Acalypha indica root extract increases post-hypoxic rat hippocampal tissue culture cell viability via phospholipase A_2 inhibition

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Abstrak

Latar belakang: *Fosfolipase A_2 (PLA_2) terlibat dalam proses inflamasi dan kematian sel pada* stroke*, dan inhibisinya dapat mendorong terjadinya neuroregenerasi. Tujuan penelitian ini adalah mempelajari pengaruh pemberian ekstrak akar Acalypha indica Linn terhadap viabilitas relatif sel dan kadar enzim PLA_2 pada kultur jaringan hipokampus pasca-hipoksia.*

Metode: *Studi eksperimental in vitro dilakukan pada 24 kultur primer jaringan sel saraf tikus Sprague Dawley dewasa yang dipajankan terhadap hipoksia dengan gas 5% O_2 / 5% CO_2 / N_2 seimbang selama 24 jam. Pascahipoksia, ekstrak Acalypha indica Linn diberikan pada 3 kelompok perlakuan, masing-masing dengan dosis 10, 15, dan 20 mg/mL, sedangkan pada kelompok kontrol tidak diberikan apapun. Setiap kelompok terdiri atas 6 sampel. Setelah inkubasi selama 72 jam, viabilitas relatif sel diukur dengan 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dan kadar enzim PLA_2 diukur dengan menggunakan metode ELISA.*

Hasil: *Kadar enzim PLA_2 kultur jaringan hipokampus tikus yang diberikan perlakuan ekstrak akar* Acalypha indica *Linn dalam dosis 10, 15, and 20 mg/mL menurun secara bermakna dibandingkan dengan kontrol (5,55 ng/mL, 6,85 ng/mL, 7,42 ng/mL vs. 7,96 ng/mL, $p < 0,05$).*

Kesimpulan: *Ekstrak akar* Acalypha indica *Linn meningkatkan viabilitas relatif sel dan menurunkan kadar enzim PLA_2 pada kultur jaringan hipokampus tikus pasca-hipoksia dengan dosis optimal 10 mg/mL.* ***(Med J Indones. 2013;22:136-40. doi:10.13181/mji.v22i3.581)***

Abstract

Background: Phospholipase A_2 (PLA_2) is involved in inflammation and cell death following stroke, and inhibition of its activity may promote neuroregeneration. This study aimed to observe the influence of *Acalypha indica* Linn root extract towards relative cell viability and PLA_2 enzyme level in post-hypoxic hippocampal tissue culture.

Methods: Experimental *in vitro* study using 24 primary neuronal cell cultures obtained from *Sprague Dawley* rat exposed to hypoxia with 5% O_2 / 5% CO_2 / N_2 balanced gas for 24 hours. Post-hypoxia, *Acalypha indica* Linn root extract was added at doses of 10, 15, and 20 mg/mL to three treatment groups. No treatment was given to the control group. Each group consists of six samples. After 72 hours of incubation, relative cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) examination, and phospholipase A_2 enzyme level was determined using ELISA.

Results: PLA_2 enzyme level of rat hippocampal tissue culture treated with *Acalypha indica* Linn root extract at 10, 15, and 20 mg/mL were significantly lower than that of control (5.55 ng/mL, 6.85 ng/mL, and 7.42 ng/mL vs. 7.96 ng/mL, $p < 0.05$).

Conclusion: *Acalypha indica* Linn root extract increases the relative cell viability and decreases the PLA_2 enzyme level of post-hypoxic mouse hippocampal tissue with the optimal dose of the extract at 10 mg/mL. ***(Med J Indones. 2013;22:136-40 doi:10.13181/mji.v22i3.581)***

Keywords: *Acalypha indica Linn, cell viability, hypoxia, neurogenesis, phospholipase A_2*

Stroke is the leading cause of morbidity and a common cause of mortality worldwide.[1] Thrombolytic is currently the primary therapy for ischemic stroke[2] due to its effect in improving neurological function; the only proven therapeutic approach that has this effect.[3] Unfortunately, this therapy has a 3-hour therapeutic window, which is very brief,[2] and increases the risk of hemorrhagic transformation.[4] Thus, neurorestorative therapy with longer therapeutic window that can improve neurological function to restore and optimize the brain function is necessary.[3,4] Neurorestorative therapy enhances the development of new nerve cells (neurogenesis) in brain tissue following ischemic stroke.[4]

