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Research Article

The effect of L-Arginine from Giant Snake Head fish (*Channa micropeltes*) on neuroinflammation and neuron damage in traumatic brain injury in rats

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Abstract

Backgrounds: Traumatic brain injury has high mortality and morbidity. The involvement of neuroinflammatory responses and neuronal damage in traumatic brain injury is the basis for the development of renewable neuroprotective agents, one of which is the amino acid L-Arginine. The amino acid L-Arginine which can be found in Giant Snake Head fish (*Channa micropeltes*) is thought to inhibit anti-inflammatory effects through the Arg-1 signaling pathway.

Aims: To prove and analyze the effect of giving L-Arginine Giant Snake Head fish on neuroinflammation and neuronal damage in a rat model of traumatic brain injury.

Methods: This randomized controlled trial (RCT) study used a posttest-only control group design. Thirty-five male rats (*Rattus norvegicus*) with the Wistar strain were randomly divided into five treatment groups, namely the normal control group (N), negative control (KN), and 3 treatment groups (A-C). The KN and A-C groups were modeled after traumatic brain injury and given L-Arginine at a dose of 0.5; 1.5; and 3 g/kgBW/day for 7 days specifically A-C. Brain tissue samples were used for the examination of TLR4, TNF-α, GSDMD, Caspase-3, and histopathological features. All data were analyzed using the Kruskall-Wallis test, except for GSDMD expression with a significance <0.05.

Results: The mean TLR4 expression in A (15.7 ± 5.35), B (12.9 ± 5.67), and C (11.4 ± 6.27) were lower and significant compared to KN, but higher and significant compared to N. The same pattern appears in the decrease in TNF-α expression, Caspase-3 expression, and histopathology of brain tissue damage. The mean relative expression of GSDMD to N in A (1.3 ± 0.53), B (1.2 ± 0.52), and C (1.2 ± 0.60) was higher than in KN, but not significant. Relative expression of GSDMD to Beta Actin, A and B have the same higher expression than C, KN, and N.

Conclusion: Expression of TLR-4, TNF-α, Caspase-3 and histopathology of brain tissue damage in traumatic brain injury model rats given Giant Snake Head fish L-Arginine were lower than without L-Arginine. Meanwhile, the expression of GSDMD in traumatic brain injury model rats when given Giant Snake Head fish L-Arginine was slightly higher than without L-Arginine.

Keywords

Channa micropeltes, L-Arginin, Neuron Damage, Neuroinflammation, Traumatic Brain Injury

Introduction

Traumatic brain injury is a brain function disorder that is the main cause of disability in the 10–49 year age group (Abbafat et al. 2020). In the United States, the mortality rate related to traumatic brain injury per 100,000 population is highest among those aged 75 years (78.5%) (Centers for Disease Control and Prevention). The Indonesian Basic Health Research in 2018 showed that the percentage of head injuries reached 11.9%. Severe head injuries reach 6–12% and cause death up to 25–37% (Tjahjadi et al. 2013).

Both acute and chronic consequences of traumatic brain injury can initiate secondary injury, which develops minutes to months after mechanical damage, which progressively contributes to neurological impairment (Bramlett et al. 2015). Immune responses that appear within minutes after injury include local signals in neurons, neuroglia, and peripheral immune cells that are required to induce the inflammatory cascade (Corps et al. 2015). During traumatic brain injury, TLR4 activate the myeloid differentiation primary response gene 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) signaling pathways, resulting in transcriptional activation. and the release of proinflammatory agents such as TNF-α. TLR4 will also activate microglia which can trigger neuronal stimulation of cell death program (apoptosis) and trigger activation of the inflammasome NACHT, LRR and PYD domains-containing protein 3 (NLRP3) which will cause the splitting of Gasdermin D protein (GSDMD) resulting in pyroptosis (Lee et al. 2013).

Existing neuroprotective agents such as progesterone, glutamate receptor antagonists, and calcium-channel blockers (CCBs) did not show statistically significant effects on functional outcome or risk of death (Pan et al. 2019). The involvement of the inflammatory response in traumatic brain injury encourages researchers to look for other agents that can act as neuroprotectors, one of which is the amino acid, L-Arginine. A study using a rat model of acute cerebral ischemia showed that a combination of low doses of lysine and L-Arginine (0.6 g/kgBW, respectively) was effective in reducing cerebral hemisphere edema. This amino acid also suppresses glutamate-induced neuronal activity in vivo (Kondoh et al. 2010).

