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The effect of addition protein, phosphatidylcholine, phosphatidylserine, and inulin on GFAP levels of acute ischemic stroke patients at Dr. Kariadi Hospital, Semarang

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ABSTRACT

Background: The occurrence of ischemia causes a loss of energy to switch to anaerobic processes resulting in acidosis due to reduced Adenosina Triphosphate (ATP). This condition makes neuron cells apoptotic. Apoptotic of several biochemical substrates in the brain, such as Glial Fibrillary Acidic Protein (GFAP) exit into the circulatory system which is associated with dysbiosis through immunological pathways.
Objectives: To determine the effect of giving enteral formula containing protein, phosphatidylcholine, phosphatidylserine, and inulin on GFAP levels in patients with acute ischemic stroke Dr. Kariadi Hospital.
Materials and Methods: This study was done in a single-blind RCT. Eighteen ischemic stroke patients were randomly divided into intervention (9 subjects) and control groups (9 subjects). The intervention group received 69 g of the powdered enteral formula three times a day for seven days. The formula contained protein (15 g), phosphatidylcholine (128 mg), phosphatidylserine (32 mg), and inulin (3 g). The subject who had diabetes mellitus received for 14 days at a dose of 34.5 g per day (7.5 g protein with additions 64mg phosphatidylcholine, 16mg phosphatidylserine, 1.5 g inulin). The control group received the standard enteral formula from the hospital, which contains (11.8 g protein without additions protein, phosphatidylcholine, phosphatidylserine, and inulin). GFAP levels by ELISA method (Enzyme-linked immunosorbent Assay) at pre and post-intervention.
Results: There was a trend of decreasing GFAP levels before and after in the intervention group towards a better direction from 8.37±4.25 to 8.30±4.9 compared with the control group which experienced an increasing trend from 5.4±1.8 to 7.5±4. There was no significant difference in GFAP levels after intervention between groups (p = 0.7).
Conclusions: The addition of protein, phosphatidylcholine, phosphatidylserine, and inulin had no significant effect on GFAP levels.

Keyword: GFAP; Protein; Phosphatidylcholine; Phosphatidylserine; Inulin

BACKGROUND

Ischemic stroke occurs when blood supply to the brain is interrupted or reduced due to a blockage. The occurrence of ischemia in cells and reduced glucose supply causes an increased glutamate expression. Glucose metabolism later switches to anaerobic processes so that it becomes acidosis due to reduced ATP. This situation prompts apoptosis or the death of nerve cells in the brain. In the event of apoptosis, some biochemical substrates may leave the brain and leak off into the circulatory system. In addition to apoptosis, there could be a reduction of proteins that play a role in maintaining the integrity of the blood-brain barrier membrane; hence membrane will encounter permeability. This results in GFAP which should be in the brain structure going out into the circulatory system.[1–4]

Glial Fibrillary Acidic Protein (GFAP) is one of the intermediate protein families, including vimentin, nestin, and is known as an intermediate filament (IF) which is a group of cell types found in the central nervous system and is responsible for maintaining the structure and migration of astroglia cells. GFAP plays a role in the mitotic process by adjusting the filamentous tissue in

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cells. Besides, GFAP also plays a role in neuron astrocyte interactions and communication between cells. Under physiological conditions, GFAP is not actively secreted by cells and is generally undetectable in the blood of healthy individuals. In a moment of brain injury, astroglia responds by producing more GFAP, which in turn will be released into the Central Nervous System (CNS) fluid and blood. GFAP is exclusively produced by astrocytes so that this protein is particularly observed in the brain. Examinations have shown that GFAP is released rapidly in cases of acute ischemic stroke, wherein levels peak within 12 hours of onset and are maintained for up to 72 hours of onset.[5]

A study by Dvorak et al which stated that serum GFAP has taken within 2-6 hours after stroke onset was significant in differentiating hemorrhagic stroke from ischemic stroke. GFAP reaches its highest level within 12 hours of stroke onset.[6] Supported by research by Puspitasari et al which states there is a significant correlation between GFAP serum levels with stroke severity scale after 1 month of stroke onset.[7] This is different from the study conducted by Neila et al, which aims to determine the difference between blood sampling time and serum GFAP levels within 24 hours of onset in patients with ischemic stroke and hemorrhagic stroke, which shows that there was no significant difference in GFAP levels in samples <6 hours, 6-12 hours, and 12-24 hours in ischemic strokes and ICH strokes.[8]

Factors that can influence GFAP include some cellular processes. In general, GFAP has an important role in the regulation between astrocytes and neurons. Changes in GFAP expression can affect synaptic function, interfere with glutamate metabolism, increase the volume of astrocytes that can expand to the surface area of neurons in contact with the astrocyte membrane, and hypoxia.[4]

Thrombolysis or *recombinant tissue plasminogen activator (*rTPA*)* may be one of his definitive therapies. However, this therapy cannot be given to all ischemic stroke patients. Given the strictness of criteria such as the time window, the time for rTPA therapy was <3 hours and a range of 3-4.5 hours after symptom onset. If the therapy is not performed according to established guidelines, one may experience a more severe risk, such as bleeding in angioedema, intracranial and gastrointestinal tract.[9] The limitations of the requirement for rTPA have made many pharmacological therapies emerged, such as neuroprotective therapies in which the addition of phospholipids given in supplement form (citicoline). Some of the most abundant types of phospholipids are phosphatidylcholine and phosphatidylserine. Phosphatidylcholine can improve the biosynthesis of membrane phospholipids which are deteriorated by an increment in free radicals during brain ischemia (neuroprotection). Besides that, the activation of the enzymes that trigger apoptosis in nerve cells will be inhibited by phosphatidylcholine so that it will affect GFAP levels.[10] Research on the effect of giving protein, neuroprotective substances (phosphatidylcholine and phosphatidylserine), and inulin on markers of brain damage, especially GFAP, is still very limited.