One mechanism of stroke is the activation of phospholipase A_2 (PLA_2) enzyme, which will produce arachidonic acid (AA),[5,6] leading to inflammation and eventually cell death.[7] There are at least 22 different PLA_2 enzymes that have been identified in various mammalian tissues, broadly classified into three families: calcium-dependent cytosolic ($cPLA_2$), secretory ($sPLA_2$), and calcium independent ($iPLA_2$).[8] Cytosolic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$) are involved in neuroinflammation and neurodegeneration.[8,9] Inflammation has been proven to inhibit basal and post-hypoxic neurogenesis.[10] In cerebral hypoxia, there is an increase in cytosolic PLA_2 ($cPLA_2$) in astrocytes and microglia.[8,11] Hippocampus,

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one of the site containing neural stem cells needed for neurogenesis,[3,12] is also very susceptible to hypoxia and a prominent site of PLA_2 expression.[13] Nowadays, all PLA_2 inhibitors that have been studied are non-specific so that the synthesis of a specific inhibitor for $cPLA_2$ is highly required.[14] Studies have shown that "knock-out" ($cPLA_2$-/-) mice had smaller infarcts and fewer neurological deficits compared to wild type following cerebral ischemia.[9] Drugs that target PLA_2 enzyme have also shown neuroprotective effects.[1,9,15]

Acalypha indica Linn (*akar kucing*) is an indigenous Indonesian plant that can easily be found in all regions of Indonesia. People have been empirically using the roots of the plant to cure nerve paralysis caused by stroke.[16] The plants have many active chemical compounds; some that have been identified are kaempferol (flavonoids), beta-sitosterol, HCN, gamma-sitosterol, and acalyphin.[17] Yolanda, et al[18] have studied *Acalypha indica* Linn's neurotherapeutic effect on post-hypoxic nerve cells. Nirmal, et al[19] have proven that acalyphin and stigmasterol exert their anti-inflammatory effects by forming PLA_2 inhibitor complexes. Decreasing the inflammatory response can increase the mobilization of endogenous adult stem cells population in the central nervous system, thereby increasing neurogenesis and neurological function.[16] This study aims to observe the effect of *Acalypha indica* Linn root extract on PLA_2 enzyme in post-hypoxic hippocampal tissue *in vitro*, and its subsequent impact on cell proliferation.

METHODS

Study design and sample

An *in vitro* experimental study was conducted on primary neuronal cell cultures, which were taken from the hippocampus of adult *Sprague Dawley* rats aged 9-10 weeks weighing 150-200 grams. The experiment was conducted at the Oral Biology Laboratory, Faculty of Dentistry Universitas Indonesia from September to October 2011. The animals were sacrificed by cervical dislocation. The total number of samples was 24, divided equally into 4 groups (1 control group and 3 treatment groups) based on Federer formula. Ethical clearance was obtained from the Committee of the Medical Research Ethics of the Faculty of Medicine, Universitas Indonesia prior to the study.

Culture of nerve cells

Nerve cells were cultured in 10-cm petri dishes that have been coated with poly-L-lysine (Nacalai Tesque, Jakarta, Indonesia) for 1 hour. The nerve cells were then seeded into $1x10^6$ cells/dish. Cultures were incubated with 5% CO_2 at 37°C. Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen) was used for culture medium and was replaced every 72 hours during the incubation period.

Cell harvest and plating

Nerve cells cultures were harvested on day 10 and identified morphologically. The number of cells was counted using trypan blue and a hemocytometer. The nerve cells were then seeded into 24 well-plates at 5×10^5 cells/well. The multiwell culture plates had previously been coated with poly-L-lysine for 1 hour.

Exposure to hypoxia

Directly after plating, nerve cell were then exposed to hypoxia by administering 5% O_2/ 5% CO_2/ N_2 balanced gas.[20] The gas were administered for 5 minutes in a vacuum container, then the vacuum container with the hypoxic cells was incubated with 5% CO_2 at 37°C for 24 hours.

