RESEARCH ARTICLE

Inhibition of Neurogenesis and Induction of Glial Scar Formation by Neuroinflammation Following Ischemic Stroke: Evaluation of BDNF, GFAP, HMGB1 and TNF-α Expressions

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Abstract

BACKGROUND: Ischemic stroke remains as a major health problem and one important process in Ischemic Stroke is neuroinflammation which has a principal role to maintain the balance of neurogenesis and neurodegeneration process in the brain. Neuroinflammation can lead to glial scar and inhibit neurogenesis processes which is needed for recovery neuron function. This study was conducted to observe the role of high mobility group box 1 (HMGB1) and tumor necrosis factor-α (TNF-α) as neuroinflammation markers to glial fibrillary acidic protein (GFAP) as glial scar marker and to brain-derived neurotrophic factor (BDNF) as neurogenesis marker in brain tissue following ischemic stroke.

METHODS: Fifteen male Wistar rats were randomized to three groups; sham group, rats receiving occlusion and terminated 180 minutes later (group A), and rats receiving occlusion and terminated after 7 days (group B). Expressions of BDNF and *BDNF* mRNA were examined using immunohistochemistry (IHC) and Reverse Transcription Polymerase Chain Reaction (RT-PCR), respectively. While GFAP, HMGB1, TNF-α were assessed using IHC.

RESULTS: Expression of BDNF was found lower in group A and group B than in sham group (5.20±1.924, 5.00±1.581, and 7.80±1.304, respectively; p=0.032). Expression of *BNDF* mRNA was found lower in group A and B than in sham group as well. While expression of GFAP was found higher in group A and B than in sham group (9.60±1.517, 11.40±2.074, and 5.20±1.48, respectively; p=0.000). Higher level of HMGB1 and TNF-α expressions were also found to in group A and group B than in sham group (9.3±1.528, 11.67±1.528, and 2.00±1.000, respectively; p=0.000 for HMGB1 and 6.33±1.155, 9.33±1.528, and 3.00±1.000, respectively; p=0.002 for TNF-α).

CONCLUSION: The presence of low BDNF levels and high levels of GFAP, HMGB1 and TNF-α markers, possibly reflects inhibition of the neurogenesis process by neuroinflammation, and induced glial scar formation in ischemic stroke conditions after than 180 hours until 7 days.

KEYWORDS: ischemic stroke, BDNF, GFAP, TNF-α, HMGB1

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Introduction

Stroke is the second leading cause of death worldwide, accounting for 11.6% of all deaths in 2019. And the most frequent type of stroke is ischemic stroke.(1) Stroke patients who survive also will experience varying degrees of disability.(2) In the past decade, neurogenesis, a process that produce new neuron from neural stem cells (NSCs), has been extensively studied. Neurogenesis process was found to increase in pathological conditions such as ischemic stroke both in animal and in stroke patients. Neurogenesis is thought to be a key process in recovery process and repair of the damaged brain region following ischemic stroke.(3)

Reactive astrocyte and glial scar formation are factors that cause the primary obstacle for recovery process in ischemic stroke. Reactive astrogliosis and glial scar yield extracellular matrix such as chondroitin sulfate proteoglycans (CSPG) which chemically attenuated neurogenesis process. (4) Glial scar formation influenced by neuroinflammation which will induce reactive astrogliosis as the first step for glial scar formation.(5) Glial scar formation process was proved in a previous study which revealed that reactive astrogliosis was induced by ischemic stroke.(6)

Reactive astrogliosis induce synthesize neurotrophin such as brain-derived neurotrophic factor (BDNF) that possess important role in neurogenesis.(7,8) BDNF can be measured in serum and was used as predictor of functional outcome in stroke patient.(9) BDNF is also said to be a promoter of neuroplasticity which is one of the processes in improving the recovery process of stroke patients.(10) Therefore glial scar and neurogenesis have become as therapeutic targets in ischemic stroke.(4)

Several endogenous neurotrophic such as nerve growth factor (NGF), BDNF, glia-derived nerve factor (GDNF) and insulin-like growth factor 1 (IGF-1) have showed the important role in neurogenesis process.(11) BDNF has critical to promote neuronal growth, survival and modulate synaptic plasticity.(12) However activated microglia by stress factor such as injury, pro-inflammatory cytokines are released and enhance gliogenesis and detrimental of neurogenesis process.(11) One of pathological conditions in ischemic stroke is glutamate excitotoxicity which can cause changes in astrocytes that is referred to as the astrogliosis. The fundamental characteristic of the astrogliosis process was the increment of GFAP levels.(13) GFAP was one of components glial scar especially in scar periphery of spinal cord injury (SCI) case (14), and the high levels of GFAP have also been reported to be associated with extensive ischemic lesions and clinical worsening.(2)

Inflammation has a principal role in maintaining the balance of neurogenesis and neurodegeneration process in the brain. One of cytokines proinflammation TNF-α was reported hampers neurogenesis process in dentate gyrus (DG).(11) TNF-α was also reported enhanced expression of GFAP in neonatal mouse brain.(15) Other pro-inflammatory markers such as HMGB1 was also believed associated to poor outcomes in traumatic brain injury (TBI) case. HMGB1 is a nuclear nonhistone protein that has a role in transcription regulation. The release of HMGB1 from necrotic cells promote increase the secretion of cytokine and activates microglia thereby propagating inflammation. (16) Attenuating neuroinflammation through inhibition of HMGB1 and TNF-α will be a very important step to induce neurogenesis and inhibit the formation of glial scars, that it will be able to improve the recovery process in ischemic stroke patients.

