The Effect of Coconut Water on Adipocyte Differentiation and Lipid Accumulation in 3T3-L1 Cells

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Summary Coconut water is reported to have lipid-lowering effects in animal studies. However, there is lack of published reports regarding its effect on adipocytes. This study observed the effect of coconut water on adipocyte differentiation and lipid accumulation in 3T3-L1 cells. The sample used in this study was mature coconut water from tall variety. Based on a preliminary study, the sample was heat-treated and added with certain amino acids as precursors for Maillard reaction to improve its original flavor. As a comparison, aromatic coconut water was used since it is highly preferred as a fresh beverage. Six samples were supplemented to 3T3-L1 cells, which were then analyzed for cell proliferation, lipid accumulation, triglyceride content, and gene expression. Arginine and vitamin C contents of the samples were also determined. The data were analyzed with ANOVA and followed by Tukey’s test. Results showed that aromatic coconut water could slightly suppress lipid accumulation, while mature coconut water had a significantly lower percentage of accumulation compared to the control sample (p<0.05). Canned and fresh samples had no significant difference in terms of lipid-lowering activity (p>0.05). Similarly, the addition of lysine and proline in canned samples did not significantly affect the cells’ differentiation. There was no significant effect on expressions of C/EBP-α and PPARγ, indicating the possibility of other pathways involved in hypolipidemic effect of coconut water. This study showed that coconut water might have potential to inhibit adipogenesis in 3T3-L1 cells due to its bioactive compounds.

Key Words coconut, maturity, tall variety, canning, fat cells

Obesity, one of the non-communicable diseases, has affected a large number of individuals in the population, which increases the risk of a variety of other metabolic syndromes, such as type 2 diabetes, cardiovascular diseases, dyslipidemia, hypertension, non-alcoholic fatty liver, etc. (1–3). Fat tissues increase both in size and number and the mechanism highly depends on the differentiation process from preadipocytes (4, 5). Adipocyte differentiation is regulated by a series of proteins, e.g. peroxisome proliferator-activated receptor (PPAR) and CCAT/enhancer-binding protein (C/EBP), where their activities can be affected by diet (4, 6, 7). Many studies have been performed to see the effects of natural food products against obesity, including natural beverage products, such as various types of tea, roselle drink, fruit juices, tomato juice, vinegar, etc. (2, 8, 7). Those studies showed that natural products have potentials as anti-obesity agents by inhibiting lipid accumulation. Many bioactive compounds contained in the products might have contributed towards the lipid-lowering effects, such as vitamins, minerals, polyphenols, etc. (1, 6).

One of the natural beverages is coconut water, where aromatic coconut water variety is highly demanded for fresh consumption. Mature coconut fruits from tall variety is more preferred by the industry due to its high fat content, which is beneficial to produce coconut milk and coconut oil. Consequently, there is a high volume of mature coconut water as by-products. Unfortunately, this type of coconut water has less preferred flavor since it is far less aromatic (9). Heat treatment could be used to improve the flavor characteristics of mature coconut water through chemical reactions, which take place during heating, such as oxidation and Maillard reaction between reducing sugars and free amino acids (10). In our preliminary study, it was found that heat treatment in the form of canning and addition of certain amino acids, such as lysine and proline, could help in improving the volatile aroma compound profile of mature coconut water.

Application of heat treatment is expected to affect the biological activities of coconut water. Several in vivo studies involving laboratory rats have managed to show coconut water, in variety of forms, could lower the level of total cholesterol, very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterols, phospholipids, free fatty acids, and triglycerides as well as...
increasing the high-density lipoprotein (HDL) cholesterol level in many sites such as in the blood, the heart, and other tissues. (11–17). A study also elaborated on the beneficial effects of both mature and young coconut water on serum lipid profile of cholesterol-fed rats (17). However, there have been no published reports discussing the influence of coconut water on 3T3-L1 adipocytes, the cell line that has been used in many studies to understand adipogenesis (18). The murine 3T3-L1 cells are considered highly useful in in vitro studies to observe preadipocyte differentiation into mature adipose cells. Moreover, these cells are considered as a close representative of adipose cells in vivo in terms of morphological and biochemical characteristics, and it is also able to release active substances, for example cytokines, enzymes, hormones, and growth factors (1). Therefore, in the present study, the effects of heated mature coconut water on 3T3-L1 cells were investigated.

MATERIALS AND METHODS

Sample preparation. Samples of fresh mature coconut water from tall variety with maturity age of 10–12 mo old were obtained from a local supplier and packed in an ice box to prevent chemical changes. Another set of samples from aromatic coconut variety was also collected from a local farm for comparison. Once the aromatic coconut fruit samples arrived in the laboratory, they were immediately dehusked and the shell was cracked to collect the water. All samples were filtered, stored in clean containers, and kept at −18°C temporarily prior to further treatment.

