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Propolis increases neuronal count in hippocampal area CA1 and prefrontal cortex in stressed rats

Kuswati*, Ety Sari Handayani*, and Zainuri Sabta Nugraha*

ABSTRACT

*Department of Anatomy,
Faculty of Medicine,
Islamic University of Indonesia
Yogyakarta

Correspondence

dr. Kuswati, M.Sc
Department of Anatomy,
Faculty of Medicine,
Islamic University of Indonesia
Jl. Kaliurang km 14,5 Yogyakarta
Phone: +62274 898444
Mobile: +62 852 284 784 87
Email : kuswatinugroho@gmail.com

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BACKGROUND

Stress induces neuronal cell damage in the hippocampus and prefrontal cortex. Propolis has a neuroprotective effect that can inhibit apoptosis and decrease neuronal cell count. This study aimed to determine the effect of propolis on neuronal cell count in hippocampal area CA1 and prefrontal cortex in Sprague Dawley rats with induced stress.

METHODS

A study of laboratory experimental design was conducted involving 24 male Sprague-Dawley *Rattus norvegicus*. The animals were randomly divided into 4 groups, i.e. controls (K), and stress groups P1, P2 and P3. Controls did not receive treatment, stress group (P1) received stress treatment, groups P2 and P3 received stress and propolis at 100 and 200 mg/kgBW, respectively. Stress and propolis were given for 14 days, followed by termination. The number of neurons in the hippocampal area CA1 and prefrontal cortex were counted. One way ANOVA was used to analyze the data.

RESULTS

The neuronal count in the hippocampal area CA1 and prefrontal cortex in the stress group (P1) was lower than in groups K, P2 and P3. There were significant differences in the neuronal count of the hippocampal area CA1 between P1 and P3 and P1 and K ($p=0.019$) and also in the neuronal count of the prefrontal cortex between P1 and P2, P3 and K ($p=0.002$).

CONCLUSIONS

This study strongly suggest that propolis inhibits the decrease in neuronal count in in the hippocampal area CA1 and prefrontal cortex of Sprague Dawley rats with induced stress. The present study suggests a potential neuroprotective effect of propolis in the prevention of neurodegenerative disorders.

Keywords: Propolis, hippocampus, prefrontal cortex, stress, rats

INTRODUCTION

The hippocampus plays an important role in learning and memory and is one of the brain regions that is affected by stress.⁽¹⁾ Rats treated with various chronic stress models, by reducing sleep duration, immobilization stress and electrical stress, have a lower number of pyramidal cells in area CA3 of the hippocampus than do rats without stress treatment.⁽²⁾ Stress administered to rats 6 hours daily for 6 weeks results in changes in the hippocampal neurons. These changes comprise a reduction in the number and length of apical and basal dendrites, so causing atrophy of the neurons in area CA3 of the hippocampus.⁽³⁾ Chronic stress plays a role in the aging process in the hippocampus, marked by changes in the expression of several genes. Exposure to stress for 10 days decreases the gene expression levels of sirtuin-1 and heat shock protein 70, but increases the gene expression levels of P-53, thioredoxin-interacting protein and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.⁽⁴⁾

In the prefrontal cortex, chronic stress increases the noradrenergic innervation and causes a decrease in working memory and interference with the connections between the prefrontal cortex and the hippocampus.⁽⁵⁾ Stress results in a decrease in the number of neurons (neuronal cell count) and B cell lymphoma 2 (Bcl-2) expression in the prefrontal cortex.^(6,7) The combination of acute and chronic stress results in cytochrome C release from the mitochondria into the cytoplasm, activation of caspase-3 and increase in the number of cells undergoing apoptosis in the prefrontal cortex.⁽⁸⁾ The results of the study by Li et al.⁽⁹⁾ demonstrates the occurrence of apoptosis and changes in the Bcl-2 associated X (Bax) and the Bcl-2/Bax ratio in the prefrontal cortex of rat models of post traumatic stress disorder (PTSD). Bachis et al.⁽¹⁰⁾ report an increase in the number of cells expressing caspase 3 in the pyramidal frontal cortex layers of Sprague Dawley rats undergoing stress. Rats that were given stress treatment by

chronic social isolation for 6 weeks underwent an increase in apoptosis, a decrease in Bcl-2 protein, an increase in Bax, and an increase in the Bax/Bcl-2 ratio in the prefrontal cortex.^(11,12) Cao et al.⁽¹³⁾ report that socially isolated rats have spatial and short term memory dysfunction, abnormal social functioning and anxiety. In addition, there was also increased active caspase 3 expression in the medial prefrontal cortex and hippocampus.

