Beneficial Effect of Ethanol Extract from Black Rice on Endothelin-1 Expression and Histological Structure of Heart during Pre-eclampsia

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ABSTRACT. In the case of pre-eclampsia, abnormality form also be found in the heart, which is later can be diagnosed as a particular symptom, hypertension. Endothelin-1, potential vasoconstrictor derived from heart, responsible to the mechanism of indirect impairment utero-spiral arteries into development of generalized endothelial dysfunction. This study was designed to identify the effect of ethanol extract from black rice in pre-eclamptic model rat, specifically related to its ability to maintain physiological activity of cardiovascular system. In this study, animal model was induced by multi-dose of tryponil sulphate 70mg/kgBW at 9th, 10th, 11th day of pregnancy, and the groups were devided into negative and positive control, treatment groups: P1, P2, P3 differed by ethanol extract dosages (12,5mg/kgBW, 25mg/kgBW, 50mg/kgBW respectively). Treatment started from day 12th to 18th of pregnancy, continued by statistical analysis to examined endothelin-1 immunohistochemistry expression using one way ANOVA and Tuckey-Test. This study indicated both the lowest expression of endothelin-1 and the normal structure of heart myofibrils, after histological examination, were identically displayed at P3 (50mg/kgBW ethanol extract) among groups. According to this study, we suggested that the ethanol extract of black rice possibly has certain compound either directly or indirectly induced cardiovascular changes during pre-eclamptic condition.

Keyword: ethanol extract, black rice, pre-eclampsia, endothelin-1
1. INTRODUCTION

Preeclampsia is a disease with clinical symptoms such as hypertension and proteinuria commonly found after 20 weeks of gestation. Indonesia Demographic and Health Survey (IDHS) estimates number of maternal mortality in Indonesia based on its causes during 2010 subsequently were 3,114 cases (27%) caused by bleeding, 2,393 cases caused by preeclampsia/eclampsia and 2,653 cases (23%) infections and 1,268 cases (11%) caused by infections (Hernawati, 2011). Preeclampsia causes the lower baby birth weight and mostly born premature. (Rahmayanti, 2010).

Preeclampsia is often referred to as "disease of theories" because currently the pathogenesis of preeclampsia is not yet well understood comprehensively. Tryponil sulphate can be used as an induction in order to obtain animal models of preeclampsia (Oktanella et al, 2017). It acts as an inhibitor of angiogenesis and is antagonistic to several growth factors, namely VEGF and PDGF which affect the placentation process (Nash, 2007) by elevate the level of sFlt-1 in maternal placenta.

Immunological adaptation failure during placental development can cause endothelial damage, one of which is due to the release of free radicals. The event was followed by the release of substances that are vasoconstrictive (endothelin-1) and the reduction of vasodilatating substances (Salindeho, 2014). Endothelin-1 is a peptide that acts as a very strong vasoconstrictor, even 10 times stronger than angiotensin II (Salindeho, 2014). This peptide is produced by endothelial and epithelial cells, macrophages, fibroblasts, and many other cell types, including cardiac myocytes (Susanti, 2011).

Conventional reatment using drugs (nifedipine, MgSO₄, etc) still be considered have negative influence both on the baby and the mother. Alternative therapies which are basically utilizing natural ingredients are chosen to minimize the mentioned side effects.

Black rice (Oryza sativa L.indica) has a pericarp, aleuron and endosperm that are dark red-blue-purple, the color indicates the presence of anthocyanin content. The content of anthocyanin in black rice ranges from 159-359 mg / 100 g. (Sa'adah, 2013). Anthocyanin is included as a flavonoid compound as an alternative medicine as an antihypertensive which has the ability to inhibit the activity of Angiotensin Converting Enzyme (ACE) (Hladunewich, 2007).

Based on this, the study was conducted with the aim to determine the effect of ethanol extract of black rice on endothelin-1 expression and histopathological features of the heart organ in preeclampsia model mice.

