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Increased Levels of Nerve Growth Factor Indicating Brain Injury in Mice Model

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Abstract

Hypoxic-ischemic encephalopathy (HIE) brain injury is one of the leading causes of death and disability worldwide. *Nerve growth factor* (NGF) is a neurotrophin that plays an important role in the natural repair and regeneration of nerves, but the previous study regarding NGF level after brain injury is still scant. This study aims to determine NGF levels in male Wistar rat models that received right Common Carotid Artery (CCA) occlusion. This study used an experimental and control design conducted in July-August 2021 at the Stem Cell Research and Development Center, Universitas Airlangga. The right CCA occlusion was performed on the Wistar mouse model in the treatment group, then placed in a hypoxic chamber and reperfusion after 60 minutes. Observations of neurology scores were carried out in the first 24 hours. After 2x24 hours the animal was sacrificed for serum NGF level measurement using the ELISA method. Statistical analysis using *t-test for independent sample*. A total of 16 male rats participated in the study. Eight rats in the treatment group were put into hemiparesis at different levels according to observations of neurological scores. Statistically meaningful differences in NGF levels were found in the treatment group compared to controls ($P < 0.05$). Average NGF levels in the treatment group were higher than in the controls. NGF levels in mice with HIE were higher than the control group, which indicates the body's natural mechanism for neuron protection following ischemic hypoxic events.

Keywords: *nerve growth factor*, ischemic hypoxia, Wistar rats

Original Research Article

Peningkatan Kadar NGF pada Model Tikus Wistar Hipoksia Iskemik sebagai Respon Paska Cedera Otak

Abstrak

Cedera otak hipoksia-iskemik ensefalopati (HIE) adalah salah satu penyebab utama kematian dan kecacatan di seluruh dunia. *Nerve growth factor* (NGF) adalah neurotrofin yang berperan penting dalam perbaikan alami dan regenerasi saraf, namun hingga kini studi terkait kadar NGF paska cedera otak masih terbatas. Penelitian ini bertujuan untuk mengetahui kadar NGF pada tikus model Wistar jantan yang mendapat perlakuan oklusi arteri karotis komunis (AKK) kanan.

Penelitian ini menggunakan desain eksperimen dan kontrol yang dilakukan pada bulan Juli-Agustus 2021 di Pusat Penelitian dan Pengembangan Sel Punca Universitas Airlangga. Oklusi AKK kanan dilakukan pada tikus model Wistar pada kelompok perlakuan, kemudian ditempatkan dalam hipoksia chamber dan dilakukan reperfusi setelah 60 menit. Pengamatan skor neurologi dilakukan pada 24 jam pertama. Setelah 2x24 jam hewan dikorbankan untuk pengukuran kadar NGF serum menggunakan metode ELISA. Analisis statistik menggunakan *t test for independent sample*. Sebanyak 16 tikus jantan mengikuti penelitian ini. Delapan tikus pada kelompok perlakuan mengalami hemiparesis dengan tingkat yang berbeda sesuai dengan pengamatan skor neurologis. Perbedaan

bermakna secara statistik kadar NGF pada kelompok perlakuan dibandingkan dengan kontrol ($P < 0,05$). Rata-rata kadar NGF pada kelompok perlakuan lebih tinggi dibandingkan dengan kontrol. Kadar NGF pada tikus dengan HIE lebih tinggi dibanding kelompok kontrol, yang

menunjukkan mekanisme alami tubuh untuk proteksi neuron pasca kejadian hipoksia iskemik.

Kata Kunci: *nerve growth factor, hipoksia iskemik, tikus wistar*

INTRODUCTION

Hypoxic-ischemic (HI) brain injury and stroke are closely related and are adverse conditions that can affect individuals of any age. Acute brain injury after cerebral ischemia due to stroke or HI brain injury, including hypoxic-ischemic encephalopathy (HIE), together is one of the leading causes of death and disability worldwide (Meloni et al, 2017; Gunawan et al, 2018, Gunawan et al, 2019; Greco et al, 2020; Hansen and Soul, 2021).

