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Protective Effects of Propolis Extract in a Rat Model of Traumatic Brain Injury via Hsp70 Induction

Steven Tandean[1*], Iskandar Japardi[1], Michael Lumintang Loe[1], Wibi Riawan[2], Julius July[3]

[1]Department of Neurosurgery, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; [2]Department of Biochemistry, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [3]Department of Neurosurgery, Faculty of Medicine, Universitas Pelita Harapan (UPH), Neuroscience Centre Siloam Hospital Lippo Village, Tanggerang, Indonesia

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***Correspondence:** Steven Tandean. Department of Neurosurgery, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia. E-mail: steven.tandean@usu.ac.id

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Abstract

BACKGROUND: Traumatic brain injury (TBI) is one of the major global health problems. Secondary brain injury is a complex inflammation cascades process that causes brain cell apoptosis. Propolis is a natural product that has neuroprotective property.

AIM: This study aimed to investigate the effect of propolis toward Hsp70 expression with apoptosis marker in brain tissue after TBI.

METHODS: Thirty-three Sprague Dawley rats were randomised into three treatments group, i.e. sham-operated controls, closed head injury (CHI), and CHI with propolis extract (treatment group). In the treatment group, propolis was given 200 mg/kg per oral for 7 days then harvested brain tissues after sacrificed by cervical dislocation at day 8. We investigated Hsp70, Caspase 3, apoptosis-inducing factor (AIF), and TUNEL assay expression using immunohistochemistry staining. Statistical test using one-way ANOVA test and Tukey HSD as post hoc test.

RESULTS: Mean of positive Hsp70 stained cells in group 1 was 6.82 ± 2.14, group 2 was 3.91 ± 2.26, and group 3 was 9.64 ± 3.53 with a significant difference of Hsp70 expression distribution within groups (p = 0.0001). Mean of positive caspase 3 stained cells in group 1 was 5.45 ± 2.30, group 2 was 13.82 ± 2.44, and group 3 was 7.03 ± 1.54 with a significant difference of caspase3 expression distribution within groups (p=0.0001). Mean of positive AIF stained cells in group 1 was 5.36 ± 2.11, group 2 was 12.82 ± 1.40, and group 3 was 8.09 ± 1.81 with a significant difference of AIF expression distribution within groups (p = 0.0001). Mean of positive TUNEL assay stained cells in group 1 was 4.82 ± 2.04, group 2 was 11.55 ± 1.51, and group 3 was 7.64 ± 1.96 with a significant difference of TUNEL test expression distribution within groups (p = 0.0001).

CONCLUSION: Propolis may protect brain cell from apoptosis after injury by maintaining Hsp70 expression in addition to antioxidant and anti-inflammatory.

Introduction

Traumatic brain injury (TBI) is one of the major global health problems. Based on the WHO report, mortality and morbidity caused by TBI have increased significantly and also make an economical problem for families and societies [1]. Incidence of TBI was varied between regions, and the commonest is road traffic injuries. Estimation of worldwide TBI is 69 million (95% CI 64-74 million) lives each year from all causes with the severest burden in Western Pacific and Southeast Asian countries [2].

Brain contusion, haemorrhage, and axonal shearing are primary brain injury that occurred directly after injury and preventable. Secondary brain injury occurred later and activated complex inflammation cascades, excitotoxicity, and reactive oxygen species (ROS) that will cause apoptosis of brain cells. Apoptosis can be activated by caspase-dependent and caspase-independent pathway. Activation of the intrinsic and extrinsic pathway from caspase-dependent pathways will activate caspase 3 and trigger apoptosis process [3], [4].

Propolis was used as traditional medicine and had been investigated for its efficacy through researches. The main components of propolis are caffeic acid phenethyl ester (CAPE) that proved had antimicrobial, antioxidant, anti-inflammatory, cytotoxicity, and neuroprotective effects. Researches proved that the antioxidant effect of propolis by increased malondialdehyde (MDA) level and reduce superoxide dismutase (SOD) and glutathione peroxidase (GPx) level. CAPE from propolis also inhibits apoptosis by preventing caspase 3, nitric oxide synthase, and cytochrome productions [5], [6]. This study aimed to investigate the effect of propolis toward Hsp70 expression with apoptosis marker in brain tissue after TBI.

