RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v54n4.2930
Majalah Kedokteran Bandung. 2022;54(4):242–248

Majalah Kedokteran Bandung (MKB)

Received: August 1, 2022
Accepted: December 21 2022
Available online: December 30, 2022

Nutmeg Extract Alters Mitochondrial Genes Regulation of Skeletal Muscle Fibers in Aging Rats

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Abstract

Nutmeg as one of Indonesia native plants has already been used widely in herbal treatment. It has been demonstrated that nutmeg presents activities related to mitochondria functions. Nutmeg potentially has the effect of peroxisome proliferator activated receptors ϒ (PPAR ϒ) which contributes to myogenesis, and may alters mitochondrial homeostasis in respiration and biogenesis. In this study, how nutmeg extract alters oxidative process in skeletal muscle of aging rats was explored. Twenty aging Wistar male rats aged 80 weeks old were divided into two groups (control and nutmeg treatment). Nutmeg extract was administered for 12 weeks using a gavage. After that, the soleus muscles were collected, weighted, frozen, and stored at -800C until used. Nutmeg was observed to increase the COX1 (1.9 folds, $p<0.01$), COX2 (1.6 folds, $p<0.05$), and COXIV (2.8 folds, $p<0.01$) gene expressions, in parallel with increased protein carbonyl levels (1.5 folds, $p<0,01$). Nutmeg treatment also significantly increased the expressions of antioxidant endogen COQ7 (2 folds, $p<0,01$) and PDSS2 (2 folds, $p<0,01$) in the soleus muscle. Taken together, nutmeg extract may improve mitochondrial respiratory oxidative activities in type I aging skeletal muscle.

Keywords: Aging, mitchondria, nutmeg, sarcopenia

Introduction

Between the ages of 30 and 70, skeletal muscle function and mitochondrial activity decline, resulting in a 25–30 percent drop in functional capability. The progressive loss of skeletal muscle mass and function, collectively known as sarcopenia, is one of the main findings of aging. There is limited information regarding the mechanism and etiology of sarcopenia and it is still not well understood, but the previous study highlighted some factors such as mitochondrial depletion, alteration of protein synthesis, and loss of the ability of reparative satellite cells. While many possible strategies have been suggested, one of the targets for the maintenance and improvement of cellular functions in sarcopenia is mitochondria.[1,2] Sarcopenia, aging problems, and frailty have mostly been linked to 1mitochondrial malfunction and diminished oxidative capacity in the skeletal muscle. In muscle, aging is linked to a decrease in mitochondrial content and function.[3,4] Studies comparing younger and older subjects, on the other hand, have shown mixed results. The synthesis rate of skeletal muscle contractile and mitochondrial proteins in human skeletal muscle decreases in aging process, which could affect muscle metabolic capacity. In aged muscles, oxidative enzyme activity and the quantity of mRNA transcripts encoding mitochondrial proteins are also lowered.[3,5]

Oxidative damage has been suggested to be a

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major cause of age-related skeletal muscle decline and also become the basis main mechanism of aging abnormalities such as sarcopenia[6] Furthermore, counteracting the negative effects of oxidative damage may become one of the prevention and treatment strategies. Specific identification of antioxidant molecules or agents that can counteract oxidative damage should be further explored. In other words, if antioxidant molecules can counteract oxidative damage, they can also play an important role in preventing the development of age-related diseases, including inactivating processes. Interventions to enhance endogenous antioxidant defenses (e.g., antioxidant dietary supplements or herbal) are of particular interest and worth exploring further. [7]

Nutmeg is one of the herbs that is easily found in Asia and other tropical areas. Nutmeg is commonly used as a spice and also in traditional medicine. Nutmeg (Myristica fragrans) is believed and is reported to have good effects on health, including as an antioxidant, antimicrobial, and anti-inflammatory. Myristica fragrans have an antioxidant effect because some of its components easily donate hydrogen atoms or electrons to bind with reactive oxygen species (ROS).[8] In the previous study, peroxisome proliferator-activated receptors α/γ (PPAR α/γ) ligand activity can be increased after nutmeg extract treatment.[9] The receptor of PPARγ plays a key role in altering the body's glucose homeostasis regulation and insulin sensitivity. Interestingly, there is limited information regarding the role of nutmeg extract regulates the homeostasis of mitochondria gene expression in aging skeletal muscle. Hence, this study was conducted to explore the role of nutmeg extract in altering the oxidative process in the skeletal muscle of aging rats.