BDNF reduces neuroinflammation on hippocampus of type 1 diabetic mice (17), but there are not many studies that observe the effect of neuroinflammation on BDNF expression in ischemic stroke cases. Therefore, this study was conducted to evaluate the effects of TNF-α and HMGB1 as pro-inflammatory biomarkers to BNDF expression, a neurotrophin lead to neurogenesis and GFAP a biomarker for glial scar formation in ischemic stroke.

Methods

Animal Preparation and Group Division

The study design was an experimental laboratory using fifteen male Wistar rats aged 3–4 months, weighing 200–260 grams were obtained from the food security and agriculture department, Bandung, Indonesia. The rats were acclimated for one week before the stroke model was established. They were kept in cages, under a 12-hour dark-light cycle. Food and water were available *ad libitium* at a temperature of 22–24°C, with a consistent humidity level of 55±5%. After that, the samples were randomized into three groups using a random table method. The number of samples was determined using the Lemeshow formula. The first five rats were under anesthesia without occlusion on the left common carotid artery (CCA) (sham group). The second five rats were occluded on the left CCA for 180 minutes then sacrificed (group A). The last five rats were occluded on the left CCA for 180 minutes and following until seven days then sacrificed (group B). The experiment was performed

in the pharmacology laboratory of the Faculty of Medicine, Universitas Brawijaya. The experimental protocol was approved by The Animal Ethics Commission of Faculty of Veterinary, Universitas Brawijaya (Protocol No. 219-KEP-UB-2023).

Ischemic Stroke Model

Wistar rats were anesthetized using ketamine 80 mg/kg BW and Xylazine 10 mg/kg BW intraperitoneally. Rats were anesthetized, and turned to supine position and fixed to the surgical table using adhesive tape. Furthermore, a small incision was made on the midline of neck approximately 3-4 cm and explored until trachea can be seen. Subsequently explore gently to find left common carotid artery, which accompanies with the vagal nerve. The left common carotid artery was isolated from the vagal nerve and connective tissue gently. After the left common carotid artery was separated from vagal nerve, the left common carotid artery was blocked using a small bulldog clamp for 180 minutes. After 180 minutes, bulldog clamp was removed and close back the neck incision. The assessment of ischemic stroke was evaluated by assessing the motor deficits on right extremities of both right forelimb and right hind limb using the foot fault scoring (FFS) value from the ladder run walking test (LRWT) after 180 minutes of occlusion. The scoring of foot fault scoring was assessed using 7 categories: 1) Score 0/Total miss: 0 were given when the limb did not touch the rung and the rat falls, a fall was defined as limb fell between rung and disturb body posture and balance; 2) Score 1/Deep slip: The limb was initially placed on a rung and then limb slipped off rung than a fall occurred; 3) Score 2/Slight slip: The limb was placed on a rung than slipped off during weight bearing, but did not result in a fall and continue a coordinated gait; 4) Score 3/Replacement: The limb was placed on a rung, but before weight bearing the limb on the rung the rat quickly lifted and placed on another rung; 5) Score 4/Correction: The limb toward for one rung, but was then placed on another rung without touching the first one or when the limb is placed on a rung, but the animal removes the foot and repositions it on the same rung; 6) Score 5/Partial placement: The limb was placed on a rung with either wrist or digits of the forelimb or heel or toes of the hindlimb; and 7) Score 6/Correct placement.(18)

Immunohistochemistry (IHC) Examination for the Measurement of GFAP, BDNF, HMGB1, and TNF-α Expressions