Material and Method

Propolis extract

Propolis collected at East Java, Indonesia was washed to clean all residual dirt. Propolis was mixed with 70% ethanol (100 gr in 500 cc water) with 50 rpm for 24 hours. Macerate filtrate was evaporated using rotary evaporator within 2 hours. Every 100 gr propolis produced 50 ccs of propolis extract.

Application of TBI

This research used 33 male Sprague Dawley rats with weight 250-400 gr and separated into three groups viz. group 1 as the negative control, group 2 as the positive control, and group 3 as the treatment group. TBI was made by using modified Feeney's weight-drop model. After general anaesthetic with ketamine HCl intramuscular (100 mg/kg body weight), mid frontal of rat' skull was exposed and dropped with 40 mg metal mass from 1.5 m height. Group 3 were given with 200 mg propolis extract/kg from oral for 7 days. At day 8, all rats were sacrificed by cervical dislocation after general anaesthesia. Brain samples were fixed with 10% formalin then contusion area of brains was processed to paraffin-embedded for immunohistochemistry (IHC) staining purpose. This research has been approved by the Health Research Ethical Committee Medical Faculty of Universitas Sumatera Utara / H. Adam Malik General Hospital.

Immunohistochemistry staining

IHC staining of proBDNF, Caspase3, Hsp70, and apoptosis-inducing factor (AIF) from paraffin-embedded of lesion cortical brain used the avidin-biotin-peroxidase complex method. 5 mm thickness paraffin-embedded sections were dewaxed, rehydrated, and microwave for 10 minutes. 3% H2O2 was used to block the activity of endogenous peroxidase then rinsed with phosphate-buffered saline (PBS). The tissue section was incubated with normal rabbit serum and incubated with a monoclonal antibody (Santa Cruz) at room temperature. These samples were rinsed with PBS and incubated with secondary antibody for 30 minutes. Tissue sections were rinsed twice with PBS and developed with 0.05% 3, 3 diamino-benzinetetrahydrochloride and slightly counterstained. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method was used to analysis apoptotic DNA fragmentations from all samples. All samples were evaluated by one pathologist (blinded) and the first author (not blinded). Positive signal of IHC was found in the cytoplasm and counted with a binocular microscope with 100x magnify in 10 high power fields.

Statistical analysis

All counts were showed in mean and standard deviation. One-way ANOVA test was used to compare between groups and Tukey HSD for post hoc test. Significant Differences when $P < 0.05$.

Results

HSP70 expression

Mean of positive Hsp70 stained cells in group 1 was 6.82 ± 2.14, group 2 was 3.91 ± 2.26, and group 3 was 9.64 ± 3.53. Distribution of Hsp70 expression within groups has a significant difference ($p = 0.0001$).

Caspase 3 expression

Mean of positive caspase 3 stained cells in group 1 was 5.45 ± 2.30, group 2 was 13.82 ± 2.44, and group 3 was 7.03 ± 1.54. Distribution of caspase3 expression within groups has a significant difference ($p = 0.0001$).

Apoptosis-inducing factor (AIF) expression

Mean of positive AIF stained cells in group 1 was 5.36 ± 2.11, group 2 was 12.82 ± 1.40, and group 3 was 8.09 ± 1.81. Distribution of AIF expression within groups has a significant difference ($p = 0.0001$).

TUNEL assay expression

Mean of positive TUNEL assay stained cells in group 1 was 4.82 ± 2.04, group 2 was 11.55 ± 1.51, and group 3 was 7.64 ± 1.96. Distribution of TUNEL test expression within groups has a significant

difference (p = 0.0001).

