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THE EFFECTS OF ETHANOL EXTRACTS OF *Centella asiatica* LEAF ON SERIAL SERUM BRAIN DERIVED NEUROTROPHIN FACTOR (BDNF) CONCENTRATION OF RATS (SPRAGUE DAWLEY) FOLLOWING CHRONIC STRESS

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ABSTRACT

Centella asiatica is considered herbal plant for increasing memory performance. Brain-derived neurotrophin factor (BDNF) has a significant role in memory formation process, while stress causes memory impairment. Objective: This study aimed to investigate the effects of ethanol extracts of *Centella asiatica* leaf on serum BDNF concentration of rats that was taken serially before and after chronic electrical stress. Materials and Methods : Twenty male rats (Sprague Dawley) were divided into four groups: control/aquades group and groups treated with different doses (mg/kg) of *Centella asiatica* :150 (CA150), 300 (CA300) and 600 (CA600). Each rat underwent memory exercise for nine days before and after electrical stress and oral administration of ethanol extracts of *Centella asiatica* for twenty-eight days. Blood sampling was taken serially from rats' tail for four times : (1) before memory exercise, (2) after memory exercise (before stress), (3) after chronic stress, and (4) after memory exercise (following chronic stress). Concentration of serum BDNF was assessed using ELISA. Results: There was no significant difference in serum BDNF concentration between groups in first and second serum sampling, which was prior to chronic stress and administration of different treatments. However, there was significant difference in third and fourth serum sampling between groups. Mean concentration of serum BDNF (ng/ml) in third and fourth sampling for control group, CA150, CA300, and CA600, respectively were 1.88+0.21 &1.93+0.24; 2.29+0.13 & 2.01+0.22; 2.29+0.08 &1.86+0.11; 2.71+0.70 and 2.99+0.27 ($p<0.05$). Conclusion: Ethanol extracts of *Centella asiatica* leaf increases serum BDNF concentration in rats after chronic stress.

Key words: stress, memory, *Centella asiatica*, BDNF

INTRODUCTION

Centella asiatica is herbal plant, growing in moist places in Asian countries. *Centella asiatica* is widely used as herbal plants in traditional medicines in many countries in Asia. Some important chemical constituents found in *Centella asiatica* are triterpenoids and flavonoids (Zheng *et al.,* 2007). Some studies highlighted asiatic acid and asiaticocide, which are parts of triterpenoids properties in *Centella asiatica*, that have functions in wound healing, brain stimulating effects, treatments of hypertension and microangiopathy, actions on gastric ulcer, and potent antioxidant and anticancer activity (Pittela *et al.,* 2009, Krishnamurthi e*et al.,* 2010). The effects of *Centella asiatica* to enhance memory performance in rats are affected by the duration of administration (Sari *et al.,* 2011).

Stress may induce structural and functional alterations in the central nervous system and particularly in hippocampus (Venero *et al.,* 2002). Hippocampal function is disturbed by the effect of chronic stress through such mechanisms as neuronal remodeling by dendritic retraction (Sousa *et al.,* 2000), suppression of synaptic activity and plasticity (Morris *et al.,* 2003), and altered neurogenesis (Leuner *et al.,* 2006; Astari *et al.,* 2012). In rats, chronic stress caused an impairment in the performance of spatial memory task in eight-arm radial maze (Leuner *et al.,* 2006; Sari, 2011)**,** Y-maze (Mclaughlin *et al.,* 2009), and also water

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maze (Li *et al.,* 2007, Sari *et al.,* 2012). There are variety of experimental conditions in order to produce chronic stress, including 21 days of predator stress combined with high-fat diet (Baran *et al.,* 2006)**,** six days of activity stress combined with food restriction (Lambert *et al.,* 1998), one month of chronic, unpredictable stress (Sousa *et al.,* 2000)**,** and daily tone-footshock for 21 consecutive days (Trentani *et al.,* 2002).

