DOI 10.7603/s40790-014-0009-0

GSTF International Journal of Psychology (JPsych) Vol.1 No.1, March 2014

Ethanol Extracts of *Centella asiatica* Leaf Improves Memory Performance in Rats after Chronic Stress via Reducing Nitric Oxide and Increasing Brain-Derived Neurotrophic Factor (BDNF) Concentration

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Received 31 Dec 2013 Accepted 10 Feb 2014

Abstract*—*Background*: *Centella asiatica* is herbaceous plant that has medicinal value to improve learning and memory. Brain-derived neurotrophic factor (BDNF) has a significant role in memory formation process, while oxidative stress causes memory impairment. *Objective*: This study aimed to investigate the effects of ethanol extracts of *Centella asiatica* leaf on memory performance and serum BDNF (neurotrophins) and Nitric Oxide(NO) concentration of rats following chronic electrical stress. *Materials and Methods* : Twenty male rats (Sprague Dawley) were divided into four groups: control/aquadest group and groups treated with three different doses (mg/kg) of *Centella asiatica* :150 (CeA150), 300 (CeA300) and 600 (CeA600). Memory performance was tested using probe test in Morris Water Maze before and after oral administration of ethanol extracts of *Centella asiatica* leaf followed by electrical stress for 28 days. At the last day of memory exercise following chronic stress, blood sample was taken from periorbital veins of all rats. Concentration of serum BDNF and total NO was assessed using ELISA. Data were analyzed using one way ANOVA. *Results*: Treatment groups had higher percentage in probe test water maze and serum BDNF concentration after chronic stress. Mean time percentage (%) of probe test in water maze performance for control group, CeA150, CeA300, and CeA600 were 53.33±2.43, 55.78±4.16, 63.33±4.07, and 73.55±3.29 ($p<0.05$). Mean concentration of serum BDNF (ng/ml) for control group, CeA150, CeA300, and CeA600 were 1.52±0.89, 1.49±0.20, 1.73±0.15, and 2.16±0.20 ($p<0.05$ vs control group). Control group had higher serum NO concentration compared to all treatments groups. Mean concentration of serum NO (µmol/L) for control group, CeA150, CeA300, and CeA600 were 5.94±0.52, 3.98±0.35, 3.06±0.33, and 2.82±0.21 ($p<0.05$). *Conclusion*: Ethanol extracts of *Centella asiatica* leaf increases memory performance and serum BDNF concentration, and also decreases Nitric Oxide levels in rats after chronic stress.

Keywords- learning and memory; brain-derived neurotrophic factor; nitric oxide; Centella asiatica.

I. INTRODUCTION

Chronic stress is known to impair memory performance. In rats, chronic stress caused an impairment in the performance of spatial memory task in eight-arm radial maze[1,2,3,4,5], Y-maze[6], and also water maze[7,8,9]. There are variety of experimental conditions in order to produce chronic stress, including 21 days of predator stress combined with high-fat diet[10], six days of activity stress combined with food restriction[11], one month of chronic, unpredictable stress[12], and daily tone-footshock for 21 consecutive days[13].

Stress may induce structural and functional alterations in the central nervous system and particularly in hippocampus [14]. Hippocampal function is disturbed by the effect of chronic stress through such mechanisms as neuronal remodeling by dendritic retraction [8,12,15], suppression of synaptic activity and plasticity[16,17,18], and altered neurogenesis[19,20,21].

Activity-dependent changes in synaptic strength are considered mechanisms underlying learning and memory. It is not only the structural changes of neurons in cerebral cortex, particularly in hippocampus, that alter learning and memory performance, but molecular substances that affect activity-dependent changes in synaptic strength also play important roles[18]. 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 [22]. BDNF is a member of the neurotrophin family, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and NT-4/5[23]. In addition to its neurotrophic effects, neuronal plasticity [22] and its regulation are the primary functions of BDNF [24].

