NUTMEG EXTRACT POTENTIALLY ALTERS THE CHARACTERISTICS PROFILE OF ADIPOSE TISSUE 3T3-L1 CELL LINES

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Abstract

Metabolic syndrome (MS) is currently emerging globally and increases the risk of dangerous chronic diseases. Obesity involving inflammatory mediators secreted by adipocytes appears to be the major cause. While white adipose tissue (WAT) functions as energy storage, brown adipose tissue (BAT) involves in thermogenesis and is known to have the ability to dissipate energy. Currently, researchers are developing BAT through the browning mechanism of adipose tissue to prevent and cure MS. The extract derived from nutmeg, a tropical seed of Myristica fragrans, has a peroxisome proliferator-activated receptor γ (PPARγ) agonistic effect which is known to stimulate brown adipocytes development. PPARγ activation stimulates the expression of uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2), and uncoupling protein 3 (UCP3). In the present study, we explore nutmeg-induced stimulation of UCP1, UCP2, and UCP3 protein level characterization shift in 3T3-L1 cell lines which indicates the browning mechanism of adipose tissue exerted by nutmeg. Nutmeg was first extracted and 3T3-L1 cell lines taken from ATCC were used which were grown in Dulbecco's Modified Eagle Medium (DMEM). Cells were treated with nutmeg extract (NuSE) with dose: 0.01; 0.1; 1; and 10 ppm. Western blot and ImageJ software were used for protein analysis and all experimental data were analyzed using SPSS. Results showed that NuSE stimulates the increase of UCP1 and UCP2 protein levels significantly at a 10-ppm dose. This suggests that polyphenol-containing malignant in NuSE has the probability to regulate PPARγ which potentially alters characteristics of white adipose tissue in 3T3-L1 cell lines.

***Keywords**: Nutmeg, uncoupling proteins, browning mechanism, adipose tissue, metabolic syndrome, 3T3-L1 cell lines*

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INTRODUCTION

Metabolic syndrome (MS), a cluster of diseases that increases the risk of cardiovascular diseases, is currently a major global health problem. Obesity, as one of its causes, is getting an increase in number.[1] According to WHO, 1.9 billion adults were overweight and 650 million of those were obese in 2016. This leads to an estimated increase in the prevalence of coronary heart disease by 97%, cancers by 61%, and type 2 diabetes by 21% in 2030.[2] Adipocytes and their associated inflammatory mediators are known to act as signaling processes in overweight and obesity pathogenesis.[3]

In addition, adipocytes secrete various adipokines which contribute to the development of obesity-associated insulin resistance that leads to atherosclerosis and MS.[4] Moreover, inflammation of adipose tissue stimulates the accumulation of macrophages which gives rise to impaired adipocyte functioning as well as insulin resistance and subsequent MS.[5,6] White Adipose Tissue (WAT), which functions to store energy in a form of triglyceride and to maintain the energy homeostasis of the human body,[7] has turned out to be an active endocrine organ producing adipokines such as adiponectin, leptin, visfatin, omentin, resistin, and TNF- α.[8]

Stefanie Carobbio et al suggest that obesity-induced metabolic problem occurs as a consequence of the mismatch between adipose tissue demands needed by positive energy balance and storage space supply determined by adipose tissue expandability.[9] The accumulation of the immoderate amount of visceral fat typically induces the dysfunction of adipose tissue expandability, therefore, amassing ectopic triglyceride which accounts for MS and cardiovascular diseases.[10]

Meanwhile, the Brown Adipose Tissue (BAT), another type of adipose tissue, controls energy balance by producing heat through non-shivering thermogenesis (NST)[11] in which energy expenditure occurs.[12] Uncoupling protein 1 (UCP1) or thermogenin, a typical inner mitochondrial membrane protein, has an immense role in BAT thermogenesis.[13] UCP1 increases mitochondrial proton conductance,[14] thus dissipates the proton gradient across the inner mitochondrial membrane, uncouples the electron transfer system from ATP synthesis, and induces energy to produce heat.[15] Due to its considerable ability to dissipate energy, BAT is presently developed as a fascinating way to prevent and cure MS and its subsequent morbidities.[12,16]

Consequently, in searching for groundbreaking therapeutics for obesity, additional signaling molecules that stimulate brown adipocyte development have been identified.[17] Some mechanisms such as norepinephrine signaling through the β3-adrenergic receptor (ADRB-3)[18,19] and β1-adrenergic receptor (ADRB-1)[20] activation, fibroblast growth factor 21 (FGF21) activation,[21] peroxisome proliferator-activator receptor-α (PPAR-α) activation,[22] apelin-APJ signalin,[23] sirtuin 5 (SIRT5) regulation,[24] and peroxisome proliferator-activator receptor-γ (PPAR-γ) activation[25] have been shown to stimulate brown adipocytes development. In the search for molecule activators of BAT and brite or beige cells, dietary[26] and pharmacological[27] treatments are being investigated.