Activity-dependent changes in synaptic strength are considered mechanisms underlying learning and memory. There are not only the structural changes of neurons in cerebral cortex and hippocampus that affect activity-dependent changes in synaptic strength, and alter learning and memory performance, but also molecular substances (Morris *et al.,* 2003). Many studies suggest that Brain-derived neurotrophic factor (BDNF) has a significant role in the process of learning and memory, such as development of patterned connections, growth and complexity of dendrites in the cerebral cortex (Yamada et al., 2002). BDNF is a member of the neurotrophin family, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and NT-4/5 (Leibrock *et al.,* 1989). Recent experimental evidence supports the role of BDNF in memory processes: memory acquisition and consolidation are associated with an increase in BDNF mRNA expression and the activation of its receptor TrkB (Mizuno *et al.,* 2003). BDNF/TrkB signal transduction pathways may also participate in the process of learning and memory during chronic stress (Li *et al.,* 2007). A study indicates that chronic mild repeated stress results in decrease of BDNF mRNA and protein (Shi *et al.,* 2010).

Therefore, we have designed an investigation to study the neuroprotective effect of herbaceous cognitive enhancer, *Centella asiatica*, on serum BDNF concentration, that is believed to play an important role in learning and memory process, in chronic stressed-rats. Serum BDNF concentration is measured serially in order to assess the biochemical entities of learning and memory following chronic stress and different dose of *Centella asiatica* treatments.

MATERIALS AND METHODS

A. Animals Experimental

Twenty male Sprague Dawley rats (1 month old, 100 – 120 grams) were randomly divided into four groups: aquades (KN), CeA 150 mg/kg (KP1), CeA 300mg/kg (KP2), and CeA 600 mg/kg (KP3). Two animals were placed in the same house with food and water available ad libitum and maintained on a 12-h light:12-h dark cycle. The experiments were approved by Medical and Health Research Ethics Committee Faculty of Medicine Gadjah Mada University.

B. Administration of Ethanol Extracts of *Centella asiatica*

Ethanol extract of Centella asiatica was obtained using maceration methods from *Integrated Testing and Research Institute* of Gadjah Mada University. In order to prepare the various dose-dependent preparations : 150 mg/kg, 300 mg/kg, and 600 mg/kg, ethanol extracts of Centella asiatica was freshly diluted with sterile aquadest. Ethanol extracts of Centella asiatica were administrated orally for 28 consecutive days with weekly weight-adjusted dose.

C. Stress Procedure

After oral administration of *Centella asiatica,* each rat was given electrical stress. The rodent electrical stressor (TW-0313) consisted of a box containing an animal space placed on a grid floor connected to a shock generator. Test rats received one session of electrical stress of total 10 min/day in the rodent electrical stresser in which inescapable footshocks were given [0.8 mA of electrical footshock in intensity and 10 s in duration with 15 s interval). Footshock stress was given chronically for 28 consecutive days.

D. Blood Collection

Blood was collected serially for four times. First blood sampling was collected at the beginning of study. Before receiving any treatment, each rat underwent memory exercise in Morris water maze everyday for nine consecutive days. Second blood sampling was collected an hour after final memory exercise in day nine. In the next day, each rat received oral administration of *Centella asiatica* and electrical stress according to its treatment group for twenty-eight consecutive days. Third blood sampling was collected an hour after final treatment in day twenty-eight. Memory exercise after chronic stress and treatment was performed for nine days. Fourth blood sampling was collected an hour after final memory exercise in day nine.

Blood was collected serially from rats' tail veins. Rat's tail was warmed with water for dilation of the vessels. An assistant held the tail to keep it steady and applied pressure at the base of the tail to further encourage dilation. A 26 gauge needle was used to enter the vein. After blood collection, a sterile gauge was applied to the puncture site with pressure to stop the bleeding. From whole blood sample, serum was made by centrifugation 1500 rpm, 4 ^{0}C, 10 minutes. Serum was kept in -800C until measurement of BDNF concentration.

E. Measurement of Serum BDNF Concentrations

Serum concentration of BDNF was assessed using Rat BDNF ELISA Kit (Boster Immunoleader, Cat. EK0308).

F. Statistic

Results were expressed as mean ± SE. Differences between groups were analyzed by ANOVA and t-Test using the SPSS software. Difference between groups were considered statistically significant at a P value <0.05.