After a traumatic brain injury, neurotrophins that play an important role in the repair and regeneration of nerves are regulated again. Among the various neurotrophins, NGF becomes the most investigated in traumatic brain injuries both preclinically and clinically. NGF exerts survival and axonal growth effects on developing neurons as well as on mature neurons as well as angiogenic effects on endothelial cells and regulates hematopoietic cells. NGF expression was found to be positively correlated with the severity and outcome of traumatic brain injury in children, and its regulation after traumatic brain injury was associated with better neurological outcomes (Ginberg et al, 2018). Compared to other neurotrophins, the concentration of NGF is higher. The NGF protein increases from the first week and persists for 7 weeks (Cheng et al, 2016). NGF has been used only in several case report in developed country, there is lack of reports and studies in Indonesia regarding (NGF) in brain injury. This agent has been used for hypoxic-ischemic injury and showed a favorable outcome, but the result is still vary (Fantacci et al, 2013; Widodo et al, 2016).

A better understanding of the pathophysiology of HI brain injury became an important condition for the development of new and effective neuroprotective therapies. This study aimed to determine whether there were increases in NGF levels in the control rat model and those who received the right communist carotid artery occlusion treatment.

METHODS

Design

This study used an experimental design of treatment and control.

Time and Place

Research activities were carried out at the *Stem Cell* Research and Development Center, Universitas Airlangga in July-August 2021. The adaptation phase is carried out for 1 week, while the treatment phase and observation of symptoms are for 48 hours.

Ethical Eligibility

This research has received a certificate of Ethical Eligibility, Faculty of Veterinary Medicine, Universitas Airlangga No. 2.KE.019.02.2018.

Animal Trials

The preparation of experimental animals used 22 Wistar strain male white rat *Rattus norvegicus* aged 6-month-old in good health, never been used in previous studies, and not infected with any disease when the study began. This experimental research used Wistar rat experimental animals obtained from the *Stem Cell* Development Center, Universitas Airlangga which were randomly grouped. The rats were classified into two, namely the control group and the treatment group. In the control group, no intervention is carried out. In the treatment group, a right CCA ligation was performed. The rats were then placed for 45 minutes in hypoxic atmospheric chambers of 8%O₂ and 92%N₂ (Wilson, 2015; Hamdy et al, 2020). Post-hypoxia reperfusion is performed by opening the CCA ligation (after 60 minutes). After 2x24 hours the rat was sacrificed. NGF levels were measured in blood serum determined by enzyme-linked immunosorbent assay (ELISA) examination. The NGF reagent used in this study was E-EL-RB1337, Elabscience, China which was read with SPECTROstar® Nano plate reader, BMG Labtech, Germany. Results are expressed in units of ng/mL and categorized in numerical scales. All instruments used in this research had been calibrated.

Ligation Procedure

Before ligation, Wistar rats were anesthetized with 10% xylazine/acepromazine. After anesthesia, the rat's fixation is carried out on the dissection board. Then the ventral neck fur was shaved and sterilized using iodine and alcohol 70%. A scalpel was used to make an incision on the cervical regional midventral, in the middle of the neck, on the upper edge of the sternum (about 1-1.5 cm long). Then the submandibular glands were released using *ophthalmic forceps*. There will be access to the sternocleidomastoid muscles and the sternohyoid muscles. Gently pull the sternocleidomastoid muscle deep inside the sternohyoid muscle (until the sternohyoid muscle appears), and there will be the CCA wrapped by fibrous connective tissue, which contains the vagal nerve inside. The CCA was carefully separated from the attached tissue using *ophthalmic forceps*. The right CCA is tied twice with silk thread 4-0, the two stitches are next to each other, then the surgical wound is sutured back with silk stitches. All of the procedures were performed by the veterinarian in the lab.

Neurology score

Clinical proof of brain ischemia was carried out through neurological examination evaluated on a six-point scale, classified as 0: No neurological

deficit; 1: Failed to extend the left front foot completely. It indicates a mild focal neurological deficit; 2. Rotate to the left. This means a moderate focal neurological deficit; 3. Fall to the left. It indicates a severe focal neurological deficit; 4. Not walking spontaneously and lowering the level of consciousness; 5. Death due to brain ischemia (Bachour et al, 2016). If the animal's score is 0 or 5, it will be excluded from the study and the dead animal will be buried.

