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Effect of Gotu Kola (*Centella Asiatica*) Extract Toward Expression of Caspase 3 of Hippocampus Pyramidal Cells on Dementia Model Rats Induced by Trimethyltin

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Abstract*—Hippocampus is part of brain that has an important role in the process of memory. Hippocampus is very sensitive to oxidative stress. Oxidative stress in the hippocampus can cause apoptosis of neurons associated with the pathogenesis of dementia. Gotu kola (Centella asiatica) has antioxidants effect that can prevent apoptotic cells. The aim of the study was to find out the effects of gotu kola extracts toward expression of caspase 3 in CA1 and CA2-CA3 regions of hippocampus on trimethyltin (TMT)-induced dementia rats. The rats were divided randomly into six groups consisting of 5 rats of each.The normal group was given oral CMC-Na solution and injection of 0.9% saline intraperitonealy (ip); the negative control group was given oral CMC-Na solution and TMT dissolved in 0.9% saline (ip); the positif control group was given oral 200 mg/kg body weight (bw) of citicoline solution and TMT; and the extract groups were treated with oral 100, 200, and 300 mg/kg bw, respectively, of gotu kola extract and TMT. The extract and citicoline solutions were given at day 1 up to day 35 of experiment, whereas the TMT chloride was injected as a single dose of 8 mg/kg bw at day 8 of experiment. On the 36 th day the rats were sacrificed. The hippocampus was taken to observe caspase 3 expression in CA1 and CA2-CA3 regions with immunohistochemical staining. The data of a percentage of the number of cells expressing caspase 3 in CA1 and CA2- CA3 regions were statistically analyzed using one-way anova continued by Tukey-HSD test at significant level 0.05. The injection of TMT could signifcantly increase caspase 3 expression in the CA1 and CA2-CA3 regions of hippocampus. Administration of gotu cola extract dose 100, 200, and 400 mg /kg bw could reduce caspase 3 expression and significantly different compare to negative control. Gotu kola extract may have antiapoptotic effect on the hippocampal pyramidal cells of the dementia model rats induced by trimethyltin.

Keywords—hippocampus, caspase 3, gotu kola (centella asiatica), dementia, TMT

I. INTRODUCTION

Dementia is a neurological syndrome with a large impact on health and quality of life [1]. The hippocampus is the main tissue involved in the pathophysiology of dementia [2]. Hippocampus is one of the components in the brain that has an important role in the process of memory [3] that is very sensitive to oxidative stress [4]. The increased oxidative stress in the hippocampus causes degeneration of neuron asassociated with the pathogenesis of dementia [5]. Oxidative stress will induce protein damage related to apoptotc process which will result in cell death [6]. Increased oxidative stress activates the permeability transition in the inner membrane of the mitochondria, causing the death of neurons that induce apoptosis [7]. Previous research showed that the mechanism of apoptosis is mediated by caspase 3 activity. Caspase 3 is activated by the release of cytochrome c in the cytosol in mitochondria [8]. Caspase 3 has important role as executors of cell [9].

Gotu kola (Centella asiatica) contains triterpenoid saponin glycosides such as centella saponin, asiaticoside, madecassoside, asiatic acid and madecassic acid [10]. The most prominent bioactive compounds in gotu kola is asiaticoside which have antioxidative and neuroprotective effects [11]. Antioxidants have a protective effect against oxidative stress by neutralizing free radicals [12]. Antioxidants stop the chain reaction to free radicals by eliminating free radical intermediates through the release of 1 hydrogen atom and the release of 1 electron so that free radicals become stable and not reactive [13]. The neuroprotective effect of Centella asiatica can protect mitochondria from oxidative stress so that it can inhibit the executing caspase activity in apoptosis [14]. This study aims to determine the effect of gotu kola extract on caspase 3 expression in pyramidal cells in the hippocampus.

II. MATERIAL AND METHOD

A. Animals

A total of 30 adult Sprague Dawley rats (180–200 g) obtained from the animal house of BPPOM, Jakarta, Indonesia, were used in the study. The rats were placed individually in cages under standard conditions (24–26 °C, 55–65 % humidity, natural 12/12 hour light/dark cycles) with ad libitum access to food and water. The rats were acclimatized for 6 days before treatment administration. The experimental protocol and animal handling procedures were approved by the Ethics Committee of Universitas Ahmad Dahlan (approval number 011804050).

