ORIGINAL ARTICLE

Fibroblast growth factor 2 improves cognitive function in neonatal rats with hypoxic ischaemic brain injury

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Abstract

Objective: To determine the effect of fibroblast growth factor 2 on cognitive function in neonatal rats with hypoxic-ischaemic brain injury.

Methods: The randomised controlled study was conducted from January to June 2011 at Mersin University, School of Medicine, Experimental Animals Research Laboratory and Physiology Behaviour Laboratory, Mersin, Turkey. It included 7-d-old male rats that were randomised into four groups: fibroblast growth factor 2-20, fibroblast growth factor 2-40, control and sham. All the rats, except those in the sham group, were kept in a hypoxia chamber containing 8% oxygen for 2 hours following ligation of the right carotid artery. After hypoxic-ischaemic brain injury was induced, 20 ng g-1 or 40 ng g-1 of fibroblast growth factor 2 was administered via the intraperitoneal route. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling method was used to evaluate neuronal apoptosis. The Morris water maze (MWM) test was administered to the rats at age 14 weeks.

Results: Of the 78 rats on the study, 18 (23%) were in the sham group, while the other three groups had 20 (25.6%) rats each. The number of apoptotic neurons in the right hemisphere in the experimental groups was significantly lower than in the control group ($p=0.004$ and $p<0.001$). The number of apoptotic neurons in the right hemisphere in the fibroblast growth factor 2-40 group was significantly lower than in the fibroblast growth factor 2-20 group ($p<0.001$). Moreover, fibroblast growth factor 2improved Morris water maze test cognitive performance in a dose-dependent manner.

Conclusion: Fibroblast growth factor 2 treatment reduced neuronal apoptosis and improved cognitive functioning in neonatal rats with experimentally-induced hypoxic-ischaemic brain injury.

Keywords: Fibroblast growth factor 2, Hypoxic-ischaemic brain injury, Newborn. (JPMA 66: 549; 2016)

Introduction

Hypoxic-ischaemic encephalopathy (HIE) is a major cause of neonatal disability and mortality.[1] Favourable outcomes are being obtained with therapeutic hypothermia (TH), but disability and mortality still occur in severe cases despite TH.[2] As such, more efficacious treatment methods for HIE are needed.

Fibroblast growth factor 2 (FGF2) is 1 of the 22 members of the FGF family.[3] FGF2 was first described as a stimulant factor for cell division in fibroblasts, and it was recently reported that FGF2 was also important for the growth, renewal and functioning of many tissues and organs.[4] FGF2 exists throughout the central nervous system (CNS) pre- and post-natally, and plays a key role in cell proliferation and differentiation. FGF2 is also thought to protect neurons from free radicals, nitric oxide, excitatory amino acids, hypoglycaemia, hypoxia and ischaemia.[5-9] Although the neuro-protective effect of FGF2 is well understood, its effect on neonatal hypoxic-ischaemic brain injury (HIBI) has not been adequately investigated.

The current study was planned to determine if neuronal apoptosis can be reduced and if cognitive functioning improves following administration of 2 different doses of FGF2 to newborn rats with experimentally-induced HIBI.

Materials and Methods

The randomised controlled study was conducted from January to June 2011 at Mersin University, School of Medicine, Experimental Animals Research Laboratory and Physiology Behaviour Laboratory, Mersin, Turkey. After approval from the university's Animal Experiments Ethics Committee, the study included rats that were randomised into four groups: FGF2-20, FGF2-40, control and sham. Considering that 7-d-old male rats are usually preferred in neonatal HIBI model, we included 7-d-old male healthy rats, and excluded rats older/younger than day 7. Also, female rats were excluded.

In order to provide the study with sufficient statistical power, it was planned to include a minimum of 8 rats per group during the Morris water maze (MWM) test. In

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addition, it was planned to decapitate at least 8 rats per group to evaluate neuronal apoptosis. As such a minimum of 16 rats were considered to be included in each group. Moreover, considering that the rats might die during the procedures, it was planned to include 4 additional rats in FGF2-20, FGF2-40, and control groups, and 2 additional rats in the sham group.