Animal studies with the administration of the drug cytoline have the potential to reduce acute brain damage and improve functional recovery, even when given several hours after an ischemic event because the phosphatidylcholine in cytoline can increase the biosynthesis of membrane phospholipids degraded by increased free radicals during brain ischemia (neuroprotection). Besides, cytoline has been shown to restore mitochondrial ATPase and Na1 / K1 ATPase activity to inhibit phospholipase A2 activation which triggers apoptosis of nerve cells.[11,12] so that will affect the levels of GFAP.

A Japanese study was conducted to investigate the effect of phosphatidylserine extracted from soybeans (Soy-PS) on the cognitive function of an elderly group with memory complaints. Oral administration of Soy-PS improves memory function, especially in the elderly with memory complaints. These effects were similarly observed at low doses (100 mg/day) and high doses (300 mg/day).[13]

Fulfillment of protein intake in stroke patients is often given enteral formula with high calories high protein diet which operates to preserve adequate nutritional intake to speed up the recovery period as the enteral formula contains high protein. Dysphagia, unconsciousness (coma), or what is known as enteral feeding is one alternative to meet the nutritional needs of stroke patients by providing nutrients in the form of enteral formula. The protein content in the enteral formula can improve body metabolism through appetite regulation and/or other mechanisms that can control body weight and body composition.[14] Several studies have proven the benefits of enteral formula protein in increasing protein consumption, muscle mass and possibly

increasing body mass index so that it can improve the motor function abilities of patients.[15–17]

Prebiotics is one of the nutrients needed in stroke patients in addition to phospholipids and protein. One type of which is named inulin. Prebiotic bacteria such as Bifidobacteria and Lactobacilli will ferment inulin to produce Short-Chain Fatty Acid (SCFA) and its fermented products in the form of propionate, butyrate, acetic acid, L-lactate, CO2, and hydrogen. Directly or indirectly, the gut-brain axis is influenced by SCFA. The blood-brain barrier can be penetrated by SCFA mediated by monocarboxylate transporter (MCT) and improves the integrity of the blood-brain barrier by increasing the expression of tight junction proteins. SCFA will improve inflammation in nerves by modulating levels of neurotrophic factors, improving morphology and glial cell function, and strengthening neurogenesis.[18] Previous research conducted by Liu et al explained that administering 10% inulin for 4 weeks can change the composition of the gut microbiota and improve gut function in obese mice.[19] The same thing was done by Chuncai et al which stated that the administration of NAC, inulin, and a combined therapy improved cognition in castrated rats. RX-fed rats treated with either the vehicle or testosterone had a significantly increased number of GFAP positive cells. This increment was attenuated by treatment with NAC, inulin, or the combined therapy.[20]

Enteral nutrition has been shown to reduce infectious complications and improve outcomes in patients with severe TBI. Increasing immune cell activity and the integrity of the intestinal barrier, most likely preventing immune dysfunction and bacterial translocation, are the benefits of early feeding, respectively. The nervous system and endocrine changes following enteral feeding can help reduce massive inflammation through vagus nerve-mediated mechanisms. In a journal published by Luyer in Bansal et al, it is shown that enteral feeding, especially dietary fat, stimulates the release of cholecystokinin and binds to cholecystokinin receptors which ultimately leads to activation of the efferent vagal system. This activation triggers an increase in acetylcholine at the gut synaptic level and decreases TNF-α production by way of acetylcholine binding to α-7 nicotinic receptors on macrophages and other immune cells. Therefore, the positive immunologic benefits of early enteral feeding after TBI may also be a result of decreased inflammatory cytokine production by way of intraluminal feeds inducing vagus nerve activity which can thus affect the effectiveness of GFAP levels.[21]

This study aimed to determine the effect of giving enteral formula containing protein, phosphatidylcholine, phosphatidylserine, and inulin on GFAP levels in ischemic stroke patients. The intervention group received 69 g of the powdered enteral formula containing 15 g of protein, 128 mg of phosphatidylcholine, 32 mg of phosphatidylserine, and 3 g of inulin dissolved in 250 ml of warm water, given three times a day for seven days.

Giving 69 g of powdered enteral formula because it contains high protein [22 g per 100 g in solid form][22]. The intake of phosphatidylcholine in the enteral formula also covers 60-90% of the daily requirement for phosphatidylcholine for adults and 3x1 enteral formula feeding for 7 days is following the therapeutic dosage for cytolin [23] where the administration of cytolin 2000 mg has a restorative effect.[24] According to the results of the study, it was shown that the administration of enteral nutrition within 7 days could reduce the mortality rate by 5.8% and survive more than the group who started giving nutrition late.[25] Feeding 69 g of enteral formula has been consistent with the therapeutic dose of phosphatidylserine, whereas administration of 100 mg/day of phosphatidylserine has provided improvement.[26] A dose of 0.5 g has been shown to increase the diversity of probiotic bacteria in the gut[27] which will produce short-chain fatty acids, namely SCFA. Inulin in the enteral formula has met the inulin dose for adults, which is 5-15 g /day and 0.5 g of inulin has been shown to increase the number of probiotic bacteria in the intestine which will produce short-chain fatty acids such as SCFA. In subjects suffering from diabetes, the treatment was carried out for 14 days at a dose of 34.5 g per day. The control group was given standard enteral formula from the hospital (11.8 g protein).