To examine the expressions of GFAP, BDNF, HMGB1 and TNF-α on brain tissue, IHC examinations were performed. Paraffin block from rat brain was cut using a microtome in 1.5 cm in front of bregma, paraffin block was cut at 4 μm in coronal sections. Brain slice was deparaffinization and rehydration by immersing the slides in xylene twice for 3 minutes each, followed by 100% ethanol for 3 minutes, and then washing in PBS for 5 minutes. The area around the slide sections was cleaned with a wipe, then the slides were immersed in a 3% hydrogen peroxide solution (v/v absolute methanol) for 15 minutes at room temperature. The slides were washed with PBS for 5 minutes, three times. Then cleaned up the area around the slide sections with a wipe, and the primary antibodies (GFAP antibody (Cat. No Sc-33673; Santa Cruz Biotechnology, Dallas, TX, USA), BDNF antibody (Cat. No. Sc-65514; Santa Cruz Biotechnology), HMGB1 antibody (Cat. No. Sc-135809; Santa Cruz Biotechnology) and TNF-α antibody (Cat. No. Sc-52746; Santa Cruz Biotechnology)) were added and incubated overnight at 4°C, accordingly. The slides were washed with PBS for 5 minutes, three times. Next step added the Avidin-Biotin Complex reagent (Cat. No. Sc-516216; Santa Cruz Biotechnology) on the slides then incubated at room temperature for 30 minutes then the slides were washed again with PBS for 5 minutes, three times. Subsequently, 3,3'-diaminobenzidine chromogen (Nichirei Biosciences, Tokyo, Japan) was added at room temperature for 10 minutes. The slides were washed with distilled water for 5 minutes, three times. The slides were immersed in a counterstain solution and washed with tap water. The last step was mounting by placing the tissue sections on glass slides and covering them with permanent mounting media. This assessment was performed in Biochemistry Laboratory, Universitas Brawijaya.

Real-time RT-PCR Analysis for the Measurement of *BDNF* mRNA Expression

The variable to depict expression of *BDNF* gene was *BDNF* mRNA which was assessed using RT-PCR. For *BDNF* mRNA expression, total RNA was extracted using RNAsimple Total RNA Kit (Cat. No. 4992858; Tiangen, Beijing, China) according to the manufacture's instruction. Reverse transcription reactions were conducted on 2 μg of RNA of nucleic acids from each sample using RT-PCR Product Series (Cat. No. 4992226/4992227/4992251; Tiangen). Real-time PCR reactions conducted using Forget-Me-Not™ EvaGreen® qPCR Master Mix (Low ROX) (Catalog No. 31045-1mL, 31045-5mL, 31045-20mL; Biotium, Fremont, CA, USA). Primer pairs used for real-time PCR were *BDNF* Forward: 5'- CAA AAG GCC AAC TGA AGC- 3'; *BDNF* Reverse: 5'-CGC CAG CCA

ATT CTC TTT- 3'. For PCR reaction, the RNA template was thawed on ice, and the FastKing-RT SuperMix and RNaseFree ddH_2O were also thawed for 5 times at room temperature (15-30°C). Cycling step: Enzyme activation at 95°C holding time 2 minutes, number of cycle was 1. Denaturation at 95°C, for 2-5 seconds. Annealing/extension at 60°C for 20-30 seconds, with number of cycles was 40. All procedures RT-PCR perform in Research Center for Vaccine Technology and Development, Universitas Airlangga, Surabaya.

Statistical Analysis

Statistical analysis was performed using the software package SPSS (version 24.0) (IBM Corporations, Armonk, NY, USA). The normality and homogeneity of each dataset was tested. Shapiro-Wilk test and Levene test, and comparisons between three independent groups were analyzed by the ANOVA test. The significance level was set to $p<0.05$ and data were presented as mean±standard deviation (SD).

Results

Motoric Deficit After Left CCA Occlusion

To prove that left CCA occlusion for 180 minutes had sucessfully caused ischemic in rat's brain, an evaluation based on motoric deficits on right extremities both right forelimb (RFL) and right hind limb (RHL) which assessed using FFS value of LRWT was performed. The results indicated that there was motoric dysfunction in form of disturbance of gait as an effect of paralysis of RFL and RHL. The FFS value of RFL and RHL after left CCA occlusion for 180 minutes were lower significantly in group A and group B than sham group (2.41±1.698 (group A) and 2.00±0.000 (group B), with $p=0.000$) for RFL and (1.41±1.543 (group A) and 1.22 ±0.441 (group B), with $p=0.000$) for RHL (Table 1).

Table 1. Mean of FFS RHL and RFL.

Group	FFS	
	RFL	RHL
Sham	6.00±0.000	6.00±0.000
Group A	2.41±1.698	1.41±1.543
Group B	2.00±0.000	1.22±0.441
p -value	0.000*	0.000*

Data presented in Mean±SD. *$p<0.005$ is considered to be significant, analyzed with ANOVA test.

BNDF Expression was Lower in Ischemic Rats Model than Control

BDNF was one of neurotrophins which synthesized by neuron and influence several functions in central nervous system (CNS) such as cell growth, plasticity, differentiation process and dendritic formation. In this study, it was found that the BDNF expression levels lower significantly in both group A and group B compared to sham group. The lowest BDNF expression level was found in group B. The mean BDNF levels in group A, group B and sham group were 5.20±1.924, 5.00±1.581, and 7.80±1.304, respectively, with $p=0.032$ (Table 2). In Figure 1, from the IHC results with 400x and 1000x magnifications, it could be seen that the amount and spread of BDNF positive neurons (brown colored cells) was found to be lower in group A and group B compared to sham group.