Treatment with *Acalypha indica* Linn extract

Each treatment groups of nerve cells cultures were given *Acalypha indica* Linn root extract at 10, 15, and 20 mg/mL. The control group was given nothing. *Acalypha indica* Linn root extract was obtained from the Department of Pharmacy, Faculty of Medicine Universitas Indonesia. The plates were re-incubated with 5% CO_2 at 37°C for 90 hours.

Measurement of relative cell viability and proliferation

Relative cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). MTT assay was performed in duplicate. PLA_2 enzyme level was measured using Mouse Phospholipase A2, PLA_2 ELISA Kit from EIAab (PT. Indogen Intertama, Jakarta, Indonesia). PLA_2 level examination was performed in duplicate. The MTT examination was performed on cells, while the PLA_2 enzyme level examination was done on the supernatant. MTT examination was performed to confirm the findings of the our previous study.[18]

Data analysis

Normality of data was tested using Shapiro-Wilk test. Due to abnormal distribution of data, comparison of data means was analyzed using non parametric test (Kruskal-Wallis) test and continued with Mann-

Whitney test. Data processing was performed using Statistical Package for the Social Sciences (SPSS) version 11.5 software.

RESULTS

Results of relative cell viability were in accordance with our previous study.[18] Relative mean PLA_2 enzyme level in the control group was 7.96 ± 0.060 ng/mL (n = 6). In groups treated with *Acalypha indica* Linn extract at doses of 10, 15, and 20 mg/mL, mean PLA_2 enzyme level was 5.55 ± 0.970 ng/mL (n=6), 6.85 ± 0.590 ng/mL (n = 6), 7.42 ± 0.090 ng/mL (n = 6), respectively (p = 0.04 in all treatment groups vs control; Figure 1).

The lowest level of PLA_2 enzyme was seen in group treated with extract at dose of 10 mg/mL, and increased significantly in groups treated with extract at doses of 15 and 20 mg/mL (p = 0.016 and 0.04, respectively). Significant difference was also found in the PLA_2 enzyme level between groups treated with extract at doses of 15 and 20 mg/mL (p = 0.04; Figure 1).

DISCUSSION

MTT test results were in accordance with the author's previous study,[18] indicating that *Acalypha indica* Linn root extract has an effect on increasing the number of viable post-hypoxic nerve cells with an optimum dose at 10 mg/mL.

The mechanism of the increased cell proliferation is thought to be due to the decrease in PLA_2 enzyme activity by acalyphin which will in turn decrease the inflammatory response and mobilize the endogenous stem cells to proliferate.[19,21] This mechanism is investigated in this research reflected by the measurement of PLA_2 enzyme level. Our results indicated that the PLA_2 enzyme level in all treatment groups were significantly lower than control, with the lowest PLA_2 enzyme level found at the dose of 10 mg/mL. This result showed that *Acalypha indica* Linn root extract decreased the PLA_2 enzyme level in post-hypoxic nerve cell culture with the optimum dose at 10 mg/mL. This result also complies with the MTT assay result.

In this study, PLA_2 data were obtained from the supernatant of the nerve cells culture, therefore the detected PLA_2 enzymes were non-specific and most likely be the PLA_2 enzyme secreted by cells ($sPLA_2$). Other than $sPLA_2$, $cPLA_2$ is also regarded as the inflammatory precursor in cerebral hypoxia.[8,11,14] Further research based on this finding is needed to examine the level of $cPLA_2$ enzyme in post-hypoxic hippocampal tissue using a more specific marker.