Statistics

The data obtained from the entire control and treatment group were carried out a normality test using Shapiro Wilk, followed by a *T-test analysis test for independent samples*. Statistical test using statistical package *for the social sciences* (SPSS) 21.0 program software

RESULT

Animals were kept for a period of seven days for adaptation. The number of experimental rats used was 22. Animal mortality reached 27% (3) rats in the control group and 27% (3) rats in the treatment group. The exact cause of death is unknown but it is suspected to be due to a severe neurological disorder. A total of 16 rats were finally able to complete the study. Characteristics of experimental animals can be seen in Table 1.

Table 1. Characteristics of the subjects of the study

Weight Loss (BB)	Group	
	Control	Treatment
Early BB (g)		
Average	203.4	206.4
Standard Deviation	4.307	5.476
Final BB (g)		
Average	199.0	204.3
Standard Deviation	4.629	5.599

Clinical proof of the occurrence of brain ischemia is carried out by an examination of the neurological score which is an evaluation with a

six-point scale. In this study, it was found that all rats experienced hemiparesis with different levels as described in Table 2.

Table 2. Neurology Score

No	Rat Group Treatment							
	1	2	3	4	5	6	7	8
Post-24-hour neurology score	1	2	2	2	1	1	1	1

The NGF variable data were tested for normality using the Shapiro-Wilks method. The results of the NGF variable data normality test in each group showed a normal distribution of data.

Homogeneity tests using the Levene test show data with homogeneous variations. Statistically meaningful differences in NGF levels were found in the treatment group compared to the control

group. The average NGF levels in the treatment group were higher than those in the controls

(Table 3) with the distribution of NGF level values seen in figure 1.

Table 3. Comparison of control group NGF levels with treatment

Group	N	Average	SD	95%CI	Mean Difference	P
Control	8	0.557	0.096	-0.2164-0.003	0.108	0.049*
Treatment	8	0.653	0.066	-0.2165-0.002		

*Meaningful P<0.05 with T-test for independent sample

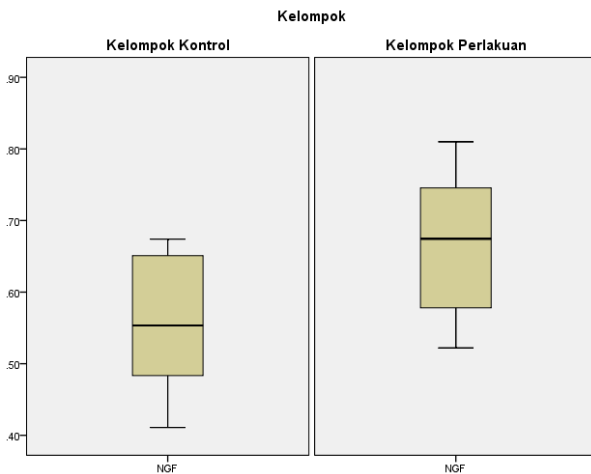


Figure 1. Box-plot image of NGF levels in control and treatment groups

DISCUSSION

Acute brain injuries after cerebral ischemia due to stroke or hypoxia-ischemia, including Ischemic Hypoxic Encephalopathy, together are one of the leading causes of death and disability worldwide (Meloni, 2017). HIE appears in 1-6 per live birth and reaching 26 per 1000 live births in developing countries. About 15%-20% of cases of brain ischemia die during the neonatal period and 30% of those who survive to suffer from neurodevelopmental disorders (Namusoke et al, 2018).

In response to the brain damage, the brain can heal itself through a variety of compensatory processes known as neuroplasticity. During the brain remodeling processes, altered growth factor signaling, synaptogenesis, angiogenesis, neuron cell proliferation, gliogenesis, and cell structural changes can remodel the brain to improve functional recovery (Sims et al, 2022). Some of the developing therapies were directed to stimulate axon growth and provide a protective effect through the secretion of neurotrophic factors such as GDNF, *Brain-Derived Nerve Factor* (BDNF), *Nerve Growth Factor* (NGF), and neurotrophin 3 (Manni et al, 2013; Calza, 2021). Exogenous NGF has a part in neuron plasticity, regeneration and

also inhibition of neuronal apoptosis after brain injury (Lin et al, 2022; Sims et al, 2022).

