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Dioscorea esculenta increase cytochrome c oxidase 1 expression and adenosine triphosphate in diabetic rats

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Abstract.
BACKGROUND: The low cytochrome c oxidase (COX)-1 activity was associated with reduction of adenosin triphosphate (ATP) production in diabetes mellitus (DM) individuals. Interestingly, the secondary metabolite of *Dioscorea esculenta* (lesser yam) fermentation in gut can increase ATP production.
OBJECTIVES: The aim of this study was to evaluate the effect of Lesser yam diet on ATP level and COX-1 expression in type 2 diabetic rats.
METHODS: Thirty Wistar rats were divided into 5 groups: (1) normal rats (N), (2) diabetic rats (DM), (3) diabetic rats with lesser yam 200 mg/kg BW (DMT1), (4) diabetic rats with lesser yam 400 mg/kg BW (DMT2), (5) diabetic rats with lesser yam 800 mg/kg BW (DMT3). The diabetic rats were induced by nicotinamide and streptozotocin and had plasma glucose more than 126 mg/dL. ATP was measured before and after 4 weeks of intervention. COX-1-was determined at skeletal muscle, heart, liver, brown adipose tissue and kidney after intervention using immuno-histochemistry (IHC).
RESULTS: Fasting blood glucose was reduced in all intervention groups compared to DM group ($p = 0.016$). ATP level was significantly increased in DMT1 group and slightly higher in DMT2 and DMT3 compared with the negative control ($p > 0.05$). After the intervention, COX-1 protein expression was higher in kidney, liver and skeletal muscle in diabetic rats received lesser yam compared to DM group ($p < 0.05$).
CONCLUSION: In this study we found that lesser yam reduced fasting blood glucose, increase plasma ATP and expression of COX-1 protein.

Keywords: Type 2 diabetesmellitus, ATP, cytochrome-c oxidase-1, *Dioscorea esculenta*

1. Background

Impairment in energy metabolism due to decreased mitochondrial activity has been observed in type 2 diabetes mellitus [1, 2]. Several studies have found a reduction in ATP synthesis both ininsulin-resistant and type 2 diabetes mellitus individuals [3–5]. The ATP reduction was caused by reduced activity of mitochondria enzyme for oxidative phosphorylation; particularly cytochrome C-oxidase (COX) [6]. COX is a key mitochondria enzyme, which is important for ATP generation through redox-linked proton pump [7, 8]. In human, COX consists of 13 subunits with subunit 1 and 2 act as catalytic region of COX enzyme [8]. Reduced activity of COX enzyme has been reported by Akude et al. [9] and Morino et al. [10] indicating the impairment in energy metabolism in diabetes condition.

Lesser yam (*Dioscorea esculenta*) is a traditional food and ubiquitous in Java, Indonesia. It is reported that resistant starch in local Indonesian lesser yam is 10.4 mg/dry weight [11]. Resistant starch is component in diet which has

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the highest yield of butyrate by colonic fermentation compared with non-starch polysaccharides [12]. Butyric acid is secondary metabolite product of gut fermentation which has an activity as histone deacetylase (HDAC) inhibitor [13]. Inhibiton of HDAC by butyrate has been shown in improving energy metabolism through activation of several transcription factors important in mitochondrial biogenesis, which will ameliorate insulin sensitivity in diabetic condition [13–15]. Therefore, in this study we want to investigate whether lesser yam has potential for improving energy metabolism in diabetic condition.

2. Methods

2.1. Animals and induction of diabetes

Male Wistar rats aged 2 months old with body weight between 180–200 gram were purchased from Unit Pengembangan Hewan Percobaan (UPHP), Institut Pertanian Bogor, Indonesia. Thirty rats were placed inside individual plastic cages with 12 hours light cycle. Rats were adapted 1 week prior to study. After adaptation, rats were fasted overnight before induction of type 2 diabetes mellitus according to Rabbani et al. [16]. In brief, rats were injected with nicotinamide (230 mg/kg body weight) and after 15 minutes, streptozotocin were introduced to rats intraperitoneally (65 mg/kg body weight). Rats with glucose serum level reached more than 126 mg/dL were selected for the study. Streptozotocin and nicotinamide were purchased from Sigma (USA). This study was approved by the Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada, Indonesia with number of etichal clearence Ref : KE/FK/641/EC.