One source of L-Arginine is animal protein, Giant Snake Head fish (*Channa micropeltes*). These fish have 15 types of amino acids, they are threonine, valine, methionine, L-leucine, leucine, phenylalanine, histidine, lysine, L-Arginine, aspartic acid, serine, glutamate, glycine, alanine, and tyrosine (Pratama et al. 2020). Research on the neuroprotective effects of L-Arginine from animal is still very limited, while Giant Snake Head fish are widely available in Indonesia. This study aimed to observe the effect of L-Arginine from Giant Snake Head fish on neuroinflammation, pyroptosis, and apoptosis in patients with traumatic brain injury. These variables were observed through the expression of TLR-4, TNF-α, Caspase-3 and histopathology of brain tissue damage.

Methods

Inclusion and exclusion criteria

This research was an experimental laboratory with a post test only group design. The study population was male rats (*Rattus norvegicus*) with the Wistar strain aged 2–3 months and weighing 150–200 grams. Sampling using consecutive sampling method based on the inclusion and exclusion criteria.

The inclusion criteria in this study included: Wistar strain rat (*R. norvegicus*), body weight 150–200 grams, age 6–8 weeks, male sex. Exclusion criteria in this study were rats showing signs of piloerection, the hair looked dull, rough, greasy, and falling out, skin looked loose, body weight decreased drastically after adaptation, eyelids slightly closed, eyes sunken, red discharge around the eyes (chromodakriorrhea), loose stools, runny, and smelly, more aggressive then passive, don't want to eat, don't want to drink, often sleeps in cages, often squeaks when held, sneezes, looks pale, has wheezing breath, and dead rats during the study going on.

Production of L-Arginine from Giant Snake Head fish

Giant Snake Head fish isolate was manufactured by using modified techniques of centrifugal time and freeze-drying (FD) processes. The extraction process is carried out for 60 minutes, then the extract results are flowed into the separator machine to separate the parts that are not used, the process lasts for 20 minutes. The crude extract of Giant Snake Head fish was then purified by protein purification by adding ammonium sulfate. Precipitation with salt uses the principle of salting out. The increased salt concentration causes water to be released from the protein which causes the attachment of hydrophobic bonds from one protein to another protein and produces a precipitate.

The crude extract produced in this process is first tested using liquid chromatography-mass spectrometry (Liquid Chromatography – mass spectrometry or LC-MS) to detect its amino acids. Samples were hydrolyzed with 6N HCL at 110 °C for 24 hours in the presence of 1% phenyl. The hydrolyzate is derivatized, dried, and dissolved with the sample solvent (water). The sample derivatives were then analyzed with LC-MS/MS. The area under the peak of each amino acid in the chromatogram is calculated and compared with the amino acid standard and reported as the number of residues per thousand amino acids. After obtaining the pure protein, L-Arginine was isolated using liquid chromatography-tandem mass spectrometry. The chromatographic Rf value shows almost the same value between the L-Arginine isolate compared to the reference L-Arginine, so it can be concluded that the resulting isolate is same as pure L-Arginine.

Experimental protocol

Test animal laboratories at Laboratorium Pusat Antar Universitas, Gadjah Mada Yogyakarta University applied good

test animal laboratory practices which can refer to the latest The Guide for the Care and Use of Laboratory Animals or to the Regulation of the Minister of Agriculture Number 44/Permentan/OT.140/5/2007 of 2007 concerning Guidelines for Good Veterinary Laboratory Practice.

The room used for testing met the requirements of temperature, humidity, light, and noise according to the needs of the test animal's life, namely the room temperature is set to 22° ± 3 °C, with a relative humidity of 30–70%, and lighting 12 hours bright 12 hours dark. The room must always be kept clean. Rats are given food and drink according to laboratory standards and are given ad libitum.

Rats are kept in cages made of waterproof, strong, and easy-to-clean materials, the rearing room is free from noise. The area of the cage per rat according to the National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). The rats were randomly assigned to each group according to the grouping of the test animals. After randomization, there was no significant difference in mean body weight between groups.

The traumatic brain injury model was made using the weight-drop method. Mice will be anesthetized with a combination of ketamine 75 mg/kgBB and xylazine 2.5 mg/kgBB intramuscularly, then shave their heads and use 70% alcohol to clean. The next process is the scalp is opened to reveal the calvaria. Induction of head injury using an iron cylinder weighing 450 grams (4 mm in diameter) was dropped at an angle of 900 from a height of 100 cm 1 time.

After the experiment, the rats need to be sacrificed. Before the rats were sacrificed, anesthesia was performed first. Rats are handled carefully without causing fear, then rats are sacrificed with one of the techniques in a separate place from other animals, and it is guarded so that no animals live around it. Euthanasia is carried out by competent personnel and accompanied by a confirmation process to ensure death. Mice were decapitated following an injection of anesthetic drugs (ketamine 75mg/kg body weight and xylazine 2,5 mg/kg body weight) and then destroyed through the burning method.