Methods

In this study, we used nutmeg (*Myristica fragrans*) extract that has been purified from safrole and myristicin owing to their hallucinogenic and hepatotoxicity effects. We use free safrole and myristicin nutmeg extract in this study to limit the possible sedative effect of nutmeg. We collected nutmeg-dried seeds from Maluku and West Java during the dry season. The powdered seed was then extracted with 225 l ethanol 95% at room temperature using a pilot scale extractor with a circulator rate of 150–200 rpm for 30 minutes and followed by an evaporation process at a temperature range of 40–60°C and pressure range of 400–500 mmHg.[10]

Extract (900 g) was prepared to remove safrole using pilot scale column chromatography. The column consists of a mesh size top and bottom of 70230 silica as a stationary phase. In the removal step, 130 L of the n-hexane: ethyl acetate (9:1) mixture was used as the mobile phase at a flow rate of 6 L min, and the eluate was eliminated. The residue was then eluted with 100 L of methanol, and all the extracts were collected, evaporated, and analyzed for safrole concentration. The safrole concentration of the extract was determined by a high-performance liquid chromatography system & #40; Waters 2998 & #41; analyzed. The analysis was performed using a C18 column (LiChroCART 2504 LiChrosphere 100 18e (5 μm) (EMerck)). The mobile phase consisted of a 27:73 (v v) mixture of water (component A) and methanol (component B). Chromatographic separation was performed at room temperature (25 ° C, maintained in air conditioning) with an injection volume of 20 μl. The data collected at the photodiode array detector wavelength of 200-400 nm was used for analysis. [10]

All the animal experiment procedures in this study were approved by Health Research Ethics Committee Faculty of Medicine Universitas Padjadjaran No 386/UN6.KEP/EC/2019. Total of 14 male Wistar rats at the age of 80 weeks with body weight 400-450 gram used in the present study were purchased from Biofarma Laboratory. Every 3–4 rats were kept in a cage (50 cm x 47cm x 45cm) with food and water available *ad libitum*. Environment was maintained under a 12 h light-12 h dark cycle and room temperature. After experiencing the adaptation process for 2 weeks, the rats were divided into two groups: control group and treatment group. Nutmeg extract was given by gavage to the treatment group for 12 weeks, meanwhile control group was given water and PGA 2% at the same time. The dose administered was counted individually for each rat–as a conversion of the human dose (500 mg/day) of 0.9 mg/day/kg body weight.[10] The rat was sacrificed using isoflurane anaesthesia. Using a precision vaporizer with induction chamber and waste gas scavenger, the gas anesthetic will be administered slowly up to >4.5% in 100% oxygen and continued until lack of respiration for >1 minute is observed. The soleus muscle were removed, weighed, and rapidly frozen in liquid nitrogen and stored at -80°C until use. Outcome of this experiment is the molecular alteration in mitochondrial dynamic

and skeletal muscle fiber.

This study use Trizol reagen from Invitrogen to analyze total RNA from muscle tissue. Onestep PCR kit (Bioline, USA) was use in this study. Specific primers for COX I, COX II, COX IV, PGC1α, MYHC1, COQ7, and PDSS2 were used. Primer sequences and annealing temperatures are shown in Table 1. Normalization using β-actin mRNA levels as an internal control for the results of PCR were analyze for each samples.

Protein carbonyl assessment that was used is Protein Carbonyl Content Assay Kit Sigma Catalog Number MAK094. Carbonyl content is analyzed by the derivatization of protein carbonyl carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) lead to the formation of stable dinitrophenyl (DNP) hydrazone adducts, which can be analyzed spectrophotometrically at 375 nm, reflects to the carbonyls present. All samples and standards was run in duplicate. Samples can be prepared in any suitable lysis buffer and centrifuged to remove any insoluble material. Samples should be diluted with water to a protein concentration of 10 mg/mL. ROS can induce damage to DNA, lipids, and proteins. The oxidation of proteins results in the production of stable carbonyl groups, which can be used as a measure of oxidative injury. The Protein Carbonyl Content Assay measures carbonyl content by determining derivatization of protein carbonyl groups with 2,4-dinitrophenyl- hydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts, which can be detected spectrophotometrically at 375 nm, proportional to the carbonyls present. All procedures followed the manufacture recommendations as decribed: 100 uL of DNPH Solution to each Sample. Vortex and incubate for10 minutes at room temperature.; Add 30 uL of the 87% TCA Solution to each Sample. Vortex and incubate on ice for 5 minutes. Centrifuge Samples at 13,000 × g for 2 minutes. Remove supernatant being careful not to disturb the pellet and add 500 uL of ice-cold acetone (not included) and place in a sonication bath for 30 seconds. Incubate at -20 °C for 5 minutes, and then centrifuge samples at 13,000× g for 2 minutes. Carefully remove acetone from pellet. Remove acetone once more to remove free DNPH. 200 uL of 6 M Guanidine Solution is added to pellet and sonicate briefly. Most proteins will resolubilize easily in the Guanidine Solution. Transfer 100 uL of each Sample to the 96 well plate. Final step, we measure sample with the absorbance at 375 nm (A375).

