The Effect of *Centella asiatica* on Brain Malondialdehyde Levels of Aged Rats

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Abstract: Background: In 2050, the number of elderly with 65 years of age or more is estimated to reach 1.5 billion. To better anticipate this problem, a shift of paradigm, from chronological to biological aging, is needed. Aging is a multifactorial process closely related to oxidative stress, a phenomenon in which the rate can be indicated through its secondary metabolite level, malondialdehyde (MDA). Objective: This study examines the effect of a well-known traditional medicinal plant used for its anti-inflammatory properties, *Centella asiatica* (CA), to brain MDA levels in aged Sprague-Dawley rats. Methods: The aged male rats were divided into three groups: negative control, positive control (vitamin E 6 IU), treatment (CA leaves ethanolic extract 300 mg/kg), with one additional group of untreated young rats. Throughout 28 days, each rat was given the corresponding treatment. The brains then were collected to be studied using the Lipid Peroxidation (MDA) Assay Kit. One-way ANOVA is the choice of the statistical analysis method. Results: We found that the level of MDA in the brain tissues of the treatment group rats had a lower value compared to that of the control group, although statistically insignificant (P = 0.5683). Unquestionably, the MDA concentration in the vitamin E-treated rats is the lowest of all. Conclusion: These results implied that CA may exhibit an antioxidative effect on aged rats which could hinder an aging process, if not prevent it.

1 INTRODUCTION

Today, the world is in a state of demographical shift. In 2050, the number of elderly with 65 years of age or more is estimated to be 1.5 billion; four times the number of that in 2010 (WHO, 2011). Being the fastest among all age groups, this increasing rate of older population will be felt mostly in developing countries such as Indonesia (Jones, 2010). As the elderly number grows, its financial weight on national health service sector will also continue to rise because older people are more susceptible to external and internal stress; a result of declining physiological function (Cesari, Prince et al., 2016, WHO, 2015). Besides, one should also consider accompanying diseases, e.g. depressive disorders and anxiety, as the main cause of quality of life deterioration. To better anticipate this imminent problem, a shift of paradigm, from chronological to biological aging, is urgently needed (Cesari, Prince et al., 2016). One way to address this challenge is by changing the focus of therapy; from just lengthening lifespans into increasing health span (Ho, So et al., 2010, WHO, 2015).

Aging is a multifactorial process closely related to oxidative stress. The theory of free radical aging stated that aging process is caused by an imbalance of an oxidative and antioxidative process (Finkel and Holbrook, 2000, López-Otín, Blasco et al., 2013). One biological consequences of the cellular oxidative stress is lipid peroxidation, a phenomenon in which the rate can be indicated through its secondary metabolite, malondialdehyde (MDA) (Lieberman, Marks et al., 2013). One of the most important organs which is vulnerable to the harmful effects of lipid peroxidation is brain, for it is

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composed of high concentrations of polyunsaturated fatty acids (PUFAs). In a study done by Dei, Takeda et al. (2002), an increase of MDA levels with age had been demonstrated in the cytoplasm of neurons and astrocytes. To fulfill a high demand of energy, brain also consumes a large amount of oxygen. However, compared to other organ, it relatively lacks antioxidant defenses, such as a lower activity in glutathione peroxidase and catalase, making it more vulnerable to oxidative stress. (Kedar, 2003). Therefore, protecting the brain from excessive oxidative damage might ameliorate the balance between pro-oxidants and antioxidants, hence promoting a healthier aging process.

One preventive effort to ensure this healthy aging is reflected in phytotherapy, known as herbal medicine, which utilizes therapeutic potential of a certain plant (Ho, So et al., 2010). *Centella asiatica* (CA), a medicinal tropical plant from the family *Apiaceae* used commonly in Southeast Asia, had shown to have neuroprotective and cognitive-enhancement effect which could play an important role in aging (Dev, 2009, Mukherjee, Kumar et al., 2007, Tiwari, Singh et al., 2008, Veerendra Kumar and Gupta, 2003). However, there were only a limited number of researches examining the antioxidative properties possessed by this plant, especially its role in brain aging and lipid peroxidation. The animal subjects which were used was also limited to a single breed of rat; not to mention the lack of comparison with a proven exogenous antioxidant.

In the present study, we compared the brain MDA levels between CA-treated aged Sprague-Dawley rats and their younger counterparts. The antioxidative properties of CA on aged rats were also compared to a well-known antioxidant agent, vitamin E. We hypothesized that aged rats which were treated with CA extract would have a lower level of brain MDA compared to those untreated, thus raising the potential of CA as an antioxidant which could promote a healthier aging process.

2 METHODS

2.1 Study Design and Subjects

The subject used in this experiment, the male Sprague-Dawley rats, is a distinct outbred albino rat used commonly in nutritional and medical research settings. These rats were obtained from the National Institute of Health Research and Development, Ministry of Health Republic of Indonesia. Sprague-

Dawley rat has an elongated head structure and a tail longer than its body. These rats are first bred by R. W. Dawley from the Sprague-Dawley Animal Company in Wisconsin, United States in 1925. Their docile characteristics make them easy to handle.