BDNF plays an important role not only in the formation, but also in the retention and/or recall, of spatial memory [25]. 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 [26]. Genetic as well as pharmacologic deprivation of BDNF or TrkB impairs learning and memory [22,24,27]. Activation of TrkB/PI3-K and protein

Published online: 19 May 2014

synthesis signaling pathway by BDNF in the hippocampus is important for spatial memory [28]. BDNF/TrkB signal transduction pathways may also participate in the process of learning and memory during chronic stress [7].

Another pathway believed to play significant roles in learning and memory is the nitric oxide pathway. Nitric oxide is synthesized by the action of three isoforms of the enzyme nitric oxide synthase (NOS) from the amino acid L-arginine in the presence of many cofactors [29]. A number of reports have demonstrated that NO possesses potent anti-inflammatory properties, where as an equally impressive number of studies suggest that NO may promote inflammation-induced cell and tissue dysfunction. Indirect effects of NO are those reactions mediated by NO-derived intermediates such as reactive nitrogen oxide species derived from the reaction of NO with oxygen or superoxide and a reproduced when fluxes of NO are enhanced [30]. Nitric oxide has been implicated in a number of functions such as apoptosis of neuronal cells[31], memory and learning [32,33], regulation of the cerebrovascular system[34], certain pathologies of neuropsychiatric disorders such as schizophrenia and major depression[29,35] stress-related diseases [36], Alzheimer's disease[37], neurodegeneration [38], cerebral ischemia, stroke, neuroinflammation [39] and also memory impairment and oxidative stress in hypoxia-induced rats[40,41]. Nitric oxide is a very labile molecule and is oxidized to nitrite and then nitrate within a few seconds where it is produced. Therefore, the stable oxidation end products of NO, i.e. nitrite (NO^{2-}) and nitrate (NO^{3-}), can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of NO production [29].

Centella asiatica is a creeping herbal plants, 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 [42]. 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 [42,43,44]. Studies showed administration of *Centella asiatica* extracts might improve learning and memory function by the enhancing neuronal dendrites in growth spurt rats[45,46] and altering amyloid β pathology in the brains and modulating components of the oxidative stress responses in neurodegenerative mice[47,48,49]. These recent studies showed the protective effect of *Centella asiatica* against behavioral, biochemical and mitochondrial dysfunction in rodents [50].

II. MATERIALS AND METHODS

A. Animals

Twenty male Sprague Dawley rats (1 month old, 100–120 grams) were randomly divided into four groups: rats that are treated with aquades (Control group), *Centella asiatica* 150 mg/kg (CeA150), *Centella asiatica* 300mg/kg (CeA300), and *Centella asiatica* 600 mg/kg (CeA600). 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 seconds in duration with 15 seconds interval). Footshock stress was given chronically for 28 consecutive days.