HIBI was induced according to the Levine-Rice model.[10] Inhalation anaesthesia was administered to all the rats for <5min, and then midline neck incision was performed and the right carotid artery was located via microscopic guidance. The right carotid artery in each rat, excluding those in the sham group, was tied with 6.0 silk suture. Next, all rats, excluding those in the sham group, were placed in a hypoxia chamber containing 8% oxygen for 2 hours. They were then removed and administered the treatment according to group. The FGF2-20 group was given 20 ng g-1 of FGF2 via the intraperitoneal route immediately after HIBI was induced. FGF2 (recombinant human FGF basic 146 aa, cat. no. 233-FB-025) solution was prepared, so as to contain 10μg mL-1 in phosphate buffered saline (PBS) containing 0.1% bovine albumin. The FGF2-40 group was given 40 ng g-1 of FGF2 via the intraperitoneal route immediately after HIBI was induced. Rats in the control group were given PBS 0.1 mL containing 0.1% bovine albumin via the intraperitoneal route immediately after HIBI was induced. In the sham group, the right carotid artery was located, but not tied following neck dissection, and hypoxia was not induced.

Following this procedure, all the rats were placed beside their mothers for a 2-hour recovery period. Then, 10 rats from each group were euthanised via cervical dislocation and were decapitated. Brains were removed and analysed for neuronal apoptosis using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) method. MWM test was administered to the remaining rats at age 14 weeks.

Rat brains were examined by a pathologist blinded to the groups and to which carotid artery was tied. Two samples from the subthalamic nucleus, hippocampus, and parietal cortex were obtained. The TUNEL method (in situ apoptosis detection kit, Biogen, catalog no S7101) was used to detect deoxyribonucleic acid (DNA) fragmentation in neurons.

Coronal brain sections 5 m thick were deparaffinised and treated with alcohol, and were then incubated with protein kinase K at room temperature for 15min. After this procedure, endogen peroxidase activity was quenched with 2% water (H_2O_2). Slices were than incubated at 37°C for 60min in a moist chamber with 50μl of terminal deoxynucleotidyl transferase (TdT) buffer. Finally, the reaction was visualised with a streptavidin-biotin-peroxidase complex and diaminobenzidine. TUNEL-labelled slides were counter-stained with 1% methyl green.

Apoptotic cell counting was performed in hippocampus, subthalamic nucleus, and parietal cortex of both the right and left hemispheres. In evaluating numeric density, total TUNEL positive stained neurons were calculated in five high power fields (5 x 400) under the light microscope.

MWM tests were initiated when the rats were aged 14 weeks and the tests were performed for 5 days. MWM tests were performed by the physiologists who were blinded to the groups. Rats were brought to the behavioural experiments laboratory for habituation 2 days before starting the MWM test. A tank 42cm deep was filled with water at 22°C. During all MWM tests the rats were placed in the tank by the same investigator. MWM tests were performed between 0900 and 1400 hrs in all groups. The tank's image was transmitted to a computer screen and was divided into 4 equal quadrants, namely the west, north, east, and south quadrants. During the first 4 days of the experiment, a 15-cm diameter platform was adjusted to a height of 40cm, so that it could not be observed from outside the tank, and was placed in the middle of the east quadrant. On the first day of the experiment, all rats, starting from the western quadrant and proceeding clockwise were placed in the water once in each quadrant with their heads turned toward the wall of the water maze. On the second, third, and fourth days of the experiment, the first placements were in a different quadrant each day, proceeding clockwise from the first placement of the first day. The rats were expected to find the hidden platform in ?60s after they were dropped into the water. Rats that failed to locate the platform within 60s were guided by hand to find the platform and were made to stay there for 15 seconds. Platform finding time (PFT) was recorded for each d/quadrant. The hidden platform in the east quadrant was removed on day 5 of the experiment, and then all rats were dropped into the water in the west quadrant and were made to remain in the water for 60 seconds. Time (s) spent in the east quadrant (which previously contained the platform) was recorded.