Propolis is one of the substances that are expected to be able to protect neurons from damage due to stress exposure.⁽¹⁴⁾ Propolis contains chrysin, caffeic acid phenethyl ester (CAPE) and caffeoylquinic acid (CQA) that have neuroprotective effects.^(15,16) Previous studies have proven that propolis may inhibit apoptosis in cell cultures of SH-SY5Y neurons (a human neuroblastoma cell line) that were exposed to staurosporin, by inhibiting caspase-3 activity and apoptosis through the intrinsic pathway.⁽¹⁷⁾ The aqueous extract of propolis decreases the production of lactate dehydrogenase (LDH) in cultures of the PC12 pheochromocytoma cell line as a result of exposure to H₂O₂. This shows that the aqueous extract of propolis has protective effects on the mitochondria and cell membranes of neurons.⁽¹⁸⁾ Pinocembrin is one of the flavanones found in propolis and honey with actions useful in preserving cognitive function.⁽¹⁹⁾ In a recent animal study, pinocembrin ameliorated cerebral damage caused by global cerebral ischemia-reperfusion.⁽²⁰⁾ These neuroprotective effects were attributed to pinocembrin suppressing damaging biomarkers including oxidative stress and inflammation.

One study found that Chinese propolis inhibits neuronal cell death induced by endoplasmic reticulum stress that has been implicated in the pathogenesis of neurodegenerative and ischemic disorders.⁽¹⁷⁾ Long term administration of chrysin at 30 mg/kg showed reduced pathological changes and attenuated the chronic cerebral hypoperfusion induced cell loss in the cortex and hippocampal CA1 area of rats.⁽²¹⁾ However, there is no

information available on the effect of propolis administration on the neuronal cell count in the hippocampal and prefrontal cortex areas.

The present study aimed to evaluate the effect of propolis administration on the neuronal cell counts in the hippocampal area CA1 and the prefrontal cortex in Sprague Dawley rats receiving social isolation stress exposure.

METHODS

Research design

This was an experimental laboratory study with posttest only control group design. The study was conducted at the Research Laboratory, Faculty of Medicine, Universitas Islam Indonesia (FK UII) from March to December 2016.

Research subjects

The study subjects were 4 month-old male *Rattus norvegicus* of the Sprague -Dawley strain, weighing 200-300 grams. The number of subjects required were calculated with the formula of Charan and Biswas⁽²²⁾ as follows:

$E = \text{total number of animals} - \text{total number of groups} = 10 - 20$

$\text{Total number of animals} = 20 - \text{total number of groups} = 20 - 4 = 16$

From the above calculation the number of subjects obtained was 16 rats. This method of sample size calculation is rather crude and should only be used if it cannot be done by power analysis. All experiments were performed in accordance with the standard guidelines for the care and use of laboratory animals.

Preparation of propolis extract

The propolis preparation used in this study is a product available in the free market, with 1 ml containing 600 mg propolis. To 0.1 ml of the propolis preparation 0.9 ml distilled water was added thus giving a concentration of 60 mg/ml. This dilution was used to make up doses of 100 mg/kgBW and 200 mg/kgBW to be administered to the rats, in accordance with the study of Lee et al.⁽²³⁾

Stress procedure and propolis treatment

In this study the social isolation stress model was applied by holding one rat per cage so that there was no interaction with the other rats.⁽²⁴⁾ This stress treatment was given for 14 days. The rats were divided by block randomization into 4 groups, each consisting of 4 rats (n=4). The groups comprised one control group (K), one stress group (P1) and two propolis groups (P2 and P3). The control group did not receive treatment, the stress group (P1) received stress treatment, while groups P2 and P3 received stress treatment and oral propolis administration by gastric tube at doses of 100 and 200 mg/kgBW, respectively. The control group and the stress group (P1) received distilled water.

Sample preparation

On day 15 the rats were terminated and underwent transcardial perfusion. The rats were anesthetized by an intramuscular injection of ketamine at 100 mg/kgBW. The transcardial perfusion used normal saline at 100-200 ml until the returning perfusion fluid was clear. After perfusion, the brain was carefully dissected out and the portions containing the hippocampus and the prefrontal cortex were fixed in phosphate-buffered formalin for 24 hours.

The portions of the brain containing the hippocampus and the prefrontal cortex were made into paraffin blocks and sectioned at 4 µm. The sectioning was done at the portion containing the hippocampus. From one rat brain 3 sample sections were made at distances of 240 µm.

