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Research Article
Glutathione Supplementation Reduces MMP-9 Levels and Infarct Area in Rats Models of Acute Ischemic Stroke

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Abstract

Background and Objective: Ischemic stroke occurs when blood vessels vascularizing the brain are blocked and unable to receive oxygen or glucose. Matrix metalloproteinases (MMPs) have been implicated in the pathophysiology of stroke. MMP-9 is known as an early marker related to incidence of stroke. While standard therapy for stroke is unable to repair damaged brain tissues, glutathione (GSH) inhibits oxidative stress activity and reduces excess MMP-9 levels in order to avoid pathological angiogenesis in ischemic stroke. This study was conducted to demonstrate the effect of GSH on MMP-9 levels and infarcted areas after acute ischemic stroke compared with standard therapy. **Methods:** This experimental study used a post-test only control group design. Twenty male Wistar rats were equally divided into 4 groups and orally treated with placebo, 0.72 mg aspirin/100 g body weight, 21.6 mg GSH/100 g body weight or GSH+aspirin for 7 day following induction of ischemic stroke by unilateral cerebral artery occlusion. Serum MMP-9 levels were measured by ELISA and infarct size (area) was measured by cresyl violet staining. **Results:** Both MMP-9 levels and infarct area were significantly reduced ($p<0.05$) in all treatment groups versus control group (placebo). GSH+aspirin therapy showed the greatest reductions. **Conclusion:** Combining GSH and aspirin significantly decreased MMP-9 levels and infarct area after acute ischemic stroke compared to standard therapy alone.

Key words: Glutathione (GSH), matrix metalloproteinase-9 (MMP-9), ischemic stroke, infarction, aspirin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

According to the 2008 Global Burden of Disease Project, the prevalence of degenerative diseases will be higher than that of infectious diseases by 2030. The prevalence of degenerative diseases is estimated to increase to 87%, with stroke becoming a main contributor. Stroke is currently the third highest cause of death in developed countries, after heart disease and cancer. Of these, 15.4% of deaths occurred in the total stroke population and 42.9% of them were caused by ischemic stroke[1]. In Indonesia, the prevalence of stroke is 43.1% in the population over 75 years of age and 0.2% in those aged 15-24 years. Furthermore, the incidence of stroke between men and women is almost the same[1-3].

Ischemic stroke occurs when blood vessels vascularizing the brain are blocked and cannot receive oxygen or glucose[4-6]. In turn, neuronal function stops, causing neurological damage and deficits. Symptoms of neurological deficits that occur depend on the affected area in the brain[7]. However, neuronal function can return to normal if blood flow is immediately restored[7,8].

MMPs have been implicated in the pathophysiology of stroke. Increased levels of MMPs have been shown to increase neuronal death, brain damage, edema and hemorrhage[4,9,10.] MMP-9 is known to be an early marker of stroke incidence that can be detected by Enzyme Linked Immunosorbent Assay (ELISA)[11,12] and is rapidly regulated during ischemic stroke[4,6]. MMP-9 is a proteolytic enzyme involved in the process of angiogenesis following the occurrence of ischemic stroke[13]. Angiogenesis is the process of formation of new blood vessels from the vascular endothelium that physiologically progress during the inflammation process. Angiogenesis can be pathological when it is uncontrolled, resulting in damage to the anatomy of blood vessels[14,13].

Studies in rats have shown that MMP-9 levels in brain peak at 24 h after ischemic onset and can last up to 72 h thereafter. MMP-9 in plasma has also been positively correlated with stroke severity according to the National Institutes of Health Stroke Scale (NIHSS)[15]. An increase of MMP-9 in infarcted and peri-infarct tissues is also involved in the progression of widespread infarction. Thus, MMP-9 may also be used as a biomarker for brain ischemia[16,17]. Excessive activation of MMP-9 has a damaging effect on the blood-brain barrier and leads to inflammation[4,18,19]. Therefore, its inhibition may be a potential therapeutic target[16,20].

Standard stroke therapy currently involves antiplatelet (aspirin), thrombolytic and anticoagulant drugs. These drugs relieve the symptoms of stroke by lysing blockages in blood vessels leading to the brain. However, this standard therapy cannot successfully repair damaged brain tissue due to the lack of oxygen to the brain. Furthermore, aspirin increases the risk of gastrointestinal bleeding, especially in the stomach[8,21,22]. However, studies have shown that the acid-base balance of the stomach can be maintained by administration of glutathione (GSH)[23,24]. GSH is a powerful antioxidant that plays an important role in the brain by recycling free radicals involved in oxidative stress. Protection against oxidative stress occurs directly through the oxidation of GSH in mitochondria[23,24]. Inhibition of oxidative stress activity is also known to decrease excessive MMP-9 levels, thus preventing the occurrence of pathologic angiogenesis. Inhibition of pathological angiogenesis can, in turn, prevent neuronal death and ultimately result in the reduction of disability and death after ischemic stroke[13,25].