MATERIALS AND METHODS

This research is experimental research with a randomized control trial in a single-blind manner where the research subjects do not know what therapy is being obtained. The research subjects were 18 ischemic stroke patients aged >18 years, 9 men and 9 women who were divided into the intervention group (9) and the control group (9)

by randomization. This research was conducted for four months (February-May). The sample size was calculated by taking into account the minimum sample size by using the experimental sample size formula for clinical trials with the following formula:*****

$$n_1 = n_2 = 2\left(\frac{(Z_\alpha + Z_\beta)\ SB}{x1\text{-}x2}\right)^2$$

The results of previous studies showed that the level of GFAP in patients with ischemic stroke was 9.46 ng / mL (mean) and the standard deviation was 4.6 ng / mL.[28] The minimum difference considered significant (x1-x2) was 5.13 ng / mL.[28] If the error of type I is set at 15% (α=0.15) then Zα=1.440. The amount of type II error is set at 20% (β=0.20) then Zβ=0.842 and research power is 80%, the sample size calculation is as follows:

$$n = 2\left(\frac{(1.440 + 0.842)\ 4.6}{5.13}\right)^2$$
$$= 8.3 \approx 9 \text{ ischemic stroke patients/group}$$

When the sample was selected with the inclusion criteria of ischemic stroke patients with attack onset, not more than 72 hours as evidenced by an MSCT head scan and aged >18 years, and the exclusion criteria for ischemic stroke patients who had a head injury in the last 3 months, subarachnoid hemorrhage, brain tumor, infection of the central nervous system, psychiatric disorders, complications of acute or chronic renal failure, a history of reactions to enteral formula, hematemesis and / hematemesis + melena and those receiving citicoline. The researcher randomized the subjects by dividing the research subjects into the control group or the intervention group. Researchers provided an informed consent sheet for consent to be the study sample to the patient's family.

GFAP levels were checked at the beginning and the end of treatment. Examination of clinical characteristics is carried out by physical examination and a doctor examines neurological examination. The MSCT scan was performed to determine whether the patient had a head injury in the last 3 months, subarachnoid hemorrhage, brain tumor, infection of the central nervous system, psychiatric disorders, patient medical records to determine blood laboratory results (routine hematology, blood sugar, lipid profile) and complications. acute or chronic renal failure, history of reactions to enteral formula, hematemesis and / hematemesis + melena, and those receiving citicoline. During the study, researchers assessed the daily intake of food consumed by patients using the 24-hour recall method for 7 days. How to determine the patient's nutritional adequacy level through the average intake divided by the patient's requirement then multiplied by 100. The control group was given enteral formula based on the standard RS conventional enteral formula according to the character/comorbid of the ischemic patient (11.8 protein without the addition of phosphatidylcholine, phosphatidylserine, and inulin). The intervention group was given enteral formula containing 15 g of protein, 128 mg of phosphatidylcholine, 32 mg of phosphatidylserine, 3 g of 69 g of powdered enteral formula inulin, each dissolved in 250 ml of warm water and given three times a day for seven days. In the intervention group suffering from diabetes, the treatment was conducted for 14 days at a dose of 34.5 g per day. This provision is differentiated because the enteral formula given contains a high enough glycemic index which will have side effects in diabetes mellitus patients, so the administration will be reduced but it is carried out for a longer period. To avoid any side effects during the intervention, the researchers observed the patients every day.

The blood samples used were whole blood vein samples taken from the median cubital vein. The 6 ml blood sample was taken using a red vacutainer. The blood was then exported to the central laboratory of Dr. Kariadi for centrifuge and stored at –20ºC until testing. After the serum was successfully collected, the serum was sent to the Faculty of Medicine Undip GAKI laboratory for testing using the ELISA GFAP E-EL-H1888 kit.

This study has received Ethical Clearance approval by the Medical Research Ethics Commission FK UNDIP / RSDK with Number 503 / EC / KEPK-RSDK / 2020.

Statistical analysis was performed on a computer using the SPSS for windows version 20.0 program. Bivariate analysis was performed with the $\chi 2$ test or with the Fisher-Exact test to determine the change (Δ) in serum GFAP levels and the characteristics of the categorized subjects. The result is significant if $p < 0.05$. Multivariate analysis on confounding variables (age, sex, hypertension, diabetes mellitus, obesity status, dyslipidemia, history of stroke, rTPA therapy,

duration of intervention, and length of treatment) was carried out if any variables in the bivariate test were found to be different, with p <0.05 using General Linear Model.