2. MATERIAL AND METHOD

2.1. Animal model of pre-eclampsia

Animal model used in this study aged 3 months with average body weight ranging from 150-200 grams. Experimental groups were divided into five: control group, positive group, preeclampsia group (P1, P2, P3) with consecutive dosages of ethanol extract of black rice are: 12.5 mg/kg body weight, 25 mg / kg body weight, and 50 mg / kg body weight.

Multiple low dose of tryponil sulphate (70mg / kg BW) was used as drug-induced pre-eclamptic condition, injected intraperitoneally on 9th-11th days (Nash, 2007). Blood-tension and urinalysis were performed before and after tryponil sulphate injection, higher blood-tension and proteinuria were performed before and after tryponil sulphate injection, higher blood-tension and proteinuria positive indicated that the animal model of pre-eclampsia has been successfully established.

2.2. Ethanol extraction

Black rice is mashed into powder then macerated with 96% ethanol. Then stir using a stirrer with a speed of 300 rpm for 4 hours then allowed to stand for up to 24 hours. After 24 hours, maserat filtered. Maserat was weighed and the extract immersion was calculated (Maulida, 2015). The test was continued with Liquid Chromatography and Mass Spectometer (LC-MS) to assure that black rice extract contained anthocyanin. Black rice ethanol extract was given by oral administration using gastric sonde on pregnancy day 12th-17th.
2.3. Histological Examination
Myocardial tissue was fixed in 10% neutral buffered formalin for 48 h. Fixed tissue was processed routinely and embedded in paraffin. Paraffin sections (5 μm) were cut and mounted on glass slides and stained with Hematoxylin and Eosin (H&E) and Masson’s trichrome stains and examined under a light microscope. Myofibril of heart was analyzed using H&E-stained sections and cardiac fibrosis was analyzed using Masson’s trichrome stain with the help of ImageJ software.

2.4. Analisa Data
All values were expressed as the mean ± SE. One-way analysis of variance test followed by Bonferroni’s correction was carried out to test for any differences between the mean values of all groups. Differences between groups were assumed as significant if p < 0.05.

3. RESULTS AND DISCUSSION

3.1. Endotelin-1 Expression
Endothelin-1 is a very strong vasoconstrictor derived from peptides (Salindeho, 2014). In the case of preeclampsia, endothelin-1 plays an important role in controlling maternal blood-pressure, if preeclampsia occurs then endothelin-1 will increase continuously so that blood pressure will be higher. The results of endothelin-1 expression in preeclampsia model mice that have been given ethanol extracts in black rice are presented in (Figure 1). Endothelin-1 expression is indicated by the presence of brownish color expression in the cytoplasm of endothelial cells in the endocardial lining of the heart. The brown color shows a bond between anti-endothelin-1 antibodies and endothelin-1 protein.

Figure 1. Endothelin-1 expression in preeclampsia model heart (Rattus norvegicus) using magnification 400x.
Description: A (normal mouse); B (preeclampsia mice); C (P1-therapeutic dose of black rice ethanol extract 12.5 mg / kg BW); D (P2-therapeutic dose of black rice ethanol extract 25 mg / kg BW); E (P3-therapeutic dose of black rice ethanol extract 50 mg / kg BW)

This is in accordance with Table 1, the mean expression of endothelin-1 in the heart of preeclampsia model which had been evaluated using immunoratio. Groups A, B, C, and D indicate different notations, this indicates that preeclampsia therapy using ethanol extract of black rice has a different effect at each dose.
| Groups | Average Endotelin-1 expression (%)±SD |
|--------|--------------------------------------|
| CN     | 23,0650 ± 2,507^a                     |
| PC     | 70,8775 ± 6,004^d                     |
| P1     | 55,1075 ± 2,372^c                     |
| P2     | 44,3675 ± 3,443^b                     |
| P3     | 23,7800 ± 1,071^a                     |