GFAP Expression was Higher in Ischemic Rat Model than in Control

In this study, the GFAP expression level was found to be significantly higher in group A and group B than in sham group. The highest GFAP expression level was found in group B. The mean GFAP levels in group A, group B and sham group were 9.60±1.517, 11.40±2.074 and 5.20±1.483, respectively, with $p=0.000$ (Table 2). From IHC staining results with 400x and 1000x magnification, it also found that amount and spread of GFAP positive neurons, cells with brown color, were found to be higher in group A and group B compared to sham group (Figure 2).

TNF-α Expression was Higher in Ischemic Rat Model than in Control

Expression level of TNF-α was found to be higher in group A and group B compared to sham group. The mean expression level of TNF-α in group A, group B and sham group were 6.33±1.155, 9.33±1.528 and 3.00±1.000, respectively, with $p=0.002$ (Table 2). The highest expression level of TNF-α was found in group B. From IHC results, it also was observed amount and spread of TNF-α positive neurons (cells with brown color) were found to be higher in group A and group B than sham group (Figure 3).

HMGB1 Expression were Higher in Ischemic Rat Model than in Control

Expression level of HMGB1 was found higher significantly in group A and group B compared to sham group. The mean expression levels of HMGB1 in group A, group B and sham group were 9.33±1.528, 11.67±1.528 and 2.00±1.000, respectively, with $p=0.000$ (Table 2). The highest expression

Table 2. Expression levels of BDNF, GFAP, TNF-α and HMGB1.

Parameter	Sham Group	Group A	Group B	*p*-value
BDNF	7.80±1.304	5.20±1.924	5.00±1.581	0.032*
GFAP	5.20±1.483	9.60±1.517	11.40±2.074	0.000*
TNF-α	3.00±1.000	6.33±1.155	9.33±1.528	0.002*
HMGB1	2.00±1.000	9.33±1.528	11.67±1.528	0.000*

Data presented in Mean±SD. *p<0.05 is considered to be significant, analyzed with ANOVA test.

levels of HMGB1 were found in group B. From IHC staining results, it was also found amount and spread of HMGB1 positive neuron (cells with brown color) were found to be higher in group A and group B than sham group (Figure 4).

BDNF mRNA Expression were Lower in Ischemic Rat Model in Control

The results of the PCR analysis demonstrated that *BDNF* mRNA expression was lower in group A and group B than sham group. The means expression of *BDNF* mRNA in group A, group B and sham group were 0.0545±0.0335, 0.0000±0.0000, and 1.0025±0.0615, respectively (Table 3). Unfortunately, the *BDNF* mRNA expression in group B was unidentified.

Correlation between the Independent Variables (TNF-α, HMGB1) and the Dependent Variables (BDNF and GFAP)

Correlation analysis was further performed to investigate the correlation between each parameters. In this study, there was negative correlation between TNF-α (r=-0.36; p=0.375) and HMGB1 (r=-0.605; p=0.112) as inflammatory markers to BNDF, which meant that the higher TNF-α and HMGB1 level, the lower BNDF expression, but this correlation did not statistically significant. In other hand, there was positive correlation between TNF-α (r=0.189; p=0.654) and HMGB1 (r=0.530; p=0.117) to GFAP, where the higher TNF-α and HMGB1 level, the higher GFAP expression, but this result also did not statistically significant (Table 4).

