitrogen for 2 h. The rats were then removed from the hypoxic chamber and returned to their mothers. To confirm the presence of HIBI and neuronal apoptosis, three rats were taken from all groups 2 h after removal from the hypoxic chamber, euthanized via cervical dislocation, and decapitated. Apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP end labeling (TUNEL), caspase-3, and immunohistochemistry. Then, three additional rats from groups A and AF were euthanized and decapitated 3 d and 2 weeks following the administration of PASCs to determine the locations of labeled stem cells.

The Morris water maze (MWM) test was administered to the rats in each group 12 weeks after inducing HIBI. After the MWM test, three rats from each group were euthanized and decapitated, and their brains were weighed and examined macroscopically. The remaining rats were observed for the development of any neoplasms and an autopsy was performed on any rat that died spontaneously; those that were still alive after 30 months were euthanized and autopsied.

This study was conducted by the Mersin University School of Medicine Experimental Animals Research Laboratory. The Mersin University Ethics Committee for Animal Experiments approved the study protocol.

Preparation of pluripotent astrocytic stem cells in culture

Neonatal 1-d-old rats were used to prepare pluripotent astrocytic cells in culture. Neonatal rats were euthanized in a carbon dioxide chamber and placed in a container filled with 70% ethanol. The cranium of each rat was cut and the contents of the cranium were removed, washed with medium, and cleaned of blood and other tissues. The olfactory lobe, cerebellum, and basal ganglia were removed. The remaining cortical parts of the brain were diluted with medium and cultured. Cells were highly confluent after 16 d. BrdU was added to the medium to label astrocytes until they were harvested. During harvesting, the cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA), counted, and then centrifuged at 3000 rpm for 10 min, after which the supernatant was removed and the cells were prepared at 50 000 cells μL^{-1}.

Administration of pluripotent astrocytic stem cells and fibroblast growth factor-2

Astrocytic stem cells prepared at a concentration of 100 000 cells per 2 μL were administered to the motor cortex of the right hemisphere using a Hamilton injector 5 d after inducing HIBI. FGF-2 (recombinant human FGF basic 146 aa, cat. no. 233-FB-025) solution was prepared at a concentration of 10 $\mu g\,mL^{-1}$ in PBS containing 0.1% bovine albumin. FGF-2 at a concentration of 10 $ng g^{-1}$ was administered intraperitoneally in group AF following the administration of PASCs.

Histopathological evaluation

Rat brains and ligated carotid arteries were evaluated by a pathologist blinded to the groupings of the study. In a routine follow-up procedure, brain tissues were incubated in alcohol, xylol, and paraffin solutions. Sections 5-μm thick were obtained from the paraffin blocks and stained with hematoxylin and eosin histochemical dyes. Preparations were examined via light microscopy (Nikon Eclipse 80i, Tokyo, Japan). Morphological changes in neurons within the coronal sections were observed by routine staining.

TUNEL method

The TUNEL method (*in situ* apoptosis detection kit, Biogen, cat. no. S7101, Istanbul, Turkey) was used to visualize the destruction of DNA in neurons. Coronal brain sections 5-μm thick were incubated at room temperature with protein kinase K for 15 min following deparaffinization and reconstitution in alcohol. 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogene, and methyl green was used for background staining. Sections were covered with a sealing substance. Tonsil sections with marked germinal centers were used as positive controls.

Caspase-3 method

Following the deparaffinization and alcohol reconstitution of 5 -μm coronal brain sections, rabbit and caspase-3 antibodies (1:100 dilution, Neomarkers, RB-1197-B0) were applied using the Avidin–Biotin complex immunoperoxidase method. The Lab-Vision Ultravision Large Volume Detection System anti-polyvalent horseradish peroxidase (HRP) biochemistry kit was used for immunohistochemical staining, and Mayer's hematoxylin was used for background staining. Tonsil sections with marked germinal centers were used as positive controls.

Evaluation of apoptosis

After TUNEL staining and caspase-3 immunohistochemical staining, coronal brain sections were evaluated under a light microscope. Apoptotic cells were counted in the hippocampus, sub-thalamic nucleus, and parietal cortex in both the right and left hemispheres. To evaluate numerical density, total numbers of TUNEL or caspase-3 positive-stained neurons were calculated in five high power fields (5×400) under a light microscope.