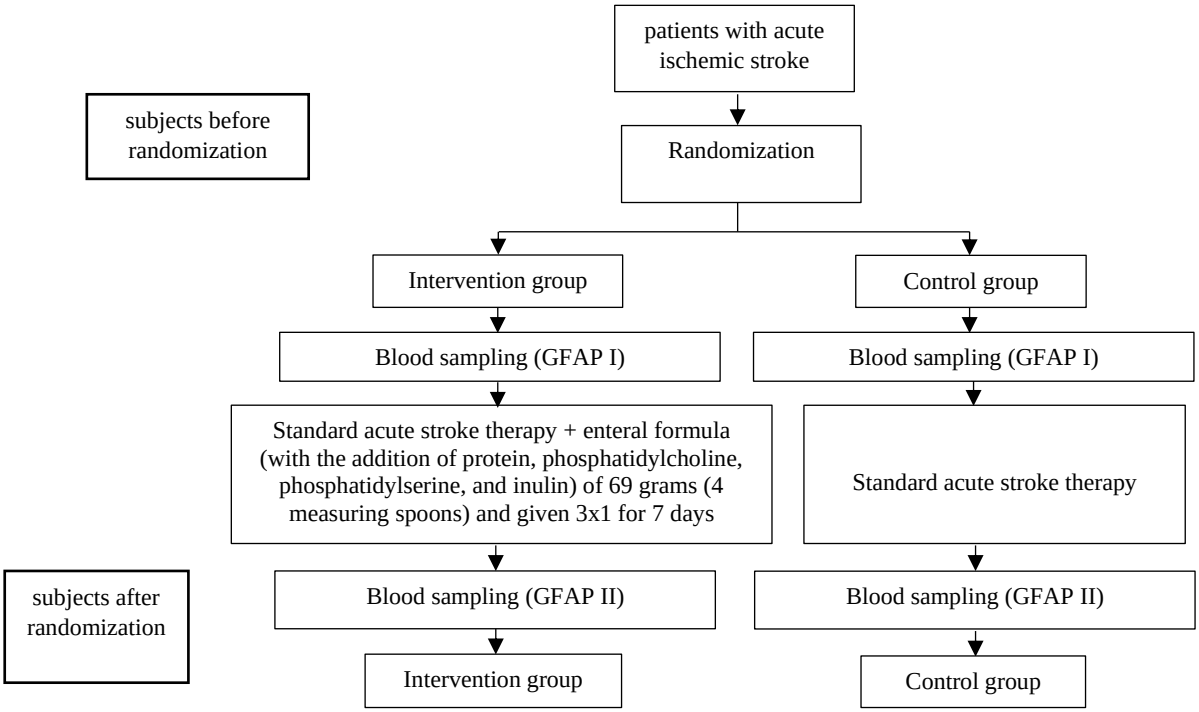

Figure 1. Flow chart of the research from the beginning to the end of the study

RESULTS

Characteristics of Research Subjects

In the implementation of the study, 8 out of 26 patients were found to have dropped out or lost to follow-up. Some of the things that caused the drop-out included patients who were not willing to continue the study (5) and died (3).

A total of 18 acute ischemic stroke patients were the subjects in the study. There were no significant differences in the categories of gender, age, BMI, obesity status, hypertension, diabetes mellitus, dyslipidemia, smoking, rTPA therapy, history of stroke, duration of intervention, and length of stay (p> 0.05). However, there were differences in the mean age category (p = 0.01).

Average Adequacy Level of Energy, Protein, Fat, Carbohydrates, Phosphatidylcholine, Phosphatidylserine, and Inulin Intake

Table 2 reveals that there is a significant difference in the adequacy of energy, protein, carbohydrate, phosphatidylcholine, phosphatidylserine, and inulin intake (p<0.05). There was no significant difference in the adequacy of fat intake between groups (p>0.05).

Difference of Mean and Change (Δ) GFAP levels

There was no difference in the mean GFAP level before the study between intervention group 8.37±4.25 and the control group 5.4±1.8 with a value (p = 0.08). These results designate that the conditions of the two groups before the intervention are corresponding (homogeneous). In the intervention group, the average GFAP level managed to decrease from 8.37±4.25 to 8.30±4.9, whereas in the control group, there was an increment from 5.4±1.8 to 7.5±4. This disparity shows that the intervention given affects even though this difference is not statistically significant (p = 0.9). Changes in Δ levels of GFAP in the intervention group also tended to decrease -0.07±2.26 compared to the control group which tended to increase 2.13±2.84 but not statistically significant (p> 0.05).

Table 1. Characteristics of Research Subjects in the Two Research Groups

Characteristics	Group		*p*
	Intervention (n=9)	Control (n=9)	
Gender			
a. Male	4 (44.4%)	6 (66.7%)	0.6[a]
b. Women	5 (55.6%)	3 (44.4%)	
Average age (years)	50.5 ± 14.8	65.5 ± 8.2	0.01[b]
Age Category (years)			
a. > 60	3 (33.3%)	7 (77.8%)	0.1[a]
b. <60	6 (66.7%)	2 (22.2%)	
Average BMI	25.2 ± 4	23.8 ± 2.6	0.3[b]
Obesity (BMI> 25)			
a. Yes	4 (44.4%)	2 (22.2%)	0.6[a]
b. Not	5 (55.6%)	7 (77.8%)	
Hypertension			
a. Yes	8 (88.9%)	7 (77.8%)	1[a]
b. Not	1 (11.1%)	2 (22.2%)	
Diabetes mellitus			
a. There is	3 (33.3%)	5 (55.6%)	0.6[a]
b. Not	6 (66.7%)	4 (44.4%)	
Dyslipidemia			
a. Yes	7 (77.8%)	8 (88.9%)	1[a]
b. Not	2 (22.2%)	1 (11.1%)	
Smoke			
a. Yes	4 (44.4%)	6 (66.7%)	0.6[a]
b. Not	5 (55.6%)	3 (33.3%)	
RTPA therapy			
a. Yes	2 (22.2%)	2 (22.2%)	1[a]
b. Not	7 (77.8%)	7 (77.8%)	
Stroke History			
a. Yes	3 (33.3%)	3 (33.3%)	1 [a]
b. Not	6 (66.7%)	6 (66.7%)	
Duration of Intervention			
a. 7 days	6 (66.7%)	9 (100%)	0.2 [a]
b. 14 days	3 (33.3%)	0 (0%)	
Length of Hospitalization			
a. <7 days	6 (66.7%)	3 (33.3%)	0.3 [a]
b. ≥ 7 days	3 (33.3%)	6 (66.7%)	