Nutmeg, a tropical seed derived from Myristica fragrans,[28] has been believed to impart several bioactivities, one of which is anti-obesity.[29] In addition, an Encyclopedia of Traditional Medicinal Plants by Yuniarti et al states that nutmeg has antidyslipidemic and antidiabetic effects. To strengthen this, Keri Lestari et al conducted research on the type 2 diabetes mellitus rat model proving that safrole-removed nutmeg extract could grant antidiabetic and antidyslipidemic effects due to its potential natural PPARα/γ.[30] Haruya Ohno, et al declare the causative connection between PPARγ agonists and white-to-brown fat conversion in which the full agonism of PPARγ is needed to activate the brown fat gene program in subcutaneous white fat.[31] Furthermore, Linda et al conducted research in rats and mice to which PPARγ agonists were given. In the final analysis, in vivo PPARγ activation could stimulate the expression of UCP1, uncoupling protein 2 (UCP2), and

uncoupling protein 3 (UCP3) in BAT.[32] Therefore, the stimulation of UCP1, UCP2, and UCP3 protein level characterization shift in cell lines has become an indicator of the browning mechanism of adipose tissue. In this present study, we explore nutmeg as one complementary and alternative medicine (CAM) to treat MS including obesity and diabetes mellitus through its browning adipose tissue mechanisms. The aim of the present study is to investigate the effects of nutmeg on the alteration of the characteristics of WAT in 3T3-L1 cell line. First, 3T3-L1 cell lines are seeded until 70-80% confluence, next treated with nutmeg extract (NuSE) and incubated, then harvested and the expressed proteins are analyzed using electrophoresis, and at last, the result is conferred from the data. The characterization shift of UCP1,[33] UCP2,[34] and UCP3[35] proteins detected by increasing bands during the electrophoresis procedure can be used to establish the direct relationship between nutmeg extract treatment and the browning mechanism of adipose tissue.

METHODS

Preparation of Nutmeg Extract (NuSE) Nutmeg was obtained from the seeds of Myristica fragrance which were collected during the dry season from Maluku and West Java Island, Indonesia. The dried seeds were ground into 30 kilograms of powder. The powder was extracted by 225 L, 95% ethanol using a pilot-scale extractor with a rate of 150-200 rpm for 30 minutes into liquid. The liquid was evaporated at 40-60^{o}C, 400-500 mmHg into 5.2 kilograms of solid extract of the nutmeg seed. Nine hundred grams of the solid extract was prepared for safrole removal using pilot-scale column chromatography. During the removal step, 130 liters of a mixture of n-hexane: ethyl acetate (9:1) was used as the mobile phase at 6 liters/minute flow rate from which the eluates were discarded. The residue was eluted with 100 liters of methanol and then collected and dried at 50^{o}C of evaporation. After the removal step, analysis for safrole residue was conducted by using a high-performance liquid chromatography system. Lastly, safrole-free nutmeg extract was ready to be used for the treatment.

Cell culture and adipocyte differentiation Murine 3T3-L1 (ATCC® CL¬-173™) preadipocytes were obtained from American Type Culture Collection (ATCC) with catalog number 30-2002 and biosafety level 1 (Manassas, VA). Cells have been tested and found negative for ectromelia virus (mousepox). Cell lines from ATCC have been thoroughly authenticated using cytochrome C oxidase I gene (COI analysis) to rule out inter-species contamination and short tandem repeat (STR) profiling to distinguish between individual human cell lines and to rule out intra-species contamination. Cells should be kept in the liquid nitrogen vapor phase if continued storage was necessary. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/l glucose and supplemented with 10% v/v calf serum in a humidified 5% CO2 atmosphere at 37°C. The 3T3-L1 cells were cultured to ~70-80% confluence in a 24-well plate. Cells were treated with or without NuSE in proliferation for 3 days with dose: 0,01; 0,1; 1; and 10 ppm.