Hematoxyllin eosin staining was done according to the protocol at the Research Laboratory of FK UII, which briefly was as follows. The slide containing the tissue section was placed in a staining jar, deparaffinized, then washed twice in distilled water. Subsequently the slide was placed in the hematoxyllin solution for 10 minutes, washed 3 times in distilled water, and checked under the microscope. If the hematoxyllin staining was adequate, the slide was washed in running water. Subsequently the

Table 1. Distribution of mean neuronal cell counts in hippocampal area CA1 and in the prefrontal cortex, by treatment groups

Variable	Treatment groups				p value
	K (n=4)	P1 (n=4)	P2 (n=4)	P3 (n=4)	
Neuronal cell count of hippocampal area CA1	61.69 ± 9.89	40.08 ± 2.59	51.86 ± 11.11	58.49 ± 14.61	0.019
Neuronal cell count of prefrontal cortex	86.80 ± 14.50	50.03 ± 4.76	88.11 ± 22.44	89.63 ± 21.55	0.021

K=control group (non-stress); P1=stress group; P2=stress group + propolis 100 mg/kgBW; P3=stress group + propolis 200 mg/kgBW

preparation was placed in eosin and washed twice in distilled water. The slide was placed in the alcohol series of 70%, 80%, 90%, 95%, and 100% then dried with tissue paper. The dried slide was placed in xylol I, II and III for 5 minutes each, then mounted and observed under the microscope.

Neuronal cell counts in hippocampus and prefrontal cortex

The preparation was observed under the light microscope at 400 x magnification. The cell count was performed in the whole hippocampal area CA1 and prefrontal cortex. Subsequently the mean number of cells was calculated for one preparation per field of view with the formula: Mean cell count = number of cells in the area under observation/number of fields of view.

Data analysis

To compare the mean cell count per field of view between the groups, one-way ANOVA was used, followed by a post hoc test to determine the differing groups. A p-value of less than 0.05 was considered statistically significant.

Ethical clearance

This study was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Islam Indonesia under No. 03/Ka.Kom.Et/70/KE/V/2016.

RESULTS

The resulting neuronal cell counts in hippocampal area CA1 and prefrontal cortex are

presented in Table 1. The results of one way ANOVA showed a significant difference in neuronal cell count of hippocampal area CA1 between the four groups ($p=0.019$). The results of post hoc analysis showed significant differences between group P1 and the control group and group P3, respectively (Table 2). Significant differences in neuronal cell counts were also found in the prefrontal cortex ($p=0.021$). The results of post hoc analysis showed significant differences between group P1 and the groups K, P2 and P3, respectively (Table 3). There were no significant differences between the control group K and groups P2 and P3.

Table 2. Results of post hoc analysis of mean neuronal cell counts of hippocampal CA1

Group	Group	p value
K	P1	0.003
	P2	0.139
	P3	0.620
P1	P2	0.093
	P3	0.013
P2	P3	0.331

K=control group (non-stress); P1=stress group; P2=stress group + propolis 100 mg/kgBW; P3=stress group + propolis 200 mg/kgBW

Table 3. Results of post hoc analysis of mean neuronal cell counts of prefrontal cortex

Group	Group	p value
K	P1	0.001
	P2	0.898
	P3	0.783
P1	P2	0.001
	P3	0.001
P2	P3	0.898

K=control group (non-stress); P1=stress group; P2=stress group + propolis 100 mg/kgBW; P3=stress group + propolis 200 mg/kgBW

DISCUSSION

The results of this study showed that in the stress groups receiving propolis treatment (P2 and P3) the mean neuronal cell counts were higher than in the stress group receiving distilled water (P1). This showed that propolis treatment may inhibit the decrease in neuronal cell counts in hippocampal area CA1 and prefrontal cortex as a result of stress exposure. One study evaluated the effect of administration of propolis and caffeic acid phenylethyl ester (CAPE) on brain tissue of rats exposed to ionizing radiation and showed that propolis and CAPE would reduce the oxidative damage in the radiation-injured brain tissue.⁽²⁵⁾

The results of the study by Reis et al.⁽²⁶⁾ showed that supplementation with oil extract of propolis (OEP) at doses of 10, 30 and 50 mg/kgBW administered intraperitoneally for 1 hour produced anxiolytic and antidepressant effects and improved locomotion in rats. Another study showed that an aqueous extract of propolis decreased the production of LDH in cultures of neuronal-like PC12 cells as a result of exposure to H₂O₂.⁽¹⁸⁾ This showed that aqueous extract of propolis has protective effects on mitochondria and neuronal cell membranes. In mice with scopolamine-induced neurotoxicity, administration of propolis at 100 mg/kgBW may improve memory, and inhibit acetylcholine esterase activity in the cerebral and hippocampal cortex.⁽²⁷⁾ This showed that propolis increased memory through inhibition of acetylcholine esterase activity in the hippocampus. Therefore, propolis has the pharmacologic potential of a neuroprotective agent and may be used for treatment of amnesia in the elderly, Alzheimer's disease (AD) and other neurodegenerative diseases.