**Note:** notations a, b, c, and d indicate a significant difference between treatments (p <0.05)

The highest level of average Endotelin-1 expression among all groups can be found in positive control group. This result assure that multiple low dose of tryponil sulphate induction can cause the condition of preeclampsia. Tryponil sulphate induction will inhibit remodeling of the spiral arteries that occur at that time. Spiral artery remodeling is an invasion of the trophoblast into the muscle layer by the spirals causing degeneration of the muscular layer resulting in distension and vasodilation of the spiral arteries which results in a decrease in blood pressure. (Winkjosastro, 2002).

In preeclampsia remodeling failure occurs, causing the spiral arteries to become stiff and hard so that the spiral arteries do not experience distension and vasodilation so that utero placental blood flow decreases and hypoxia and placental ischemia occur (Nhofer and Stepan, 2008). An ischemic placenta stimulates the formation of free radicals, the hydroxyl radical (-OH) which is considered a toxin. Hydroxyl radicals damage the cell membrane to fat peroxide and then will damage the nucleus and endothelial cell proteins, causing endothelial dysfunction (Cunningham, 2010). Endothelial dysfunction causes a decrease in vasodilator (NO), as well as an increase in vasopressor materials such as endothelin-1. The increase in endothelin-1 as a strong vasoconstrictor causes vasocostriction in the blood vessels so that it increases the blood pressure in the rat model of preeclampsia.

In groups C and D showed different notation with group A, indicating that the therapy with a dose of 12.5 mg / kg BW (C) and 25 mg / kg BW (D) has not significantly decreased endothelin-1 expression. Group E shows the same notation as group A, so it can be said that group E with a therapeutic dose of 50 mg / kg body weight is the optimum dose.

Decreased endothelin-1 expression is caused by anthocyanins that will accumulate into endothelial cells with target cell membranes so as to protect endothelial cells from the influence of free radicals and balance extra cell and intra-cell fluids, then maintain Nitric Oxide (NO) as a strong vasodilator and reduce endothelin-1. (Kusumastuti, 2014). A decrease in endothelin-1 will cause blood vessels to undergo vasodilation, causing a decrease in blood pressure.
3.2. Heart Histology

The results of the study of the effect of ethanol extract of black rice (Oryza sativa L. indica) on the histopathological picture of the heart organ using the Hematoxylin Eosin (HE) staining method are presented in Figure 2. Group A looks normal in myocyte and myofibril forms marked with clear boundaries between cells, myocytes have a nucleus located in the middle and myofibrils or regular heart muscle fibers (Kuehel, 2003).

In group B the positive control showed a picture of the heart experiencing preeclampsia, namely myocyte damage marked by a change in myocyte nucleus to picnosis with a dark blue myocyte nucleus and a shrinking of the nucleus which is the beginning of necrosis, damage from irregular myofibril structure and marked hypertrophy with thickening of myocytes (Widyanti, 2012). Core shrinkage results from DNA condensing into a solid mass and causing an increase in acid in the nucleus so that it can easily attract hematoxylin eosin dyes. The core that experiences picnotics can turn darker in color. Suramin is negatively charged so it is easy to bind to proteins. In this case suramin binds to blood plasma protein, then it will be secreted through the kidneys in the form of urine. Suramin gives glomerular dysfunction effect so that it will increase renin secretion from kidney.

Rennin increase in the kidneys will stimulate the formation of angiotensinogen which will then turn into angiotensin I and then by ACE will be changed to angiotensin II as a vasoconstrictor. When angiotensin II is formed, it will stimulate preproendothelin, which then by the enzyme endopeptidase is converted to proendothelin and subsequently by Endothelin Converting Enzym (ECE) is converted into endothelin-1 as a strong vasoconstrictor and an increase in aldosterone and antidiuretic hormone.