Western blot analysis (WB) 3T3-L1 preadipocytes left untreated or induced with NuSE for the indicated times were washed with Phosphate-Buffered Saline (PBS) and scraped into Sodium Dodecyl Sulfate (SDS) lysis buffer (60 mM Tris-HCl, pH 6.8, 1% SDS) for whole-cell lysates. The cells were harvested and processed in vitro extraction. 24 well were added by 120 µL/well of lysis solution. Lysis solution consists of lysis buffer (Radioimmunoprecipitation (RIPA) buffer) and sample buffer in a ratio of 1:1. Protein inhibitors and Dithiothreitol (DTT) were added later in the ratio of 1:100. All samples were heated at 96oC for 5 minutes and snap-frozen on the ice for 2-3 minutes. The same amount of protein was loaded in all cases, and β-actin was used as a loading control, 10 µL of protein were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for two hours and a half. SDS-PAGE was transferred to a nitrocellulose membrane (GE Healthcare) by electrophoresis for half an hour. 2% blocking reagent that

consists of 0,25% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline Tween-20 (PBST) was added. Membrane immunoblotting was conducted using primary antibodies Caspase-3 catalog number 14220 and Caspase-9 catalog number 9508 from Cell Signaling with a dilution of 1:300 then incubated at 4oC overnight. The signals were imaged by LI-COR Odyssey. Protein band thickness was analyzed by using ImageJ software (NIH). Blots were stripped by stripping buffer from Thermo Scientific and reprobed using β-actin as an internal control for protein level monitoring.

Statistical Analysis Experimental data were analyzed using IBM SPSS Statistics 25 and results are presented as the mean ± standard error of the mean from 3 independent experiments (3 wells for each set of data). One-sample T-Test was used for statistical analysis with $p<0.05$ and $p<0.001$ were considered statistically significant.

RESULT

Western Blot result of UCP1, UCP2, UCP3 expression in 3T3-L1 cell lines was shown in Figure 1. Based on the observation, UCP1 protein level fluctuated as NuSE dose increased from 0.01; 0.1; 1; to 1 ppm with an increasing trend. Notably, based on statistical analysis, UCP1 protein level was only significantly increased (2.5 folds) with NuSE 10 ppm treatment compared to the control group ($p<0.05$) (Figure 2). Additionally, it was only UCP1 protein level that exclusively increased (1.4 folds) in 3T3-L1 cell line which was beforehand treated with dexamethasone as a positive control. However, UCP2 and UCP3 protein level remained constant with dexamethasone treatment.

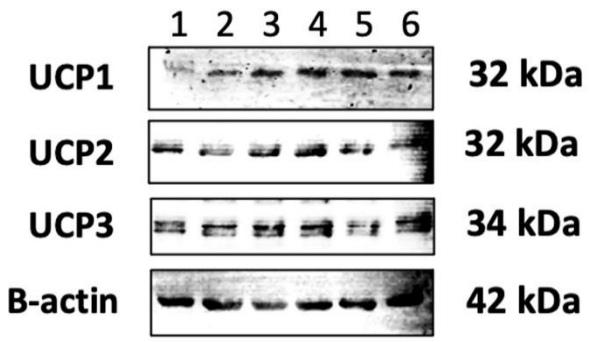

Figure 1. NuSE induced the stimulation of UCP1, UCP2, and UCP3 characterization shift in 3T3-L1 cell lines. Representative Western Blot showing the stimulation of UCP1, UCP2, and UCP3 characterization in 3T3-L1 cell lines treated with NuSE with doses of 0,01;0,1;1;10 ppm and with standard medium differentiation for 3 days. β-actin was used as internal loading control.

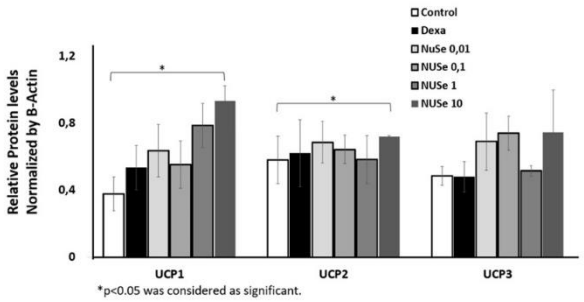

Figure 2. A graph showing UCP1, UCP2, and UCP3 protein level characterization shift in 3T3-L1 cell lines. Data were normalized with β-actin and presented as the mean ± standard error of the mean. *$p<0.05$ was considered significant. Data were tested with One Sample T-Test with 95% confidence interval.