Propolis administration at a dose of 200 mg/kgBW to Wistar rats exposed to aluminum nitrate could inhibit lipid peroxidation in the liver, kidneys and neurons.⁽²⁸⁾ Furthermore, propolis treatment could increase the concentrations of the antioxidant glutathione in brain tissues. Propolis treatment could inhibit the decrease in ATPase in

the liver, kidneys and brain. Propolis treatment could restore acetylcholine esterase activity in the brain after exposure to aluminum nitrate. Addition of an ethanolic extract of propolis to neuronal cell cultures which had been exposed to the neurotoxic agents staurosporin and hydrogen peroxide, could inhibit the production of reactive oxygen species (ROS) and caspase-3 activity.⁽²⁹⁾ In rats with neurotoxicity induced by exposure to lead (Pb) for 4 weeks and receiving oral propolis treatment, it was shown that propolis could inhibit the neurotoxic effects of Pb. This was apparent in the normal acetylcholine esterase activity and the decrease in malondialdehyde (MDA) concentrations in the brain. Propolis increased the activities of reduced nicotinamide adenine dinucleotide (NADH), succinate dehydrogenase (SDH) and cytochrome C oxidase in mitochondria.⁽³⁰⁾

Propolis contains CAPE that has been proven to have neuroprotective effects. CAPE inhibits neurodegeneration in dopaminergic neurons in the striatum and prevents the loss of dopamine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. The neuroprotective effect of CAPE is associated with decreased inducible nitric oxide synthase (iNOS) and caspase 1 expression. In vitro, CAPE inhibits the neurotoxicity induced by MPP⁺ (1-methyl-4-phenylpyridinium), the neurotoxic oxidation product of MPTP, and inhibits the release of cytochrome C and apoptosis inducing factor (AIF) from the mitochondria.⁽¹⁵⁾ CAPE reduces the neurotoxicity due to acrolein in HT22 murine hippocampal neuronal cells. CAPE inhibits ROS production by neuronal cells of the hippocampus as a result of acrolein exposure. Therefore, CAPE has antioxidant effects. Administration of CAPE restores decreased glutathione levels from acrolein exposure in hippocampal neuronal cultures. CAPE inhibits the activation of p38 and JNK 1.⁽³¹⁾

Propolis contains caffeoylquinic acid (CQA), an antioxidant component with neuroprotective effect in cultures of 1–42

treated SH-SY5Y neuroblastoma cells. CQA increases the viability of 1–42 cells treated with SH-SY5Y. CQA increases the expression of glycolytic enzyme mRNA (phosphoglycerate kinase-1; PGK1) and intracellular ATP concentrations. CQA improves spatial learning and memory in mice.⁽¹⁶⁾

Chrysin decreases the activities of superoxide dismutase and lipid peroxidase, and the levels of nitric oxide, tumor necrosis factor alpha, and interleukin-1 β . Administration of chrysin inhibits the expression of Bax, Bcl-2 and caspase-3.⁽³²⁾

The limitation of the present study is that only a histological examination was performed with counting of the number of neuronal cells in the brain and no biochemical analysis that may support the results of this study. The clinical implication of these study results is that propolis may be used to inhibit or prevent neuronal damage due to degeneration caused by stress exposure. Further studies should be done to study the pharmacologic effects and the toxicity of propolis in order to support its benefits as a neuroprotective agent in brain tissue.

CONCLUSION

Administration of propolis may inhibit a decrease in neuronal cell count in hippocampal area CA1 and prefrontal cortex in rats with social isolation-induced stress. This study provides useful information for considering propolis as an alternative treatment choice for neurodegenerative disorders.

CONFLICT OF INTEREST

There were no conflicts of interest in the present study.

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CONTRIBUTORS

K contributed to the design of the study, wrote the protocol and wrote the first draft of the manuscript. ZSN contributed to revising the protocol. ESH revised the manuscript. All authors read and approved the final manuscript.



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