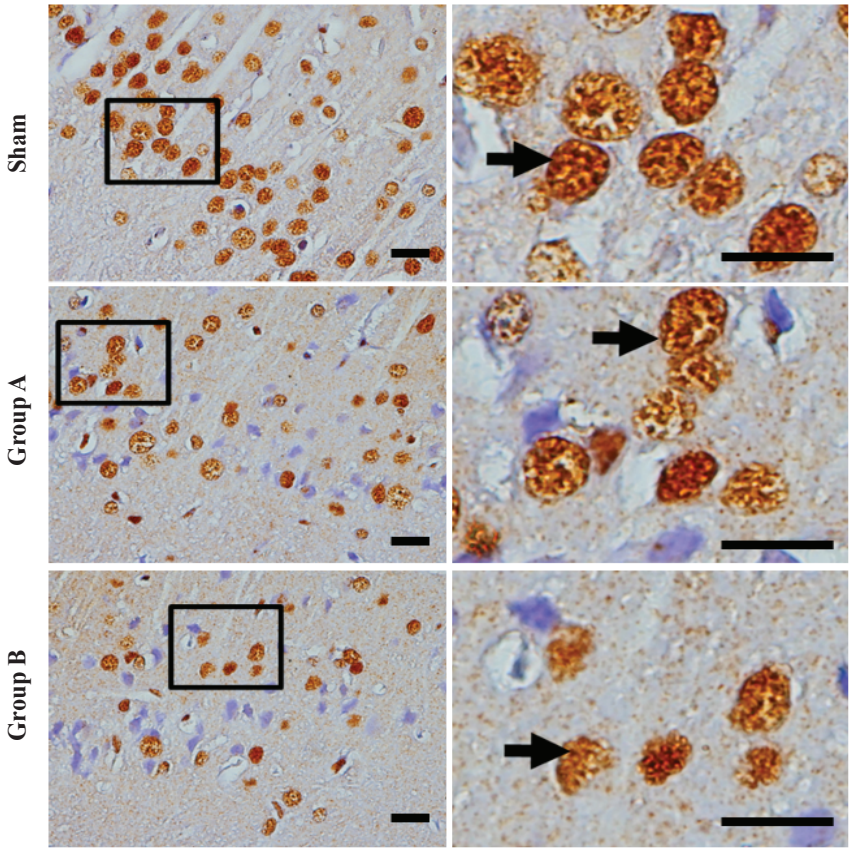

Figure 1. Expression level of BDNF in neurons (brown colored cells) was assessed using IHC staining. The lowest expression level of BDNF was found in group with ischemic stroke (group B) while expression level of BDNF in group A (control positive) was found higher than sham but lower than group B. The highest expression level of BNDF was found in the sham group. Black box: spread of BDNF positive neuron. Black arrow: BNDF positive neuron. 400x magnification to see spread of BDNF positive neurons, 1000x magnification to identify BDNF positive neurons. Black bar: 0.025 mm.

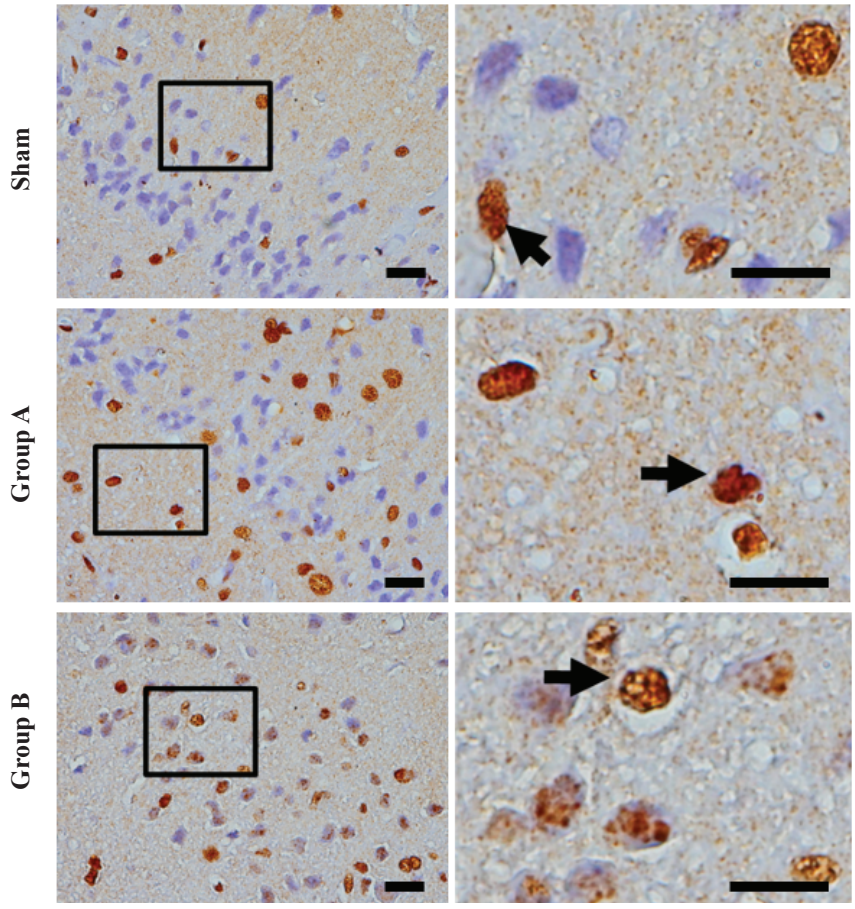

Figure 2. Expression level of GFAP in neurons (brown colored cells) was assessed using IHC staining. The expression of GFAP in group B has the highest result compared to other groups. The lowest expression of GFAP was found in sham group, while expression of GFAP in group A was found higher than sham but lower than group B. Black box: spread of GFAP positive neuron, black arrow: GFAP positive neuron. 400x magnification to saw spread of GFAP positive neuron, 1000x magnification to identify GFAP positive neuron. Black bar: 0.025mm.