Data was analysed using SPSS 11.5. The normalities of continuous measurements were examined with Shapiro-Wilk test. Variance analysis was used to identify differences between measurements on day 1, 2, 3, and 4 in each group. Inter-group differences according to the day were also examined via variance analysis. Homogeneity of the variances was determined using the Levene test. One-way analysis of variance (ANOVA) was used when variance was homogenous and the Welch test was used when it was

not. Tukey's and Games-Howell tests were used for paired comparisons. Additionally, inter-group differences in time spent in the east quadrant on day 5 were analysed using one-way ANOVA and the Welch test. Paired samples t test was used to identify differences between right and left hemisphere apoptotic neuron counts within each group. One-way ANOVA and the Welch test were used to determine inter-group differences between right and left hemisphere measurements. Descriptive statistics were recorded as mean ± standard deviation (SD). The level of statistical significance was set at p<0.05.

Results

Of the 78 rats on the study, 18 (23%) were in the sham group, while the other three groups had 20 (25.6%) rats each. In all, 4 (5.12%) rats died due to various reasons. In total, 40(54%) rats were euthanized and decapitated for neuronal apoptosis assessment. The MWM test was performed on the remaining 34 (43.5%) rats at age 14 weeks; 8 (23.5%) each in the sham, control, and FGF2-20 groups, and 10 (29.4%) in the FGF2-40 group.

There were significantly more apoptotic cells in the right hemisphere than in the left hemisphere in all groups except the sham group (Table-1). The number of apoptotic neurons in the right hemisphere in the sham, FGF2-20, and FGF2-40 groups was significantly lower than in the control group (p<0.001, p=0.004, and p<0.001, respectively). The right hemisphere apoptotic neuron count in the sham group was significantly lower than in the FGF2-20 and FGF2-40groups (p<0.001 and p=0.001, respectively). The right hemisphere apoptotic neuron count in the FGF2-40 group was significantly lower than in the FGF2-20 group (p<0.001).

Table-1: The number of TUNEL-positive apoptotic neurons, according to group.

Group	Right hemisphere	Left hemisphere	P0
Sham (n=10)	1.30 ± 0.8	1.30 ± 0.9	1.000
Control (n=10)	10.30 ± 2.0	3.90 ± 1.2	<0.001
FGF2-20 (n=10)	7.30 ± 0.9	3.20 ± 1.6	<0.001
FGF2-40 (n=10)	3.90 ± 1.37	1.70 ± 0.9	0.001
P1	<0.001	<0.001	
P2	<0.001	0.028	
P3	0.001	0.783	
P4	0.004	0.695	
P5	<0.001	0.001	
P6	<0.001	0.010	

Data given are mean ± SD.
P0:Comparison between the number of apoptotic neurons in the left and right hemispheres.P1:Comparison between the sham and control groups.
P2: Comparison between the sham and FGF2-20 groups.
P3: Comparison between the sham and FGF2-40 groups.
P4: Comparison between the control and FGF2-20 groups.
P5: Comparison between the control and FGF2-40 groups.
P6: Comparison between the FGF2-20 and FGF2-40 groups.
SD: Standarddeviation
FGF2: Fibroblast growth factor 2.
TUNEL: Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labelling.

Table-2: Mean PFT during MWM testing on d 1-4.