[a] *Fisher-Exact*; [b] *Independent T-Test*

Table 2. Adequacy Level of Patient Intake at Research

Variable (%)	Intervention	Control	*p*
	Mean ± SD	Mean ± SD	
Energy Adequacy Level	95 ± 18.4	75.7±11.9	0.018[b]
Protein Adequacy Level	94.1 ± 17.6	69.1±11.7	0.003[b]
Adequacy Level of Fat	74.3 ± 21.2	75.9±14.8	0.9[b]
Adequacy Level of Carbohydrates	106.9 ± 20.5	78.8±14.4	0.004[b]
Adequacy Level of Phosphatidylcholine	72.7±8	25.2±3.4	0.0[b]
Adequacy Level of Phosphatidylserine	112.3±12.3	49±10.2	0.0[b]
Adequacy Level of Inulin	92.4±7.9	24.1±7.6	0.0[b]

[b]*Independent T-Test*

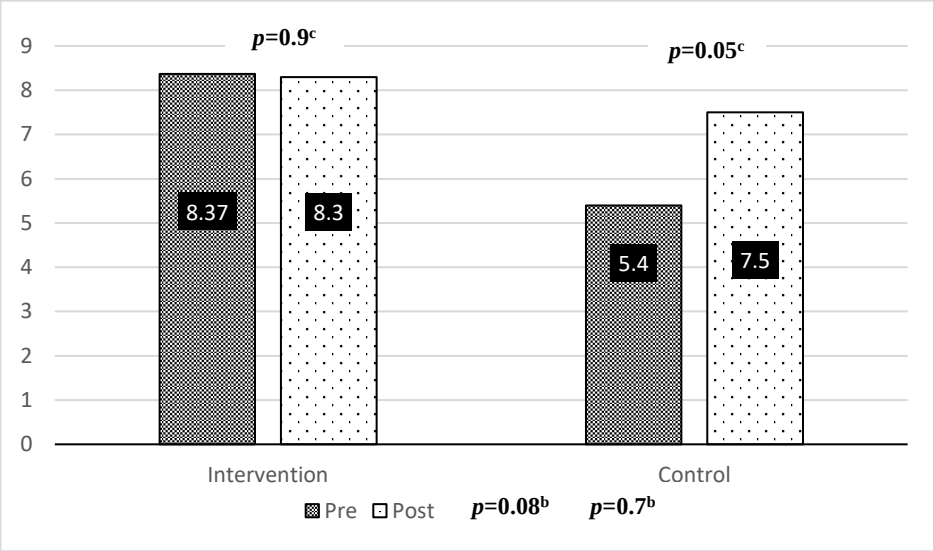

Figure 2. Average GFAP Levels Between Groups

[b] *Independent T-Test*; [c] *Paired T-Test*

Sub-Group Analysis of Inter-Group GFAP Levels

Median GFAP levels before the study in the intervention group DM subgroup 11.3 (2.4 – 12.4) with the control group 6 (3.9 – 8.1) was not statistically significant (p = 0.3). These results indicated that the state of the two groups before the intervention was in identical condition. In the DM sub-group in the intervention group, the median GFAP level increased from 11.3 (2.4 – 12.4) to 12.6 (2.1 – 12.8), it was the same as the control group which also increased from 6 (3.9 – 8.1) to 7 (3.4 – 15.6) and statistically, this difference was not significant (p>0.05). In the DM subgroup, the change in GFAP levels in the intervention group also decreased from 0.4 (-0.3 – 1.3) the same was the case with the control group 1 (-0.5 – 7.9) nonetheless, this difference was not statistically significant.

Median GFAP levels before the study in the non-DM subgroup of the intervention group 9.6 (1.1-12.3) with the control group 4.1 (2.8-6.7) were not statistically significant (p = 0.1). These results indicated that the condition of the two groups before the intervention is in the same condition. In the non-DM subgroup the intervention group median GFAP level there was a tendency to decrease from 9.6 (1.1-12.3) to 7.3 (0.8-14.2), in contrast to the control group which there was an increase from 4.1 (2.8-6.7) to 5.9 (3.8-8.2), and statistically, this difference was not significant (p>0.05). In the non-DM subgroup, the change in Δ GFAP levels in the intervention group also tended to decrease -0.2 (-3.5 – 3.4) is different the case with the control group which there was an increase of 0.8 (-0.5 – 4.9) however, this difference was not statistically significant.