Aldosterone regulates the volume of extracellular fluids, aldosterone will reduce NaCl excretion by increasing reabsorption in the kidney tubules while antidiuretic hormone will result in less urine being excreted outside the body so that urine becomes concentrated. The increase in NaCl concentration and urine concentration will be diluted again by increasing the volume of extracellular fluid which in turn will increase the volume and blood pressure (Keman, 2014).

Hypertension will trigger high ROS production, causing lipid peroxidation. Lipid peroxidation will form in long chains that cause damage to the organization of cell membranes resulting in damage to the structure and function of heart cell membranes (myocytes). Increased blood pressure after suramin induction causes ventricular remodeling by increasing the number of sarcomeres in myocardial cells, resulting in an increase in ventricular wall thickness (hypertrophy).

Group C which was given therapy of ethanol extract of black rice at a dose of 12.5 mg / kg BW showed a histopathological picture approaching group B, namely positive control, namely the nucleus of picnotic myocytes, myocytes with no clear borders, the presence of myocyte hypertrophy, and irregular myofibril structure. Group D with the administration of ethanol extract of black rice 25 mg / kg BW showed a change that is a decrease in the myocyte nucleus that is experiencing hypnotism but there is still a myocyte hypertrophy. Changes in cardiac histopathology in group E with the treatment of ethanol extract of black rice at a dose of 50 mg / kg BW were close to group A, namely by decreasing the number of nuclei that experienced picnotics, as well as myofibril structure and myocyte thickness which were close to normal.
Figure 2  Histopathological picture of the myocardial heart organ in the ventricular sinister rat (Rattus norvegicus) model of preeclampsia with a 400x magnification

Description: A (normal mouse); B (preeclampsia mice); C (therapeutic dose of black rice ethanol extract 12.5 mg / kg BW); D (therapeutic dose of black rice ethanol extract 25 mg / kg BW); E (therapeutic dose of ethanol extract of black rice 50 mg / kg BW). Normal myocyte nucleus (IN), picnotic myocyte nucleus (IP), myofibrils (MF) and hypertrophy (H).

Repair of heart muscle cell damage in groups C, D, and E caused by the content of anthocyanin contained in black rice can reduce blood pressure by
inhibiting ACE so that angiotensin I cannot be converted to angiotensin II, where angiotensin II functions to increase the activity of the sympathetic nervous system, vasoconstriction of vascular smooth muscle and increase water and sodium retention. (Kusumastuti, 2014). The absence of angiotensin II results in preproendothelin not being formed so that endothelin-1 is also not formed. When angiotensin II and endothelin-1 cannot be formed it causes vasodilation of arterioles in the circulatory system so that it can reduce blood pressure then the heart load will decrease. The response to a decrease in blood pressure is regression of hypertrophy by decreasing the number of sarcomeres in myocardial cells and then there is no ventricular wall thickness (hypertrophy). The myocyte nucleus undergoing picnosis undergoes repair through cell regeneration. Stem heart cells will carry out mitosis to maintain stem cell populations and form heart muscle cells, smooth muscle cells and endothelial cells, leading to the formation of new capillary vessels and increased heart function that can restore heart tissue to normal. The effect of therapeutic dose was proven in group C dose 12.5 mg / kg body weight and group D dose 25 mg / kg body weight had not been able to improve the histopathological picture in the rat heart (Rattus norvegicus) preeclampsia model, while a dose of 50 mg / kg body weight in group E was seen the repair of heart muscle cells that resemble group A is characterized by regular myofibril structure and reduced cell necrosis.

CONCLUSION
Treatment of black rice ethanol extract in preeclampsia mice can reduce endothelin-1 expression and improve the histopathological changes of rat heart.

SUGGESTION
In further studies, it is necessary to investigate the therapeutic effect of ethanol extracts of black rice (Oryza sativa L.indica) on organs and other parameters that support the condition of preeclampsia for curative and preventive efforts.

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