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Effect of Sweet Purple Potato Extract (*Ipomoea batatas* L) on Zebrafish (*Danio rerio*) by Diet Induced Obesity

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KEYWORDS

Sweet purple potato Extract (*Ipomoea batatas* L.)
Zebrafish (*Danio rerio*)
Diet induced obesity

Abstract The effect of a high-fat diet on zebrafish as an alternative experimental animal in studying lipid metabolism is not fully understood. In this research, sweet purple potato extract (SPPE) which has anthocyanin have used as an anti-obesity property. Zebrafish aged 8 mpf was divided into five groups: Non-DIO (control negative), DIO (Diet-Induced Obesity, control positive), and three experimental groups (DIO A, DIO B, DIO C). Each group consisted of five Zebrafish. All groups have been fed freshly hatched Artemia for 40 days where the Non-DIO's amount was 5 mg Cyst/fish one time per day, and the other groups' amount was 60 mg Cyst/fish three times per day. The experimental groups were exposed to sweet purple potato extract three times per day by different SPPE doses: DIO A by 80 ppm, DIO B by 120 ppm, and DIO C by 160 ppm. The results show that there was no significant difference between the positive control group and the experimental groups in term of feeding intake, BMI, blood glucose, and total cholesterol. However, based on molecular parameters, there was a declining trend on PPAR-γ level, although, the adiponectin level remained stable. We suggest that anthocyanin does not affect feed intake, BMI, blood glucose level, cholesterol, and expression of PPAR-γ and adiponectin of zebrafish with diet-induced obesity.

Introduction

Obesity is a chronic metabolic disease that is a high-risk factor for the incidence of diabetes, dyslipidemia, stroke, cardiovascular diseases such as cardiomyopathy, hypertension, coronary heart disease, heart failure, and arrhythmias and osteoarthritis (Hruby & Hu, 2015; Poirier Paul et al., 2006). Obesity causing by an imbalance between energy intake and chronic energy use is influenced by many factors, including environmental factors, diet, eating behavior, and physical activity (Cecchini et al., 2010; Hruby & Hu, 2015). Adipocyte or adipogenesis maturity is influenced by transcription factors such as LPL (lipoprotein lipase), C/EBP (CCAAT/enhancer-binding protein), and PPAR (peroxisome proliferator reactivated receptors) (Ntambi & Young-Cheul, 2000). In general, PPARs plays an essential role in energy homeostasis and obesity development. PPAR-α and PPAR-δ are essential for lipid breakdown, while PPAR-γ works for lipid accumulation and adipogenesis (Den Broeder et al., 2015). In fish and mammals, PPAR-γ is the primary regulator in adipogenesis, where its functional shift may increase the risk of diabetes and cardiovascular disease (Wafer et al., 2017). In zebrafish, PPAR-γ signaling pathway controls lipid metabolism, including lipid uptake, transport, storage, and disposal (Walczak & Tontonoz, 2002). PPAR-γ expression quantity in zebrafish is almost the same as PPARs-α, especially in the liver hepatocytes (Ibabe et al., 2005). Therefore, it is crucial to measure the expression of PPAR-γ concerning the influence of

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SPPE. Adipocyte tissue secretes several mediators named adipokines, such as adiponectin, leptin, and resistin (Graf et al., 2013). Anthocyanin is commonly found in plants such as cherry, raspberry, blackberry, blueberry, red corn, eggplant, tomato, purple sweet potato, red yam, and others (Oancea & Oprean, 2011). Anthocyanin itself has several biological activities, for example as antioxidants (Kähkönen & Heinonen, 2003), anti-inflammatory (Wang & Mazza, 2002; Youdim et al., 2002), anti-atherosclerosis (Fuhrman et al., 2005; Xia et al., 2005), anti-diabetic (Grace et al., 2009; Nizamutdinova et al., 2009; Rojo et al., 2012; Jang et al., 2013), and anti-obesity (Lee et al., 2014). Consuming these substances may normalize adipocyte hypertrophy induced by high-fat foods because of the increase of lipoprotein lipase activity in skeletal muscle, the decrease of visceral adipose tissue, and the suppress of lipid accumulation in adipocytes by extensive inhibition of transcription factors that regulate lipogenesis (Kim et al., 2012; Tsuda et al., 2004). Zebrafish are increasingly used as models of human diseases because their organs and tissues show similarities in terms of structure and functions. Moreover, they are easy to manipulate genetically and easy to breed and test. In lipid metabolism research, zebrafish has similarities to those of human in lipid metabolism by validating the anti-obesity effects of natural products (Hasumura et al., 2012; Hölttä-Vuori et al., 2010; Meguro et al., 2015; Oka et al., 2010, 2010; Stewart et al., 2012, 2010; Tainaka et al., 2011). The aim of this research is to examine the effects of ethanolic extract from sweet purple potatoes on zebrafish induced by a diet of obesity.