Table 1: All markers (Hsp70, caspase 3, AIF, and TUNEL assay) expression in A) Negative control group; B) Positive control group; C) Propolis treatment group

Marker	Groups	N	Mean	Standard deviation	P
Hsp70	Negative control	11	6.82	2.136	0.0001
	Positive control	11	3.91	2.26	
	Propolis treatment	11	9.64	3.53	
Caspase 3	Negative control	11	5.45	2.30	0.0001
	Positive control	11	13.82	2.44	
	Propolis treatment	11	7.82	1.54	
Apoptosis inducing factor (AIF)	Negative control	11	5.45	2.30	0.0001
	Positive control	11	13.82	2.44	
	Propolis treatment	11	7.03	1.54	
TUNEL assay	Negative control	11	4.82	2.04	0.0001
	Positive control	11	11.55	1.51	
	Propolis treatment	11	7.64	1.96	

Discussion

Propolis composes of flavonoids, phenolic acid, steroid, essential oils. However, CAPE is the major component for antioxidant and anti-inflammatory effect. Secondary brain injury can be prevented based on these properties [6]. Spinal cord injury experiment using rat model with the administration of 200 mg/kg intraperitoneally reduce caspase-3 and cathepsin B expression compared to trauma group significantly [7]. Other animal experiment using rats that kept at 40°C for 12 hours to give heat stress then administrated propolis extract orally as a supplement showed low caspase-3 and malondialdehyde (MDA) expression with high glutathione (GSH) expression close to control group [8].

Our study showed that group 3 with propolis administration was significantly lower caspase-3 expressions than group 2 although not as low as group 1 and for AIF expression, group 3 was significantly lower than group 2 and as low as group 1. So propolis extracts had been proven to inhibit caspase-dependent and independent pathways. This study showed propolis could reduce AIF level in the treatment group similar to the negative control group compare to caspase level, so propolis is more prominently in inhibiting independent pathway. All these processes will decrease apoptotic events downstream and finally reduce the apoptosis in the brain cells. Apoptosis is programmed cell death that caused by several conditions, including brain injury. Apoptosis is the final result of caspase-dependent and independent pathways process including nuclear condensation, cell body shrinkage and fragmentation of DNA. The typical morphological phenotype of apoptotic cells is the reduction of cell volume. TUNEL assay can detect apoptotic cells that undergo vast DNA degradation during the late phase of apoptosis.[9] Traumatic brain injury experiment using rat model with 200 mg/kg propolis extract every day were significantly increased Bcl-2 level but decrease TNF-α, apoptosis, and necrosis expression [10], [11]. Our study also showed that propolis could reduce positive TUNEL assay level significantly in group 3 compared with group 2 and has a positive TUNEL assay level as low as group 1. This TUNEL assay proved that propolis could reduce apoptosis after traumatic brain injury.

Hsp70 has been proved to have protection properties in brain injury and cerebral schema. Hsp70 can prevent the process of caspase-dependent and independent pathways by inhibiting the release of cytochrome c, Apaf-1, and caspase3. Neuroprotective properties of Hsp70 by decreasing apoptotic events downstream [12], [13]. Animal experiment with turkey pout that administrated herbicide intravenously then fed with propolis showed normalised Hsp70 expression to a similar level of the control group [14]. Our experiment showed that group 3 had Hsp70 level significantly higher than group 2 but no significant different than group 1. This means propolis can normalise the Hsp70 level similar to normal condition or negative control group. Propolis treatment group in our study showed lower caspase-3, AIF, and TUNEL assay expression with higher Hsp70 expression than group 2 that significant different ($p < 0.05$). Hsp70 expression between group 1 and group 3 was not different significantly which mean propolis has the ability to increase Hsp70 level in the treatment group similar to the control group without trauma. We suggested that propolis can preserve Hsp70 expression in the injury area. Based on this, we proposed that propolis's neuroprotective properties also from Hsp70 induction, besides from anti-inflammatory and antioxidant properties.

In conclusion, apoptotic markers (caspase 3, AIF, and TUNEL assay) reduced significantly by propolis treatment compare to injury groups ($p < 0.05$). Propolis also can preserve Hsp70 expression similar to the control group. So it was shown that propolis has a potential effect in protecting brain cells from injury by preserving Hsp70 expression in addition to anti-inflammation and antioxidant properties. However, further clinical researches are needed for clinical use of propolis.

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