D. Memory Test

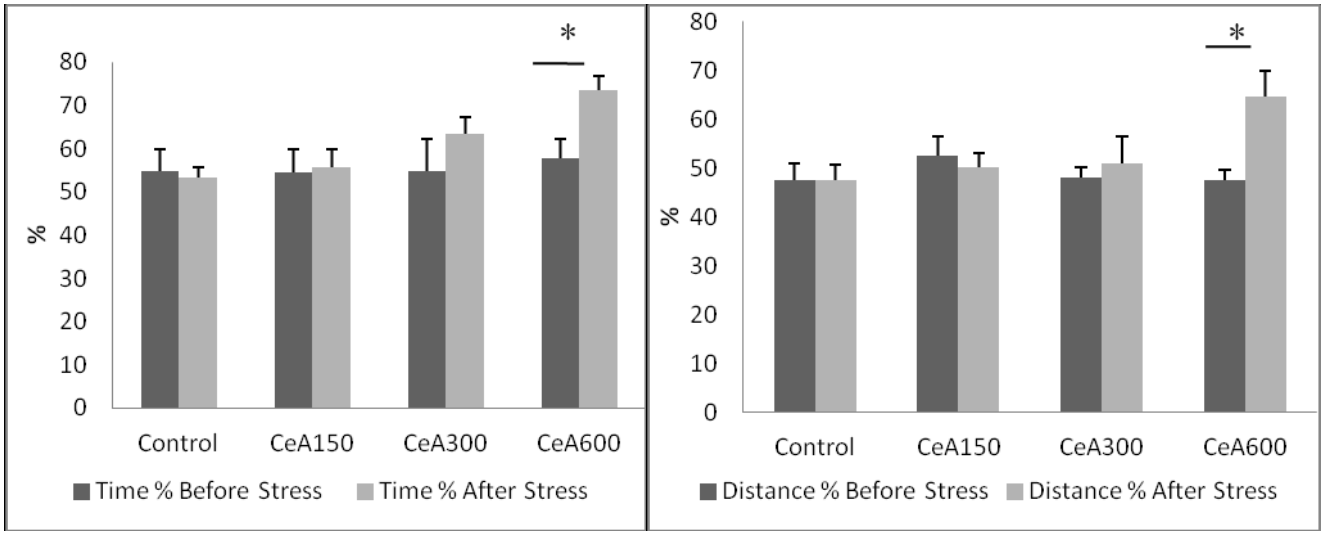
Memory was assessed using Probe Test in Morris Water Maze (circle pool with 1.8 meter in diameter and 0.4 meter in height). Water maze was conducted nine days before and after 28-days treatments, the first eight days for finding hidden platform exercise and one last day for probe test. On day nine (24 hr following the last hidden platform trial), a probe trial was conducted in which the platform was removed from the pool. This was accomplished by measuring the percentage of time spent in target quadrant compared to total time of ninety seconds and distance swam in target quadrant compared to total distance swam for each trial. These assessments provided a second estimate of the strength and accuracy of the memory of the previous platform location [51].

E. Measurement of Serum BDNF and NO Concentrations

An hour after final probe test, blood sample was collected from rats' periorbital veins. From whole blood sample, serum was made by centrifugation 1500 rpm, 4^0C, 10 minutes. Serum concentration of BDNF was assessed using Rat BDNF ELISA Kit (BosterImmunoleader, Cat. EK0308). Serum concentration of NO was assessed using Total NO/Nitrite/Nitrate Assay (Parameter™, Cat. KGE001, SKGE001, PKGE00).

F. Statistical Analysis

Result were expressed as mean ± SD. 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.



(a) (b)

Figure 1. Comparison of probe test in Morris water maze before and after chronic electrical stress : (a) Mean percentage of time spent in target quadrant, (b) Mean percentage of distance swam in target quadrant. The performance of each rat was tested 24 hours after the final training day in a probe trial (90sec) during which the platform was removed.*p<0.05.

III. RESULTS

A. Memory Test

The result of probe trial in Morris Water Maze before and after chronic electrical stress for mean percentage of time spent in target quadrant (%) for Control, CeA150, CeA 300, CeA600 respectively were 54.89±5.01 and 53.33±2.43, 54.44±5.5 and 55.78±4.16, 54.89±6.6 and 63.33±4.07, 57.78±4.29 and 73.55±3.29 ($p<0.05$)(Figure 1(a)). The assessment of memory test from probe trial was not only obtained from mean percentage of time spent, but also from mean percentage of distance swam in target quadrant. Mean percentage of distance swam in target quadrant (%) before and after chronic electrical stress for Control, CeA150, CeA300, and CeA600 respectively were 47.65±3.33 and 47.49±3.21, 52.63±2.98 and 50.06±3.79, 48.09±5.5 and 50.92±4.16, 47.63±5.26 and 64.75±2.64 ($p<0.05$) (Fig 1(b)).

B. Concentration of Serum BDNF and NO

Mean concentration of serum BDNF (ng/ml) after chronic electrical stress for Control, CeA150, CeA300, and CeA600 were 1.52±0.89, 1.49±0.20, 1.73±0.15, and 2.16±0.20 ($p<0.05$vsControl group) (Fig 2). Mean concentration of serum NO (µmol/L) after chronic electrical stress for Control, CeA150, CeA300, and CeA600 were 5.94±0.52, 3.98±0.35, 3.06±0.33, and 2.82±0.21 ($p<0.05$) (Fig 3).