Morris water maze test

A standard MWM test was used to evaluate rats' learning and memory function [24]. MWM testing began upon the completion of postnatal week 12 and was performed for 5 d. A tank 42-cm deep was filled with water at 22 °C. During the first 4 d of the experiment, a hidden platform 15 cm in diameter was adjusted to a height of 40 cm and was placed at the midpoint of the east quadrant. Rats that could not find the platform within 60 s were guided manually to the platform and were allowed to remain on the platform for 15 s. The platform finding time (PFT) was recorded for each trial for 4 d. The hidden platform in the east quadrant was removed on day 5 of the experiment. All of the rats were then placed in the water in the west quadrant and were left in the water for 60 s. Time (s) spent in the east quadrant, which previously contained the platform, was recorded.

Statistical analysis

Statistical analysis was performed using SPSS v. 11.5 for Windows (SPSS, Inc., Chicago, IL). For rats with adequate data, mean ± SD values were calculated, and p values <0.05 was accepted as statistically significant. MWM test data for day 1–4 were evaluated using repeated measures variance analysis (repeated measures ANOVA), and MWM test data for day 5 were evaluated via variance analysis (ANOVA).

Results

During the application of hypoxia, two rats from group M, two rats from group AF, and one rat from group A died. A rat from group M died 16 d after the establishment of HIBI; this rat from group M had significantly retarded growth compared to the other rats, and the exact cause of death was unable to be determined, despite autopsy findings. A total of 24 rats were euthanized and decapitated. MWM tests were performed on the remaining 12 rats in group S, 12 in group M, 11 in group A, and 13 in group AF.

Histopathological findings

TUNEL and caspase-3 staining indicated the presence of between 8 and 12 apoptotic cells in each brain region of the rats in each of the HIBI-induced groups (groups A, AF and M); however, only 0–2 apoptotic cells were present in each brain region of rats in the sham group. This finding indicated that the HIBI model applied in our study was successful; however, statistical analysis of these findings was not performed because only three rats from each group were decapitated. The examination of brain sections from the three rats in groups A and AF that were decapitated on day 3 following the administration of stem cells showed that cells with BrdU-positive nuclei were observed only at the injection sites. No cells with BrdU-positive nuclei were observed at the injection sites of the brain sections from the three rats in groups A and AF that were decapitated 2 weeks after the administration of PASCs, whereas stem cells with BrdU-positive nuclei were observed in the sections that included regions close to the hippocampus (Figure 1). Similar numbers of stem cells (1–4 cells in group A, 1–4 cells in group AF) with BrdU-positive nuclei were present in the brain sections from rats in groups A and AF. Only three rats from each group were decapitated to determine the localization of stem cells; due to the limited number of rats decapitated, we were unable to perform statistical analysis.

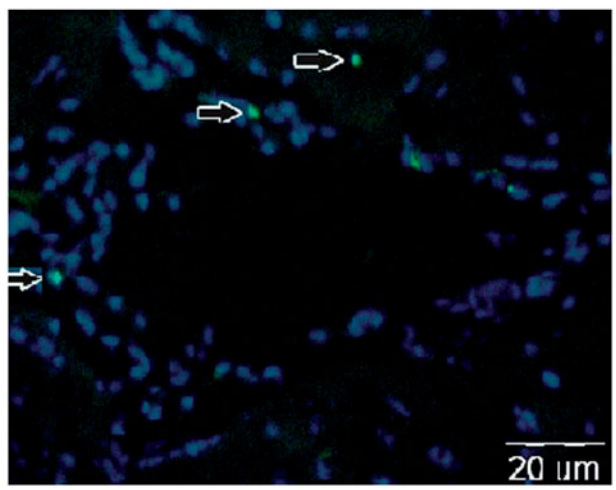

Figure 1. BrdU-labeled cells surrounding the necrotic area in a section including the hippocampus in the brain of a rat 2 weeks following administration of PASCs (BrdU: bromodeoxyuridine).