There were 5 research groups, normal control group, negative control, and treatment with doses of A, B, C. The L-arginine dosage were 0.5 g/kgBW; 1.5 g/kgBW, and 3 g/kgBW. Researchers gave L-arginine orally using a probe once a day, for 7 days. After 7 days, all samples were terminated for brain organ harvesting. The rat brain that had been taken was then processed into histopathological preparations.

TLR-4 expression

TLR4 expression was observed using the immunohistochemical technique. Histopathological preparations were incubated using rabbit polyclonal anti-TLR4 (ABclonal, Inc) and a secondary antibody labeled with biotin-conjugated. Observations were made on a light microscope with 400× magnification and positive cells were counted in 10 visual fields and the average was found. Positive cells show brown-stained cytoplasm.

TNF-α expression

TNF-α expression test was carried out using the immunohistochemical method. The deparaffinized preparations were then washed using phosphate-buffered saline (PBS) and then soaked in 3% H2O2 for 15 minutes at room temperature. Next, the preparations were labeled with unspecific protein sequences, primary antibodies (rabbit polyclonal anti-TNF-α), secondary antibodies (conjugated biotin-labeled), and Streptavidin-Horse Radish Peroxidase (SA-HRP). Staining using Mayer's Hematoxylin which was incubated for 10 minutes and washed using distilled water and then dried. TNF-α expression was observed using a light microscope. Cells that express TNF-α will show brown cytoplasm.

Evaluation of apoptosis

Observation of apoptosis was carried out by observing Caspase-3 expression. Caspase 3 double staining was performed with frozen rat slices fixed in 4% paraformaldehyde solution. The fixation process was followed by washing using PBS and then incubated in 3% H2O2 in methanol at room temperature. This method requires two types of antibodies, namely the first antibody (purified rabbit anti-active caspse-3 monoclonal antibody; ABclonal, Inc.) and the second antibody (Universal-LSAB TM Kit).

Evaluation of pyroptosis

Pyroptosis can be observed through caspase activation, GSDMD cleavage, or by inhibiting components of the pyroptotic pathway. Pyroptosis involves cleavage of GSDMD (53 kDa), resulting in a 30 kDa N-terminal fragment that can be detected using western blotting. The required reagents are included in the Western Blotting Application Solutions Kit. The solution was prepared by deionized reversed osmosis (RODI). The network to be tested is soaked first using a regulatory medium within a predetermined

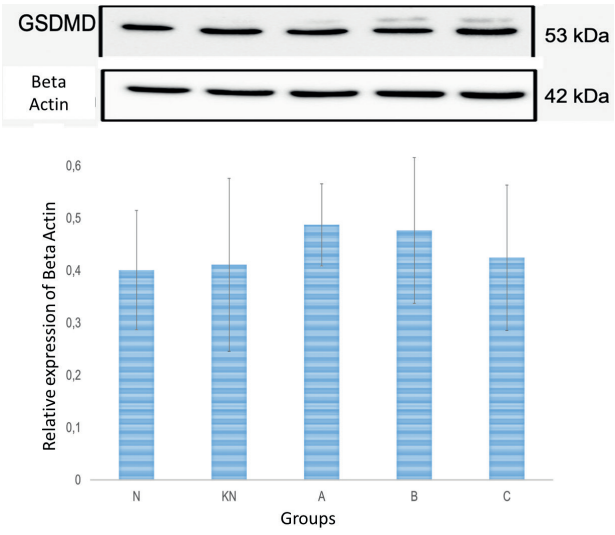

Figure 1. Western blotting analysis and relative expression of GSDMD to Beta Actin.