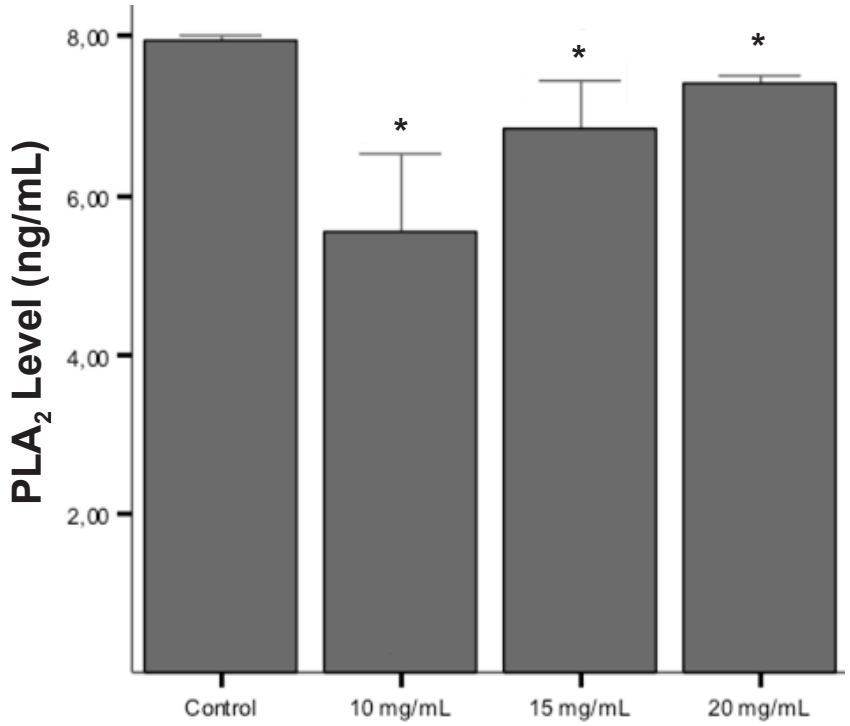

Figure 1. Comparison of PLA_2 enzyme level among control and treatment groups given Acalypha indica Linn *root extract with consecutive doses of 10, 15, and 20 mg/mL. Data was given in means ± SD from 6 different samples.*
** showed significant difference compared to countrol group*

Structurally, Nirmal, et al[19] have shown that acalyphin exerts its anti-inflammatory effects by forming an inhibitory complex in Asp-49, Lys-69, and Gly 30 from the active site of $sPLA_2$ Russell viper, and with Ca^{2+} ion from the active site of $sPLA_2$ bovine pancreas. The mechanism of this inhibitory complex formation has not been investigated in the central nervous system, thus further research is needed to assess whether or not in the central nervous system *Acalypha indica* Linn root extract would generate a similar $sPLA_2$ inhibitory complex mechanism similar to that in Russell's viper and bovine pancreas.

In this study, the doses used were 10, 15, and 20 mg/mL based on experiences from previous studies.[16] It was discovered that the optimum dose of *Acalypha indica* Linn root extract to increase relative cell viability and decrease PLA_2 enzyme level was 10 mg/mL. This result suggests that a lower dose than 10 mg/mL might give the same or better result, hence further study to determine the lowest optimum dose of *Acalypha indica* Linn. root extract for neurorestorative therapy post-hypoxia is needed.

In addition to reducing the inflammatory response, *Acalypha indica* Linn's root extracts may also increase cell viability by increasing neurotrophic factors such as BDNF, VEGF, and bFGF; which are required for the survival of neuroblast.[3] Increased neurotrophic factors as a mechanism of increased relative cell viability caused by *Acalypha indica* Linn root extract has not been studied, hence further research is needed to assess whether *Acalypha indica* Linn root extract can enhance post-hypoxic cell viability through enhancement of neurotrophic factors by examining the levels of growth factors (e.g. BDNF).

The limitation of this research lies in the morphologic identification of the neural cells. This method cannot fully guarantee 100% identification of neural cells with certainty because other cells also such as glial cells and fibroblasts also reside in the hippocampus, which may be identified through this method. Thus, further research is needed with more precise identification of the neural cells using specific markers or antibodies such as neuron-specific enolase type 2.

From this research we can conclude that *Acalypha indica* Linn root extract at doses of 10, 15, and 20 mg/mL with the optimum dose of 10 mg/mL can increase relative cell viability of rat hippocampal cells post-hypoxia, *in vitro*.

Acknowledgments

The research was funded by *Hibah Riset Unggulan Universitas Indonesia* (RUUI) 2011 contract number 1318/H2.R12/PPM.00.01 *Sumber Pendanaan*/2011. The authors would like to thank the expert consultant of this research, dr. Nurhadi Ibrahim, PhD for all his help and support. We would also like to thank the staff of the Oral Biology Laboratory, Faculty of Dentistry Universitas Indonesia for their work during the research; and Department of Physiology, Faculty of Medicine Universitas Indonesia for their support.

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