2.2. Preparation of lesser yam flour

Lesser yam flour was prepared according to Richana and Sunarti [17]. In brief, lesser yam was purchased from local market and peeled, washed and cutted in small pieces. Lesser yam then dried in the oven (50° C for 24 hour), milled,sifted (80 mesh), and stored in vacuum plastic bag. Resistant starch, soluble fiber and insoluble fiber were analyzed in lesser yam flour using a method conducted by Goñi et al. [18].

2.3. Experimental study

Rats then divided into 5 groups including a non-diabetic control rats (N), diabetic control rats (DM), diabetic rats with lesser yam 200 mg/kg body weight (DMT1), diabetic rats with lesser yam 400 mg/kg body weight (DMT2), diabetic rats with lesser yam 800 mg/kg body weight (DMT3). The dose of 400 mg/kg body weight was used based on recommended daily consumption of fiber in normal weight human for 25 gram a day/62.5 kg of adult weight. We used 200 mg/kg body weight was used a the half dosage of normal dose and 800 mg/kg body weight was used as the doubled dose of normal fiber dosage. Those additional two dosages were used in order to evaluate whether this intervention was dose dependent. The flour was incoorperated in the diet of rats. The composition of the animal diet were presented in Table 1. Body weight and food intake were recorded.

2.4. Laboratorium analysis

After 4 weeks of intervention, rats were euthanized with cervical dislocation under anesthesia. Skeletal muscle, heart, liver, brown adiposed tissue, and kidney were taken and stored immediately in 10% (v/v) of liquid formalin buffer. Tissue from skeletal muscle, heart, liver, brown adipose tissue and kidney were paraformaldehyde-fixed prior to immunohistochemistry analysis. The tissue then embedded and sectioned by microtom (3 μm), and incubated at 50°C overnight. Paraformaldehyde was removed from the tissue by addition of xylol I, xylol II, and xylol III then rehydrated in alcohol with different concentration (absolute, 95% and 75% v/v), 3 minutes each.

After the sections were cooled down, the sections were blocked with universal tracking antibody and incubated with COX-1 antibody for 1 hour. Secondary antibody streptavidin peroxydase and S-(2-aminoethyl)-L-cysteine (AEC) were added as a substrate. Diaminobenzydine (DAB) was used as chromogen. The hematoxylin meyer was used as

Table 1
Animal diet

Composition	Semi Purified Diet	Lesser yam flour 200 mg/kg body weight (DM T1)	Lesser yam flour 400 mg/kg body weight (DM T2)	Lesser yam flour 800 mg/kg body weight (DM T3)
Casein	24%	24%	24%	24%
DL-Metionine	0.30%	0.3%	0.3%	0.3%
Cornstarch	61%	52.67%	44.33%	38%
Vitamin Mix (AIN 93)	1%	1%	1%	1%
Mineral Mix (AIN 93)	3.5%	3.5%	3.5%	3.5%
Choline Chloride	0.20%	0.2%	0.2%	0.2%
Agar-agar	5%	5%	5%	5%
Corn oil	5%	5%	5%	5%
Lesser yam	0%	9.33%	16.67%	23%

counterstain, and mounted with E-Z mount. COX-1 antibody was purchased from Cusabio (China). Expression of COX-1 was calculated based on the amount of cells expressing the protein stained using microscope. Blood was drawned from orbital cynus of rats at day 5 of induction and after 4 weeks of intervention. Blood was centrifused immediately to collect serum. Glucose was measured using enzymatic reaction of GOD-PAP (Dyasis, Germany). ATP was measured using Enzyme Linked Immunosorbant Assay (ELISA) method (Bluegene).

2.5. Statistical analysis

Statistical analysis was done using GraphPad Prism 6 for Windows (GraphPad Software La Jolla, California USA). Analysis of variance (ANOVA) was used to compare the effect of each interventions (DMT1, DMT2, DMT3 and DM) followed by Tukey HSD. Minimum significance value was set at $\alpha = 0.05$.