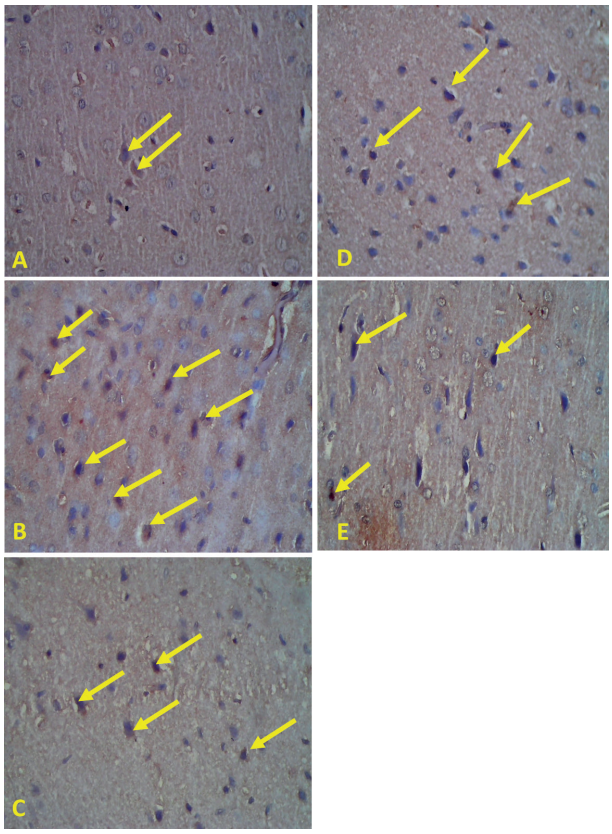

Figure 2. TLR4 immunohistochemical staining with a microscope magnification of 400×. Description: TLR4 expression in the cerebral cortex area shows TLR4 expression in astrocytes (yellow arrows). **A.** Control group normally expressed 5%; **B.** Negative control group expressed 20%; **C.** Group A expressed 15%; **D.** Group B expressed 15%; **E.** Group C expressed 10%.

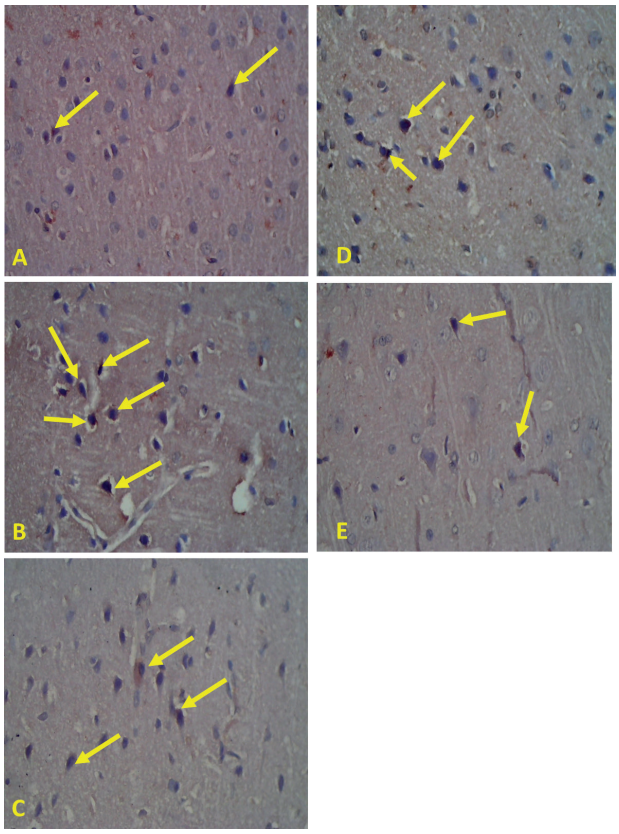

Figure 3. TNF-α immunohistochemical staining with a microscope magnification of 400×. TNF-α expression in the cerebral cortex area shows TNF-α expression in astrocytes (yellow arrows). **A.** Control group normally expressed 5%; **B.** Negative control group expressed 15%; **C.** Group A was depressed 10%; **D.** Group B expressed 10%; **E.** Group C expressed 5%.

period of time. Then the tissue was washed using PBS. Prior to the western blot test, the tissue was extracted first by lysing the cells using an SDS sample buffer. Then sonication was carried out to reduce the viscosity of the sample. The sample volume injected into the SDS PAGE gel was 20 µl. After that, it was transferred into the nitrocellulose membrane.

Histological examinations

Staining of histological preparations was initiated by deparaffinization in xylol solution 3 times for 15 minutes. The rehydration process to remove xylol is by placing the preparation in an absolute alcohol solution for 2 minutes, then putting it back into a 95% to 70% alcohol solution for 1 minute. Finished rehydration, followed by washing the preparations with running water. Then stained with hematoxylin for 10 minutes. The next step is washing again with running water for 2 minutes. Eosin staining process for 1 minute, then the preparations were washed with running water. After the eosin staining was complete, the histological preparations were dehydrated in 95% alcohol and absolute alcohol twice for 2 minutes. Then soak the preparations using xylol 2 times for 2 minutes, then the preparations are covered with a cover glass using permount adhesive, and labeled.

Statistical analysis

TLR4 and TNF-α expression were observed by immunohistochemical examination. Gasdermin D cleavage to assess pyroptotic activity was detected using western blotting. The data obtained were statistically processed using the Statistical Package for the Social Sciences (SPSS) version 23 application. Moewardi Hospital provided full approval for this research (Ethical Clearance Number: 665/VI/HREC/2021).