Table 1. Mean PFT during MWM testing on days 1–4.

	PFT (mean ± SD)				
	Day 1	Day 2	Day 3	Day 4	Differences in PFT between day 1 and 4 (mean ± SD)
Group M	46.7 ± 11	38.5 ± 12	26.7 ± 10	24.0 ± 14	22.6 ± 15
Group A	50.7 ± 10	38.1 ± 21	36.3 ± 21	32.3 ± 22	18.3 ± 17
Group AF	49.0 ± 15	34.0 ± 15	22.9 ± 14	21.7 ± 16	27.3 ± 12
Group S	43.1 ± 10	21.6 ± 7	14.2 ± 6	9.2 ± 5	33.8 ± 14
p	0.596	0.073	0.01*	0.007*	0.098

MWM, Morris water maze; PFT, platform finding time.
*Significant differences between day 3 and day 4 were observed only in groups S and A.

Morris water maze test

The mean PFT was calculated separately for each group on day 1–4 of MWM testing. The mean PFT decreased significantly in each group over time ($p < 0.001$). On day 2–4, the mean PFT was shortest in group S, followed by group AF. In group A, the PFT was significantly longer than group S on day 3–4 ($p = 0.01$ and 0.007, respectively) (Table 1). On day 5 of the MWM test, the time spent in the eastern quadrant (which previously contained the platform) was longest in group S (19.8 ± 8.0 s), followed by groups AF (18.4 ± 9.0 s), A (15.9 ± 7.5 s), and M (15.9 ± 4.3 s); however, the differences between groups were not significant ($p = 0.51$).

Gross examination of rat brains and brain weights

At week 14, the brains of the rats in the sham group were observed to be normal, whereas the right hemispheres of the brains of the rats in the other groups were smaller, with irregular contours. The most severe effects were observed in the rats in groups M and A. The mean brain weight in group S (1641 mg) was highest, followed by those in group AF (1427 mg), group A (1357 mg), and group M (1269 mg). Statistical analysis was not performed on brain weight measurements because only three rats from each group were euthanized and decapitated.

Autopsy findings

Only one rat in group M died, 9 month post-HIBI, and one rat in group A died 11 months post-HIBI; both were autopsied. Of the remaining rats, all were autopsied 30 months post-HIBI. Changes associated with aging were observed in all the autopsied rats; however, no benign or malignant neoplasms were observed in any of the rats' brains or other organs.

Discussion

The present findings demonstrate that following the administration of PASCs in rats with experimentally induced HIBI, PASCs migrated to the injured brain regions; however, cognitive functioning did not improve. Additionally, the administration of FGF-2 with PASCs had a positive effect on cognitive functioning, but the effect was not significant. Perinatal HIE remains an important problem, despite scientific and technological advancements. Stem cell therapy is a recently developed treatment for HIE, and relevant experimental studies have begun [17,19,25]. Diverse types of cells, including embryonic stem cells, neuronal stem cells, bone marrow cells, mesenchymal stem cells, and cells harvested from the umbilical cord can be used for stem cell administration [16,17,19,25]. In the present study PASCs, which have been shown to transform into neurons or glial cells, were used.

Van Velthoven et al. studied the generation of new cells in the brain following experimentally induced HIBI in 9-d old neonatal rats and reported that self-cell generation in the brain was suppressed on day 1 after the induction of HIBI but began to increase on day 2 and peaked on day 3 [17]. These findings suggest that cell death was predominant, and cell reproduction was suppressed, on day 1 following HIBI; the creation of an environment suitable for cellular reproduction began on day 2; and a suitable environment for cellular reproduction was established on day 3. Based on these findings, in the present study, stem cells were administered 5 d after HIBI was experimentally induced.

Stem cells in animals with experimentally induced HIBI can be administered through the veins, peritoneum, arteries, heart, or brain [26,27]. More stem cells reach the brain when administered through the heart or internal carotid artery compared to administration through the veins [26]. A recent study reported that stem cells administered intranasally reach the brain in rats with experimentally induced HIBI [28]; however, as the anatomic structures of rats differ from those of humans, intranasal cell administration in humans might not have the same result. In the present study, stem cells were administered directly into the brain to ensure that all administered stem cells reached the brain.