B. Extraction of Gotu Cola

Gotu kola powder was obtained from CV Merapi Farma, Yogyakarta, Indonesia. Five hundreds grams of powder was weighed and put into an electric stirred macerator. Powder was macerated twice with 2.5 L of 70% ethanol (Sigma-Aldrich, Inc., St. Louis, USA) for 24 hours. The maserate was then filtered with a Buchner funnel until separated from the pulp. The resulting filtrate was concentrated under reduced pressure at 40 °C in a rotary evaporator (Heidolph, Germany). The extract was eventually dissolved in the sodium carboxymethyl cellulose (CMC-Na) solution prior to oral administration to the rats.

C. Experiment Design

The treatment was carried out for 35 days. All groups except the normal group were injected by TMT solution at dose 8 mg / kg bw on 8th day of treatment. The normal group (Normal) was given an oral solution of CMC-Na 1% (p.o) and intra-peritonial (i.p) injection of 0.9%. NaCl. The negative group (TMT) was given a CMC-Na solution (p.o). The positive group (Citicoline) was given a citicoline dose of 200 mg / kg bw (p.o). The extract groups were given gotu cola extract dose of 100, 200, and 400 mg / kg bw (p.o), respectively. On the 36th day the rats were sacrificed using CO2 gas inhalation and the brain was taken for imunohistochemical observations.

D. Caspase 3 Immunohistochemical Staining

The right cerebral hemispherium of brain was separated and put into a tissue pot containing 10% formalin in phospat buffered saline (PBS) for 6 days. The hippocampus was then carefully separated and embedded in paraffin blocks as standard procedur in Laboratory of Pathology Anatomy, Universitas Gadjah Mada, Indonesia. The paraffin blocks were sectioned using microtome (Leica) at a thickness of 3 μm.

Each hippocampus was represented by three sections, which was taken from the middle part of any given hippocampus. Sections of hippocampus were placed on poly-L-lysine slides in order to prepare for immunohistochemical staining. The immunohistochemical staining of caspase 3 was conducted according to manufacturer's procedure (Starr Trek Universal Detection System Protocol, Biocare Medical, Concord, California) in Laboratory of Pathology Anatomy, Sardjito Hospital, Indonesia, using primary antibody (Rabbit Polyclonal Anti-Caspase 3 Antibody, Lab Vision Co., USA). A section with no active caspase 3 expression was also prepared to serve as a negative control.

The percentage of cells expressing caspase-3 in the cytoplasm or nuclei or the both was determined by counting the total number of apoptotic cells in cornu ammonis area of 3 slices of hippocampus (x400 magnification), per the total number of cells in the same area multiplied with 100%.

The percentage of apoptotic cells expressing caspase 3 were statistically analyzed using a one-way analyze of variance (Anova). The differences between groups were analyzed using a post-hoc Tukey HSD test. The significance level was set at $p < 0.05$.

III. Results and Discussion

Protection of hippocampus cells from degeneration is a useful preventive strategy for dementia. The prevention of dementia usually includes compounds that can prevent oxidative damage to neurons. Gotu cola has antioxidative compound that potentially prevent the cell death through antiapoptotic mechanism. The purpose of the present study was to observe the effect of gotu cola extract toward the expression of caspase 3 of hippocampus pyramidal cells. Caspases are fundamental factors of the mammalian apoptotic mechanism and caspase-3 is the main executioner of apoptosis.

The result of the study reveals that the protein caspases-3 was stained mainly in the cytoplasms of pyramidal cells in the both CA1 and CA2-CA3 regions (Figure 1 and 3). One way Anova of the data percentage of pyramidal cells expressing caspase-3 per three slices of hippocampus shows that a significant main effect ($p<0.05$). Tukey HSD test of these data showed that the percentage of the CA1 and CA2 CA3 pyramidal cells expressing protein caspase 3 in the TMT-treated group were significantly higher than in the normal group ($p<0.05$). The administration of gotu kola extract at dose 100, 200 and 300 mg/kg bw could decrease the percentage of the CA1 and CA2-CA3 pyramidal cells expressing protein caspase 3. There were no significant difference among the extract groups. The administration of citicolin could also prevent the expression of caspase 3 in the both of CA1 and CA2-CA3 cells. The results of statistic anaysis of the data are shown in Figure 2 and 4.