Results

TLR4 expression

Immunohistochemical examination showed TLR4 expression in astrocyte cells in the cerebral cortex of a traumatic brain injury rat model. Cells expressing TLR4 will show brown cytoplasm. The results of the Kruskall-Wallis comparative analysis of TLR4 expression showed a p value <0.001 so it could be concluded that the administration of Giant Snake Head fish L-Arginine had a significant effect on TLR4 expression in traumatic brain injury rat models. The Mann-Whitney post hoc test (Table 1) also showed that administration of L-Arginine in Giant Snake Head

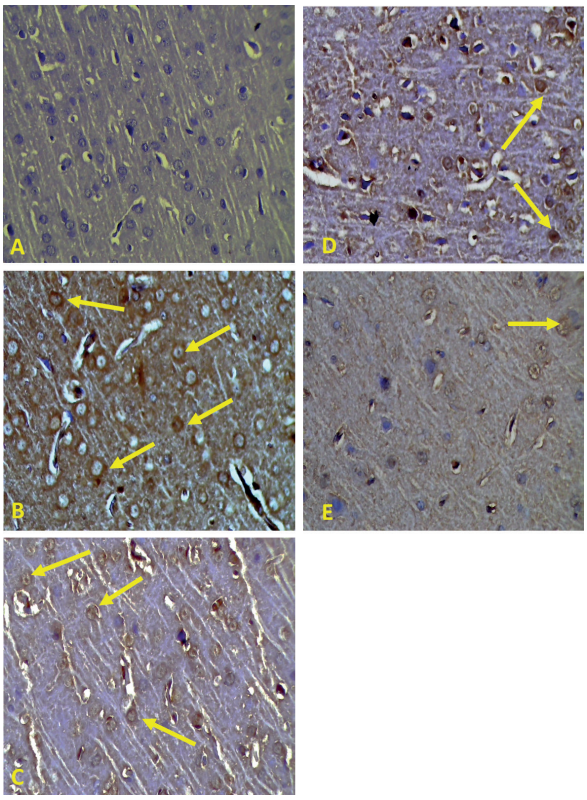

Figure 4. Immunohistochemical staining of Caspase-3 400× microscope magnification. Picture of caspase 3 expression in the cerebral cortex area shows a picture of caspase 3 expression in the cytoplasm of neuron cells (yellow arrow). **A.** The normal control group showed a score of 0; **B.** The negative control group shows a score of 4; **C.** Group A shows a score of 3; **D.** Group B shows a score of 2; **E.** Group C shows a score of 1.

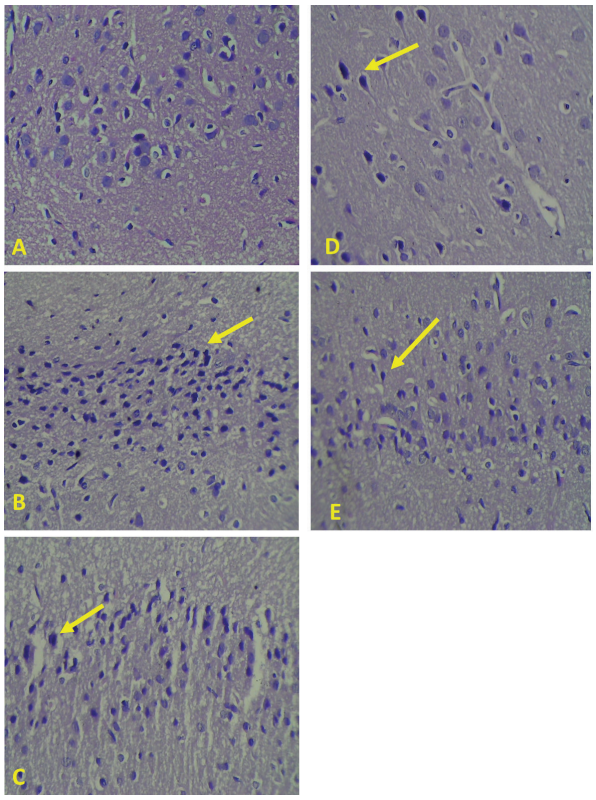

Figure 5. Histopathological picture of brain tissue damage with HE staining, 400× microscope magnification. Description: Histopathological picture of the cerebral cortex area shows degeneration of neuron cells (yellow arrows). **A.** The normal control group shows a score of 0; **B.** The negative control group shows a score of 2; **C.** Group A shows a score of 2; **D.** Group B shows a score of 1; **E.** Group C shows a score of 1.

Table 1. Mann-Whitney post hoc test on TLR4 expression.

Groups	p
Normal Control Group	
vs Negative Control Group	0,001**
vs Group A	0,001**
vs Group B	0,001**
vs Group C	0,009**
Negative Control Group	
vs Normal Control Group	0,001**
vs Group A	0,174
vs Group B	0,048*
vs Group C	0,027*
Group A	
vs Normal Control Group	0,001**
vs Negative Control Group	0,174
vs Group B	0,315
vs Group C	0,164
Group B	
vs Normal Control Group	0,001**
vs Negative Control Group	0,048*
vs Group A	0,315
vs Group C	0,478
Group C	
vs Normal Control Group	0,009**
vs Negative Control Group	0,027*
vs Group A	0,164
vs Group B	0,478

Details: * Significant at α = 0.05; ** Significant at α = 0.01.

fish at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW had a significant effect on TLR4 expression.