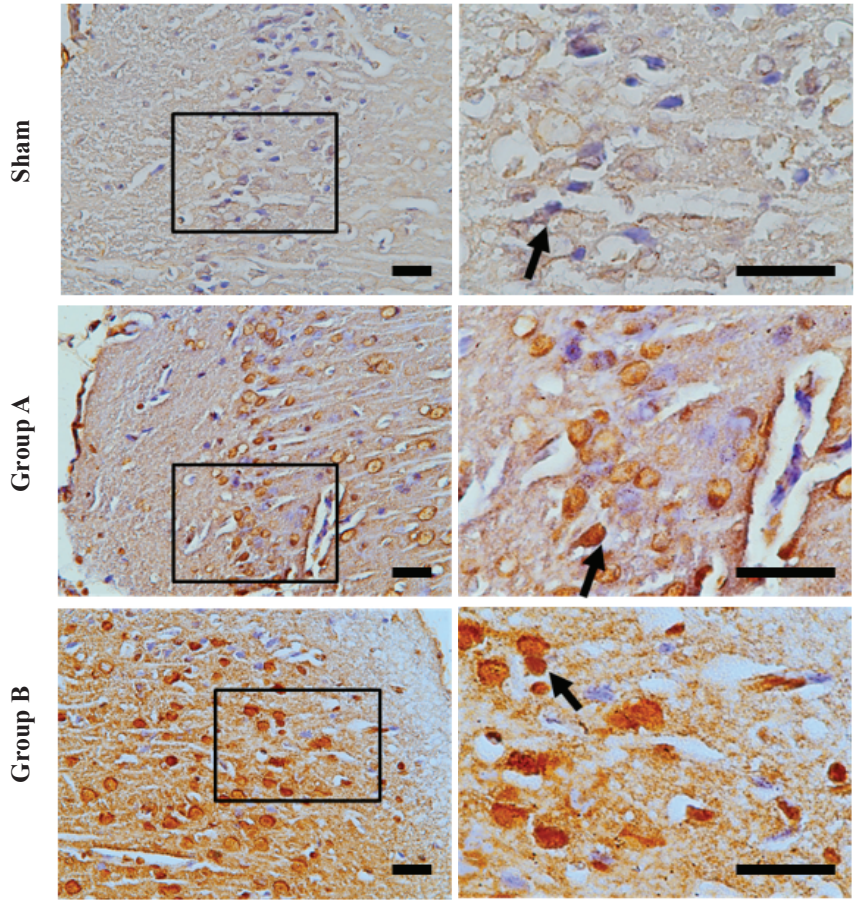

Figure 3. Expression level of TNF-α in neurons (brown colored cells) was assessed using IHC staining. The highest expression of TNF-α was found in group B. The lowest expression of TNF-α was found in the sham group. Expression level of TNF-α in group A was higher than sham but lower than group B. Black box: spread of TNF-α positive neuron, black arrow: TNF-α positive neuron. 400x magnification to see spread of TNF-α positive neurons, 1000x magnification to identify TNF-α positive neurons. Black bar: 0.025mm.

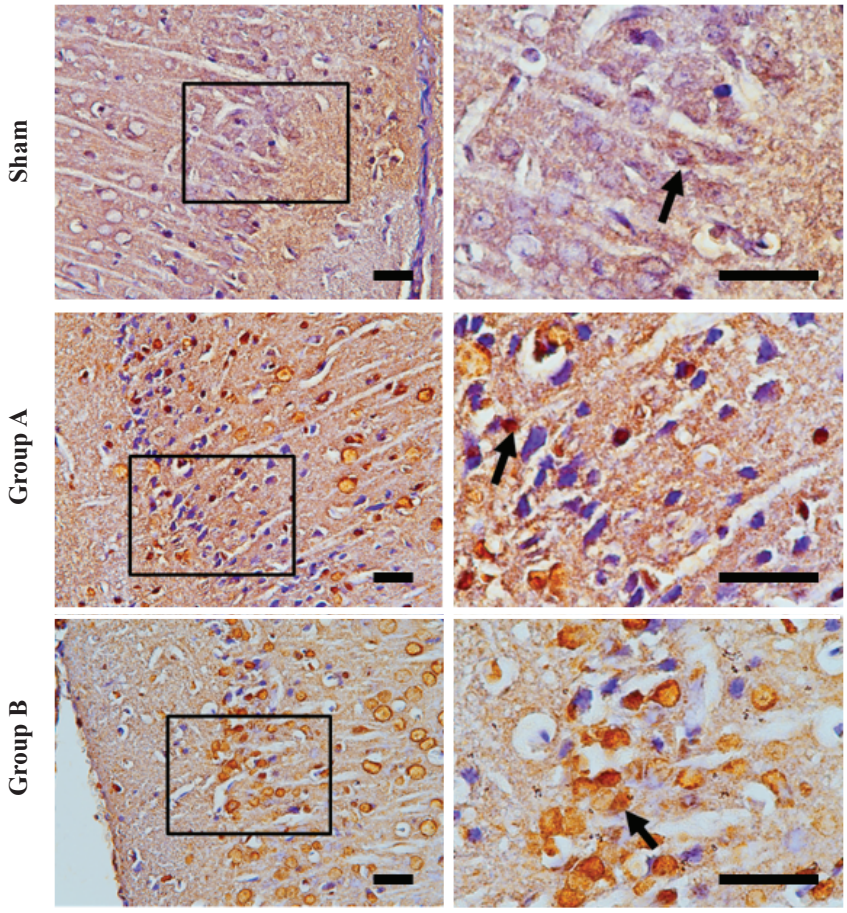

Figure 4. Expression level of HMGB1 in neurons (brown colored cells) was assessed using IHC staining. The highest expression of HMGB1 was found in group B. The lowest expression of HMGB1 was found in the sham group. The expression level of HMGB1 in group A was higher than sham but lower than group B. Black box: spread of HMGB1positive neuron, black arrow: HMGB1 positive neuron. 400x magnification to saw spread of HMGB1 positive neuron, 1000x magnification to identify HMGB1positive neuron. Black bar: 0.025mm.