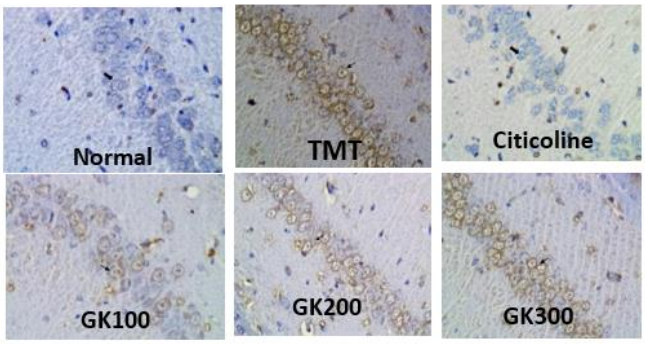

Fig. 1. Microscopic feature of caspase 3 immunohistochemical staining in the CA1 hippocampus pyramidal cells (400x magnification).on TMT-induced dementia models rat. The caspase 3 protein was expressed as brown spot in the cytoplasm cell (thick black arrow). Normal cells are showed as blue cells.(thin black arrow).

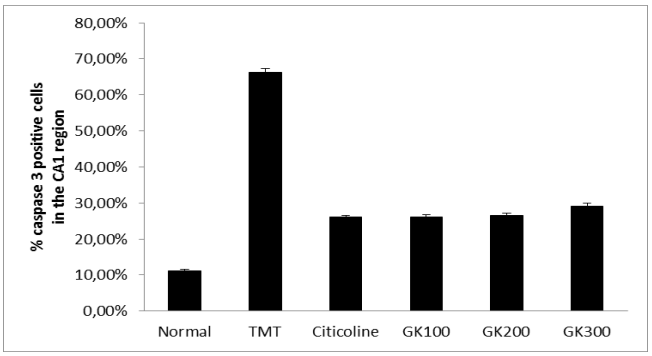

Fig. 2. Mean ± SEM of the percentage of CA1 cells expressing caspase 3 in normal, TMT injected, gotu kola extract and citicoline-administered rats. Results of one-way ANOVA: n = 5; * p <0.05 significantly different compared to the TMT group.

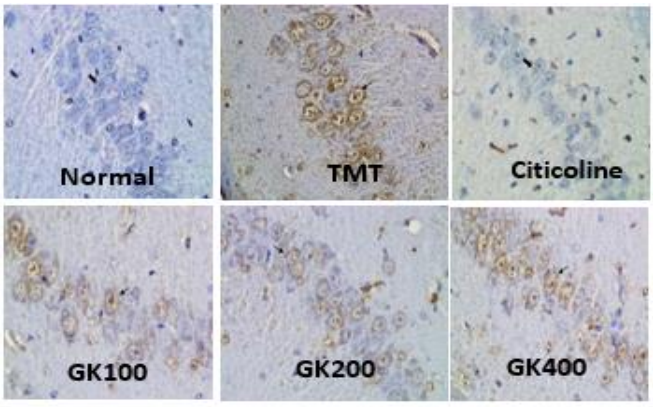

Fig. 3. Microscopic feature of caspase 3 immunohistochemical staining in the CA2-CA3 hippocampus pyramidal cells (400x magnification) on TMT-induced dementia models rat. The caspase 3 protein was expressed as brown spot in the cytoplasm cell (thick black arrow). Normal cells are shown with thick black arrow (thin black arrow).