TNF-α expression

Immunohistochemical examination was also performed to assess the expression of TNF-α in astrocyte cells in the cerebral cortex of the traumatic brain injury rat model. The effect of Giant Snake Head fish L-Arginine on TNF-α expression showed a significant difference ($p<0.001$). Based on the results of the Kruskall-Wallis comparative test, it can be concluded that administration of L-Arginine from Giant Snake Head fish had a significant effect on TNF-α expression in traumatic brain injury rat models. The Mann-Whitney post hoc test (Table 2) also showed that administration of L-Arginine in Giant Snake Head fish at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW had a significant effect on TNF-α expression.

Gasdermin D expression

Gasdermin D relative expression compared to the normal control group had homogeneous data ($p > 0.05$) but not normally distributed (p value < 0.05). The results of the Kruskall-Wallis comparative test showed $p = 0.829$ ($p > 0.05$), so it can be concluded that L-Arginine administration

Table 2. Mann-Whitney post hoc test on TNF-α expression.

Groups	p
Normal Control Group	
vs Negative Control Group	0,001**
vs Group A	0,005**
vs Group B	0,030*
vs Group C	0,254
Negative Control Group	
vs Normal Control Group	0,001**
vs Group A	0,031*
vs Group B	0,023*
vs Group C	0,001**
Group A	
vs Normal Control Group	0,005**
vs Negative Control Group	0,031*
vs Group B	0,253
vs Group C	0,007**
Group B	
vs Normal Control Group	0,030*
vs Negative Control Group	0,023*
vs Group A	0,253
vs Group C	0,091
Group C	
vs Normal Control Group	0,254
vs Negative Control Group	0,001**
vs Group A	0,007**
vs Group B	0,091

Details: * Significant at α = 0.05; ** Significant at α = 0.01.

Table 3. Mann-Whitney Post Hoc Test on GSDMD Relative Expression Compared to the Normal Control Group.

Groups	p
Negative Control Group	
vs Group A	0,277
vs Group B	0,949
vs Group C	0,749
Group A	
vs Negative Control Group	0,277
vs Group B	0,749
vs Group C	0,482
Group B	
vs Negative Control Group	0,949
vs Group A	0,749
vs Group C	0,949
Group C	
vs Negative Control Group	0,749
vs Group A	0,482
vs Group B	0,949

of Giant Snake Head fish did not have a significant effect on the Gasdermin D relative expression compared to the normal control group in traumatic brain injury rat models (Table 3). The Mann-Whitney post hoc test also showed that administration of L-Arginine in Giant Snake Head fish at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW, did not have a significant effect on the Gasdermin D relative expression compared to the normal control group (Table 4).

One Way Anova comparative test showed that L-Arginine administration of Giant Snake Head fish did not have a significant effect on the Gasdermin D relative expression compared to Beta Actin in traumatic brain injury rat models. The Bonferroni post hoc test also showed that L-Arginine in Giant Snake Head fish at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW had no significant effect on the Gasdermin D relative expression compared to Beta Actin.

Table 4. Bonferroni Post Hoc Test on GSDMD Relative Expression Compared to the Beta Actin.

Groups	Mean	p-value	Confidence Interval 95%	
			Lower Bound	Upper Bound
Normal Control Group				
vs Negative Control Group	-0,0	1,000	-0,221	0,201
vs Group A	-0,1	1,000	-0,297	0,125
vs Group B	-0,1	1,000	-0,286	0,135
vs Group C	-0,0	1,000	-0,234	0,187
Negative Control Group				
vs Normal Control Group	0,1	1,000	-0,201	0,221
vs Group A	-0,1	1,000	-0,287	0,135
vs Group B	-0,1	1,000	-0,277	0,145
vs Group C	-0,0	1,000	-0,224	0,197
Group A				
vs Normal Control Group	0,1	1,000	-0,125	0,297
vs Negative Control Group	0,1	1,000	-0,135	0,287
vs Group B	0,0	1,000	-0,200	0,221
vs Group C	0,1	1,000	-0,148	0,273
Group B				
vs Normal Control Group	0,1	1,000	-0,135	0,286
vs Negative Control Group	0,1	1,000	-0,145	0,277
vs Group A	-0,0	1,000	-0,221	0,200
vs Group C	0,1	1,000	-0,159	0,263
Group C				
vs Normal Control Group	0,0	1,000	-0,187	0,234
vs Negative Control Group	0,0	1,000	-0,197	0,224
vs Group A	-0,1	1,000	-0,273	0,148
vs Group B	-0,1	1,000	-0,263	0,159

Detail: * Significant at α = 0,05.