Materials and methods

Ethical Approval

This research conformed to the ethical guidelines No. 291/EC/KEPK/07/2016 established by the Ethical Commission of Health Research of Medical Faculty of Universitas Brawijaya, Malang Indonesia. Anthocyanin Extract: Anthocyanin was extracted from the sweet purple potato of Gunung Kawi, East Java, Indonesia using ethanol 96% maceration. The sample was macerated for 14 hours, with 0.01% HCl in ethanol. The extraction was continued by using a chromatography column with polyamide CC-6 resin, then applied water and ethanol as eluent. The result of anthocyanin was 0.132g / 100g of samples (fresh weight). Antioxidant capacity was measured by DPPH, IC50, and LCMS spectrum methods (Sujadmoko, 2013).

Diet Induced Obesity

Artemia *nauplii* (newly hatched brine shrimp) was given to all groups, Non-DIO group was 5mg/day/fish, and the other groups were 60mg/day/fish. Artemia containing high calory amount, 22% of fat, 16% of carbohydrate, and 44% of protein, is an excellent feed to induce obesity (Oka et al., 2010). Each mg of Artemia contains approximately 5 calories, with around 80% of the feed was consumed by Non-DIO's fish, and DIO's groups consumed 50% of the feed. So that the average number of calories intake in Non-DIO group was 20 cal/day, while in DIO groups were 150 cal/day (Oka et al., 2010).

Zebrafish

In this research, 8 mpf wild type zebrafish was provided by the Laboratory of Pharmacology, Faculty of Medicine, Universitas Brawijaya. Initially, zebrafish were acclimatized under 60L aquarium with neutral pH, 25-29°C range temperature, aerated, and filtered water (Reed and Jennings, 2011) for one week. Zebrafish were divided into five groups (5 fishes/group): Non-DIO, DIO, DIO A (DIO+80 ppm SPPE), DIO B (DIO+120 ppm SPPE), and DIO C (DIO+160 ppm SPPE), each group under 3.4L aquarium. Non-DIO was fed one time per day, and the other groups were fed three times per day. DIO A, DIO B, and DIO C were given anthocyanin extract three times a day.

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Food Intake

Food intake was measured in day 39, three times a day by the same person (Hasumura et al., 2012; Meguro et al., 2015). The method was carried out: Non-DIO group was fed with artemia 5 mg/fish/day, and the other groups were fed with artemia 60 mg/fish/day. Then, 15 minutes later, all experimental groups (DIO A, DIO B, and DIO C) were given with SPPE in three different dosages: 80 ppm, 120 ppm, and 160 ppm consecutively. In 105 minutes after that, all fishes were transferred to the newly washed aquarium using fish net carefully. The old aquariums that still contained water, feeding residue, and fish's feces then left until the feces had been precipitated in the base of the aquariums (Ostrander, 2000)**.** The feces then siphoned manually by pipette and placed to a different container (Ostrander, 2000). After that, the feeding residues had taken from the aquarium by 120 µm plankton net (three times filtration). Then the feeding residues inside the plankton placed to the watch glass for measuring the weight by analytical balance. The total weight of watch glass and feeding residue then reduced by watch glass weight, and that was the real weight of feeding residue. Food intake of zebrafish was the result of initial feeding weight reduced by feeding residue weight.

Body Mass Index

Body mass index measurement has been conducted in every week: day 0, 14, 20, 27, 34, and 40 on every fish in each group (Hasumura et al., 2012). The equipment for BMI measurement were calipers, calibrated analytic balance, an empty watch glass, and petri dish filled with an anesthetic solution (Tricaine 0/168 mg/ml) (Westerfield, 2007). Fish was caught using fishnet then carefully placed into petri dish until it did not respond to an external stimulus. The fish length was measured from the tip of the head to the tip of the tail using calipers, and then the fish was placed into analytic balance to measured its weight.

Blood Glucose

A blood sample has been taken after 40 days of treatment by cutting off the tail of each fish in all groups. Blood glucose value has been measured by using a handheld blood glucose meter. This procedure has been done by using tricaine 0.168 mg/ml as an anesthetic agent similar to BMI measurement procedure (Pedroso et al., 2012).