The rats were divided into two groups according to their age; young rats (8-12 weeks old) and aged rats group (20-24 months old). The aged rats were further divided into three final groups according to the treatment given; negative control (water as placebo), positive control (vitamin E), and treatment (*Centella asiatica* ethanolic leaves extract) group. In total, there were 4 experimental groups.

To differentiate individual rats in every group, a color-coding system was used; each rat possessed a distinct mark on a certain part of its body. The rats have initial weights ranging from 183 to 308 g for the young rats, and 333 to 490 g for the aged, all in healthy state. Using Federer's formula, a minimum of 24 subjects was needed to achieve the optimal sample size. However, to anticipate the possibility of subject exclusion due to death or other unforeseen causes, a total of 27 rats were used.

2.2 Extract Preparation

Centella asiatica (CA) leaves were dried under the sunlight until the water content fully evaporated and grinded to small fractions. The active substances of these grinded particles were then extracted by soaking them to a solvent, ethanol, for 24 to 48 hours repeatedly. To obtain and separate the active substances from its solvent, a rotary evaporator was utilized. Subsequently, the percentage of active substances contained in the viscous solution produced from this process was measured using gravimetric analysis.

2.3 Treatments

Prior to the 28-day treatment, all rats underwent a one-week acclimatization at the experiment room, adapting to a $24^{\circ}C$ temperature and a light-dark cycle of 12:12 with lights on at 9.00 PM. Throughout the study, all groups were fed daily with 10 g of standard pelleted chow (protein 18.5-20.5 %; fat ± 4%; fiber ± 6%; calcium ± 0.9%; phosphor ± 0.7%) and provided with water *ad libitum*.

After the aged rats were randomly distributed into the three groups, the following treatment was started at day-1 and ended at day-28 accordingly; water as placebo (negative control), CA leaves ethanolic extract with 300 mg/kg bodyweight dosage (treatment), and 6 IU of vitamin E (positive control).

All treatments were given twice daily. As for the young rats group, no additional treatment was given (water as placebo).

2.4 Termination

In the last day of treatment (day-28), all rats were sedated under ketamine and xylazine prior to termination and brain collection procedure. The brains were weighed, put in a sterile container, and preserved in a -20°C container.

2.5 Outcomes

2.5.1 Tissue Homogenate

One hundred milligrams of tissue from each brain was dissolved with 1 ml of 0.01 M phosphate-buffered saline (PBS) with a pH of 7.4 before homogenized. It was then centrifuged at 3500 rpm for 10 minutes. Then, the supernatant was obtained and kept in a -20°C container.

2.5.2 MDA Calculation

Four hundred microliters mixture of water, MDA standard, and supernatant were put into each of two 1.5 mL tubes. Into every tube, 200 μL of trichloroacetic acid (TCA) 20% was added, then vortexed and centrifuged at 5000 rpm for 10 minutes. After the supernatants were transferred to 2 mL tubes, 400 μL of thiobarbituric acid (TBA) 0.67% was added before the tubes were incubated for 10 minutes in a water bath with a temperature of 96-100°C. Following the incubation, the tubes were left out in the air until they reached room temperature before their wave absorbance at 530 nm were measured using a spectrophotometer. The final MDA concentrations were calculated based on the MDA wave absorbance standard curve.

2.6 Statistical Analysis

The data acquired were processed and analyzed through GraphPad Prism ver. 7.00 statistical software. The results are shown as mean ± SEM. Shapiro-Wilk normality test was performed to see if the data came from a Gaussian distribution. Ordinary one-way ANOVA was the chosen parametric test, followed by Tukey's multiple comparisons test with a single pooled variance as the follow-up. The statistical significance was defined as a P value of <0.05.

2.7 Ethical Consent

The study protocol and the usage of rats as experimental subjects was approved by the Health Research Ethical Committee, Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo Hospital in December 2016 with the registration number 1016/UN2.F1/ETIK/2016.

3 RESULTS & DISCUSSION

Of all 27 healthy male rats involved at the beginning of the study, only 21 rats were alive at the time of termination. In different periods, each of the 6 rats appeared sick initially, and then died for unknown reasons.

From our data, brain MDA concentration was found to be lowest in those treated with vitamin E (positive control) with a mean and SEM of 3.12 ± 0.39 nmol/L. In the negative control group consisting of untreated aged rats, the MDA concentration measured 3.78 ± 0.44 nmol/L, closely followed by the young rats at 3.70 ± 0.21 nmol/L as the second highest. With a mean difference of only 0.29 nmol/L with the vitamin E-treated group, CA-treated aged rats had brain MDA concentration of 3.41 ± 0.25 nmol/L (Figure 1).