Heat treatment of the coconut water samples involved canning process. Coconut water was pre-heated to 72°C and held for 1 min before it was poured into clean cans. Heating was continued to 80°C and maintained for 1 min to exhaust the air before the cans were sealed. Seamed cans were then sterilized in a steam retort at 121°C for 20 min. Once sterilization was completed, the cans were immediately cooled down in an icy water bath.

A total of six samples were used in this study: M (fresh mature coconut water), MC (canned mature coconut water), MCL (canned mature coconut water added with lysine), MCP (canned mature coconut water added with proline), A (fresh aromatic coconut water), and AC (canned aromatic coconut water).

All coconut water samples were concentrated prior to cell study using a rotary evaporator operated at 72 mbar and 40°C for 40 min. This step resulted in highly concentrated coconut water samples that had lost 94.95 ± 2.02% of its weight. Concentrated coconut water was then diluted with nuclease-free water, and stored in small aliquots for one time use at −20°C as the stock solution. Prior to cell study, stock solutions were thawed and diluted in growth medium following the pre-set concentrations.

3T3-L1 cell culture and differentiation. 3T3-L1 cell line (ATCC, USA) was maintained in high-glucose Dulbecco’s modified eagle medium (DMEM) (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere with 5% CO₂. Cells were subcultured every 5–6 d at 70–80% confluence. Cells plated were 3–10 passes from the original vial at a concentration of 3×10⁴ cells/well in 24-well collagen-coated plates. For the differentiation process, cells were grown up to 100% confluence in the initial culture medium for 4 d. At this point (Day 0), cells were switched to differentiation medium (DMEM, 10% FBS, 10 μg/mL insulin (Thermo Fisher Scientific, USA), 0.5 μM dexamethasone (Thermo Fisher Scientific, USA), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, USA)) for 3 d. On Day 3, the dexamethasone and IBMX were removed, thus leaving maturation medium with only insulin for an additional 6 d, with the change of medium performed every 2 d. On Day 9, cells were ready to be harvested and analyzed.

Coconut water samples were added together with differentiation medium and maturation medium at pre-set concentrations. Control sample was prepared by using the same volume of nuclease-free water.

Cell proliferation. Cell proliferation was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The 3T3-L1 cells were seeded in 96-well plate at 1×10⁴ cells/well. Culture medium that contained different concentrations of sample was added to each well and the wells were incubated at 37°C and 5% CO₂. After 48 h, 10 μL of 5.5 mg/mL MTT (Thermo Fisher Scientific, USA) was added into each well and the plate was returned into the incubator for 2 h. Next, the media was removed and cells were lysed with mixture of dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, USA) and ethanol (Thermo Fisher Scientific, USA) with the ratio of 1 : 1. The plate was kept in the dark for 5 min and the absorbance was detected at 570 nm using a microplate reader (Tecan, Switzerland).

Oil Red O staining. On the ninth day after induction of differentiation, 10% formalin (Thermo Fisher Scientific, USA) was added to replace the culture media and the plate was left at ambient temperature for 1 h. Each well was washed with 60% isopropanol (Thermo Fisher Scientific, USA) and allowed to air dry. Cells were incubated with Oil Red O working solution (Sigma-Aldrich, USA) for 15 min and washed 4 times with distilled water. Next, the cells were viewed using a Nikon Eclipse TS 100 microscope at a magnification of 20×. To quantify the intracellular lipids, the stained lipid droplets were further dissolved in absolute isopropanol. The absorbance of the extracted dye was measured using a microplate reader at 492 nm.

Triglyceride analysis. Nine days after induction of differentiation, 5% Triton-X (Sigma-Aldrich, USA) was added to each well after removal of maturation media. The plate was sonicated for 10 min and cooled down quickly. Sonication was repeated 3 times to ensure all cells were lysed. Cell lysate was collected and centri-
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Supernatant was transferred to a microtube and diluted with distilled water before transferred to a 96-well plate. The cellular triglyceride content was determined by a commercial triglyceride quantification colorimetric/fluorometric kit (BioVision, USA) according to the manufacturer’s protocol and the absorbance was measured by a microplate reader at 570 nm.

Gene expression. The RNA of the cells was extracted using a commercial GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) and purified with DNase I, Amplification Grade (Thermo Fisher Scientific, USA) following the manufacturer’s protocols. The first strand of cDNA was synthesized from 200 ng of RNA using a commercial RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. Five hundred nL of cDNA was then subjected to real-time PCR amplification with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) as the dye. Real-time PCR (Bio-Rad CFX96™ Real-Time System, USA) was used with the conditions set at: 5 min at 95˚C for initial denaturation, followed by 39 polymerase chain reaction (PCR) cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 30 s, and 78˚C for 10 s. The level of cycle threshold (Ct) was normalized using β-actin as a reference gene and reported as relative to unstimulated cells.