RESULTS AND DISCUSSION

Mean serum BDNF concentration in first, second, third, and fourth blood collection for all treatment groups are shown in Table 1. The data show that there was no significant difference in the first blood collection between groups. It means all rats had similar baseline in serum BDNF concentration prior to treatments. In the second blood collection, which was collected after the first memory exercise, there was no significant difference in serum BDNF concentration between groups. This result shows similar response to memory exercise in all groups before different treatments were given, in which mean serum BDNF level of all four groups was higher after the first memory exercise.

There was significant difference between groups in serum BDNF concentration in third and fourth blood collection (Figure 1). In third blood collection, which was collected right after chronic electrical stress and different treatments to the groups, all treatment groups (KP1, KP2, and KP3) had significant higher serum BDNF level compared to control group (KN). While in fourth blood collection, which was collected after final memory exercise, only KP3 had significant higher serum BDNF level compared to the other groups.

Table 1. Mean Serum BDNF Concentration in Serial Blood Collection

	Mean Serum BDNF Concentration (ng/ml)			
	1	2	3	4
KN (Aquades)	1.928 ± 0.258	2.446 ± 0.127	1.88±0.21	1.93±0.24
KP1 (CeA 150 mg/kg)	1.892 ± 0.205	2.056 ± 0.311	2.29±0.13*	2.01±0.22
KP2 (CeA 300 mg/kg)	1.718 ± 0.098	2.156 ± 0.238	2.29±0.08*	1.86±0.11
KP3 (CeA 600 mg/kg)	1.514 ± 0.159	2.102 ± 0.25	2.71±0.70**	2.99±0.27#

* P < 0.05 vs KN group. **P < 0.01 vs KN group. # P < 0.01 vs KN, KP2, and KP3 groups.

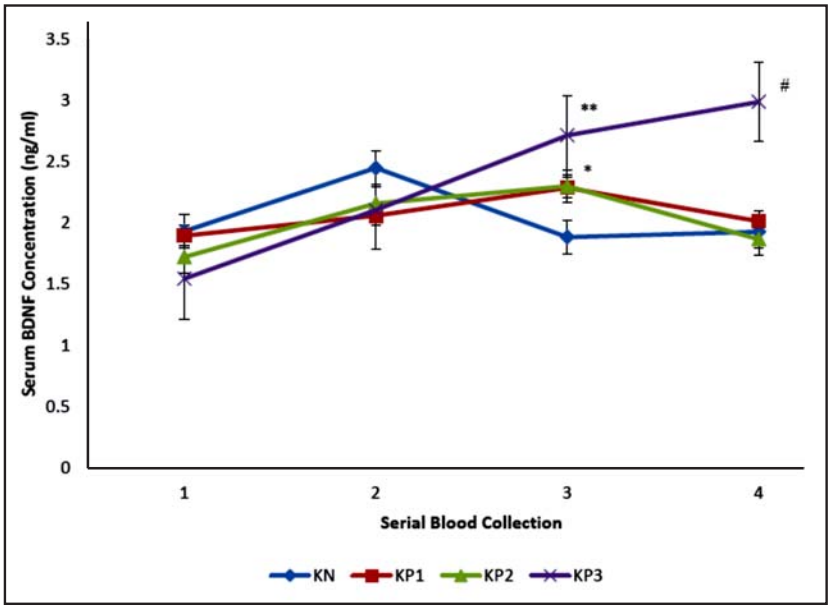

Figure 1. Serum BDNF level in serial blood collection. * P < 0.05 vs KN group. **P < 0.01 vs KN group. # P < 0.01 vs KN, KP2, and KP3 groups.

To our knowledge, this is the first study that reports serum BDNF level in serial blood collection following pre-stress memory exercise, oral treatment of *Centella asiatica* and chronic stress, and post-stress memory exercise. We used serum to measure BDNF concentration using ELISA method because according to previous studies serum BDNF is not affected by the handling of blood sample, its absorbancies are identical with those from whole blood to determine BDNF, and its storage is more stable that has been postulated for more than 12 months at -20 ^{0}C (Elfving *et al.,* 2009). In this study, the samples were stored at -80 ^{0}C for less than 3 months, and therefore our samples are not likely to be affected from the storage conditions. Another study suggests that measurement of blood and plasma BDNF levels using ELISA method correlated with brain-tissue BDNF levels, including hippocampus and prefrontal cortex, across species (Klein *et al.,* 2010). Therefore, we measured BDNF level in serum in order to discover its level in hippocampus.