3. Results

Lesser yam starch contains $14.29 \pm 0.16\%$ of resistant starch, $15.10 \pm 0.06\%$ of soluble fiber, and $19.69 \pm 0.21\%$ of insoluble fiber per 100 gram of dry weight. As seen in Fig. 1, rats in DMT2 and DMT3 groups have gained less weight compared to DM control ($p = 0.033$ and $p = 0.031$ respectively). In addition, rats in DMT2 and DMT3 groups have lower food intake compared to control and diabetic control groups (Fig. 1). Fasting serum glucose was significantly lower in all lesser yam treated groups compared with DM control ($p < 0.0001$). There is no significant difference ($p > 0.05$) in fasting serum glucose level between DMT1, DMT2 and DMT3, indicating that all lesser yam intervention groups are effective in lowering serum glucose level in diabetic condition.

Plasma ATP level was not significantly different in the pre test condition (Fig. 1). However, after 4 weeks of intervention, DM group had significantly lower ATP level ($p = 0.0025$) compared to Control group and slightly lower in all lesser yam groups (Fig. 1). Furthermore, DMT1 group has the highest ATP level compared with other lesser yam group (DMT2 and DMT3).

An Immunohistochemistry was done to analyze the effect of lesser yam intervention to protein expression of COX-1 (Fig. 2). Five organs including liver, heart, brown fat, skeletal muscle and kidney were collected from rats after interventions. From those organs, only liver, skeletal muscle and kidney that were affected by lesser yam intervention. After 4 weeks of intervention, DMT1 increased COX 1 expression in kidney ($p = 0{,}016$); DMT2 increased COX 1 expression in liver ($p = 0.001$) and kidney ($p = 0.009$); DMT3 increased COX1 expression in liver ($p = 0.037$), kidney ($p = 0.001$) and skeletal muscle ($p = 0.032$).

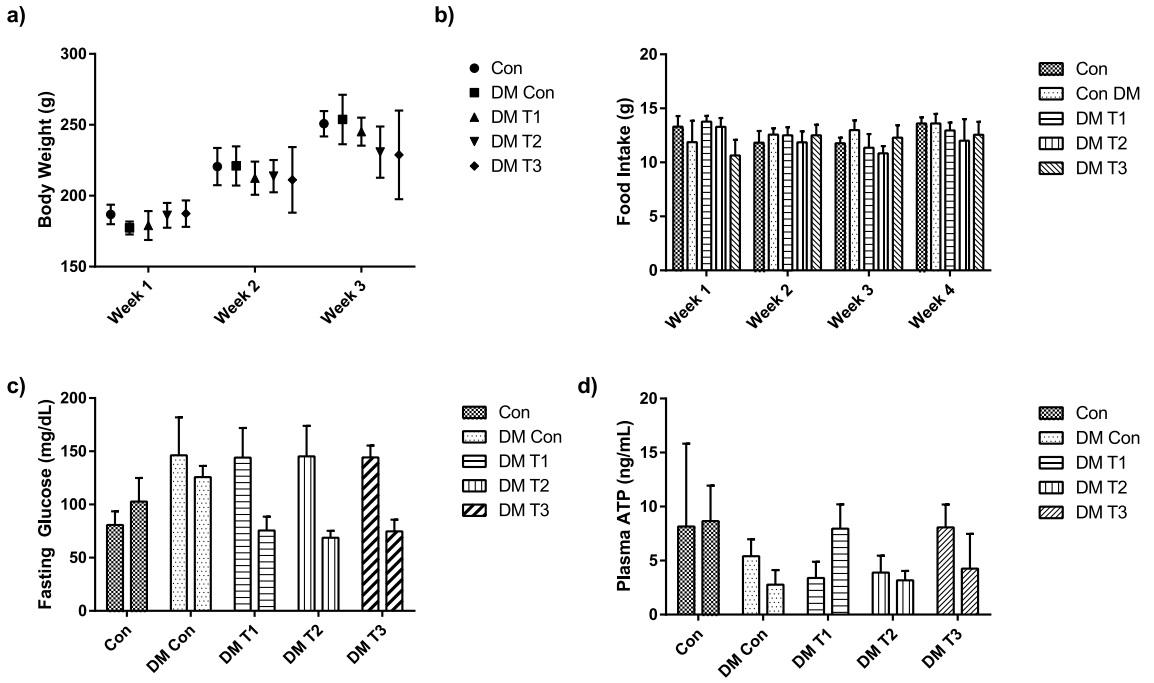

Fig. 1. Administration oflesser yam (*Dioscorea esculenta*) in the diet prevent body weight gain (a), decrease food intake (b), reduce fasting serum glucose (c) and increase plasma ATP level (d) of diabetic rats.