Arginine and ascorbic acid content. Several samples were selected for analysis of arginine and ascorbic acid content. Determination of the arginine content was performed following the method for amino acid analysis set by the European Communities (19). Free amino acids contained in the sample were extracted with diluted HCl (0.1 mol/L) and separated by ion-exchange chromatography. Determination was carried out by reaction with ninhydrin followed by photometric detection at 570 nm. Meanwhile, ascorbic acid content of the samples was analyzed following the modified method that measured absorbance of samples at 520 nm after being reacted with 2,6-dichlorophenolindophenol and 2,4-dinitrophenylhydrazine (20).

Statistical analysis. Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., USA). The results were presented as means±SD from three independent replications. Differences among samples were analyzed using One-Way ANOVA followed by Tukey’s test. Statistical significance was set at \( p<0.05 \).

RESULTS

Cell proliferation

To determine if coconut water could inhibit the proliferation of preadipocytes, the cultured pre-confluent 3T3-L1 cells were treated with coconut water samples at concentrations of 0, 5, 10, 50, and 100 \( \mu \)g/mL for 48 h. After 2 d of incubation, cell proliferation was measured using the MTT assay. The results indicated that 48 h exposure of cells to coconut water significantly caused the reduction of pre-adipocyte proliferation in most of samples starting from 5 \( \mu \)g/mL (data not shown). The highest concentration where all treatments showed statistical differences from untreated cell was 50 \( \mu \)g/mL (data not shown); therefore, this concentration was set as the highest possible limit for further experiments.

Lipid accumulation and triglyceride content

Adipocyte differentiation could be observed by intracellular accumulation of lipid droplets. All samples were prepared in three working concentrations of 5, 15, and 45 \( \mu \)g/mL and tested. Differentiation of preadipocytes into mature adipocytes is marked by the transition from a fibroblast-like shape to a rounded structure that shows accumulation of lipid droplets (8). Differentiated 3T3-L1 cells were subjected to Oil Red O staining. Figure 1 provides representative images of Oil Red O-stained cells at the highest concentration of 45 \( \mu \)g/mL and it shows that all samples could slightly suppress lipid accumulation in comparison to untreated cells (control). The results that were observed under a microscope were further supported by quantitative data obtained by spectrophotometric analysis of Oil Red O-stained cells that were eluted with isopropanol, as shown in Fig. 2.
Many studies on adipocyte differentiation have provided knowledge on functions of PP AR-g and C/EBP-a genes on the molecular regulation of the process. Both transcriptional factors are highly important at the early stage of adipocyte differentiation and they are known to be the keys to regulate promoters of adipogenic genes (4, 8). PP AR-g is often labeled as the ‘master regulator’ in differentiation of adipocytes that works by controlling expression of proteins involved in adipogenesis and lipid metabolism (7). Moreover, PP AR-g promotes differentiation and proliferation of adipocytes, leading to an increase in fat deposit (5). Together with PP AR-g, C/EBP-a is also an adipogenic transcription factor that activates a series of genes during adipocyte differentiation (8).

A reduction in mRNA expression of adipogenic transcription factors indicates a decline in formation of lipid droplets, which are the major marker of adipocyte differentiation (8). PP AR-g and C/EBP-a are the main transcriptional regulators of adipogenesis and they are needed to synthesize many adipocyte functional proteins (6). Reduction of expression of PP AR-g and C/EBP-a was stated to reflect disturbance in adipogenesis during transcription (18).

Expression of PPAR-g and C/EBP-a was analyzed using quantitative real-time PCR to observe whether fresh and canned coconut water could have an effect. Four samples were selected to be analyzed: fresh mature coconut water (M), canned mature coconut water (MC), canned mature coconut water with addition of proline (MCP), and fresh young aromatic coconut water (A). The first three samples were selected to observe if both heating and addition of amino acid could bring an effect on the cell activity, while the fourth one was selected as a comparison sample since it is commonly consumed as fresh coconut water in the market. The samples were introduced into the analysis at two concentrations: 15 and 45 μg/mL. Figure 4 shows that there was no significant difference of all analyzed samples compared to control (untreated cells). The data was calculated as relative to β-actin gene expression of the control sample.