Memory exercise in this study was conducted using Morris water maze which requires the rats to swim in order to accomplish the task. This task in Morris water maze was not only for memory test purpose, but also for regular physical exercise (Terry, 2009). We found there were increasing serum BDNF level of all groups in second blood collection, which was collected after the first exercise in Morris water maze (Figure 1).

It is known that regular physical exercise also has beneficial effects for brain function, such as better memory performance (Radak, 2001), decreasing oxidative damage (Radak, 2001), enhancing capillarization for hippocampal neurogenesis (Fabel, 2001), and also increasing neurotrophin production (Radak, 2006). Unfortunately, the beneficial effects of training are proven reversibly in the brain, since detraining down regulates neurotrophin level and memory performance (Radak, 2006).

In this study, we proposed role of *Centella asiatica* in inducing BDNF concentration after chronic electrical stress. We found that *Centella asiatica* significantly increased serum BDNF concentration in all *Centella asiatica* treatment groups after chronic stress compared to control group. We also found that *Centella asiatica* significantly increased serum BDNF concentration after memory exercise following chronic stress only in KP3 group. Based on this study, we concluded that the effect of *Centella asiatica* is increasing BDNF level after stress but maintenance in increasing BDNF level is dose dependent.

BDNF and its receptor TrkB play important role during stress injury. Reduction of BDNF mRNA and protein expression were observed in the CA3 and the dentate gyrus of the hippocampus after repeated immobilization stress (Givalois *et al.,* 2001), together with increased expression of TrkB mRNA (Nibuya *et al.,* 1999), may play a role in neuronal plasticity process as an compensatory adaptation to the stress response. On the contrary, after a series of acute injuries, such as cerebral ischemia, epilepsy, or cerebral trauma, the up regulation of BDNF mRNA, as well as the augmentation of TrkB mRNA were observed in cerebral cortex and hippocampus (Rage*et al.,* 2002).

Multiple chronic stresses change the protein expression and its gene regulation, as well as the post-synaptic density (PSD) composition in the hippocampus neuron (Sun *et al.,* 2006, Liu *et al.,* 2004). The main component of PSD is Fyn that is the molecular basis of learning and memory by its participation in synaptic plasticity (Kojima *et al.,* 2007). BDNF/TrkB signaling and NMDA receptors are very important for spatial memory formation. Fyn may play a key role in this interaction by linking TrkB with NR2 (Mizuno *et al.,* 2003). Other study by same group also demonstrated contribution of BDNF inducing TrkB/Phosphatidil Inositol 3-Kinase (PI3-K) signaling pathway is critical for spatial learning in the radial arm maze (Mizuno *et al.,* 2003). The TrkB signaling downstream effectors involved in BDNF/TrkB signaling pathway, such as such as p-Akt, p-GSK-3², and p-mTOR in the hippocampus of rats, are also subjected to change following immobilization stress (Fang *et al.,* 2013).

Centella asiatica was found to be able to increase serum BDNF level and maintain its increasing level at certain dose. An active component of *Centella asiatica* that has known as a cognitive enhancer is asiaticoside. Asiaticoside is also reported function as a dementia-treating agent (Kumar *et al.,*2011). In the previous studies using *Centella asiatica* as substance to ameliorate cognitive and memory function, the data was based on brain morphological improvement after treatment, such as enhancement of neuronal dendrites in growth

spurt rats (Mohandas *et al.,*, 2006; Mohandas *et al.,* 2009), the thickness of pyramidal layer in CA1 hippocampus (Sari e*et al.,* 2012), and alteration of amyloid ² pathology in the brain of Alzheimer's disease animal models (Dhanasekaran *et al.,* 2009). *Centella asiatica* was also proven to modulate components of the oxidative stress response in neurodegenerative mice (Kumar *et al.,* 2011). In this study, we add the biochemical evidence of *Centella asiatica* that is able to increase serum BDNF level in chronic stressed-rats that may support the amelioration in memory function.

CONCLUSION

Centella asiatica ethanol extract treatment increases and maintains serum BDNF concentration after chronic stress.

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