Differences of gene expression and protein between control and treatment group were analyzed with One-Way Analyze of Variance (ANOVA) test using SPSS V.13.. Data were expressed as mean ± standard error minimum

Table Primer PCR and Cycles

Name of Primer	Gen Code (Pubmed)		Sequence	Tm (C)	Time (sec)	Cycles	Product (bp)
ß-actin	NM_031144.3	F	tgg aga aga ttt ggc acc a	60	40	35	193
		R	cca gag gca tac agg gac aa				
PGC-1alpha	XM_017599391.1	F	CGC ACA ACT CAG CAA GTC CTC	62	90	37	263
		R	CCT TGC TGG CCT CCA AAG TCT C				
COX4	NM_017202.1	F	CTC CCA TCT TAT GTT GAT CG	51	40	35	144
		R	GTA CAA TTG GAC TTT CTC ATC C				
COX1		F	GGA GCA GTA TTC GCC ATC AT	55,3	40	35	244
		R	CGG CCG TAA GTG AGA TGA AT				
COX2		F	ACT TGG CTT ACA AGA CGC TAC A	56,7	40	35	162
		R	TCT TGG GCG TCT ATT GTG CTT				
COQ7	NM_012785.3	F	TGG AGG AGG ACG CTG AGA AG	57	40	35	309
		R	GTG ACA CAA CCC CAA ACA CC				
PDSS	NM_001014249.1	F	CTC GGC ACC CGT AGT CTT AC	58	40	35	122
		R	CAG CTT TGA TTG TTT CCC GCA				

(SEM). Statistical significance was designated at p <0.05. Every animal subject was included to analyse.

Result

Twenty 80-week male Wistar rats were sacrificed and soleus muscles were analyzed. All the animal subjects were in healthy condition located in individual cage. No inactive nor sick animal subjects were found in this study. There were no adverse effect of nutmeg extract treatment observed during routine welfare assessment in this study.

One of the indicator of mitochondria quantity in skeletal muscle through gene expression of cytochrome c oxidase subunit 1, 2 and IV. COX1, COX2 and COXIV are the component of the respiratory chain in OXPHOS system. Nutmeg extract increased COX1 gene expression by 1.9 folds ($p<0.01$), increased COX2 by 1.6 folds ($p<0.05$) and increased COX IV by 2.8 folds ($p<0.01$) in soleus muscle (type I muscle) after 12 weeks nutmeg treatment. Increase COX1, COX2 and COX IV provide some insight that nutmeg extract may increase mitochondrial activity from coordination of three genomes significantly in soleus muscle. We also observed that nutmeg extract show not significant changes of PGC1α gene expression.

Skeletal muscle is one of the tissue that undergo high oxidative metabolisme both in type I and type II muscle. There are age-dependent increases of cytochrom oxidase deficient in myocytes in skeletal muscle and reduce electron transport chain complexes activity in skeletal muscle mitochondria. Higher level of COX1, COX2 and COX IV may reflects higher mitochondria level and probably the respiratory oxidative process within soleus muscle. We then analyse the level of protein carbonyl content as one of the marker of oxidative stress. Increase protein carbonyl can be considered as increasing oxidative metabolisme or decreasing antioxidant capacity in skeletal muscle. Protein carbonyl content were significantly higher in nutmeg

We measured the gene expression of COQ7 and PDSS2 after nutmeg treatment, there is significant increasement of COQ7 by 2 folds ($p<0,01$) and PDSS2 by 2 folds ($p<0,01$) in soleus muscle. Increasing gene expression of COQ7 and PDDSS2 in this type of muscle which have abundant mitochondria after nutmeg treatment may reflect the improvement of endogenous antioxidant induced by nutmeg or as adaptive response to increase of oxidative stress.. Increase protein carbonyl can be considered as increasing oxidative metabolisme or decreasing antioxidant capacity in skeletal muscle. Protein carbonyl content were significantly higher in nutmeg group both in type I soleus muscle ($p<0,01$). Protein carbonyl was increased significantly 1.5 folds by nutmeg treatment in soleus muscle of aging rat.