**DISCUSSION**

**Lipid accumulation**

Figure 2 indicates that sample M showed a reduction of intracellular lipid droplets during adipogenesis at 15–30% in comparison with the untreated cells (set as 100%). Several studies reported that both young and...
mature coconut water from West Coast Tall variety were able to significantly reduce lipid levels in blood and tissues of hypertensive and diabetic mice (12, 15). Hypolipidemic effects of coconut water had been investigated in a study that found both young and mature coconut water from West Coast Tall variety could counteract the negative effects of cholesterol feeding in mice (16). Decreased values were reported in lipid levels and fat accumulation in blood and tissues, which were hypothesized due to several biologically active components that are contained in coconut water, for example L-arginine, ascorbic acid, and minerals such as calcium, magnesium, and potassium. Furthermore, coconut water was shown to have similar effects with lovastatin, a lipid-lowering drug, in lowering the serum lipid levels in mice fed with fat-cholesterol enriched diet (17).

An experiment observed that mice fed with both tender and mature coconut water from West Coast Tall variety had lower total triglyceride and total cholesterol in their serum and tissues, most possibly due to its L-arginine content (17). The effect of diet containing coconut protein with 24.5% of arginine in reducing hyperlipidemia induced by high-cholesterol diet was reported in another study (21). Furthermore, a study reported that dietary supplementation of L-arginine to diabetic rats had decreased hypertriglyceridemia and serum LDL cholesterol level (22). Table 1 shows that coconut water from both tall and aromatic varieties had arginine in their composition. The differences between sample M and A were most possibly due to the natural differences between both varieties.

Figure 2 shows that at similar concentration, heated mature coconut water (MC, MCL, and MCP) tended to have less inhibitory effect on lipid accumulation compared to the fresh sample (M). When industrial processing is applied, beneficial components contained in the coconut water could possibly be reduced, leading to a decline in several of its therapeutic activities as shown in an experiment studying the effect of coconut water from different varieties and different processing methods on oxidative stress level in lung fibroblasts (23). The effect of processing was also seen in terms of arginine content in coconut products, as shown in an experiment that reported a 10% reduction of arginine content in coconut meat from West Coast Tall variety where free arginine was observed to be 40% reduced by the heat treatment, due to possible Maillard reaction between free amino acids and reducing sugars (25). Similar to the present study, canning process was found to slightly reduce arginine content of mature coconut water (Table 1).

Another factor that is possibly involved in hypolipidemic activity of coconut water is its vitamin C content (15–17). An experiment observed that vitamin C, administered at 60 mg/kg of body weight, possessed hypolipidemic effect and it could decrease the total serum cholesterol and triglycerides in mice fed with cholesterol-rich diet (26). Table 1 shows that higher amount of ascorbic acid was present in fresh aromatic coconut water than in fresh mature coconut water. In another study, it was found that coconut water from young Dwarf varieties had approximately two times the amount of ascorbic acid as compared to coconut water from yellow Malaysian Tall variety (23).

Table 1. Arginine and ascorbic acid contents of coconut water samples.

| Coconut water sample | Arginine (mg/100 mL) | Ascorbic acid (µg/mL) |
|----------------------|----------------------|-----------------------|
| M (fresh, mature)    | 2.02                 | 1.80                  |
| MC (mature, canned)  | 1.85                 | 1.80                  |
| A (fresh, aromatic)  | 3.14                 | 2.92                  |

Triglyceride content
Fresh mature coconut water sample (M) did not show significant difference in triglyceride content compared to the control, while heated mature coconut water samples (MC, MCL, and MCP) could slightly suppress the effect lower than that of M. Heat treatment has been associated with reduction of bioactive components in coconut water, which might affect its biological effects (27).

Figure 3 shows that interestingly, mature coconut water from tall variety was seen to have no significant difference in terms of hypolipidemic activity with the coconut water from aromatic coconuts, which is the type of coconut water more preferred by the consumers and often consumed as fresh beverage.

Furthermore, addition of lysine and proline in the samples MCL and MCP did not cause significant effect in reducing lipid accumulation compared to the canned sample without addition of any amino acids (MC), reflecting the low importance of the two amino acids in lipid metabolism, even though they could help in improving the aroma characteristics of the canned mature coconut water.

Gene expression
There are variety of transcription factors that have been identified to be the key regulators of the adipogenesis process, such as CCAAT-enhancer binding protein (C/EBP-α, -β, and -δ), peroxisome proliferator-activated receptor (PPAR-γ and -β/δ), and helix-loop-helix (HLH) (SREBP-1c) families, where interrelated actions between those factors result in regulation of adipocyte differentiation (28). Therefore, there was a possibility that the effect of coconut water on adipogenesis of 3T3-L1 was regulated by a more complex pathway involving not only the two genes used in the study, thus resulting in no significant differences among samples as shown in Fig. 4.

As a conclusion, this study suggested that mature coconut water from tall variety may have the potential in lipid-lowering effect by suppressing lipid accumulation in 3T3-L1 cells, similar to the effect shown by aromatic coconut water. Thermal processing in the form of retort canning and addition of the amino acids lysine
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