Table 3. Sub-group analysis of GFAP levels between groups

Sub Group	Kelompok	n	Before	After	p	Δ
			Median (minimum-maximum)	Median (minimum-maximum)		Median (minimum-maximum)
DM	Intervention	3	11.3 (2.4 – 12.4)	12.6 (2.1 – 12.8)	0.2[d]	0.4 (-0.3 – 1.3)
	Control	5	6 (3.9 – 8.1)	7 (3.4 – 15.6)	0.1[c]	1 (-0.5 – 7.9)
	p		0.3[b]	0.8[e]		0.3[b]
Non DM	Intervention	5	9.6 (1.1-12.3)	7.3 (0.8-14.2)	0.7[c]	-0.2(-3.5 – 3.4)
	Control	5	4.1 (2.8-6.7)	5.9 (3.8-8.2)	0.2[c]	0.8 (-0.5 – 4.9)
	p		0.1[b]	0.4[b]		0.2[b]

[b] *Independent T-Test* ; [c] *Paired T-Test*; [d] *Wilcoxon*; [e] *Mann-Whitney*

GLM (General Linear Model)

Table 4 shows that there is no significant difference in GFAP levels controlled by the age variable (p = 0.3)

Table 4. General Linear Model

Variable	Covariat	*p*	R^2	Lower	Upper
(Δ) GFAP levels	Age	0.5	0.1	-0.14	0.079

DISCUSSION

Subject characteristics data revealed that there were no significant differences in the categories of gender, age, BMI, obesity status, hypertension, diabetes mellitus, dyslipidemia, smoking, rTPA therapy, history of stroke, duration of intervention, and length of stay (p>0.05). However, there were differences in the mean age category (p = 0.01). Stroke is often referred to as a disease suffered by older people. Currently, there was a trend that young people suffer from a stroke. The rising number of stroke cases at a young age can be prompted by a not-healthy lifestyle, such as smoking, alcohol consumption, frequent eating low-fiber foods such as fast food or junk food, and lack of exercise which will lead to hypertension. The habit is usually worsened by people in Central Java who are accustomed to consuming sweet foods or high glucose levels and foods containing coconut enteral formula which can trigger atherosclerosis and thrombosis, which results in reduced oxygen supply to the brain and can lead to stroke. The outcomes of the different test results for the adequacy of energy intake, carbohydrate intake, and fat intake between the intervention group and the control group showed significant results (p <0.05) but not for fat intake (p> 0.05). The exception is because one of the interventions given to the control group was standard enteral formula from the hospital, where before the 7th day and the patient had gone home, the researchers could not bring the enteral formula to continue therapy at home for one reason or another. Unlike the case with the intervention group before the 7th day; however, the patient had gone home, the researcher could provide the enteral formula to continue therapy at home. It could also be caused by monitoring food consumption before the study was completed. Researchers carry out a recall via cellular media such as WhatsApp because when the study took place coincided with the COVID-19 pandemic, which caused the patient to be unwilling to be visited by the researcher. If the patient during the study was still in the hospital, there was a standard portion for each type of diet. However, it is different from patients who have not returned home before the 7th day. There is also another possibility, such as when researchers do a recall, patients tend to overestimate or underestimate the food they eat. The size of the household measurement also varies, so it can affect recall results. There is also another possibility, such as when researchers do a recall, patients tend to overestimate or underestimate the food they eat. The size of the household measurement also varies, so it can affect recall results. There is also another possibility, such as when researchers do a recall, the patient tends to overestimate or underestimate the food consumed. The size of the household measurement also varies, so it can affect recall results.

The distribution of serum GFAP levels at the start and end of the study found that all patients were at abnormal levels (>0.11 ng/ml).[7] This could result in the presence of high-fat content or other anomalies in the sample that could prompt a matrix effect, which would interfere with detection. The hemolyzed blood sample contains the HRP analog (Horseradish Peroxidase), which can make a false-positive result. The hemolyzed sample releases a substance from the red blood cells, which can be mistaken for HRP. HRP is the critical reagent used for ELISA. Therefore, once the sample is hemolyzed, detection will be affected. This is one of the factors that can influence GFAP.

Other factors that can affect GFAP occur in several cellular processes. The drastic decrease in oxygen in the brain causes the brain to undergo anaerobic metabolism so that the availability of ATP is low. Hypoxic conditions cause neuron cell death in minutes because the brain is a part that is very sensitive to hypoxic states. Hypoxia triggers a series of pathways that lead to decreased energy and the extracellular release of glutamate. Neurons and glial cells can die through various causes such as excitotoxicity, cellular edema, oxidative stress, and inflammation. Hypoxia increases the formation of reactive oxygen species (ROS) which results in oxidative stress on cells. Increased levels of ROS are a major cause of brain tissue damage after hypoxia. ROS damage tissue directly through the modification of cellular proteins, lipids, and DNA. ROS indirectly interferes with cellular signaling and the regulation of gene expression. Astrocytes play an

especially important role in the communication between astrocytes-neurons through the release of several neurotrophic factors to maintain central nervous system homeostasis. Astrocytes also play a role in maintaining homeostasis in the environment around neurons. Recent research has shown that astrocytes are immunocompetent in the brain. If there is a disturbance in the brain that causes damage, the astrocytes will be activated and move quickly to the site of damage (astrogliosis). On excessive activation, it turns out that astrocytes increase the production of several substances, one of which is a glial fibrillary acidic protein (GFAP).[29]

Mean baseline GFAP serum levels in the intervention group 8.37±4.25 and control group 5.4± 1.8 with a value (p = 0.08). The final study serum GFAP levels in the intervention group were 8.30±4.9 and in the control group was 7.5±4. These numbers showed that the intervention given still affects even though the difference is not statistically significant (p = 0.9). In Figure 2, the intervention group declined -0.07±2.26 compared to the control group, which increased 2.45±2.86. This difference was statistically significant (p = 0.03).