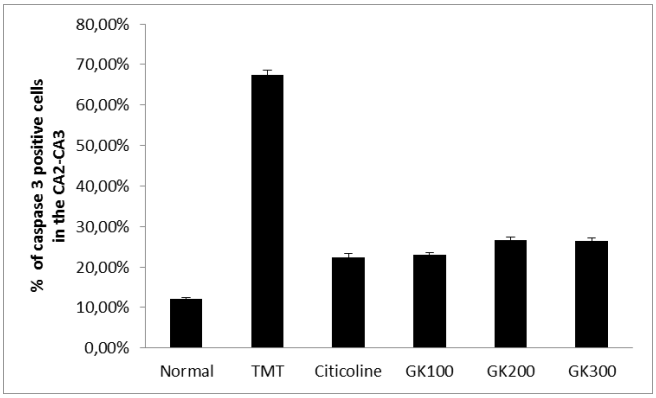

Fig. 4. Mean ± SEM of the percentage of CA2CA3 cells expressing caspase 3 in normal, TMT injected, gotu kola extract and citicoline-administered rats. Results of one-way ANOVA: n = 5; * p <0.05 significantly different compared to the TMT group.

In this study showed that the TMT injection caused the increase of caspase 3 expression in the CA1 and CA2-CA3 regions Previous research proved that injection of TMT causes degeneration in pyramidal cells in CA1 and CA2-CA3 regions [15]. These results are also in line with Yuliani's (2015) study which exhibited that TMT increases caspase 3 expression in pyramidal cells. TMT is a strong neurotoxic compound that selectively induces neuronal death in the hippocampus [16]. The precise mechanism of TMT effects are not entirely clear however. In general the toxicity of organotin compounds related to the amount and length of alkyl groups attached to the tin and has a high specificity in neurons [17]. Previous in vitro and in vivo studies reported that TMT-mediated neuron damage was induced by derivatives of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [18]. Dimethyltin, result of demethylation of TMT, will bind directly to protein called stannin (SNN). SNN is an 88 amino acid protein encoded by cDNA of TMT-sensitive cells. It may be localised in the outer mitochondrial membran and endoplasmatic reticulum membrane. SNN interacts with regulatory proteins involved in the promotion of apoptosis and activates caspase-3 as an effector caspase for neuronal damage in the hippocampus. Selective lesions in the hippocampus have been reported to damage the recognition memory performance in humans, monkey and rodent.

Gotu kola extract has a pro-cognitive effect on humans and rodents [19], and can improve memory of mental retardation in children. Gotu kola also shows that it can prevent memory deficits due to streptozotocin induction and protect cholinergic neurons from the toxic effects of aluminum [20]. Administration of gotu kola can also reduce the production of carbonyl proteins in the brains of old mice [21] so that it is capable to prevent oxidative stress which trigger dementia. In the present study the administration of gotu kola extract can reduce caspase 3 expression in the hippocampus pyramidal cells CA1 and CA2-CA3 in rats. Asiaticoside, the main active compound contained in gotu kola, is able to provide a protective effect on neurons [22]. Asiaticoside blocks the cell death by reducing the concentration of intracellular free radicals [22] and protecting mitochondria from oxidative stress [14]. Asiaticoside and flavonoids in gotu kola play a role in the process of cell apoptosis by increasing the expression of Bcl-2 protein and reducing Bax expression [23].

The present study revealed that there was no linear dose – response relationship occured in the neuroprotective effects of gotu cola extract. Previous study reported that gotu kola has non-dose-dependent properties, especially in the ability of gotu kola to increase the thickness of the pyramidal cell layer in the hippocampus. It is possibly caused by the ability of high-dose gotu kola to stimulate the formation of pro-oxidants through an increase in free radicals in the body [24].

The administration of citicoline, in the present study, demonstrated that the rats treated with 100, 200, 300 mg/kg bw of gotu cola extract showed similar caspase 3 expression to the rats treated with 200 mg/kg bw of citicoline. Citicoline (citidine-5-diphosphocholine or CDP-choline) has been regularly used as a neuroprotective and memory enhancing drug [25]. Citicolin also decreased the number of caspase 3 positive cells and prevented the fragmentation of the DNA in the penumbra zone of the brains of rats with cerebral artery occlusion (48). It can stimulate the biosynthesis of glutathione and activates the glutathione reductase and preventing lipid peroxidation. It seems that the ablity of citicoline to block decomposition and to resynthesise of membrane phospholipids enables it to selectively and partially protects CA regions pyramidal neurons [26].

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