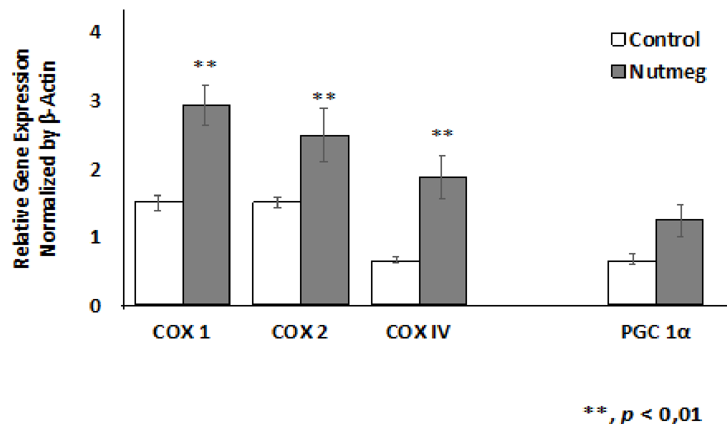

Figure 1 Nutmeg Increased COX 1, COX 2, and COX IV Gene Expression in Soleus Muscle of Aging Rats

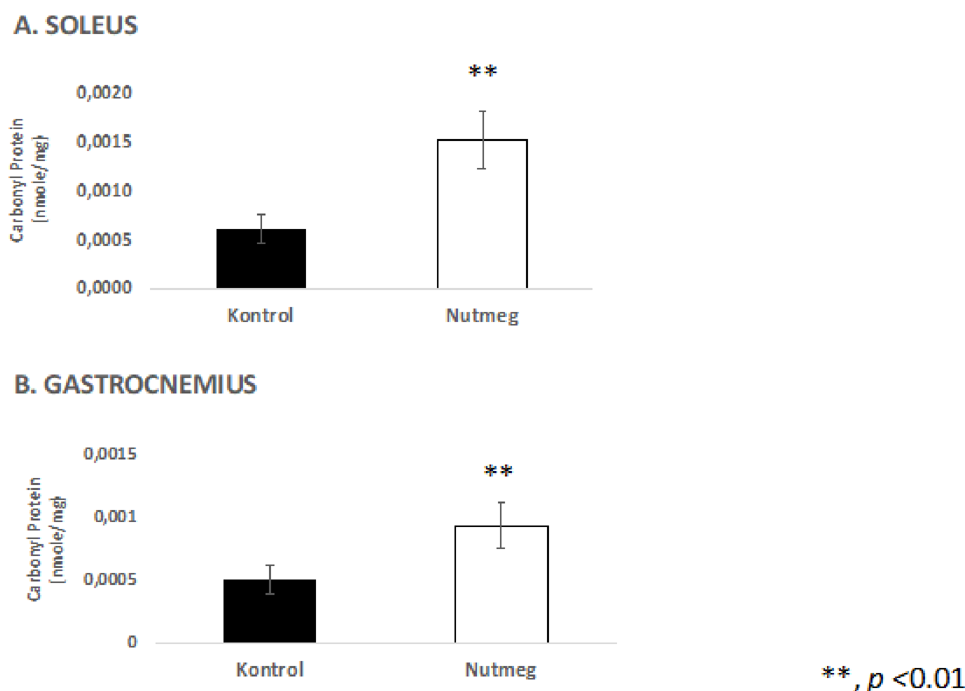

Figure 2 Nutmeg Increased Protein Carbonyl Level in Soleus Muscle of Aging Rats

Discussion

Increasing risk of fall and deaths are increased along with aging process specifically toward skeletal muscle. On the other hand, skeletal muscle function support almost all the daily life activities and highly associated with mitochondrial functions to fulfill ATP demands.

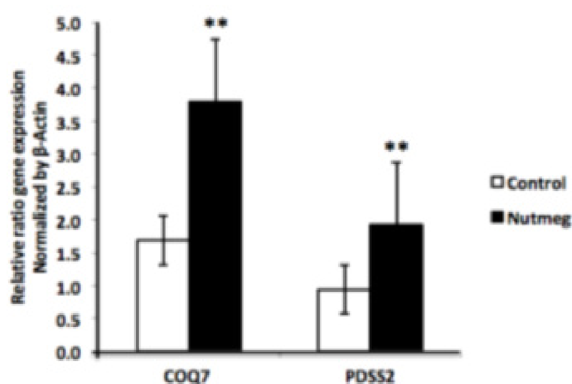

Figure 3 Nutmeg Increased Gene Expression of COQ7 and PDSS2 in Soleus Muscle of Aging Rats