IV. DISCUSSION

Our study showed that *Centella asiatica* extract treatment ameliorated memory performance after chronic stress. This amelioration is dose dependent mechanism as shown by the significant difference in the probe test in CeA600 group, but not in CeA150 and CeA300 (Fig 1). An active component of *Centella asiatica* that has been known as a cognitive enhancer is asiaticoside. Asiaticoside has been used as a dementia-treating agent [50]. We hypothesized that the active components of *Centella asiatica* modulate oxidative stress, especially NO production and BDNF concentration.

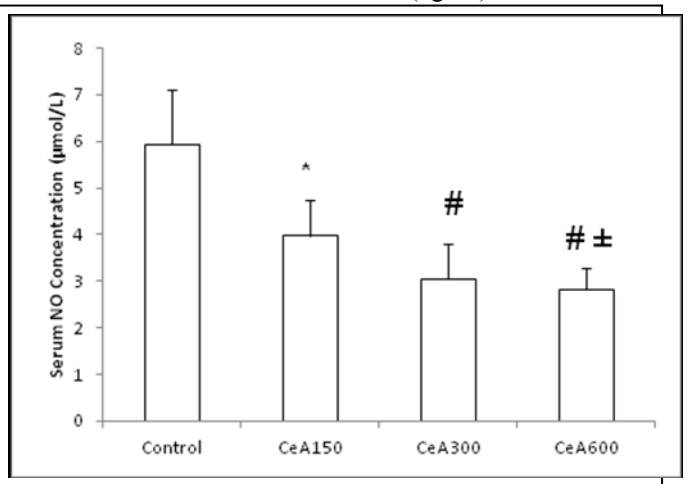


Figure 2. Serum NO concentration measured after the final probe trial following chronic electrical stress in all treatments groups. * p<0.05 vs Control group, # p<0.01 vs Control group, ± p<0.05 vs CeA150 group.

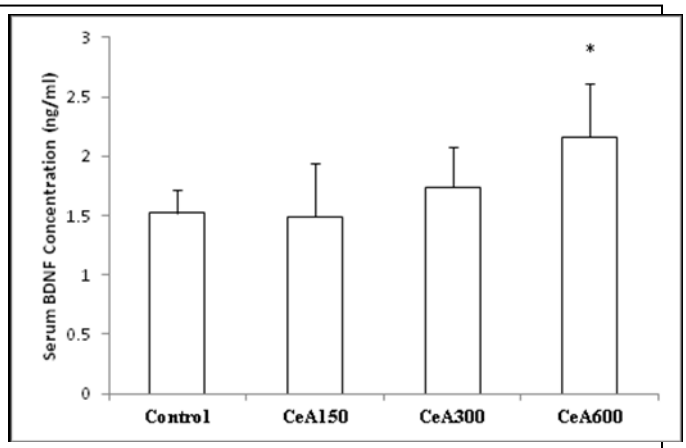


Figure 3. Serum BDNF concentration measured after the final probe trial following chronic electrical stress in all treatments groups. * p<0.05 vs Control group

CeA600 group had the lowest NO concentration (Fig 3) and the best memory performance (Fig.1&2). We found also significant NO concentration reduction in CeA150 and CeA300 groups. However, we didn't find a significant difference between NO concentration in CeA150 and CeA300 groups. The mechanism of NO concentration in inducing memory performance is still controversy. It has been reported that hippocampal response to stress is influenced by NO level [51]. NO has significant function in long term potentiation (LTP), a putative cellular mechanism of memory formation in hippocampus [52]. Level of NO, the location of NO production, the extent of oxidative stress and the type of neurodegenerative process influence the role of NO, weather leading to toxicity or neuroprotection [53]. NO acts as an antioxidant and protects against cellular injury due to H_2O_2 and alkylhydroperoxides [54,55]. It is suggested that the NO production is a physiological response to injury[56]. Meanwhile, high production of NO following the induction of iNOS expression, can interact with superoxide anion generated by the mitochondria or by others mechanisms, leading to the formation of the powerful oxidant species peroxynitrite. Peroxynitrite induces cell damage and altered neuronal physiological function [54].