Discussion

Neuroinflammation is the main process underlying the pathological reaction in ischemic stroke. Neuroinflammation process is initiated with release of danger-associated molecular patterns (DAMPs) such as adenosine, heat shock proteins, HMGB1, interleukin (IL)-3 in injury area. The Neuroinflammation process is also induced by microglia activation in hypoxia conditions. Activated microglia will release proinflammatory cytokines such as TNF-α. Binding of TNF-α to its receptors tumor necrosis factor receptor (TNFR)1 and TNFR2 triggers neuronal death.(19)

Table 3. Mean of BDNF mRNA Expression assess using RT-PCR.

Group	*BDNF* Expression	*p*-value
Sham	1.0025±0.0615	
Group A	0.0545±0.0335	0.000*
Group B	0.0000±0.0000	

Data presented in Mean±SD. *p<0.05 is considered to be significant, analyzed with ANOVA test.

Similarly to HMGB1, non-histone DNA-binding nuclear protein, in the intracellular space plays a role in regulating transcription process (20), but in the extracellular space, HMGB1 causes recruitment of immune cells and activates TNF-α, IL-1, and IL-6 (21). HMGB1 is actively secreted by microglia and passively by necrotic neurons and mediates neuroinflammatory processes that play important role in pathogenesis of ischemic stroke. Activity of HMGB1 is mediated by its receptors, that are toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGEs). (22) During ischemic stroke CREB, a transcription factor for BDNF, will be activated. cAMP Response Element-Binding Protein (CREB) is required for the early induction for all the major BDNF transcript.(23) BDNF is one of brain neurotrophins has vital role in neurogenesis, differentiation, proliferation, neuron maturation and synaptic plasticity.(24) Expression of BDNF is influenced by neuroinflammation. Neuroinflammation is known to influence several pathways related to BNDF signaling. In pathological conditions such as ischemic stroke, abnormalities in BNDF expression have been found.(25)

In this study, expression of BDNF in brain tissue (Table 2 and Figure 1) demonstrated lower in group A (hyperacute

Table 4. Correlation between the independent variables and the dependent variables.

	BDNF		GFAP	
	r value	*p*-value	r value	*p*-value
TNF-α	0.360	0.375	0.189	0.654
HMGB1	0.605	0.112	0.530	0.117

p-value<0.005 is considered to be significant, tested with Pearson correlation test. r=-0,36 : weak negative correlation; r=-0,605 moderate negative correlation; r=0,189 weak positive correlation; r=0.530 moderate positive correlation.

phase of ischemic stroke) and B (acute phase of ischemic stroke). Likewise, with *BDNF* mRNA expression also showed lower in group A and group B (Table 3). In this study, it was also revealed higher biomarker neuroinflammation of HMGB1 and TNF-α in group A and group B (Table 2, Figure 3, and Figure 4). On the other hand, a previous study reported an increase in brain BDNF levels at 4 and 24 h after stroke although was not accompanied by change in plasma and serum.(26) The increase of BDNF level after ischemic stroke was required for recovery process in the brain. BDNF binding to the receptor tropomyosin receptor kinase B (TrkB) and p75 neurotrophin receptor (p75NTR) induced cell growth, differentiation, and plasticity.(27) The lower in BDNF expression in this study which was different from the previous study mentioned may be caused by increase of neuroinflammation factors after ischemic stroke. In this study, there was higher expression of HMGB1 and TNF-α significantly in group A and group B, that brought up presumption that lower of BNDF level and *BDNF* mRNA expression in brain tissue was caused by enhancement of neuroinflammation factors. HMGB1 was likely to induce downregulation of BDNF expression. This result, in accordance with another study conducted reporting increasement of HMGB1 expression induce downregulation BDNF significantly especially in diabetic retinal neurodegenerative cases. Even in normal rats which were induced with HMGB1 intravitreal also cause downregulation of BNDF. The decrement of BNDF as an effect of HMGB1 was suspected through interaction between HMGB1 and its receptor RAGE, that induce upregulation NF-kB and causes chronic inflammation.(27) The chronic inflammation subsequently causes decreased expression of BDNF.(28) From the correlation analysis, there is a negative correlation between TNF-α and HMGB1 to BNDF. This means that the higher the expression of TNF-α and HMGB1, the lower the expression of BNDF, but statistically not significant. To ensure the inflammatory factors that induce low BNDF expression, other examinations are needed such as administering TNF-α inhibitors or HMGB1 inhibitors.