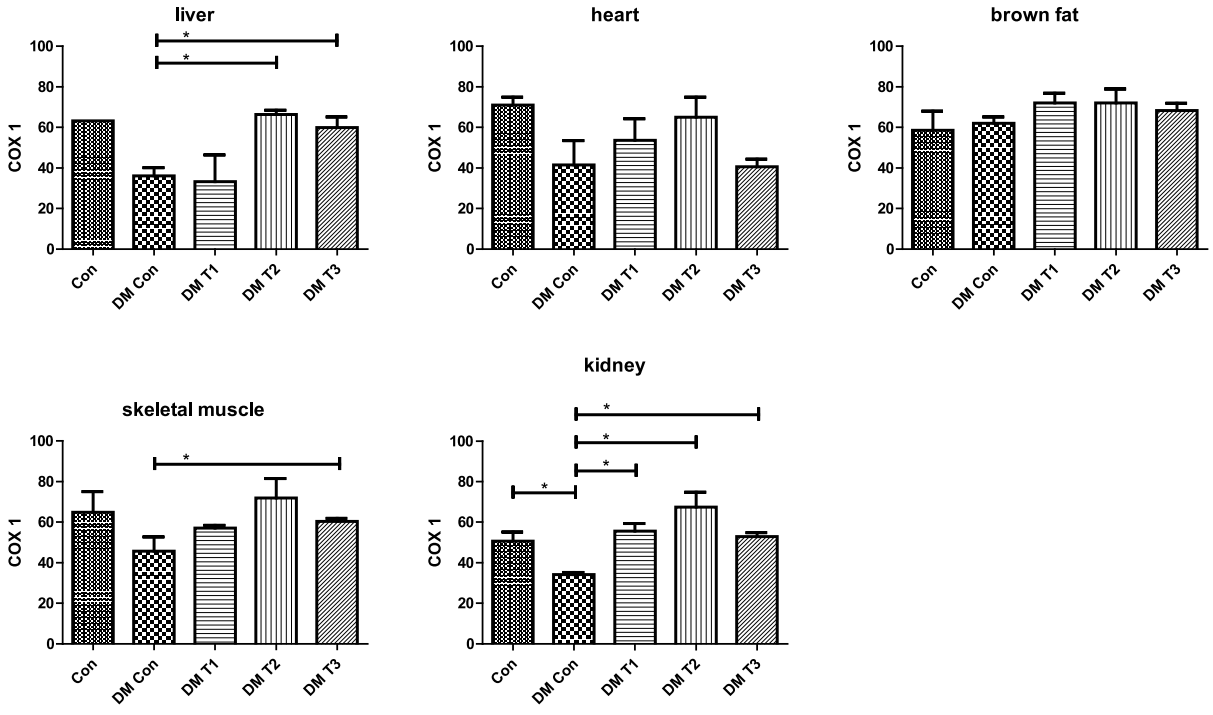

Fig. 2. Protein expression of COX-1 in various organs (liver, heart, brown fat, skeletal muscle and kidney) after interventions.

4. Discussion

Impairment in energy metabolism due to reduction in mitochondrial function was observed in T2DM and insulin resistance [19]. The disturbance of mitochondrial function leads to a reduction in oxidative capacity of mitochondria thus lowering the production of ATP [20]. This process is also followed by decreased COX1 expression in mitochondria [9, 10], which lead to the low level of ATP due to impairment in several enzymes important in phosphorylative oxidation activity [4, 20].

In the present study, we showed the potential effect of lessser yam (*Dioscorea esculenta*) in the regulation of energy metabolism, body weight and blood glucose in diabetic condition. Intervention of lesser yam can suppress appetite and halted body weight gain. Additionally, we showed that ATP production is increased significantly in diabetic group treated with low dose of lesser yam. COX 1 expression was also affected by the lesser yam intervention in kidney, liver and skeletal muscle.

