**Black Sugarcane Decoction To Reduce Rat Brain Ischemia**

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Background

There are people in Yogyakarta, who use black sugarcane decoction (BSD) to prevent stroke. BSD contains policosanol and antioxidants. It has been proven that policosanol can reduce global ischemia in Mongolian gerbils. This study aims to study the effect of BSD on brain ischemia. A rat stroke model was used. Brain ischemia was produced by a 20-minute bilateral carotid artery ligation (BCAL).

Method

Eighteen male rats (Wistar) were used. Rats were divided into three groups: BSD treated stroke model rats (group 1), Non treated stroke model rats (group 2), and sham operated rats (group 3). Sounding BSD for 1 week before BCAL. Decapitation of rats performed two hours post BCAL. Brain tissue were stained with TTC. Ischemic area analyzed using Image J softwere. Statistical analysis was conducted by using One Way ANOVA test .

Result

There were differences in the mean percentage of rat brain ischemic area between group 3 (0%), group 2 (3.13%) and group 1 (1.15%) (p value 0.001). Post hoc test showed that there was no difference between group 3 with group 1. Instead, there was a significant difference between the group 2 with other groups.

Conslusion

The provision of BSD reduced ischemic rat brain after ligation of bilateral carotid artery .

Keyword : sugarcane, brain ischemia, bilateral carotid artery ligation

Air Rebusan Tebu Hitam Menurunkan Iskemik Otak Tikus

Latar belakang

Masyarakat Yogyakarta menggunakan air rebusan tebu hitam untuk mencegah stroke. Tebu hitam mengandung policosanol dan antioksidan. Telah terbukti bahwa policosanol dapat mengurangi iskemia global pada gerbil Mongolia. Penelitian ini bertujuan untuk mempelajari pengaruh air rebusan tebu hitam pada iskemia otak. Iskemia otak dihasilkan dengan menggunakan model stroke pada tikus dengan meligasi selama 20 menit arteri karotis komunis bilateral.

Metode

Penelitian ini menggunakan delapan belas tikus jantan (galur Wistar). Tikus dibagi menjadi tiga kelompok yaitu kelompok rat stroke model yang mendapatkan sondase air rebusan tebu hitam (kelompok 1), kelompok rat stroke model yang tidak mendapatkan sondase air rebusan tebu hitam (kelompok 2), dan kelompok *sham* (kelompok 3). Pemberian sondase air rebusan tebu hitam selama 1 minggu, sebelum dilakukan ligasi bilateral pada arteri carotis. Dekapitasi kepala tikus dilakukan dua jam pasca ligasi. Jaringan otak diwarnai dengan TTC. Daerah iskemik dianalisis menggunakan Image J softwere. Analisis statistik dilakukan dengan menggunakan uji One Way ANOVA.

Hasil

Ada perbedaan persentase rata-rata daerah iskemik otak tikus antara kelompok 3 (0%), kelompok 2 (3.13%) dan kelompok 1 (1,15%) (p value 0,001). Uji post hoc menunjukkan bahwa tidak ada perbedaan antara kelompok 3 dengan kelompok 1. Sebaliknya, ada perbedaan yang signifikan antara kelompok 2 dengan kelompok lain.

Conslusion

Penyediaan BSD berkurang otak tikus iskemik setelah ligasi arteri karotis bilateral.

Kata Kunci : tebu, iskemik otak, ligasi arteri carotis bilateral

**INTRODUCTION**

 Scientific research shows that sugarcane contains policosanol. Policosanol is a long-chain of alcohol compound. Pre-clinical trials on policosanol showed that the compound had the ability to reduce the incidence of stroke through the mechanism of inhibition of platelet aggregation and a decrease in blood cholesterol levels. (1,2) It was how the benefits of policosanol as a stroke therapy was still debatable. A clinical study showed that administration of policosanol immediately after the incident of ischemic stroke would reduce the subsequent ischemic stroke.(3) Giving 200 mg/kg policosanol dosage to Mongolian gerbils which were induced stroke by bilateral carotid artery ligation (BCAL) could reduce cerebral ischemia. (4)

 In addition to policosanol, sugarcane also contains antioxidants, especially kuercetin. In vivo, the ability of kuercetin in reducing cerebral ischemia was still debated. There were controversies about the ability of kuercetin to pass through the blood brain barrier. (5) In contrast, in vitro antioxidant compounds in sugarcane could reduce oxidative stress in human HepG2 cells. (6,7)

 Based on the fact that sugarcane contains policosanol and kuercetin , as well as the usage of black sugarcane decoction (BSD) as a stroke therapy in the community, the development of rat stroke model, and brain ischemia was produced by a 20-minute bilateral carotid artery ligation (BCAL), the researchers were interested to figure out the effect of BSD to decrease ischemic rat brain with BCAL.

**METHOD**

**Research design**

 This research applied quasi experimental design using post test for the control group. The study was conducted from September until November 2015 at the Pharmacy Laboratory, Isslamic University of Indonesia.