Increased levels of MMP-9 plasma correlates with brain function during 24 h of acute cerebral ischemia in mice[15]. This study was conducted to investigate the effects of GSH administration on MMP-9 levels and cerebral tissue damage following stroke using unilateral artery cerebral occlusion (UCAO) to induce ischemic stroke in rats. Previous studies have shown that administration of 16,800 mg of GSH per month can help reduce symptoms of neurological disorders in humans and is safe to use[26].

MATERIALS AND METHODS

Animals and treatments: The current experimental study used a post-test only control group design. Male white Wistar rats were obtained from the research laboratory of integrated research and testing research service pre clinic experimental animal development (Unit 4) of Gajahmada University (Yogyakarta, Indonesia). Twenty rats were randomly and equally allocated into 4 treatment groups (I-IV), with 2 extra rats per group added in case of exclusion (5 rats/group). Ischemic stroke was induced in all rats via UCAO prior to treatment.

Before treatment, rats were adapted to laboratory conditions for 1 week to adjust to their environment at room temperature (25°C). All animals were given food in the form of pellets in standard plastic enclosures. Rats (150-200 g, 8-12 weeks of age) were selected based on overall health, lack of anatomic abnormalities and no allergies to aspirin and glutathione. This study was approved by the ethical committee of Karyadi Hospital, Semarang City, Indonesia. Induced ischemic stroke using UCAO method. Rat induced, anesthetized with ketamine before binding to the right common carotid artery for 45 min. Arterial ligation stops, then an incision wound is stitched and bandaged with sterile

gauze. Mice that lose weight up to 10% or die during the research process will be dropped out of the study. Rat blood collection and brain tissue removal were performed on day 8. Study design was as follows: Group I rats received 2 mL water, Group II received 0.72 mg aspirin/100 g body weight (BW), Group III received 21.6 mg GSH/100 g BW and Group IV received 0.72 mg aspirin+21.6 mg GSH/100 g BW. The compound was dissolved in water and administered orally by gastric gavage. MMP-9 levels in rat serum were measured by ELISA. Histopathologic examination with cresyl violet was used to determine the percentage of infarcts. Brain regions assessed included the cerebral cortex and striatum. Neuron damage was calculated based on observation with a light microscope at 1000x magnification. The presence of healthy neurons represented less infarct areas and pale coloration represented a wider infarct area.

Data analysis: Data were analyzed using SPSS. Descriptive tests were conducted to assess the characteristics of the data. Normal data were analyzed using the Shapiro-Wilk test. Significance was determined using the one-way ANOVA with post-hoc tests. Bonferroni post-hoc tests were used to identify the most influential groups. A $p<0.05$ was considered statistically significant.

RESULTS

Table 1 shows that the highest mean MMP-9 level in rat serum was in Group III (GSH alone) and the highest mean infarct area was in Group I (placebo). The lowest mean serum MMP-9 level and infarct area was found in Group IV (aspirin+GSH). Figure 1 shows measurements of the infarct area in the cerebrum cortex.

The results of data analysis showed that Group IV rats (aspirin+GSH) had the lowest levels of serum MMP-9 compared to all other treatment groups. However, Group II (aspirin alone) showed lower MMP-9 levels than Groups I (placebo) or III (GSH alone) (Table 2).

While aspirin treatment alone (Group II) was able to decrease infarct area compared to controls (Group I). GSH alone (Group III) caused more reduction in infarct size compared to aspirin. However, the most significant decrease in infarct area was observed in group IV (aspirin+GSH) compared to all other treatment groups (Table 2). As expected, control group (Group I) had little to no influence on the infarct area.

MMP-9 levels: There was a significant decrease in MMP-9 levels between Group III (GSH) and group IV (aspirin+GSH) ($p<0.05$). Group IV (aspirin+GSH) also had a significant decrease in MMP-9 levels compared to group III (GSH) ($p = 0.010$). Group II (aspirin) also differed significantly from group III (GSH) ($p = 0.007$). The results of the data analysis showed that group IV (aspirin+GSH) had the most significant effect on decreasing MMP-9 levels compared with all treatment groups. Group IV differed significantly from group I (placebo) ($p<0.05$), while group II (aspirin alone) did not differ significantly from group I ($p>0.05$).