The difference is partly because the patients in the study sample were patients who had repeated strokes (had a history of stroke), which according to the study of Ren *et al.* (2016) which examined GFAP levels in 13 subjects with hemorrhagic strokes, 23 subjects with ischemic strokes and 7 subjects with recurrent stroke reported that in patients with ischemic stroke, the mean serum GFAP level was significantly lower in subjects without a history of stroke compared with patients who had a previous stroke (p = 0.004). This increment occurred due to persistent pathophysiological processes including vascular disorders, long-term changes in BBB permeability, and damage to the brain parenchyma itself triggered by an initial stroke.[30] In this study, patients with recurrent strokes found five patients in the control group, three patients in the treated group.

This can then be caused by comorbid patient diseases such as diabetes mellitus. Research conducted by Lotosh *et al.* (2013) on experimental animals by inducing streptozotocin (STZ) showed that autoantibodies against neuron proteins, especially GFAP increased sharply on day five after being induced by streptozotocin. In this study, it can be concluded that STZ produces a toxic effect on astrocytes which causes the release of GFAP protein into the bloodstream and causes the formation of AAb (autoantibody) which is specific to GFAP protein.[31]

The COVID-19 pandemic can also cause another thing during the research, which resulted in a lack of research samples. Where in a study conducted by Zang *et al.* (2019) which examined the relationship between giving citicoline injection to 102 patients with hemorrhagic stroke and GFAP levels showed a significant relationship (p = 0.000).[28]

Another thing is made possible by the dose of enteral formula that is too small, the duration of administration, and the route of administration which can be the cause of the research results. The doses in this study were 45 g protein/day, 384 mg/day phosphatidylcholine, 96 g/day phosphatidylserines, and 9 g/day inulin. In some studies, the minimum duration of intervention given was 14 days and obtained significant results. [26,32–35]

The results obtained by the DM sub-group analysis of GFAP levels in the intervention group showed an increasing trend of 0.4 (-0.3 – 1.3) compared to the control group of 1 (-0.5 – 7.9) and statistically not significantly different (p>0.05). Unlike the case with the non-DM subgroup in the intervention group which showed a decreasing trend of -0.2(-3.5 – 3.4) compared to the control group where there was an increasing trend of 0.8 (-0.5 – 4.9) and not statistically significant (p> 0.05). This occurred because, in patients with hyperglycemia, it can provoke nerve degeneration through oxidative stress. Metabolic and oxidative factors often cause rapid changes in glial cells. A key indicator of this response was an increased synthesis of GFAP, which is an astrocytic marker. The event was evidenced by the study of Baydas *et al.* (2003) who conducted a study on 40 experimental animals divided into three groups. The first group was injected with STZ, the second group was injected with STZ + melatonin, and the last group was the control group. The results showed that the STZ-induced group caused glial reactivity, while the STZ + melatonin-injected group decreased the GFAP recativity.[36]

Research on the effect of giving protein, phosphatidylcholine, phosphatidylserine, and inulin on markers of brain damage such as GFAP is still minimal. In this study, the intervention was administered orally or in the form of powdered enteral formula with protein,

phosphatidylcholine, phosphatidylserine, and inulin. Efficiently, phosphatidylcholine and phosphatidylserine are almost entirely absorbed, which then spreads throughout the body across the blood-brain barrier and into the central nervous system. Inulin is easily dissolved in water and difficult to hydrolyze by enzymes in the digestive tract so that it can reach the large intestine as a whole. Probiotic bacteria will ferment inulin in the intestines and become short-chain fatty acids called SCFA.[10,23,27]

In this study, the intervention gave still affected even though it was not statistically significant (p> 0.05), according to research from Ji *et al.* (2018), a high protein diet given earlier can improve motor coordination. Currently, most of the hospitals have implemented facilities such as flexor and elbow extensor training, physiotherapy, for example, teaching good movement patterns and posture. However, this facility will not be optimal if there are patients with severe disabilities and a lack of cooperation between patients and hospital staff. Increasing SOD expression, restoring iNOS expression, and suppressing MDA expression are among the advantages of the early high protein diet. This suggests that oxidative stress can be inhibited if a high-protein diet is given early on so that free radicals and inflammation in the body can be reduced and can improve post-stroke clinical outcomes.[32]

Supported by research by Aquilani *et al*. (2008) which provided supplementation with hyperproteic nutritional formulas (10% protein = 40 grams of protein) in 20 patients for 21 days, it showed that the National Institutes Stroke Scale (NIHSS) scores of patients with protein administration increased on average 4.4 compared to a control group average of 3.0. However, there was an increase in motor recovery of the paretic arms and legs which were found to be better able to withstand gravity for 10 seconds (with legs outstretched) in the intervention group. The improvement in the performance of the extremities against gravity was less seen in the control group patients.[33] In this study, it has a ratio of 1: 2 related to protein administration with research conducted by Aquilani.

In a study conducted by Tykhomyrov *et al.* (2019) who examined the effect of giving CDP-choline or citicoline injections to 33 ischemic stroke patients with GFAP levels who gave citicoline injection of 1000 mg for 14 days, they decreased GFAP levels by 61% with (p = <0.01). CDP-choline can protect astrocytes and neurons, as well as increase angiogenic capacity through downregulation of angiostatin in post-ischemic patients with atrial fibrillation after acute ischemic stroke. Also, CDP-choline can increase cell proliferation, vasculogenesis, and synaptophysin levels and reduce GFAP levels in the peri-infarct area in ischemic stroke. In this study, enteral nutrition was given with a content of 384 phosphatidylcholine/day.[34]