On the contrary, NuSE treatment imparted a different pattern of effects on UCP2 protein level expression pattern in 3T3-L1 cell lines. Different from UCP1, UCP2 protein level had a decreasing trend following an increasing NuSE dose from 0.01; 0.1; to 1 ppm. However, as well as in UCP1, NuSE 10 ppm treatment significantly increased UCP2 protein level (1.2 folds) in 3T3-L1 cell lines ($p<0.05$) (Figure 2).

In the same way as UCP1, NuSE treatment also imparted an increasing trend of UCP3 protein level expression patterns in 3T3-L1 cell lines. However, unlike UCP1 and UCP2, there were no statistically significant results in UCP3 protein level increase in NuSE-treated 3T3-L1 cell lines (Figure 2).

DISCUSSION

According to the experiment results, there were alterations in UCP1, UCP2, and UCP3 protein expression patterns. UCP1 and UCP2 protein levels in 3T3-L1 cell lines

increased significantly following NuSE 10 ppm treatment ($p<0.05$) (Figure 2). UCP1 is the main protein that is involved in thermogenesis.[36] The alterations are due to the PPARγ agonist activity obtained from NuSE treatment. Coelho et al stated that PPARγ agonists are able to activate the browning of WAT, marked by UCP1 and other protein markers.[37] Macelignan (nutmeg content), which contains polyphenols, is known to play a role as a PPARγ agonist.[38] During PPARγ agonist-PPARγ receptor binding, adipogenic gene programs initiation occurs involving coactivators such as CREB-binding protein (CBP), histone acetyltransferase p300 (p300), cluster of differentiation 36 (Cd36), fatty acid-binding protein 4 (Fabp4), adiponectin, and fatty acid synthase (Fasn).[39–41] PPARγ recruits a positive regulatory domain containing 16 (PRDM16), EBF Transcription Factor 2 (EBF2), and euchromatic histone lysine methyltransferase 1 (EHMT1) and forms PPARγ/ PRDM16/ EHMT1/ EBF2 transcription complex to stimulate the expression of target genes in adipogenesis such as UCP1, UCP2, and UCP3. This process promotes the differentiation of brown and beige adipocytes from their precursors, Myf5+ and Myf5- through the browning mechanism of adipose tissue. In line with the data (Figure 1 and 2), the increasing level of UCP1 and UCP2 protein levels demonstrates the presence of the browning mechanism of adipose tissue exerted by NuSE treatment in 3T3-L1 cell line.

UCP1 and UCP2 protein level increase shown by our data (Figure 2) is paralleled with several journal reports. Lee MS et al have also observed UCP protein level increase in a dose-dependent manner in 3T3-L1 cells which were treated earlier with a polyphenols-containing substance such as epigallocatechin gallate.[42,43] A study published by Kelly et al stated that PPARγ and PPARα mediate the regulation of UCP1, UCP2, and UCP3 gene expression. Thiazolidinedione-treated rats and mice experienced an increase in UCP1, UCP2, and UCP3 mRNA expression levels.[32] This is paralleled to what we found in Figure 2. In addition, it is noted that the highest NuSE dose (10 ppm) increases UCP1 and UCP2 protein levels significantly ($p<0.05$). Therefore, NuSE needs to be administered in a higher dose to induce significant responses since low-dose NuSE just increases the protein level vaguely. In a previous study published by Lesmana et al, nutmeg extract was used as a single dose, and readjusting the dose was required to address a more optimal browning process.[36] In the present study, we have successfully complemented that previous research by readjusting the nutmeg extract into specific doses of 0.01; 0.1; 1; 10 ppm. We conclude that nutmeg in a higher dose is more effective in increasing uncoupling proteins than in the lower one. Future studies are needed to increase the dose in the interest of knowing more optimal effects of nutmeg extract.