This study used the CCA ligation method to create a hypoxic process of ischemia in the brain with experimental rat animals. Occlusion of the CCA was conducted by inserting sutures directly into the internal carotid artery and pulling the suture to disrupt the blood supply to the middle cerebral arteries. The cerebral vascular occlusion model in this study has several advantages: First, it mimics human ischemic stroke, which often originates from the occlusion of middle cerebral arteries, and shows a penumbra similar to a human stroke. In addition, the middle cerebral arteries occlusive model is characterized by a large volume of infarction and high reproducibility. Reperfusion and duration of ischemia can be precisely controlled. In addition, this procedure is relatively simple to perform and does not take time (Fluri et al, 2015).

According to Fluri et al (2015), a model of animal brain ischemia has been developed, to identify the mechanisms underlying cerebral ischemia and develop new agents for therapy. The majority of stroke experiments are carried out on small animals (for example, mice, rats, and rabbits). The use of small animals gives obvious advantages - lower cost and greater acceptance from an ethical point of view - compared to larger animals. Rats are one of the most frequently used animals in stroke studies for various reasons: the blood vessels of the brain and the physiology of rats are similar to those of humans, their medium size of the body allows easy monitoring of physiological parameters; its small brain size is well suited for fixation procedures (for example, the process of freezing in vivo for biochemical analysis); there is relative homogeneity in the strain, and most importantly, it is easy to execute reproductive studies (Tayebati et al, 2012).

This study showed results of meaningfully different NGF levels between experimental control and treatment animals. NGF levels in treated animals were higher than in controls.

Neurotrophin is a *neurotrophic growth factor* that is secreted from neuron cells, glial cells, and hematopoietic cells and is responsible for the life defense mechanisms, differentiation, and neuroprotection of neuronal tissues residing within the brain. Specifically, neurotrophin regulates synaptic plasticity and protects neurons from oxidative stress and apoptosis that can stimulate neurogenesis. Members of the neurotrophin family include NGF, brain-derived neurotrophic factor, neurotrophin (NT)-3, and NT-4/5, which are grouped based on their structural similarity with NGF, a prototype of neurotrophin (Ginberg et al, 2018).

In the experimental model of *traumatic brain injury* (TBI), regional brain changes in the expression of neurotrophin and its receptors were observed. For example, NGF reorganized after cortical trauma or injection of the NGF lentivirus gene weakens cholinergic memory deficits and expressed cognitive functions and intranasal administration of NGF reduces TBI-induced brain edema. In addition, progenitor transplantation of embryonic neurons or *bone marrow* cells after experimental TBI demonstrated therapeutic Neurotherapy effects dependent on NGF, measured by improved cognitive and neuromotor function. Also, the subpopulation derived from HUCB MNC has profound neurogenic potential through anti-inflammation, increased neurogenesis, antioxidant activity, and neurotrophin release, such as NGF (Ginberg et al, 2018).

The widespread role of the neuroprotective mechanisms of NGF, both in the central and peripheral nervous systems, has been widely reported, especially as part of neuron damage caused by severe TBI. Intranasal NGF improves perfusion and brain metabolism with the consequence of the concomitant improvement of the child's neurological function, which confirms that this neurotrophin provides a key role in the mechanism of nerve protection in the injured brain (Sims et al, 2022).

The specific role of NGF in determining the mechanisms of nerve repair in damaged brains in this study has not been clearly understood, but some evidence supports the action of this neurotrophin in modulating the biosynthesis of *doublecortin* (DCX) inside the brain. New striatum neurons can be generated when NGF is infused into the lateral ventricles of the brain and an

increase in intrastriatal neurotrophin levels shows the initial phase of induced neurogenesis. Previously, elevated levels of NGF and DCX have been shown to correlate significantly with good outcomes in patients with severe TBI. The correlation between NGF and DCX suggests that these two biological markers are involved in the mechanism of neuron repair after TBI, thus playing an important role in the reorganization of neuron connections after head trauma (Ginberg et al, 2018). The limitation of the study is the minimum length of the study and we didn't measure the other neurotrophins biomarker due to the limited capability of the human and laboratory resource.

The authors suggest for more prospective and long-term research are in needed to determine the exact role of NGF as a promising therapeutic agents for brain injury.

CONCLUSION

From the results of the study, it was found that NGF levels in the blood of hypoxic-ischemic rat's model that received right CCA occlusion treatment and reperfusion were higher than controls. This suggests the existence of a natural mechanism for the protection of neurons after a brain injury.

CONFLICT OF INTEREST

There is no *conflict of interest* in this study.

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