In a study conducted by Kataoka *et al*. (2010) which examined the effect of Soy-PS administration on 78 elderly people with mild cognitive impairment (50-69 years) were randomly selected to consume Soy-PS (100 mg or 300 mg/day) or placebo for 6 months. Administration of Soy-PS orally for 6 months improved memory function, especially delayed memory in the elderly with memory complaints. Administration of Soy-PS was able to improve memory function, especially delayed memory in the elderly with memory complaints. Increasing the memory score is also one of the benefits of giving Soy-PS. The activation of signaling proteins and receptors that are essential for neuronal survival is influenced by phosphatidylserine. Phosphatidylserine also plays a role in signaling processes such as regulating neurotransmitters. In this study, the intervention was given with enteral nutrition containing 96 phosphatidylserines/day for 7 days.[26]

The administration of inulin in Hofman's study was 8% by injection of inulin in mice which were equivalent to 40 grams of inulin in humans per day. Hoffman's research shows that 8% inulin can increase cecal content, produce more SCFA, increase the number of bacterial enzymes in the cecum, increase systemic metabolism by modulating the gut microbiome, and can reduce the characteristics of brain inflammation in early AD (Alzheimer's Disease) compared to 4% inulin. Hoffman's research also states that 8% is considered the maximum tolerable and beneficial amount for the human organism. In this study, 9 grams of inulin per day were given.[35]

Inulin is a part of prebiotics. Prebiotics is a component of food that cannot be digested by the digestive tract enzymatically, which will then fermented by microbiota in the intestine. Inulin is a substrate that progresses the diversity of beneficial microbiota in the intestine so that the production of SCFAs such as acetate, propionate, and butyrate will increase as a result of anaerobic fermentation. The gut-brain axis is influenced by

SCFA either directly or indirectly. SCFA binds to various receptors to further inhibit histone deacetylation and affect the integrity of the intestinal barrier and intestinal mucosal immunity. The interaction performed by the SCFA on various receptors can induce indirect signaling to the brain by inducing the secretion of intestinal hormones. Systemic inflammation is affected by SCFA by regulating leukin secretion and inducing differentiation of Tregs or T regulatory cells. The blood-brain barrier can be penetrated by the SCFA through the MCT or monocarboxylate transporter and improves the integrity of the blood-brain barrier. SCFA can enhance neurogenesis, function, and homeostasis of nerve cells and improve the morphology and function of glial cells found in the central nervous system. [35,37]

RTPA therapy also appears to contribute to study results. In the study by Davalos *et al.* (2002), 47% of patients were treated with rTPA and given citicoline therapy 1000 mg per 12 hours intravenously for three days, then orally were given 2x500 mg tablets per 12 hours for a total of six weeks. rTPA can rebuild blood flow in the penumbra; therefore it is difficult for citicoline to enhance the performance caused by the effects of thrombolysis. The severity of most stroke patients in Davalos *et al.*'s study was also high so that no penumbra region was susceptible to being saved by citicoline. Consequently, in the subgroup without rTPA, the efficacy of citicoline exhibited better results than the group with rTPA.[24] In this study, 4 patients received rTPA therapy.

Furthermore, it can be caused by patients who have gone home before the 7th day. Although given enteral formula to continue therapy at home, this can cause researchers to be unable to control whether or not the intervention is complete. The drugs received and the presence of comorbidities that concern the improvement of clinical outcome in ischemic stroke patients, because of these factors, a significant reduction in GFAP levels required.

A multicentre study conducted on 4023 ischemic stroke patients who were given a protein-energy supplement of 62.5 g / day for 6-7 months in 125 hospitals and 15 countries showed that routine oral protein energy supplementation of a usual hospital diet did not improve outcomes in patients admitted with recent stroke.[38] A study by Sabin *et al.* (2013) giving citicoline to 347 ischemic stroke subjects (1g / day for 12 months) showed no significant difference (p = 0.186).[39] Supported by Clark's study that gave citicoline to 453 ischemic stroke subjects (1000 mg 2x / day for 12 weeks) showed no significant difference (p>0.05).[40] A study conducted by Manor *et al.* (2013) regarding the administration of phosphatidylserine in 30 elderly with memory complaints (300 mg/day for 12 weeks) showed no significant difference in focused attention, sustained attention, visuospatial learning, spatial short-term memory (p>0.05).[41] The study by Tuncay *et al.* (2018) who performed Enteral Formula with Probiotic Content (EFPC) intervention in 23 neuro-critical care patients for 21 days was associated with more frequent dosing of subjects showing no significant difference.[42]

The results of this study emphasized that enteral formula containing protein, phosphatidylcholine, phosphatidylserine, and inulin had a good effect on GFAP levels, as evidenced by changes (Δ) in serum GFAP levels in the intervention group which decreased -0.07±2.26 compared to the control group, which increased 2.13±2.84 although it was not statistically significant (p>0.05).

In General Linear Model (GLM), it shows that there is no significant difference in (Δ) GFAP levels were controlled by age variable (p>0,05). This may be due to the lack of research samples. During the study, there was a COVID-19 pandemic which made patients tend to hesitate or fear to have themselves examined at Dr. Kariadi. Remembering Dr. Kariadi is a referral hospital in Semarang.

The limitations of this study were the shortage of sample, length of time, and dose of administration. The advantage of this study is to provide enteral nutrition therapy to stroke patients by examining brain damage markers (GFAP) because this study is still very limited, so it can add to the literature for other researchers.

CONCLUSIONS

The addition of 15 g protein, 128 mg phosphatidylcholine, 32 mg phosphatidylserine, and 9 g inulin given for seven days had no significant effect on GFAP levels.

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