Caspase-3 expression

Caspase-3 expression in the negative control group had a mean of 3.6 ± 0.79. Caspase-3 expression in group A had a mean of 3.0 ± 0.59. Caspase-3 expression in group B had a mean of 1.7 ± 0.60. Caspase-3 expression in group C had a mean of 1.3 ± 0.49. The Kruskall-Wallis comparative test on the effect of L-Arginine from Giant Snake Head fish on Caspase-3 expression showed a significant difference ($p<0.001$). The post hoc test between groups showed a p value <0.05 which indicated a statistically significant result (Table 5). Based on the description above, it can be seen that the administration of L-Arginine from Giant Snake Head fish at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW, has a significant effect on the expression of Caspase-3.

Histopathology of brain tissue damage

The lowest average rating is in the normal control group with a value of 4.0 and the highest average rating is in the negative control group with a value of 28.79. The effect of giving Giant Snake Head fish L-Arginine on the his-

Table 5. Mann-Whitney post hoc test on Caspase-3 expression.

Groups	p
Normal Control Group	
vs Negative Control Group	0,001**
vs Group A	0,001**
vs Group B	0,001**
vs Group C	0,001**
Negative Control Group	
vs Normal Control Group	0,001**
vs Group A	0,095
vs Group B	0,004**
vs Group C	0,002**
Group A	
vs Normal Control Group	0,001**
vs Negative Control Group	0,095
vs Group B	0,008**
vs Group C	0,002**
Group B	
vs Normal Control Group	0,001**
vs Negative Control Group	0,004**
vs Group A	0,008**
vs Group C	0,244
Group C	
vs Normal Control Group	0,001**
vs Negative Control Group	0,002**
vs Group A	0,002**
vs Group B	0,244

Details: * Significant at α = 0.05; ** Significant at α = 0.01.

topathological picture of brain tissue damage showed a significant difference ($p < 0.001$). Based on the results of the Kruskall-Wallis comparative analysis, it can be concluded that the administration of Giant Snake Head fish L-Arginine has a significant effect on the histopathological picture of brain tissue damage in the rat model of traumatic brain injury. From the results of the post hoc test, it was found that the administration of Giant Snake Head fish L-Arginine at doses of 0.5 g/kgBW, 1.5 g/kgBW, and 3 g/kgBW, had a significant effect on the histopathological picture of brain tissue damage.

Discussion

This research supports the evidence that administration of L-Arginine in Giant Snake Head fish has the This study used natural L-Arginine s sourced from Giant Snake Head fish. Giant Snake Head fish L-Arginine has several advantages compared to synthetic L-Arginine . First, L-Arginine sourced from Giant Snake Head fish is a form of participation by researchers in utilizing local wisdom as the newest method of brain injury therapy. Second, Giant Snake Head fish is a type of fish that is widely found in Indonesia. This type of fish has a low price, high protein level, and benefits in the process of wound healing and post-surgery. In addition, Giant Snake Head fish contains a relatively high amount of L-Arginine compared to other types of freshwater fish. It is hoped that the use of L-Arginine in Giant Snake Head fish will help popularize and preserve the use of local animal resources in the development of medical science.

Table 6. Mann-Whitney Post Hoc Test on of Histopathology of Brain Tissue Damage.

Groups	p
Normal Control Group	
vs Negative Control Group	0,001**
vs Group A	0,001**
vs Group B	0,001**
vs Group C	<0,001**
Negative Control Group	
vs Normal Control Group	0,001**
vs Group A	0,054
vs Group B	0,015*
vs Group C	0,002**
Group A	
vs Normal Control Group	0,001**
vs Negative Control Group	0,054
vs Group B	0,298
vs Group C	0,037*
Group B	
vs Normal Control Group	0,001**
vs Negative Control Group	0,015*
vs Group A	0,298
vs Group C	0,254
Group C	
vs Normal Control Group	<0,001**
vs Negative Control Group	0,002*
vs Group A	0,037*
vs Group B	0,254

Detail: * Significant at α = 0.05; ** Significant at α = 0.01.

The blood brain barrier damage due to injury will stimulate several immune responses in the brain. In the early phase of injury, DAMPs are released including HSP and HMGB1 which can be bound by TLR4. TLR4 will activate the NF-kβ signaling pathway resulting in transcriptional activation and release of proinflammatory agents (Kabadi et al. 2014; Jassam et al. 2017). NF-kβ also activates the NLRP3 inflammasome (Swanson et al. 2019). The inflammasome NACHT, LRR and PYD domains-containing protein 3 will activate caspase 1, then caspase 1 will cleave Gasdermin D (GSDMD) which causes the N-terminal domain of GSDMD to form a hole in the plasma membrane which causes pyroptosis (Jiang et al. 2020).

