Candlenut leaf methanol extract induces re-endothelialization in high-fat diet/streptozotocin-induced rats

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Abstract. This study aimed to investigate the effect of candlenut (Aleurites moluccana) leaf extract on late endothelial progenitor cells (EPCs) and endothelial cells in high-fat diet/streptozotocin-induced rats. Twenty-five male Wistar rats were randomly divided into 5 groups. A normal control group was fed a standard diet and injected with citrate buffer. A positive control (HFD/S) group was fed a high-fat diet for 4 weeks, then injected with streptozotocin. Three groups were fed a high-fat diet and injected with streptozotocin, then treated with candlenut leaf extract in concentrations of 100, 200, or 400 mg/kgBW for 4 weeks. The percentage of late EPCs (CD34⁺/CD133⁻ cells) was determined by flow cytometry. The tail artery histopathology sections were stained with hematoxylin-eosin, then the number of endothelial cells was counted. The administration of a high-fat diet and streptozotocin in rats significantly reduced the percentage of late EPCs in peripheral blood circulation and the number of tail artery endothelial cells compared to the normal controls. Candlenut leaf extract supplementation in concentrations of 200 and 400 mg/kgBW significantly increased the percentage of late EPCs and the number of endothelial cells compared to the HFD/S group. It is concluded that candlenut leaf extract can promote re-endothelialization in high-fat diet/streptozotocin-induced rats.

1. Introduction

Diabetes mellitus is a metabolic disease characterized by increased blood glucose (hyperglycemia) occurring due to abnormalities in insulin secretion, insulin action, or both. [1] Based on the World Health Organization (WHO) data, globally in 2014, an estimated 422 million adults were living with diabetes compared to 108 million in 1980. [2] The cost of diabetes to the UK National Health Service (NHS) is £9.8bn in direct costs in 2010/11 with £1bn for type 1 and £8.8bn for type 2 diabetes. About
80% of the costs are spent on treating complications. The biggest costs for complications are for excess hospitalization, cardiovascular disease also damaged kidneys and nerves. [3, 4]

Diabetes is associated with endothelial cell dysfunction and impaired neovascularization and repair mechanism of the body. [5] Endothelial dysfunction contributes to the pathogenesis and clinical manifestation of atherosclerosis in diabetes, leading to cardiovascular morbidity and mortality. It has been suggested that the improvement of endothelial dysfunction is able to prevent the development and progression of atherosclerosis [6, 7].

Endothelial progenitor cells (EPCs) play an important role in vasculogenesis, angiogenesis, and repair of damage. EPCs, which are a circulating cell population originating from the bone marrow, have an ability to restore damage caused by diabetes. [5] However, decreased EPCs number and function have been reported in patients with diabetes and other diabetes-related disorders. [8, 9] The common markers for EPCs are CD31, vascular endothelial growth factor receptor 1/2 (VEGF R1/2), Flk1, vascular endothelial cadherin (VE-cadherin), von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), CD34, CD133. [5] In this study, we evaluated CD34+/CD133+ cells as the marker for late EPCs. [10]

Candlenut tree (Aleurites moluccana L.) is a native plant to Indonesia and Malaysia. One of the important flavonoids contained in candlenut leaf is swertisin. [11] Swertisin from various other plants has been shown to have a hypoglycemic activity. [12, 13] Candlenut leaves containing swertisin have also been proved to have an anti-inflammatory activity, [14, 15, 16] and can promote wound healing. [17] This study aimed to evaluate the effect of candlenut leaf extract on the percentage of late EPCs in peripheral blood circulation and the number of tail artery endothelial cells in high-fat diet/streptozotocin-induced rats. The combination of a high-fat diet and multiple injections of low-dose streptozotocin has been proved to induce type 2 diabetes in rats. [18]

2. Materials and methods

2.1. Plant materials and extraction

Candlenut leaves were obtained from old trees, marked by lanceolate, dark green leaves, with > 20 cm long. The leaves were obtained from candlenut plantations in the West Nusa Tenggara region. The extraction process was performed according to the method of Quintão et al. (2012) with modifications. [19] Briefly, the leaves were washed with water, then dried up for 3 days, and mashed using a blender. The powder was macerated with 70% ethanol for 24 hours (x3) with continuous shaking. The filtrate was collected. The solvent was evaporated using a rotary evaporator under vacuum condition at 45°C. The crude extract obtained was stored in a closed glass bottle and coated with aluminum foil at -20°C until used.