UCP2 and UCP3 may have roles in thermogenesis[44–47] Previous studies showed that PPARγ[48] and PPARα[49] agonists may induce UCP2 and UCP3 expressions. UCP2 and UCP3 were suspected to play roles in browning adipose tissue although the exact mechanism remained unclear.[36] However, in our study we found that the effect of NuSE treatment on UCP2 and UCP3 protein expression patterns was not as significant as in UCP1. Moreover, UCP2 had a different protein expression pattern. It is shown that there is a contrarily decreasing trend in UCP2 protein levels following an increasing NuSE dose (Figure 2). There are some factors that drive these effects into nonlinear responses. One of those may be due to a hermetic response, a dynamic adaptive response, or biological plasticity of a living system in response to intermittent mild stressors, which can cause the nonlinear dose-response relationship in which low versus high doses of an agent can impart effects in opposite directions.[50–52] There are several known herbal traditional complementary medicine (TCM) that may act through hormetic dose-response mechanisms in which stimulatory effects appear at low doses and inhibitory effects appear at high doses in vitro.[53] One of the examples is the major active component of Rhizoma coptidis, berberine, which has been shown to cause an apparent hermetic effect on cells.[54] In addition, a study conducted by

Abraham Peper analyzed aspects of the relationship between drug dose and drug effect. It was concluded that the effect of a specific dose was not easy to predict owing to some rationales, such as compensatory response and tolerance level. An exceeding compensatory response compared to the normal drug effect will obtain a negative drug effect, thus making the correlation between drug dose and its effect more intricate to predict.[55] Taken together, nonlinear response of UCP protein level increase in 3T3-L1 cell lines at increasing NuSE dose might be due to those affecting factors above.

In our experiments, we used dexamethasone as a positive control. Dexamethasone has a significant role in the differentiation process of 3T3-L1 cell lines. Throughout adipocyte differentiation, there are various induced enzymes and transcription factors such as CCAAT/enhancer binding protein β (C/EBPβ), CCAAT/enhancer binding protein α (C/EBPα), and PPARγ.[56–58] Several studies have sought for methods and model systems in differentiating preadipocytes. Various chemical stimulants were found to stimulate preadipocyte differentiation, such as insulin and isobutylmethylxanthine (IBMX).[59] Insulin and dexamethasone have become a differentiation medium for 3T3-L1 preadipocytes.[60] Insulin promotes preadipocyte differentiation via a signaling network involving multiple insulin receptor substrates (IRSs). It plays specific roles by activating transcriptional events involving phosphoinositide 3-kinase (PI3K) pathway.[61,62] Whereas dexamethasone is involved in preadipocyte differentiation by regulating the expression of nuclear factors such as PPARγ, C/EBPα, and Id2. The presence of dexamethasone is really pivotal that its absence will result in poorly differentiated adipocytes.[63]

Based on our data, UCP1 was the only protein which appeared to increase 1.4 folds following the treatment with dexamethasone as positive control, while UCP2 and UCP3 protein level remain unchanged. Previous study conducted by Katharina Weber et al showed similar result to this. In the study, glucocorticoids do not significantly change UCP3 expression both in vitro and in vivo.[64] On the contrary, some other studies suggest opposite results. Emiko Kasahara et al conducted an animal study and suggested that stress-induced glucocorticoid release upregulated UCP2 expression in mice analyzed by real-time PCR.[65] Additionally, Poggioli et al concluded in their study that there was a marked drop in UCP1 mRNA levels in high fat-fed animal,[66] which was just the reverse to what we found. However, findings may vary due to different research objects, methods, confounding, and detection tools. Thus, our results could become a reference for future research development in adipocytes differentiation.

Further studies are needed to confirm whether nutmeg specifically induces the browning mechanism of white adipose tissue through PPARγ activation. PPARγ inhibitors may be used to evaluate whether it may interfere the browning mechanism. In addition, observing specific WAT genes may also be helpful. Decreasing the level of WAT genes such as AdipoQ and adiponectin may reflect that nutmeg specifically induces the browning WAT process. Furthermore, in vivo studies encompassing feeding and giving exercise training towards rats or mice could assist researchers in finding out if physical activity may enhance the nutmeg effect in the browning mechanism of WAT.

CONCLUSION

There are alterations of protein expression pattern in UCP1, UCP2, and UCP3. At NuSE 10 ppm, we found significant results in the increase of UCP1 and UCP2 protein levels ($p<0.05$). However, NuSE treatment on UCP2 and UCP3 protein expression patterns were not as significant as in UCP1 owing to several underlying factors. Otherwise, although UCP3 protein level had an increasing trend, we found no significant results in UCP3 protein expression pattern in NuSE-treated 3T3-L1 cell lines. We suggest that malignant (polyphenolic content) in NuSE acts as a hormone-like substance which plays important role in adipocyte differentiation by regulating

PPARγ. Therefore, we conclude that there is a possibility that nutmeg potentially alters the characteristics of WAT in 3T3-L1 cell lines.

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