Cholesterol Total

Cholesterol sample has been taken from visceral fat taken from abdomen dissection after 40 days (Pedroso et al., 2012). The cholesterol total was measured using colorimetric procedure following the manual kit of EnzymChrom™ AF HDL and LDL/VLDL Assay Kit (E2HL-100). The initial sample was prepared by mixing 20 µL visceral and 55 µL Precipitation Reagent into a 1.5 mL centrifuge tube and then homogenized and centrifuged for 5 minutes at 9,500 rpm. HDL sample was made on a clean tube by transferring 24 µL of the initial sample's supernatant and adding 96 µL Assay Buffer. Then, the remaining supernatant in centrifuge tube was removed so there was just pellet inside, and afterward 40 µL PBS was transferred and mixed. 24 µL of this mix and 96 µL Assay Buffer was placed and mixed in the second clean tube as LDL/VLDL sample. The Total sample was made by mixing 12 µL visceral and 108 µL Assay Buffer. Finally, The Standard sample was formed by mixing 5 µL mg/dL cholesterol and 145 µL Assay Buffer. The next step was creating the assay by transferring 50 µL of Assay Buffer ('Blank'), HDL sample, LDL/VLDL sample, Total sample, and Standard sample into wells of a transparent flat-bottom plate consisting 96 wells. For each well, 50 µL of the mixed of 55 µL Assay Buffer, 1 µL Enzyme Mix, and 1 µL Dye Reagent was added. Then, the plate was tapped to mix the assay. Subsequently, the assay was

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incubated in room temperature for 30 minutes and finally recorded the OD value at 570 nm.

Adiponectin

Adiponectin measurement includes two steps: protein isolation and ELISA measurement. Protein isolation of liver has been done refer to the manual kit of Fish Adiponectin (ADP) ELISA kit My Biosource co., Ltd., USA. The process had been begun by separated all blood vessels from the organs then been pondered. The liver organ has been finely chopped and homogenated inside PBS (100µl for 10mg tissues) by centrifugated at 5000 rpm for around 15 minutes. Afterward, the supernatants were collected and ready as a sample. This sample was used immediately with all reagents conditioned in room temperature (18-25°C) for 30 minutes. A set of Standard wells, Sample wells and Control wells were prepared by adding 50 µL of Standard, Sample, Sample Diluent according to the type of wells. Then, all wells were added by 100µl of HRP-conjugate reagent, protected with a Closure Plate Membrane, and finally incubated at 37°C for 60 minutes. Afterward, all wells were washed manually in order to remove unnecessary substances and conserve all strips. The washing procedure was conducted by filling Wash Solution (1x) for a minute then draining using absorbent paper. This procedure was repeated four times. The next step was transferring 50 µL of Chromogen Solution A and B consecutively to each well. The assay then incubated in no light condition at 37°C for 15 minutes. Finally, the process was stopped by adding 50 µL Stop Solution and the assay was read using ELISA reader after 5 until 15 minutes by OD at 450 nm.

PPAR-γ

Protein isolation of liver has been done refer to the manual kit of Fish PPAR-γ ELISA kit My Biosource co., Ltd., USA. In general, this procedure is similar to Adiponectin measurement but with a different kit. This has been begun by removing all blood vessels from the organs then been pondered. After homogenated by centrifuged, a sample was collected from the supernatants. Then the assay was prepared by transferring 50 µL of Standard to Standard wells, Sample to Sample wells, and Sample Diluent to Control wells and 100 µL of HRP-conjugate reagent to all wells. Protected by a Closure Plate Membran, the assay was incubated at 37°C for 60 minutes. The same washing process to Adiponectin procedure was conducted. Chromogen Solution A and B were transferred consecutively to each well before incubated n no light condition at 37 °C for 15 minutes. Finally, the process was stopped with 50 µL Stop Solution and kept for 5-15 minutes before measured using ELISA reader by OD at 450 nm.

Results and discussions

Table 1. is presenting the percentage of food intake for each Zebrafish group. In general, there is no significant difference between DIO A, DIO B, and DIO C in term of food consumption. In this experiment, negative control group consumed artemia nauplii 5mg/fish/day or 100% of the given food (mean 25 calories), while positive control group consumed 60mg/fish/day or 59.8% (mean 179 calories). The percentage of food consumed in DIO A is 70%, DIO B is 71%, and DIO C is 68%. The same pattern has also happened to the BMI result. Furthermore, we found that there was a decreased level of blood glucose in DIO A and BIO B groups, but an increased level in DIO C group, compared to the positive control group (DIO). The examination of total cholesterol in the visceral fat shows that there was a declining trend in total cholesterol levels in the experimental groups compared to the positive control group, but the result was not significant. Lowest total cholesterol levels are in the DIO C group. There was a declining trend on PPAR-γ level, although it was not significant compared to the positive control group. However, in this study, adiponectin level remains unchanged.