Zheng et al. [25] administered 50 000 multipotent astrocytic stem cells (MASCs) μL^{-1} into the brains of 7-d old rats with experimentally induced HIBI. The stem cells migrated to the injured brain regions and transformed into neurons and astrocytes; however, as functional tests were not performed, the effectiveness of the treatment is difficult to determine. In the present study, rats with experimentally induced HIBI were administered 100 000 PASCs $2\,\mu L^{-1}$ and the stem cells migrated to the injured brain regions; however, treatment with PASCs had no effect on learning or memory. To the best of our knowledge, Zheng et al.'s study and the present study are the only reports in the literature that describe the administration of PASCs in rats with experimentally induced HIBI. According to the results of these two studies, although PASCs administered following the induction of HIBI migrated to the injured brain regions, the treatment did not result in any functional improvements, which might have been due to the small numbers of cells administered or the type of stem cells used.

Van Velthoven et al. [17] administered mesenchymal stem cells (MSCs) harvested from mouse bone marrow into the brains of mice 3 d after the induction of HIBI. Neurons, oligodendrocytes, and astrocytes exhibited significantly increased rates of reproduction, and numbers of microglia decreased in the MSC treatment group. Double labeling showed that the majority of the reproducing cells were not the transfused cells, but were native brain cells, which

suggests that the transfused cells stimulated the reproduction of native cells in the brain via secreted factors. In addition, the results of functional tests improved in the animals to which MSCs were administered. The beneficial effects of administering stem cells to rats with experimentally induced HIBI were the result not only of the migration of stem cells to injured brain regions but also of secreted factors [17,29]. The identification of these factors could lead to the development of more effective treatments for HIBI or of treatments that do not require the administration of stem cells.

FGF-2 has protective effects against ischemic brain injury. In a study in which ischemic brain injury was induced in 3-d-old neonatal rats via the ligation of the bilateral carotid arteries, $10\,ngg^{-1}$ of FGF-2 was administered to 54 rats, physiological saline was administered to another 54 rats, and 54 rats were included in the sham group [30]. The generation of new cells in the sub-ventricular regions of the brain on day 4, 7, and 14 were compared among the three groups; in the FGF-2 group, more new cells were generated than in the other groups at all three time points. Additionally, the numbers of neurons, astrocytes, and oligodendrocytes were higher in the FGF-2 group than in the sham group. Neurons comprised the greatest proportion of reproducing cells in the FGF-2 group. FGF-2 not only stimulated cell reproduction but also stimulated the differentiation of cells into neurons, astrocytes, and oligodendrocytes [30]. Wang et al. [31] reported that the administration of FGF-2 in adult rats with ischemia that was experimentally induced via the ligation of the middle cerebral arteries resulted in increased cell reproduction and improved functional test results. FGF-2 and PASCs were administered in the present study, as FGF-2 is known to have neuronally protective effects and the literature on FGF-2 in HIBI models is scarce. In the present study, the MWM test results of rats that were administered FGF-2 + PASCs were most similar to those of rats from the sham group. Therefore, we hypothesize that FGF-2 had positive effects on HIBI, although it is difficult to discern whether the positive effects of FGF-2 were direct or mediated by PASCs.

The development of neoplasms following the administration of stem cells remains a concern [32]; thus, the rats in the present study were followed-up for 30 months in the laboratory. Rats that died spontaneously during the follow-up period were autopsied and those that were alive at 30 months were euthanized and autopsied. Changes associated with aging were observed in all of the autopsied rats; however, no benign or malignant neoplasms were observed, which indicates that the administration of PASCs and FGF-2 did not increase the risk of developing neoplasms. However, only a small number of rats were included in this study, and our findings must be carefully evaluated.

In conclusion, the present findings demonstrate that PASC administration alone did not have a positive effect on cognitive function in neonatal rats with experimentally induced HIBI. The administration of PASCs together with FGF-2 had a positive effect on cognitive functioning, although the effect did not reach the level of significance. We believe that additional research on the administration of FGF-2 to rats with HIBI is necessary.

Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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