**Animal and experimental procedure**

 The animal used in this study were 18 male rats (Rattus norvegicus of the wistar strain) that had met the inclusion and exclusion criteria. The rats were reared in the Pharmacy Laboratorium, Inslamic University of Indonesia. Inclusion criteria for this study were helthy 3-month old male rats without any defect, of 175-250 g body weight. Determination of healthy rats was based on the physical state of the rats, i.e. those with clean, not wet or sticky bristles, active movements, and appropriate cycle of eating, drinking and sleeping. Exclusion criteria of this research were sick and dying rats during the study.

 During the 1stday until the 7thday, the experimental animals were located in cages for adaptation ( 40x20x20 ) cm3 . One cage was filled by 1 rat. The inside temperature was set at room temperature. Lighting was arranged with light-dark cycle for 12 hours. Light cycle was began at 06.00 am and dark cycle was started at 06:00 pm . Pellets were given every day in the morning at 06.00 am. Drinking water was provided ad libitum.

 Subjects were divided into three groups, of which each consisted of 6 rats. The description of the group are as follows:1. Group 1 was a BSD treated stroke model rats (rats of the stroke model with a sounding of BSD for 1 week before BCAL), group 2 was Non-treated stroke model rats, group 3 was sham operated rats (the same operation without BCAL). Brain ischemia was produced by a 20-minute bilateral carotid artery ligation (BCAL).

**BCAL procedure**

 BCAL was performed on the 15th day. Stages ligation is as follows: a. Anesthesia. During surgery, anaesthetize the rats using 80-100 mg / kg im ketamine. The rat is placed in a sterile platform and keep the rat rectal temperature at 37 ± 1 ° C. b. Disinfection stage. This stage aims to prevent infection. Swipe surgical are with betadine from center of surgical site to outside (anterior surface of the rat neck). c. Incision stage. Open the anterior neck with midline vertical incision. Dissect the underlying submandibular gland. Dissect the medial of right sternocleidomastoid muscle to expose the common carotid artery (CCA). Separated the arteries carefully from the vagus nerve and connective tissue. d. Ligation stage. Use a steryl nylon suture (cutgut) to make a 20-minute bilateral carotid artery ligation . e. After ligation is complete then given analgesic therapy ie 0,1 mL 0.25% bupivacaine, frequency of one time / day (analgesic recommended for rat stroke model).

**Preparation of BSD**

 The BSD was made based on modified Asikin’s protocol ( 2014 ) as follows : a. In 100 g fresh sugarcane peel contains 500 mg policosanol. Policosanol dose was 200 mg / kg / day / rat. (4) The sugarcane peel dose was 40 g / kg / day / rat. b .One rat requires 40 g / kg / day of fresh black sugarcane peel. The number of boiled sugarcane stalks were measured by the daily need of the solution .c . Before boiling, the black sugarcane could be stored in a temperature of 10 ° C. (8)

**Preparation of the brain**

 The rat brain tissue were taken at day 15, two hours post BCAL. Decapitation of rats were performed with a trancardial perfusion technique. Brain tissues were stained with TTC (2,3,5-triphenyltetrazolium chloride). TTC staining procedure is as follow : a. make 2% TTC in 1x PBS (i.e. 2 g TTC in 100 ml 1x PBS). B. cut the brain coronally plane at 2 mm thickness, c. Incubate the sliced brain in 2% TTC in the black boxes for 15-20 min, d. Carefully aspirate TTC solution and add fresh 10% PFA solution. TTC solution should be protect from light and kept in Room temperature. The part of rats brain that were studied comprised the cerebral cortex and striatum at the first slice of brain.

**Statistical analysis**

 Ischemic area was analyzed using Image J. Statistical analysis was performed by using One Way ANOVA test .

**Ethical clearance**

 This study was reviewed by the ethical clearance committee for preclinical research, Faculty of Medicine, Islamic University of Indonesia.

**Result**

 Ischemic brain area appears white in each group using image J software. Ischemic area results are expressed as a percentage, which is a ratio between the ischemic area and the total area of ​​the brain. The mean of percentage is analyzed using One Way Anova (table 1) and post hoc test (Table 2) .





Picture 1. TTC staining results. A. Sham operated rats, B. Non-treated Stroke Model Rats, C. BSD treated Stroke Model rats

Table 1.One way Anova

|  |  |  |  |
| --- | --- | --- | --- |
| Group | Mean (%) | SD | P Value ANOVA |
| Sham operated rats | 0 | 0 | 0,001\* |
| Non-treated Stroke Model Rats | 3,13 | 0,59 |
| BSD treated Stroke Model Rats | 1,15 | 0,47 |

 These results indicated that there were differences between the mean percentage of rat brain’s ischemic area between sham operated rats (0 %), Non-treated Stroke Model Rats (3.13%) and the BSD treated Stroke Model Rats (1.15 %) (p value 0.001) . Post hoc test showed that there was no difference between the percentage of area of ischemic brain of sham operated rats and the BSD treated Stroke Model Rats. Meanwhile, there was a significant difference between the group of Non-treated Stroke Model Rats and the other groups .