2.2. Animals

Twenty-five male Wistar rats, 10 weeks old, weighing 180-200 grams were housed in clean wire cages in a standard laboratory condition (dark/light cycles 12 h/12 h, 25 ± 2°C). Rats were provided with standard diet and tap water ad libitum and acclimatized in the laboratory condition for 7 days before treatment. All experimental protocols were approved by the ethics committee of the Faculty of Medicine, Universitas Brawijaya (No. 246/EC/KEPK/06/2016).

Rats were randomly divided into five groups. A normal control group (N) was fed a standard diet and injected with citrate buffer (vehicle). A positive control group (HFD/S) was fed a high-fat diet and injected with low-dose streptozotocin. Three treatment groups were fed a high-fat diet and injected with low-dose streptozotocin, then given with candlenut leaf extract in concentrations of 100, 200, or 400 mg/kgBW (E100, E200, and E400, respectively) for 4 weeks. The extract dosages were determined based on the preliminary study (unpublished data).

2.3. Experimental protocols

2.3.1. Induction a high-fat diet and low-dose streptozotocin. Rats were fed a high-fat diet with a composition of 22% fat, 48% carbohydrate, 20% protein, and calories 44.3 kJ/kg. After 4-week high-
fat diet, rats were intraperitoneally injected with low-dose streptozotocin (Bioworld, Dublin, OH, USA) at a dose of 25-30 mg/kgBW. A week after injection, fasting blood glucose (FBG) levels were measured by a glucose oxidase method using a glucometer (Accu-Chek Active, Roche Diagnostics Limited, Germany). The blood samples used were collected from rat tail vein. Rats with FBG levels < 7.8 mmol/L were injected once again with streptozotocin at the same dose. The normal control group was injected intraperitoneally with the vehicle, citrate buffer (0.1 M, pH 4.4). Four weeks after the second injection, rats with FBG levels > 7.8 mmol/L were used in this study. [18]

2.3.2. Tissue sampling. At the end of the study period, rats were sacrificed under anesthesia with an intraperitoneal injection of ketamine at a dose of 40 mg/kgBW, then exsanguinated by cardiac puncture. The EDTA-blood was collected for isolation of the circulating EPCs. The tail artery was removed, fixed with 10% buffered formalin, and embedded in paraffin for histopathological evaluation. [20]

2.3.3. Isolation of circulating EPCs. The isolation of circulating EPCs was performed according to the method of the previous study. [20] Briefly, mononuclear cells (MNCs) were isolated using Histopaque 1.083 g/ml (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation (1600 rpm, 20°C, 30 min.). Isolated cells were then washed three times with Hanks’ balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA). CD34+/CD133+ cells were evaluated by immunostaining. Antibodies used were PE-conjugated anti-mouse CD34+ monoclonal antibody (BioLegend, London, UK) and FITC-conjugated rabbit anti-CD133 polyclonal antibody (Bioss Inc., Woburn, Massachusetts, USA). Cells were then detected by flow cytometry (BD FACSCalibur™ Flow Cytometer; BD Biosciences, San Jose, CA, USA).

2.3.4. Tail artery endothelial cells count. Hematoxylin-eosin (HE) staining was carried out on tail artery histopathology, then observed with a light microscope at 400x magnification, and scanned with Olyvia scan dot slide software. The total number of endothelial cells was calculated in the whole tail artery histopathology sections. Observations and calculations were carried out by two trained observers to ensure objective results.

2.3.5. Statistical analyses. Data are presented as the mean ± S.D. Data were analyzed with SPSS 22.0 statistical package for Windows. The significance of difference between groups was determined by one-way analysis of variance (ANOVA) and least significant difference (LSD) post-hoc test. The p-value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Percentage of late EPCs in peripheral blood circulation

Based on statistical analysis, it was found that the administration of a high-fat diet and low-dose streptozotocin in rats reduced the percentage of late EPCs in peripheral blood circulation significantly (p = 0.01) compared to the normal control group. The supplementation of candlenut leaf extract in concentrations of 200 and 400 mg/kgBW increased the percentage of late EPCs in peripheral blood circulation significantly (p < 0.05) compared to the HFD/S group (Figure 1).