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Table 1. Proportion of food intake, Body Mass Index (BMI), Blood Glucose, Total Cholesterol, Adiponectin and PPAR-γ for each Zebrafish group

Group	Food Intake *↑↥	Body Mass Index *↑↥	Blood Glucose (mg/dL) ↑↥	Total Cholesterol (mg/dL) *↥	Adiponectin (µg/dL) ↑↥	PPAR-γ (pg/mL) ↑↥
NON-DIO	0.026 ± 0.01	0.036 ± 0.01	50.60 ± 5.41	112.31 ± 3.22	8.37 ± 0.14	1393 ± 63.64
DIO	0.059 ± 0.01	0.043 ± 0.00	71.60 ± 12.66	110.18 ± 21.54	7.72 ± 1.53	1418 ± 268.70
DIO A	0.071 ± 0.00	0.046 ± 0.00	63.60 ± 7.27	99.09 ± 15.83	7.39 ± 3.142	1175 ± 17.68
DIO B	0.071 ± 0.01	0.040 ± 0.00	51.40 ± 11.68	83.74 ± 0.64	6.75 ± 3.26	1063 ± 615.18
DIO C	0.068 ± 0.01	0.039 ± 0.00	74.00 ± 32.49	80.93 ± 3.23	6.82 ± 3.01	975 ± 74.25

Note: * $p < 0.05$, oneway ANOVA.
↑ $p > 0.05$, normality test Kolmogorov Smirnov - Lilliefors Significance Correction.
↥ $p > 0.05$, normality test Shapiro-Wilk.

Based on the results of the study, it seems that the difference in zebrafish feed intake was not significant in all experimental groups when compared to the positive control group, indicating that SPPE is not able to reduce food intake in zebrafish with DIO. Small amounts of adipocytes will reduce leptin production, which will cause a hungry response to the hypothalamus. Conversely, when the condition of adipocytes is excessive, adipocytes will release leptin, which stimulates the satiety center in the hypothalamus and macrophages in the fatty tissue will release NPY (Neuropeptide Y). In the obesity condition, there is an excessive amount of leptin, and the amount of adiponectin is reduced due to the production of high adipocyte accumulation. However, in conditions of obesity, leptin cannot stimulate the center of satiety due to leptin resistance caused by disruption of the hypothalamic response to signals given by leptin (Yang & Barouch, 2007). In zebrafish, leptin does not affect adipostat and feeding behavior (Michel et al., 2016). In zebrafish, the gene that has the most role in eating behavior is NPY. NPY is an orexigenic substance that works in stimulating food intake (Yokobori et al., 2012). However, the results of this study alleged that anthocyanin did not work on the NPY pathway in inhibiting feed intake. This condition is presumably due to differences in anthocyanin structure in sweet purple potato (*Ipomoea batatas* L) with anthocyanin structure in black soybean in (Badshah et al., 2013) study on the effect of decreasing NPY expression. In sweet purple potato, the most anthocyanin structure is peonidin & cyanidin (Mohanraj & Sivasankar, 2014), whereas, in the black soybean, the most anthocyanin structure is delphinidin & cyanidin (Koh et al., 2014).

In term of BMI, there was no significant difference between the experimental groups and the positive control group. This result is supported by the results of zebrafish feed intake in the previous discussion, which showed no effect of SPPE (*Ipomoea batatas* L) on zebrafish feed intake. It is because BMI is also influenced by feed intake (Newby et al., 2003). The results also showed a decrease in zebrafish blood glucose levels in the DIO A group when compared to the positive control group and DIO B when compared to the positive control group, but conversely, there was an increase in blood glucose levels in the DIO C group when compared to the positive control group. This is related to the different SPPE doses given in each treatment group. Changes in blood glucose levels occur due to exposure to SPPE in the treatment group. One of the anthocyanin mechanisms in SPPE, in relation to the decrease

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in zebrafish blood glucose levels, is anthocyanin works by suppressing the expression of several transcription factors in the process of adipogenesis namely PPAR-γ and C / EBPα (Rosen et al., 2002). In this study, it was shown that there was a tendency to decrease PPAR- γ levels in the treatment group with SPPE exposure. This results in a decrease in the accumulation and differentiation of adipocytes (Berger, 2005), thus decreasing the production of adipokine-adipokines such as TNF-α, IL-6, and which trigger insulin resistance (Sherwood, 2011).