Therefore, the content and function of muscle mitochondria are directly related to muscle performance, risk of metabolic disease, and quality of life. In previous studies using the in vivo and ex vivo approaches, mitochondrial function of skeletal muscle decreased with age in both rodents as in humans.[11] Three primary mitochondrial functions in aging skeletal muscle are most likely correlated to this process: adenosine tri phosphate (ATP) production, ROS production and apoptosis-regulating mitochondrial function. [12] Although there are quite many evidences in the role of mitochondrial alteration during aging process, recent evidence suggests further analysis, as alterations in mitochondrial function is also influenced with different fiber type composition. [13]

The hindlimb muscle of rats consist of a slow myosin isoform (MHC I) and three fast myosin isoform (MHC IIA, IIB and IID). The rats soleus muscle have various fibre type I, IIA, IIAD and IID fibers, with a predominance of type I. [14] In this study, we use soleus muscle because it is more solid model to observe small changes stimulated by many factors since it can be harvested cleanly whole bundle to eliminate error during sampling. It has specific characteristic as follow:

approximately 40% type I, 40% type IIA and few IIB/X with higher mitochondrial content and consider slow twitch type muscle. Soleus muscle is important for maintaining posture and balance and another function that demand endurance. [14]

Previous studies in aged mice have shown that the effect of age on mitochondrial respiratory function is muscle-specific and emphasizes a central role in complex I dysfunction. Deterioration of mitochondrial quality and increased compensatory increase are observed only in type II muscles, whereas type I muscles show minimal effect on aging and compensatory mitochondrial protein content during aging. [15] COX1 and COX2 are mitochondrial encoded gene that reflects respiratory chain process by mitochondria. Increased gene expression of COX1, COX2 and COXIV significantly in aged type I muscle after nutmeg treatment may showed that there is increasing mitochondrial respiratory function (Figure 1).

Muscle dysfunction during aging process is characterized by reductive remodelling of muscle fibers and gradual impairment in all physiological functions including oxidative stress. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant systems.[16] Previous studies showed that ROS may also correlate with various signaling pathways in skeletal muscle. Moderate increase in short-term oxidants activate signaling pathways that lead to cellular adaptation and protection from further stress within muscle fibers. [17] Nonetheless, high levels of ROS synthesized over time can lead to accelerated proteolysis and ultimately cell death.

Protein carbonyl is type of protein oxidation which is induced by ROS accumulation, thus it is can well-used marker for oxidative stress. Protein sequestration in the plasma is usually analyzed as protein carbonyls content, was significantly higher in nutmeg treatment in aged oxidative and glycolitic type muscle (Figure 2). The increasement of protein carbonyl can be correlated with the increase of COX1, COX2 and COXIV as an indicator of mitochondrial respiratory function. Increased of protein carbonyl may give benefit as long as muscle cells have proper endogenous antioxidant.

Ubiquinone coenzym (coenzyme Q) is a prenylated benzoquinone which plays a fundamental role shuttling electrons from Complex I and Complex II to Complex III in the mitochondrial electron transport chain and also may have a protective roles against oxidative stress as the only lipid-soluble antioxidants which is synthesized in all organism.[18] mCOQ transcripted in skeletal muscle mostly consist of mCOQ7 followed by mCOQ8, mCOQ2, mPDSS2, mPDSS1 and mCOQ3.[19,20] Reduction of COQ7 has been associated to changes in oxidative stress, increase protein carbonyl content in type I muscle together with increase of gene expression of COQ7 and PDSS2 (Figure 3) may reflect possible adaptive respons from aged skeletal muscle after nutmeg treatment. Increase COQ7 and PDSS2 may be resulted from adaptive physiological responses toward declining mitochondrial function during aging or as a response to increasing oxidative stress. The balance between ROS and endogenous antioxidant is needed to ensure muscle cell function, in which, COQ7 and PDSS2 genes expression was involved and altered with oxidative changes.

Understanding regarding ROS increase in our system during aging, we found that nutmeg coincidentally also increase expression of gene regulated for antioxidant activity (Figure 3). This may lead us to possible beneficial ROS regulation stimulated by nutmeg. This process may indicate that nutmeg stimulated beneficial ROS in order to preserve or maintain physiological function during aging. Mitochondrial dynamic during aging remain uncertain until now, further molecular analysis to prove exact effect of nutmeg extract effect and its potential benefit still need to pursue moreforward. In addition, the chemical component of nutmeg extract still unclear undergo further analysis to make sure its efficacy and possible adverse effect.

Taken together, nutmeg may have role to stimulate gene expression of mitochondria and ROS production. Increasing ROS production together with increasing COQ7 and PDSS2 as endogenous antioxidant may reflect that this is due to physiological adaptation during aging skeletal muscle fiber type while there is increasing indicator of improvement in respiratory oxidative activity. We assume this process also involve mitochondrial dynamic and possible post translational modification in aging skeletal muscle.

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