Table 1: Matrix metalloproteinase (MMP)-9 levels and infarct area after treatment

	Group I		Group II		Group III		Group IV	
	MMP-9 levels (ng mL^{-1})	Infarct areas (%)	MMP 9 levels (ng mL^{-1})	Infarct areas (%)	MMP 9 levels (ng mL^{-1})	Infarct areas (%)	MMP 9 levels (ng mL^{-1})	Infarct areas (%)
	0.794	52.0	0.603	40	0.808	38.0	0.660	32.0
	0.785	50.0	0.605	48	0.842	40.0	0.696	40.0
	0.650	52.0	0.599	44	0.828	40.0	0.606	30.0
	0.884	58.0	0.711	50	0.845	42.0	0.589	28.0
	0.806	50.0	0.766	48	0.844	42.0	0.479	28.0
Mean	0.784	52.4	0.657	46	0.833	40.4	0.606	31.6

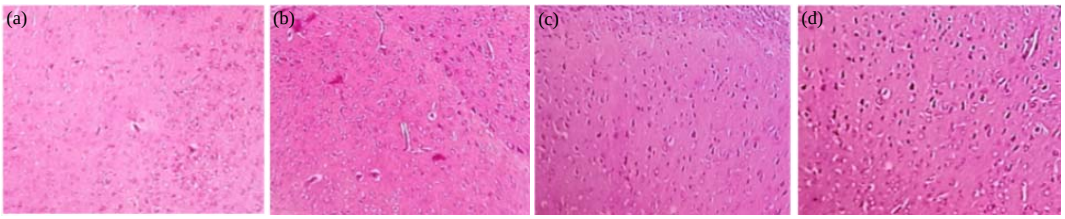

Fig. 1(a-d): Cresyl violet histopathology, (a) Group I (placebo/water), (b) Group II (aspirin alone), (c) Group III (GSH alone) and (d) Group IV (aspirin+GSH)
The most extensive nerve damage found in Group I (a), is shown the most pale color. Group IV (d) has the largest number of healthy neurons, indicated by the brightest color

Table 2: Post-hoc analysis of matrix metalloproteinase (MMP)-9 levels and infarct area between groups

	MMP-9 Levels		Infarct areas	
Groups	p-value	95% CI	Significant	95% CI
Group I				
II	0.073	-0.008-0.262	0.086	-0.62-13.42
III	1.000	-0.185-0.086	0.001*	4.98-19.02
IV	0.007*	0.043-0.313	0.000*	13.78-27.82
Group II				
I	0.073	-0.262-0.008	0.086	-13.42-0.62
III	0.007*	-0.312--0.041	0.173	-1.42-12.62
IV	1.000	-0.084-0.186	0.000*	7.38-21.42
Group III				
I	1.000	-0.086-0.185	0.001*	-19.02--4.98
II	0.007*	0.041-0.312	0.173	-12.62-1.42
IV	0.001*	0.092-0.363	0.010*	1.78-15.82
Group IV				
I	0.007*	-0.313--0.043	0.000*	-27.82--13.78
II	1.000	-0.186-0.084	0.000*	-21.42--7.38
III	0.001*	-0.363--0.092	0.010*	-15.82--1.78

*Significantly different (p<0.05), CI: Confidence interval

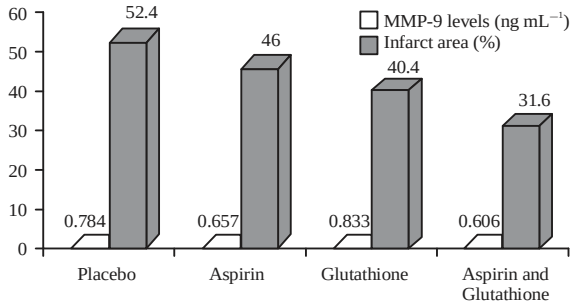

Fig. 2: MMP-9 levels and infarct areas for all treatment groups

Taken together, these data suggest that glutathione supplementation may decrease MMP-9.

Infarct area: Group III (GSH) and Group IV (aspirin+GSH) had significant decreases in infarct area compared to the placebo group ($p = 0.001$). In addition, Group IV (aspirin+GSH) had a significant decrease in infarct area compared to Group II (aspirin) and Group III (GSH) ($p<0.05$). Group II (aspirin) did not differ significantly from Group III (GSH) ($p = 0.173$). The results indicated that Group IV (aspirin+GSH) had the most significant decrease in infarct area compared to all treatment groups (Fig. 2).

DISCUSSION

Effects of GSH on serum MMP-9 levels: The role of GSH as an anti-inflammatory agent capable of decreasing MMP-9 levels in rats induced by ischemic stroke should be aided by standard therapy. Previous research has shown that inhibition of MMP activity by GSH can be enhanced by the addition of tetracycline[27]. GSH has been shown to preserve and protect brain cells from acidosis, pollutants, free radicals and harmful chemicals from metabolic waste[28-32]. The role of GSH as a major endogenous antioxidant is particularly beneficial for immune function, especially in patients experiencing inflammation[33,34]. During the process of inflammation, MMP expression is influenced by cytokines that act as inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6)[35,26,36]. The results of the data analysis showed that serum MMP-9 levels in Group III (GSH alone) did not differ significantly compared the placebo control group. Further investigation on the effect of administering various doses of GSH on serum MMP-9 levels is necessary to determine an optimal dose that is safe, since doses greater than 24 mg have been reported to disrupt liver function[27,37].