Although there is controversy, we propose that the protective effect of *Centella asiatica* may be mediated by reduction of NO production. Nitric Oxide could induce apoptotic cell death in neuronal cells leading to injury. Although the mechanism were still unclear, some pathways, such as the interaction with excitatory amino acid receptors [56], the depletion of cellular NAD^+ [57] and the activation of caspases [58] were involved in the cascade of events leading to NO-induced apoptosis. *Centella asiatica* also had been reported to prevent memory deficit in senescence mice. *Centella asiatica* improved behavioral alteration, reduced oxidative damage and mitochondrial ROS formation[50]. Synthesize of NO is determined by the action of three isoforms of the enzyme nitric oxide synthase (NOS) from the amino acid L-arginine in the presence of many cofactors [59]. Both neuronal NOS (nNOS) and inducible NOS (iNOS) had been reported to generate both NO and O^{2-}, then led to production of $ONOO^-$ and caused cellular injury *in vitro* [60]. Moreover, a study by Zhou et al. suggested that nNOS over-expression in the hippocampus is essential for chronic stress-induced depression due to hippocampal neurogenesis suppression [61]. An increase of NO due to up-regulation of iNOS also interrupts the memory consolidation by altering the cholinergic functions during hypoxia[40]. In this study, we suggested that *Centella asiatica* extract treatment reduced NO concentration, although we still need further investigation to elucidate the role of enzymes that contribute to this reduction.

Next, we quantified BDNF concentration in this study and proposed role of *Centella asiatica* in inducing BDNF concentration. We found *Centella asiatica* significantly increased the BDNF serum concentration only in CeA600 group (Fig 3). An increase of BDNF concentration in CeA600 associated with better memory performance in CEA600 group. Based on this result, we concluded that effect of *Centella asiatica* in BDNF concentration was a dose dependent mechanism. BDNF has been reported to contribute in learning and memory [7, 28]. Multiple chronic stresses alter learning and memory performance of rats, change the post-synaptic density (PSD) protein expression, gene regulation and composition in the hippocampus neurons[62,63]. The main component of PSD is Fyn that is the molecular basis of learning and memory by its participation in synaptic plasticity [64]. The increases of Fyn, BDNF, TrkB protein expression and Fyn mRNA levels may participate in the enhancement of learning and memory induced by chronic multiple stress [7]. Previous studies reported that the interaction between BDNF/TrkB signaling and NMDA receptors is very important for spatial memory formation and Fyn may play a key role in this interaction by linking TrkB with NR2B [26]. 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 [28]. We demonstrated that amelioration of memory performance after *Centella asiatica* treatment also induced BDNF elevation.

Further exploration about downstream signaling of BDNF is needed for completing this study. It is necessary also to know the localization of BDNF and its receptors in the brain in this model, so we can conclude what cells that may express BDNF. Next, immunostaining may be needed for further exploration and some culture study using cells that express BDNF also useful to observe potential role of *Centella asiatica* in regenerative medicine. We also think to use newborn rat to reduce the fault from the experiment.

In conclusion, *Centella asiatica* extract treatment increases memory performance of rat after chronic stress due to elevation of BDNF concentration and reduction of NO production.

ACKNOWLEDGMENT

We thank to Jajah Fachiroh, S.P.,M.Si.,Ph.D and Dewi Sulistyawati, Amd.AK for professional technical assistants and Mr. Mulyono for technical assistant of animal care. This study is funded by "Annual Medical Faculty Gadjah Mada University Research Grant" for BDNF concentration measurement.

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