Group	d 1 (s)	d 2 (s)	d 3 (s)	d 4 (s)	P0
Sham	39.0 ±10.5	15.9 ± 6.1	8.2 ± 4.3	9.6 ± 6.5	0.003
Control	51.8 ± 10.1	41.7 ± 18.7	41.5 ± 25.4	34.6 ± 23.6	0.260
FGF2-20	44.4 ± 12.9	30.7 ± 24.5	17.0 ± 20.1	13.1 ± 15.2	0.040
FGF2-40	45.3 ± 10.8	19.6 ± 15.9	14.8 ± 13.6	12.4 ± 10.1	<0.001
P1	0.174	0.023	0.030	<0.001	
P2	1.000	0.401	0.638	0.930	
P3	1.000	0.903	0.503	0.889	
P4	1.000	0.748	<0.001	<0.001	
P5	1.000	0.032	<0.001	0.001	
P6	1.000	0.691	0.993	1.000	

Data given are mean ± SD.
P0: Indicates if PFT differed from d 1 to d 4 within each group. P1, P2, P3, P4, P5, and P6: Indicates if PFT differed between sham and control group, sham and FGF2-20 groups, sham and FGF2-40 groups, control and FGF2-20 groups, control and FGF2-40 groups, and FGF2-20 and FGF2-40 groups, respectively.
MWM: Morris water maze
PFT: Platform finding time
FGF2: Fibroblast growth factor 2
SD: Standard deviation.

The left hemisphere apoptotic neuron count in the sham group and FGF2-40 group was significantly lower than in the control group(p<0.001 and p=0.001, respectively). The sham and FGF2-40 groups did not differ significantly in terms of the left hemisphere apoptotic neuron count (p=0.783). The left hemisphere apoptotic neuron count in the sham and FGF2-40 groups was significantly lower than in the FGF2-20 group (p=0.028 and p=0.010, respectively). Moreover, the FGF2-20 and control groups did not differ significantly with respect to the left hemisphere apoptotic neuron count (p=0.695).

After the MWM test, there were no significant differences in PFT between day 1, 2, 3, and 4 in the control group (p=0.063), whereas in the sham, FGF2-20 and FGF2-40 groups, PFT differed significantly between day 1, 2, 3, and 4(p<0.001, p=0.016, and p<0.001, respectively). PFT was significantly shorter on day 4 than on day 1 in the sham, FGF2-20, and FGF2-40 groups (p=0.003, p=0.040, and p<0.001, respectively), but not in the control group (p=0.260). There was no significant difference in PFT on day 1 among the 4 groups (p=0.174). On day 2 PFT was significantly shorter in the sham and FGF2-40 groups than in the control group (p=0.023 and p=0.032, respectively). PFT on day 3 and day 4 was significantly short in the sham, FGF2-20, and FGF2-40 groups than in the control group (Table-2).

Mean time spent in the east quadrant on day 5 was 18.4±8.8s in the control group versus 27.7±5.8s in the sham group, 26.6±5.9s in the FGF2-40 group, and 23.5±3.7s in the FGF2-20 group. The rats in the sham and FGF2-40 groups spent significantly more time in the east quadrant on day 5 than the rats in the control group (p=0.004 and p=0.015, respectively). There were no significant differences in time spent in the east quadrant on day 5 between the control and FGF2-20 groups (p=0.120).

Discussion

There is currently no adequately effective method of treatment for newborns with severe HIE. Severe disability and mortality in newborns with HIE can be reduced by only 15% via TH.[2] Therefore, new effective methods of treating HIE are needed. FGF2 is a multifunctional growth factor that exists throughout the CNS.[3] FGF2 is found in messenger ribonucleic acid (RNA)in the cortex, hippocampus, striatum, thalamus, substantia nigra, olfactory bulbs, pons, medulla oblongata, motor and sensory nuclei, and the anterior and posterior pituitary glands.[11-18] FGF2 activates high affinity tyrosine kinase receptors (FGFR1-4) in alliance with heparin or heparin sulfate proteoglycan, which in turn activates its pleiotropic effects. FGF2 plays an important role in CNS differentiation and functioning.[3]

An experimental study reported that neuron proliferation in the hippocampus was markedly reduced via intrinsic FGF2 suppression in neonatal rats, and that administration of a single 20-ng g-1 dose of extrinsic FGF2 subcutaneously increased neuron production two fold. Based on this finding, the study's researchers posited that hippocampal neurogenesis in newborn rats was regulated by environmental FGF2 levels.[19] Another study reported that FGF2 production increased 2.5 fold when neurons from the cortex of rat embryos were exposed to hypoxia. That study also reported that extrinsic administration of FGF2 reduced hypoxia-associated neuron death.[20] The results of our study were also correlating with these previous studies. Intraperitoneal FGF2 administration immediately after hypoxia caused a significant decrease in neuronal apoptosis in our study.