3.2. Tail artery endothelial cells

It was known that the administration of a high-fat diet and low-dose streptozotocin in rats reduced the number of tail artery endothelial cells significantly (p = 0.03) compared to the normal group. Candlenut leaf extract supplementation in a concentration of 100, 200, and 400 mg/kgBW increased the number of tail artery endothelial cells significantly (p < 0.05) compared to the HFD/S group, approaching the normal value (p > 0.05) (Figure 2).
Figure 1. Late EPCs in peripheral blood circulation. A. The isolated mononuclear cells were analyzed by flow cytometry. The ordinate and abscissa of graphs show the fluorescence intensity of CD34$^+$ and CD133$^+$ mononuclear cells. The number in lower right quadrant shows the percentage of CD34$^+$/CD133$^-$ cells (late EPCs). B. Bar graph represents the percentage of CD34$^+$/CD133$^-$ cells (late EPCs). Values are expressed as mean ± S.D. of 5 rats in each group. N: A normal control group; HFD/S: A positive control group given a high-fat diet and low-dose streptozotocin; E100, E200, and E400: Groups given a high-fat diet and low-dose streptozotocin, then administered with candlenut leaf extract in concentrations of 100, 200, and 400 mg/kgBW. Different notation shows significant difference ($p < 0.05$).

The administration of a high-fat diet and multiple injections of low-dose streptozotocin in rats can induce type 2 diabetes. [18, 21] A high-fat diet can induce insulin resistance. While streptozotocin can damage the DNA of pancreatic β-cell leading to insulin deficiency condition. [18] In the condition of insulin resistance, the selective inhibition of the PI3K/Akt pathway is found in skeletal muscles of obese people and subjects with type 2 diabetes, [22] also in the vessel and myocardium of obese Zucker rats. [23] This inhibition leads to endothelial dysfunction. [6, 24]

In addition, the condition of chronic hyperglycemia results in a rapid production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), causing increased microvascular permeability. [25, 26] The increase in pro-inflammatory cytokine levels also causes down-regulation of eNOS and apoptosis in endothelial cells. [27] This is in line with the result of the study, in which the number of tail artery endothelial cells in HFD/S group was significantly decreased compared to the normal controls.

In this study, the percentage of late EPCs in peripheral blood circulation in HFD/S group was significantly reduced compared to the normal controls. The result is similar to other previous studies. [8, 9] Circulating EPCs reduction in diabetic patients can reflect a shortened peripheral survival of EPCs or a poor mobilization of EPCs from the bone marrow. [8] Furthermore, a study proved that the eNOS activation is impaired in diabetes resulting in poor mobilization of EPCs. [28] This condition causes EPCs to lose the ability to restore endothelial cells damage caused by diabetes.
Figure 2. Tail artery endothelial cells. A. The representative micrographs of the hematoxylin-eosin stained tail artery from 5 independent experiments in which the same results were obtained are shown (HE, x400). Black arrow: endothelial cells. B. Bar graph represents the tail artery endothelial cells count. Values are expressed as mean ± S.D. of 5 rats in each group. N: A normal control group; HFD/S: A positive control group given a high-fat diet and low-dose streptozotocin; E100, E200, and E400: Groups given a high-fat diet and low-dose streptozotocin, then administered with candlenut leaf extract in concentrations of 100, 200, and 400 mg/kgBW. Different notation shows significant difference (p < 0.05).

In this study, the supplementation of candlenut leaf extract significantly elevated the percentage of late EPCs in peripheral blood circulation and the number of tail artery endothelial cells compared to the HFD/S group. These effects are suspected to be caused by flavonoid contained in the extract, namely swertisin, which has a hypoglycemic activity. [11, 19] Swertisin isolated from other plants has been proved to inhibit α-glucosidase [13, 29, 30] and aldose reductase. [13] Swertisin also stimulates islet differentiation, [31] and has the potential to stimulate insulin secretion. [32] Moreover, it has been proved that candlenut leaf methanol extract has a hypolipidemic activity in rats. [33] In addition, swertisin in candlenut leaf extract has been proved to have an anti-inflammatory activity, [14, 15, 16, 34] and can stimulate wound healing [17].

4. Conclusion
Thus, candlenut leaf extract was able to improve the number and function of EPCs, leading to endothelial cells regeneration in high-fat diet/streptozotocin-induced rats. Thus, it can be concluded that candlenut leaf extract has the potential to promote re-endothelialization in type 2 diabetes.

Conflicts of interest
The authors declared that there was no potential conflict of interest relevant to this article.
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