Previous study has reported the beneficial effect of lesser yam in improving fasting plasma glucose of diabetic mice [21]. This effect was particularly caused by the presence of fiber, resistant starch and inulin in lesser yam [11, 22]. Inulin and other dietary fiber component in food have been reported to increase insulin sensitivity and improvement in glucose homeostasis in animal model with diabetes [23–26]. In addition, inulin and other dietary fibers can resist human enzyme dygestion and escape to the colon where it will be fermented by the colonic bacteria to produce short chain fatty acids (SCFAs) including acetate, butyrate, and propionate [27]. After being absorbed, those fatty acids are circulated in the blood and contacted with their receptors; free fatty acid 2 and 3 (FFAR2 and FFAR3), in the cells [28–30]. Activation of FFAR2 and FFAR3 in the targeted cells have been reported to induce several function such as anti-inflammation, regulation of appetite through the release of leptin secretion and regulation of symphatetic nervous system [30]. In addition, stimulation of both FFAR2 and FFAR3 by SCFAs in the small intestine have been linked to the elevated production of glucagon like peptide (GLP)-1 and PYY, which has been correlated with decreased appetite, elevation of insulin secretion and improvement of insulin sensitivity [30–35].

In addition, butyrate has gained attention as this small molecule can inhibit histone deacetylase (HDAc); an important enzyme for down regulating gene expression through histone modification [13]. It is previously demonstrated that inhibition of HDAc by butyrate can increase the expression of transcription coactivators that are important in mitochondria biogenesis such as PGC-1α [14]. Increasing PGC-1α expression is correlated with elevated fatty acid oxidation and ATP production [36, 37].

It is well established that diabetes is associated with decreased production of cellular ATP synthesis [3, 5]. Indeed, in this study, we observed marked decrease, approximately 70%,of plasma or extracellular ATP level in diabetic rats compared to normal rats, which is followed by the reduction of COX-1 expression in liver, kidney, heart, and skeletal muscle. Although we didn't measure the intracellular level of ATP, however, extracellular ATP level reflects the cellular production of ATP [38, 39]. Extracellular ATP is an extracellular adenine compound, which is released and transported out from most of the cells under normal or in response to stress or certain stimuli in the body [40–42]. Under basal condition, only 1% of intracellular ATP that would be released into extracellular compartment although there is a favorable gradient concentration (around 10^6 fold) to induce ATP efflux from the cells [38]. This concentration is sufficient to induce certain physiological effect through activation of purinoreceptor such as apoptosis [43], regulation of glucose uptake [44–46], cell survival [42],vasoconstriction [47] and vasodilation [48].

Extracellular ATP level in the blood is regulated by two factors; i.e., the ATP release from the cells and ATP degradation by the ectoATPase in the plasma membrane of the cells [49, 50]. Impairment in ATP release and ATP catabolism can cause low level of ATP level in extracellular compartment as previously demonstrated in diabetic rats condition [51]. Diet also has an important role in increasing extracellular ATP level. For instance, ketogenic diet (very low carbohydrate diet) has been reported to induce ATP synthesis in the cells and increase the ATP level in the extracellular compartment of the cells [52]. The ability of ketogenic diet in increasing extracellular ATP level was caused by the increase activity of mitochondria in generating ATP through ketone bodies oxidation [52–54]. In this study, we showed that low dose of lesser yam from the diet is able to increase extracellular ATP level whereas high dose of lesser yam has an inverse impact on extracellular ATP level. Interestingly, low dose of lesser yam slightly increase the COX-1 expression in several organ. Why low dose of lesser yam can increase extracellular ATP level despite modest increase in the COX-1 expression is currently unclear; however,we hypothesize that the high level

of extracellular ATP in this group is independent to mitochondria function. Further study is needed to address the mechanism behind this issue.

In conclusion, we showed that lesser yam intervention in diabetic rats has ability to ameliorate fasting plasma glucose,increase extracellular ATP level and COX-1 expression. Further study is needed to evaluate the effect lesser yam on mitochondrial dysfunction in human.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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