Seo et al.[21] housed mice with HIBI in a stimulating environment (running wheels, tunnels, shelters, and toys) or standard cages for 2 months. The FGF2 level in the mice in the stimulating environment was significantly higher than in those housed in standard cages, and the mice in the stimulating environment exhibited a higher level of neuro behavioural functioning. A subgroup of the rats housed in the stimulating environment was administered anti-FGF2 and the positive effects associated with that environment disappeared. Based on these findings, the researchers suggested that the stimulating environment, as opposed to increased FGF2, resulted in improved neuro behavioural functioning in the mice with chronic HIBI. Although the study designs were different, but the findings of our study support the study of Seo et al.[21] We found that intraperitoneal FGF2 administration caused significant improvements in cognitive tests. In addition, we obtained better results in cognitive tests following high-dose FGF2 administration.

It was reported that subcutaneous administration of 10 ng g-1 of FGF2 for 7 days rats with experimentally-induced bilateral motor cortex damage resulted in less brain injury and better cognitive functioning than in those not administered FGF2.[22] A study on rats with ischaemic brain injury induced via binding the bilateral carotid arteries reported that intraventricular administration of 10 ng g-1 of FGF2 increased cell proliferation in the brain and stimulated neuron, astrocyte, and oligodendrocyte differentiation.[23] Another study on rats with ischaemic brain injury induced via binding the mid cerebral artery reported that cell proliferation in the brain increased, neuron conversion was stimulated, and neurological functional test results were better in the rats administered FGF2 nasally.[24] Our results support the findings of these studies. In our study, we found that intraperitoneal FGF2 administration led to a significant decrease in neuronal apoptosis, and significant improvement in cognitive tests.

There are only a few studies that have investigated the effects of FGF2 based on neonatal rat HIBI models. Rats with induced HIBI were given FGF2, and then nestin and growth-associated protein-43 (GAP-43) expression in the brain and cognitive functioning were investigated.[25] The researchers reported that there was significant improvement in cognitive functioning in the rats that were administered FGF2. They also reported that nestin and GAP-43 expression increased in the rats administered FGF2, and suggested that this finding might have been associated with the role of FGF2 in neuronal damage repair. In another study, neonatal rats with induced HIBI were treated with FGF2, and then neuronal apoptosis and bone morphogenetic protein 4 (BPM4) expression in the rats' brains were examined.[26] It was observed that FGF2 treatment resulted in a significant reduction in neuronal apoptosis and a significant increase in BPM4 expression. Based on these findings, the researchers posited that FGF2 inhibits neuronalapoptosis and repairs neuronal damage via increasing BPM4 expression. Similarly, in the present study a single dose of FGF2 administered immediately following hypoxia-ischaemia decreased

neuronal apoptosis in the brain and was associated with significant improvement in cognitive functioning; FGF240 ng g-1 was more effective than 20 ng g-1.

An important limitation of our study is that we were unable to demonstrate how FGF2 improves cognitive functions. However, previous studies have shown that FGF2 stimulates cell proliferation and differentiation in brain, and prevents neuronal apoptosis. FGF2 may improve cognitive functions via one or more of these mechanisms. In our study, we demonstrated that FGF2 prevented neuronal apoptosis. However, we did not examine the effects of FGF2 on cell proliferation or cell differentiation in brain. We could have elucidated this issue if we had examined cell proliferation and differentiation in brain after FGF2 administration in our study.

Conclusion

FGF2 administered intraperitoneally in neonatal rats with induced HIBI reduced neuronalapoptosis and improved cognitive test performance. Based on our findings, FGF2 shows promise for the treatment of neonatal HIBI and additional research is warranted.

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