Although the findings were not statistically significant (P = 0.5683), the decrease of brain MDA seen in CA-treated aged rats from that of untreated aged rats correlates with a similar previous finding (Kumar and Gupta, 2002). That study demonstrated a significant decrease of brain MDA in male Wistar rats treated with 200 and 300 mg/kg of CA whole-plant aqueous extract. However, the research did not provide information about the age of the rats used;
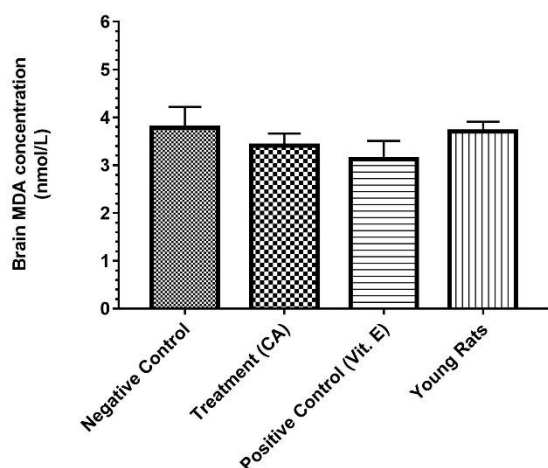
Figure 1: Brain MDA concentration (nmol/L) in different groups of aged male Sprague-Dawley rats and young Sprague-Dawley rats. Data are shown in mean ± SEM (P = 0.5683).

albeit the weight range was stated to be 200-250 g. This was almost half of the aged Sprague-Dawley rats used in current study (333-490 g). This reduction of MDA as a lipid peroxidation marker indicate that there was also a decrease in the lipid peroxidation process itself. This decrease may be due to the electron and H^+ donating capacity of flavonoids present in CA (Subathra, Shila et al., 2005). Furthermore, beside its established role as an oxidative stress indicator, MDA was also known for causing yet another secondary oxidative stress to proteins nearby. In one research which studied the interaction between MDA and bovine serum albumin (BSA), it was found that an oxidative process called protein glycooxidation played the key role. The research hypothesized that this process was one of the main cause of molecular aging (Traverso, Menini et al., 2004). Hence, by decreasing the MDA levels on brain tissue, not only the lipid peroxidation of the PUFAs will be reduced, but also the secondary detrimental damage caused by MDA to proteins will also be prevented.

In lipid peroxidation process of PUFAs, which can be found at large amount in brain tissue, chemical reactions induced by lipid peroxyl radical (LOO⁻) appear to be responsible for aging and other age-dependent diseases (Spiteller, 2007). Compared to other organs in our bodies, brain also has a higher risk for oxidative damage because (1) it requires significant amounts of oxygen per weight (approximately 20% of the total oxygen used in humans) while (2) not highly equipped with antioxidant protective mechanisms. In addition, key

ingredients behind the cause of cell membrane lipid peroxidation, Fe and ascorbate, was found to be at high concentration in brain tissue (Floyd, 1999). This means that CA capability to lower lipid peroxidation process in the aging brain could translate into a potent antioxidant effect in an organ inherently faced with a pro-oxidative state. *Centella asiatica* positive effects on brain aging have been attributed to its two major triterpene saponosides; asiatic and madecassic acides, as well as their heterosides; asiaticoside and madecassoside (Orhan, 2012).

Our data also demonstrate that vitamin E reduced MDA brain concentration in aged rats. This finding was relevant with a proven role of vitamin E as a peroxyl radical scavenger which terminates chain reactions and protecting long-chain PUFAs for important cellular signaling events (Traber and Atkinson, 2007). Nonetheless, one in vivo study showed that supplemental vitamin E given to healthy persons had no effect to the rate of lipid peroxidation (Meagher, Barry et al., 2001). A difference on the marker used on that observation (urinary isoprostane called $iPF_{2\alpha}$-VI and urinary 4-hydroxynonenal) and the fact that it measured whole-body lipid peroxidation instead of a single organ might be the cause of this contradicting finding, among many others.

Unexpectedly, in this current study, one finding raised questions; the resemblance between the brain MDA levels of young rats to that found in the untreated aged rats. Statistically, the comparison between these two groups were proven to be the most insignificant (P = 0.9987). This result was not analogous with a previous study done by Subathra, Shila et al. (2005) which displayed a significantly lower level of MDA in various brain regions of young rats when contrasted to untreated aged rats. Some plausible explanation behind these disparities are the difference in the strain of rat (Wistar vs. Sprague-Dawley), the age range of young rats (3-4 months vs. 2-3 months) and aged rats (>24 months vs. 20-24 months), and the type of CA extract used (whole-plant vs. leaves). A shorter duration of treatment in present study (28 days) could also play a key role in these different findings. Likewise, 6 rats which died in the middle of the study, thus altering the previously optimal number of subjects, might contribute to the change in mean calculations.

4 CONCLUSION

From our results, we concluded that *Centella asiatica* may exhibit an antioxidative effect on aged rats, comparable to that of vitamin E, which was demonstrated by its capacity to reduce malondialdehyde levels in aged brain rats. Despite the insignificance found, the study suggests a potential future role of *Centella asiatica* in hindering aging process, if not preventing it.

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