In traumatic brain injury there is also the opening of ion channels resulting in increased levels of [Ca^{2+}], providing a stimulus to mitochondria to activate the apoptotic pathway through the release of cytochrome c and activation of the caspase cascade. Mitochondria also produce AIF and Endo G which increase apoptosis. The increase in [Ca^{2+}] levels was also influenced by the amino acid glutamate, which increased in the first hour after injury. [Ca^{2+}] ions also stimulate apoptosis by activating calpain which can activate the caspase cascade through caspase12. Activation of the caspase cascade is also mediated by TNF-α which activates TNF receptors (TNFR1 and TNFR2) in the cell membrane resulting in activation of caspase-8, caspase-3 then apoptosis occurs (Jiang et al. 2020).

In this study, there was no significant decrease in GSDMD in the rat model of traumatic brain injury. This could be due to the changing pattern of NLRP3 expression over

time after traumatic brain injury. NLRP3 mRNA expression began to increase and reached a peak value at 6 hours of post-traumatic brain injury treatment. After 6 hours, NLRP3 expression gradually decreased within 24 hours, but then continued to increase up to 7 days, and its expression was higher than NLRP3 expression at 6 hours after treatment. Increased NLRP3 expression can also be caused by disturbances in cellular ion homeostasis, including K+ release and Ca2+ entry, and ATP outflow from damaged and lysed cells after traumatic brain injury. This condition causes an increase in the first stage of NLRP3 expression. The second-stage increase in NLRP3 after 24 hours of post-traumatic brain injury may be due to secondary injury, including cellular stress and inflammatory cascades (Jiang et al. 2018; Meloni et al. 2020).

Brain damage due to injury will result in the release of Alarmin which will be recognized by PRRs, especially TLR4. TLR4 activation will trigger microglia phagocytosis activity and release of inflammatory mediators, including TNF-α. Increased TLR4 expression was evident in neurons and astrocytes after trauma. Inhibition or elimination of TLR4 expression in traumatic brain injury shows decreased astrocyte activation, proinflammatory mediator production, and neuronal autophagy thereby inhibiting neuroinflammatory processes (Jiang et al. 2020). Another form of L-Arginine, Cationic Arginine-Rich Peptides (CARP) has been shown to reduce neuroinflammation after traumatic brain injury by suppressing TLR4 regulation and the production of inflammatory mediators beneath it such as TNF-α and IL-1ß (Jiang et al. 2018). Continued neuroinflammatory processes will result in damage to neuroglia cells through various mechanisms such as apoptosis, autophagy, or pyroptosis. Suppressing the expression of cell death effectors such as Caspase-3 in apoptosis or Gasdermin D in pyroptosis will reduce cell death and provide negative feedback to neuroinflammation. This is an opportunity to research drugs that have the potential to be neuroprotective (Wei et al. 2022).

Arginine is an essential amino acid involved in various pathways in health and disease. Arginine becomes essential in stress conditions and catabolic states when the capacity of endogenous arginine synthesis is exceeded, including critical illness conditions such as sepsis, burns, and trauma (Morris et al. 2017). Arginine also plays a key role in metabolic, immune, and reparative responses to trauma. Plasma arginine levels fall acutely after trauma within minutes to hours, while arginase activity increases. Plasma arginine levels may remain low for up to a week or longer in severe injuries (Meng et al. 2017). Accumulating evidences suggests that decreased arginine availability by myeloid-derived suppressor cells (MDSCs) is the cause of T-cell dysfunction following physical injury, coinciding with the induction of arginase I expressing MDSCs (Zhu et al. 2014; Qiu et al. 2019).

Limitations in this study include: L-Arginine s were administered via the oral route, so it takes longer time to achieve increased plasma levels compared to intravenous access. In almost all variables (TLR4 expression, TNF-α, GSDMD relative expression to the normal control group, Caspase-3 expression, and histopathological features of brain tissue damage) the distribution of primary data was found to be abnormal and inhomogeneous. In addition, the Western Blot examination uses anti-GSDMD antibodies, so that the total GSDMD is quantified, not just the activated GSDMD. Further research is necessary to provide L-Arginine administration via the intravenous route to shorten the time to achieve an increase in plasma levels compared to oral administration and use quantitative neuroinflammatory marker parameters that have a higher sensitivity.

Conclusion

Expression of TLR-4, TNF-α, Caspase-3 and histopathology of brain tissue damage in traumatic brain injury model rats given Giant Snake Head fish L-Arginine were lower than without L-Arginine . Meanwhile, the expression of GSDMD in traumatic brain injury model rats when given Giant Snake Head fish L-Arginine was slightly higher than without L-Arginine.

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