Effect of GSH on infarct area: The results of this study indicate that GSH can decrease the size of the cerebral infarction area. It is hypothesized that this effect occurs due to a decrease in the circulation of reactive oxygen species, which can prevent intracellular and extracellular damage. Prevention of DNA damage, oxidative stress, inflammatory control and apoptosis has been shown to reduce the size of a cerebral infarction[10,32,38,39]. Oxidative stress is associated with an increase in the level of nitric oxide circulating in the blood, high levels of nitric oxide have been shown to increase neuronal death[21,39-41]. The results of the present study show that administration of GSH can improve the pathogenesis of ischemic stroke by decreasing the infarct area and reducing cell death[38].

Effect of supplementation GSH (combining GSH and aspirin) on MMP-9 levels: GSH supports thiol exchange

systems (bonds between carbon and sulfur) and is known for its role in the regulation of cellular activity[23,33,42]. For example, GSH is able to maintain mitochondrial function in addition to preventing and/or repairing DNA damage[40,41,43]. GSH has an anti-inflammatory effect by stimulating the hypothalamus-pituitary-adrenal axis and stimulating the production of glucocorticoid hormones[42,44,45].

Studies have shown a positive relationship between oxidative stress and MMP-9 levels during the inflammatory process. Increased MMP-9 levels are associated with decreased levels of tissue inhibitor matrix metalloproteinases (TIMPs)[10,27]. GSH plays an important role in influencing the balance between TIMPs and MMPs. In addition, GSH reportedly inactivates MMPs when inflammatory processes are continuous[10,20,27,36].

In the present study, administration of GSH or aspirin was able to decrease serum MMP-9 levels after ischemic stroke in rats. Aspirin (standard therapy) is known to have an anti-inflammatory effect in the body by inhibiting the formation of proinflammatory mediators, such as those that influence MMP expression (IL-1β, TNF-α and IL-6)[35,36,46-49]. Furthermore, administration of GSH and aspirin together was able to reduce serum MMP-9 levels more than aspirin or GSH alone. It is known that GSH supplementation induces anti-inflammatory responses to decrease MMP-9 levels[44,45,47-49].

Effect of GSH supplementation on infarct area: The present study found that supplementation of GSH is able to significantly decrease the size of infarct areas compared to standard aspirin therapy alone. A previous report noted that MMP-9 affects the process of angiogenesis following an ischemic stroke. Uncontrolled angiogenesis plays a role in the pathology of ischemic stroke, resulting in increased thrombosis and embolization. In addition, blockage of blood vessels decreases oxygenation in the brain, which can expand the infarct area[4,9,13,50].

Aspirin (acetylsalicylic acid) is a nonsteroidal anti-inflammatory drug with antiplatelet activity that is useful in stroke therapy. Aspirin inhibits thromboxane formation during blood clotting, which improves blood flow to restricted brain regions[1,47,48]. Aspirin is known to inhibit the activity of the cyclooxygenase (COX) enzyme[47-49]. COX inhibition can decrease prostaglandins (PG) levels. Physiologically, PG protect the gastric mucosa and maintain function as a platelet aggregate[48,49]. The acid-base balance in the stomach can be maintained by giving GSH[34,51] and aspirin reacts with GSH to produce acetyl products[49]. The main function of acetyl products is to provide energy through oxidation[49,52,53]. GSH balance in the body prevents oxidative stress caused by aspirin[49,53,54]. Moreover, GSH supplementation can decrease MMP-9 levels and the infarct area in Wistar rats after ischemic stroke induction compared to standard therapy. We found that administration of GSH or aspirin alone can decrease the infarct area (Fig. 2).

Further research should be conducted in stroke patients to confirm these findings and extend its used as a therapy. Moreover, additional studies are needed to determine the optimal dose of GSH.

CONCLUSION

The results of the current study showed that GSH administration alone can decrease the size of infarct areas after ischemic stroke. However, GSH supplementation with aspirin elicited the greatest reduction in MMP-9 levels and infarct.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of supplementation glutathione (GSH and aspirin combination) that can be beneficial for rat models of ischemic stroke. This study will help researchers to reveal the linkages of MMP-9 levels and the extent of infarction in ischemic stroke that many researchers can not explore. Thus, a new theory about the combination of glutathione and possibly other combinations can be obtained.

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