Likewise with TNF-α, a pleiotropic cytokine, was found to increase 1 day after injury.(29) In this study, it was observed that decrease of BDNF and *BNDF* mRNA expression in brain tissue accompanied with increase of TNF-α expression. This result is in line with a study that demonstrated that administration of TNF-α to cell culture human umbilical vein endothelial cells (HUVEC) for 6 hours could reduce *BDNF* mRNA expression. And the influence of TNF-α on *BDNF* mRNA expression was proven by administering the TNFR1 antagonist, namely WP9QY, where TNFR1 antagonist prevented a decrease in *BDNF* mRNA expression by TNF-α. Based on this finding, the obstacle mechanism of TNF-α to *BDNF* mRNA expression was thought to be mediated by activity of TNF-α to its receptor TNFR1. Administration TNF-α to HUVEC-derived EA.hy 926 cells alone, or in combination with protein kinase C (PKC) inhibitor Go 6983 was reported to have no effect on *BNDF* mRNA expression, indicating that the downregulation of BDNF by TNF-α was PKC-independent.(25)

Neurogenesis is important for recovery process after brain injury.(30) Neuroinflammation as effect of ischemic stroke induce activated astrocyte which releases GFAP (14) and potentially attenuated neurogenesis process.(31) GFAP mRNA expression was also found to increase during ischemic stroke.(32) Upregulation of GFAP will give barrier in lesion site. GFAP is one of the biomarkers for glia scar formation. Glia scar formation involved several pathways including Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) (24) and is also induced by transforming growth factor (TGF)-β1 (33). STAT 3 also involved in regulation of GFAP gene expression.(34) Glial scar formation is one of factors attenuated axonal growth (35) and it is stimulated by neuroinflammation (36). The results of this study revealed that GFAP expression increased in brain tissue both in group A and group B.

Even the highest GFAP expression was found in group B. In this study, increase of GFAP accompanied by increased of TNF-α and HMGB1 level. That raised suspicion that neuroinflammation has potential to upregulate GFAP. This finding is in line with a study reporting that administration of TNF-α in culture astrocytes increases GFAP expression. The mechanism involved in induction of GFAP expression by TNF-α was supposed to be the activity of mitogen-activated protein kinase and extracellular signal-regulated kinase 2 (MAPK Erk2) pathway. This mechanism was proved by administering MAPK Erk2 inhibitor could inhibit TNF-α in induce increase of GFAP expression.(37) On the other hand, the role of HMGB1 in upregulation of GFAP still unclear. In this study was observed that the increase of GFAP was also accompanied by an increase of HMGB1 level. While another study reported that HMGB1 expression in neurons was markedly enhanced after a rat spinal nerve ligation. And mostly HMGB1-positive non-neuronal cells at the lesion site co-localized with GFAP.(38) This indicates two possibilities, the first was glial cells can secrete HMGB1 or the second was HMGB1 induces GFAP upregulation. Other studies reported the increase of TLR4 expression in the penumbra area both in acute stroke and chronic stroke. Administration of HMGB1, a ligand of TLR4, on astrocyte culture caused an increase of TLR4 expression and increase of TLR4 was accompanied with increase of GFAP expression.(39) This was in line with the results of this study which showed that an increase in GFAP in the ischemic area was accompanied by an increase in HMGB1 in both hyperacute and acute stroke. Enhancement of GFAP referred to increase in the density of glial scar formed. In this study, there was a positive correlation between TNF-α and HMGB1 to GFAP. This means that the higher the expression of TNF-α and HMGB1, the higher the expression of GFAP, but statistically not significant. For further research, it is necessary to add TNF-α inhibitors or HMGB1 inhibitors to ensure that high GFAP is caused by inflammatory factors.

The results of this study could be the basis for management of ischemic stroke especially in acute phase and implication in the health field is the management of ischemic stroke in acute phase should be focused on regulating neurogenesis and inhibiting glial scar formation to improve clinical manifestation in stroke patients. In this study, the measurement of GFAP, BDNF, HMGB1 and TNF-α positive neurons on IHC result was accounted manually, which may raise some bias on the study result. Therefore, for further research, it is recommended to use bioimage analysis applications to calculate the IHC result, such as QuPath.

Conclusion

The presence of low BDNF levels and high levels of GFAP, HMGB1 and TNF-α markers, possibly reflects inhibition of the neurogenesis process by neuroinflammation, and induced glial scar formation in ischemic stroke conditions after than 180 hours until 7 days.

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Authors Contribution

AW, FAR, AM, are responsible for study concepts, study design, study analysis, and manuscript review. WR are responsible for analysis of immunohistochemistry, study analysis, and statistical analysis. All authors took parts in giving critical revision of the manuscript and all authors approved the final version of the manuscript.

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