In line with the decrease in adipokine-adipokine, zebrafish blood glucose levels finally decreased in the treatment group DIO A and DIO B compared to the positive control group. However, in the two treatment groups, the changes that occurred did not differ significantly. In contrast to the treatment group DIO A and DIO B, in the DIO C group with the highest SPPE dose, the blood glucose level conversely increased beyond the blood glucose level when compared with the positive control group. This result may be caused by several factors such as metabolic rate, environment, stress level, and the level of gonadal maturity of the zebrafish studied (Zahangir et al., 2015). At surgery, it was found in the DIO C group that there was a zebrafish that had gonad maturity levels reaching level 4 (matured, gonads filling three quarters of the body cavity, eggs were seen) (Tester & Takata, 1953), and two zebrafish with maturity levels gonads reach level 4 in the positive control group. The egg production process will trigger an increase in zebrafish blood glucose levels (Zahangir et al., 2015), and according to (Kordi & Tamsil, 2010) during the reproduction process most metabolic results will be focused on gonadal development so that this might lead to high blood glucose levels in the DIO C treatment group. In addition, increased blood glucose levels and feed intakes can also be caused by various types of zebrafish stressors, such as physical stressors (handling, fishing, net chasing, confinement), social stressors (dominant and submissive interactions between fish), and environmental stressors (dissolved oxygen, pH, salinity, xenobiotic exposure) (Larson et al., 2006). The difference in genetic variation of each fish causes different vulnerabilities to stressors, so the stress levels of each fish are different (Clark et al., 2011).

In this study, there were also suspected side effects from tricaine methanesulfonate (TMS) exposure that repeated at zebrafish to increase blood glucose levels and feed intake in each group. TMS was used for fish anesthetics on days 0, 14, 20, 27, 34, and 40 (for the process of measuring fish BMI) (Hasumura et al., 2012). TMS exposure can increase cortisol levels, followed by an increase in blood glucose levels of fish (Carter et al., 2011). Changes in total cholesterol levels which tend to decrease are caused by the administration of anthocyanin-containing SPPE (Teow et al., 2007). Anthocyanins have antioxidants, antiproliferative, anti-inflammatory effects (Bowen-Forbes et al., 2010), and cholesterol concentration reduction (Graf et al., 2013). Also, repeated exposure to tricaine methanesulfonate (anesthetics used for fish BMI measurements on days 0, 14, 20, 27, 34, and 40) can trigger an increase in blood glucose levels in fish (Carter et al., 2011) which allegedly can also affect cholesterol metabolism in fish. The relationship between administration of anthocyanin exposure with a decrease in total cholesterol levels in zebrafish is caused by anthocyanin administration which has the effect of reducing protein expression at the lipogenic transcription factor level, one of which is SREBP-1c (Sterol Regulatory Element Binding Protein) - 1c) (Lee et al., 2014) where SREBP-1c is one of the primary transcription regulators of cholesterol metabolism which will initially act to increase but then reduce cellular cholesterol levels (Hölttä-Vuori et al., 2010). Besides,

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anthocyanins from SPPE can also activate AMPK (Takikawa et al., 2010), which can inhibit cholesterol synthesis, which then causes a decrease in cholesterol levels (Towler & Hardie, 2007). The decrease in cholesterol levels was associated with the decrease in total fat cholesterol of in visceral zebrafish in all treatment groups. However, this cholesterol reduction in the experimental groups, statistically, is not highly significant, DIO A ($p = 0.797$), DIO B ($p = 0.261$), and DIO C ($p = 0.081$). Anthocyanins in SPPE may cause this reduction, although, the result data may be inconsistent (Basu et al., 2010).

Conclusions and suggestions

In summary, the exposure of SPPE (Ipomoea batatas L.) to zebrafish age 8 mpf with DIO is not affecting food intake and BMI nor reduce blood glucose level, but there is a tendency of blood glucose level decrease. Moreover, comparing the experimental groups and the positive control group, it seems that anthocyanins of SPPE can decrease total cholesterol and PPAR-γ levels, although the number is not very convincing. However, adiponectin level remains unchanged for all DIO groups. In the future, it is crucial to know the effect of given SPPE dose on the measurement results because it still contains other substances. It is also essential to measure the effect of SPPE on the expression of other molecular parameters such as PPAR-α and PPARB-β in other adipocyte organs.

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