Table 2.*Post Hoc test*

|  |  |
| --- | --- |
| group | Sig. |
| Sham operated rats | Non-treated Stroke Model Rats | 0,015\* |
|  | BSD treated stroke model rats | 0,184 |
| Non-treated Stroke Model Rats | Sham operated rats | 0,015\* |
|  | BSD treated stroke model rats | 0,000\* |
| BSD treated stroke model rats | Sham operated rats | 0,184 |
|  | Non-treated Stroke Model Rats | 0,000\* |

**Discussion**

 TTC is a water-soluble dye. This compound will bind with the enzyme dehydrogenase and the cofactor NAD in the mitochondria. A healthy tissue is dark red whereas mitochondrial damages in ischemic areas of the brain are white because they will not be stained by TTC. (9) BCAL causes ischemic brain. The cells undergoing necrosis will swell. Intracellular organelle and plasma membrane will break, so some enzymes are going out to the plasma, one of them that going out is the lactate dehydrogenase (LDH). (10) The enzyme will lead to plasma so it can be measured. Compared to other enzyme, Lactate dehydrogenase is more sensitive to describe the incidence of ischemic brain. This enzyme can be used to assess the incidence of stroke. (11)

 Theories about increasing time of the enzyme levels in the blood ara vary. Eelevated levels of this enzyme are varies from 8 hours to several days after onset of stroke. Lactate dehydrogenase levels in brain tissue would reach the top of 48-120 hours post stroke. In the first hours of stroke there is a different levels of lactate dehydrogenase between brain cortex and the subcortical in ischemic lesions. (11)

 There was an increase in LDH activity under occlusion for 1 hour in the carotid artery.(12) There was an increase in the LDH activity in pyramidal neurons of the hippocampus and layers II, III and V of the brain cortex of the Mongolian gerbils after 7 minute of ischemia. LDH activities fickle, i.e. LDH levels began to appear at the 7th minute ligation, and declined in the first 2 hours post-ligation. This condition will become normal again 7 days after ischemia (reperfusion period). (13)

 The results show that a 20-minute of BCAL can cause ischemic area of ​​the cortex and striatum. TTC staining shows an ischemic area of brain of non-treated stroke model rats. The ischemic area occurrs after 2 hours post BCAL. The results support the other research, stating that bilateral carotid artery occlusion for 30 minutes and reperfusion for 1 hour led to global ischemic on Sprague-Dawley rats brain. The ligation of bilateral carotid arteries for 30 minutes and 1 hour reperfusion causes infarcted area on TTC staining. (9)

 Ischemic area of the BSD treated stroke model rats are smaller than non-treated stroke model rats. These results show that the black sugarcane can be used as neuroprotectant. Sugarcane is a source of antioxidants. (14) Phenolic concentration in molasses is 381 ug / g molasses. The phenolic compounds contain cathecin (16.42 mg / g molasses) and quercetin 3-O-glucosyl-xyloxide (25.27 mg / g molasses). Catechin functions as an antioxidant. (15) It is known that quercetin has anti-tumor, anti-thrombotic, anti-inflammatory and anti-apoptotic effect. Quercetin plays an active role in the cellular mechanisms act as the inhibitor for as phosphatidylinositol- 3 kinase, protein kinase C, xanthine oxidase and NADPH diaphorase, that makes quercetin has a neuroprotective effect. (5) High levels of these phenolic allow the use of these compounds in the prevention and treatment of diseases caused by oxidative stress because molasses is able to protect the cells, even more protective than the α-tocopherol. (6,7) Phenolic in the black sugarcane, can inhibit the lipid peroxidation on the brain so it can be used as neuroprotectant. (16)

 In addition, there are phenolic compounds derived from sugar cane wax called D- 003 . These compounds have antioxidant properties. The compound contains a octacosanoic compound (C28). D - 003 is able to inhibit plasma lipid peroxidase. (17,18) Toxicity test of sugarcane molasses proven that the compound is safe because there is no increase in levels of LDH. (19)

 The results are consistent with research by Molinga et al . 1999. Provision of policosanol 200 mg / kg in stroke-induced-Mongolian gerbils can reduce cerebral ischemia. (4) The giving D - 003 200 mg / kg can reduces cerebral edema and clinical signs in Mongolian gerbils with ligation of bilateral carotid artery. (18)

 Limitation of this study is the use of cutgut threads to tie the bilateral carotid arteries. It is necessary to ensure that the bond strength equal to each other. The results of this study can be used as a scientific basis for the community that the black sugar cane can be used as a neuroprotektan to prevent stroke. Further research is necessary to determine the therapeutic dose, toxicity test of black sugarcane decoction, and development of ligation technique using a clamp artery.

**Conclusion**

The provision of BSD reduced ischemic rat brain after BCAL .

**Conflict of interest**

Competing interests: No Relevant disclosures

**Acknowledgement**

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