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Alteration of Skeletal Muscle's Satellite cell Differentiation Gene in Young Rats by Nutmeg Supplementation

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Increasing and maintaining skeletal muscle mass known to have beneficial effects to maintain stability of body's metabolism in young adult, while reduction of skeletal muscle strength and mass associated with physical disability and increased morbidity and mortality. Skeletal muscle growth involves activation of satellite cells. This study investigated the effect of Nutmeg seed extract free safrole and myristicin (Nutmeg) on skeletal muscle mass and activation of satellite cells in soleus and gastrocnemius muscle.Ten male Wistar strain rats ages 6 week were divided into 2 groups randomly. Nutmeg extract were given to treatment group for 12 weeks. By the end of treatment period, soleus and gastrocnemius muscle were isolated and subjected for gene expression analysis. Nutmeg administration increased gastrocnemius muscle mass (p=0.025) and soleus muscle mass (p=0.028). In soleus muscle, Nutmeg significantly increase Akt (p=0.007) and *MyoD* gene expression (p=0.037) but not the *Myf5* gene expression (p=0,221). While gastrocnemius muscle of the Nutmeg group showed higher expression on Akt gene (0.038). However, no difference were observed in gastrocnemius *Myo D* (p=0,081) and *Myf5*(p=0,323) mRNA expression. It suggest, that Nutmeg extract increase *MyoD* expression through activation *Akt* pathway mainly in Soleus muscle.As the conclusion, Nutmeg extract administration increase protein synthesis in skeletal muscle through satellite cell activation partly via *Akt* and *MyoD* gene expression.

Keywords: Skeletal muscle, Nutmeg, autophagy, satellite cells.

Skeletal muscle plays a major role in physical activities, another important skeletal muscle role is maintaining metabolic homeostasis especially glucose metabolism[1]. Research in the elderly community in Korea showed a greater risk of developing type 2 diabetes mellitus in groups with lower fat- muscle mass ratio[2–4].

Skeletal muscle mass affected by various conditions such as aging, cachexia, physical activity, denervation and burning. Gradual and complete reduction in skeletal muscle mass and strength associated with physical disabilities, decrease independence and increasing morbidity and mortality[5–7]. Therefore, skeletal muscle mass is an important factor that can affect quality of life.

Skeletal muscle mass changed by various stimuli such as exercise, nutrition and micronutrient balance[8,9]. The combination of exercise and proper nutrition proved can induce beneficial molecular signaling pathways to increase and maintain muscle mass. Skeletal muscle mass regulated by the processes of muscle protein synthesis, which lead to muscle hypertrophy and muscle protein breakdown (atrophy) and this process is influenced by physical activities, nutrition such as amino acids, growth factors, glucose and insulin[10–12].

IGF-AKT/mTOR is the main pathway to regulate the skeletal muscle mass by increasing protein synthesis and suppress the protein degradations[13]. Previous studies showed that various physical activities can increase the IGF-1, Akt and mTOR expression in human and its downstream pathways to induce skeletal muscle hypertrophy[14,15]. Notably, the mTOR is known as the main regulator in skeletal muscle regulation. The mTOR regulate the process of protein translation and also known as one of the major pathways to regulate autophagy[16]. Autophagy is a process of self-eating the damaged cellular organs, recycling proteins, protect cells from cellular stress or nutritional limitations and to regulate cell death pathway. Therefore, the autophagy prevents the accumulation of damaged organelles and maintain the myofiber homeostasis.

In skeletal muscles, autophagy needed to maintain the muscle mass and the myofiber integrity [17,18]. However, excessive autophagy can lead to muscle wasting[19]. The activated mTOR, through AKT and AMPK signaling can suppress the autophagy process[20,21]. Previous studies showed resistance training combined with endurance exercise effective to prevent autophagy.

Akt pathways also involved in the satellite cell activation by increasing the expression of *MyoD*[22,23].*MyoD* is a family member of myogenic regulatory factors (MRF) that play a role in regulating the process of myogenesis and muscular hypertrophy. *MyoD* is highly expressed in satellite cells during differentiation period. Satellite cells activated when muscle regenerated and differentiated, thus satellite cells play an important role in the process of muscle regeneration and maintain muscle mass by producing new fiber muscle[24].

Recent studies in natural components showed a promising effect to stimulate the IGF-Akt pathways as alternative supplements to increase and maintain skeletal muscle mass. Nutmeg or often called Nutmeg with the scientific name Myristica Fragrans is a typical Indonesian plant that is from the Maluku islands, has many benefits such as anti-inflammatory, analgesic, anticancer, antioxidant, hypoglicemic and antidiabetic [25–30]. Nutmeg seeds have good potential for the treatment of type 2 diabetes with increasing activity through the mechanism of PPAR γ agonist [31,32]. The activity of PPAR γ agonist in Nutmeg seeds by increasing insulin sensitivity is expected to activate the AKT and mTOR signaling pathways to increase protein synthesis and muscle mass. Previous research showed that the Nutmeg extract treatment able to maintain muscle mass and prevent sarcopenia in old rats by increasing IGF-1 expression followed by a cascade of Akt / mTOR signaling pathway which leads to increase myogenesis and muscle regeneration.[33] Age differences affect the regulation of skeletal muscle protein synthesis process. Changes in metabolism process such as higher fat deposition repress myogenesis in elder age.[34] Consequently, effect of administration of nutmeg in young rats' skeletal muscle needs to be determined. Therefore, we investigate the Nutmeg regulation on the skeletal muscle hypertrophy through the Akt/mTOR pathways and the involvement of satellite cell in skeletal muscle hypertrophy.

METHODS

Animal Experiments

12 male Wistar rats aged 6 weeks, obtained from the Animal Facility of PT Biofarma Indonesia. Rats were kept at 24°C under a 12-hour light - dark cycle, and humidity were adjusted to 55%, with food and water ad libitum for 12 weeks in Animal Laboratory, Physiology Division, Faculty of Medicine, Universitas Padjadjaran. All experimental protocols and methods were approved by the Ethics Committee, Faculty of Medicine, Universitas Padjadjaran Number 28/UN.6.KEP/EC/2019.

The rats were randomly divided into control and treatment group. The Nutmeg extract was given to the treatment group for 12 weeks by gavage. The Nutmeg extract used in this study was free safrole and myristicin. Nutmeg extract as

Glucopala Caplet were obtained from the Faculty of Pharmacy Laboratory, Padjadjaran University batch number FP08.A1604.001[31,35–37]. The dose given to the rats in the treatment group was converted from human dose (300 mg / day) with final dose 8.1 mg/kg/day.Nutmeg seed extract was given once a day. The rat sacrificed using overdose isoflurane, then the soleus and gastrocnemius

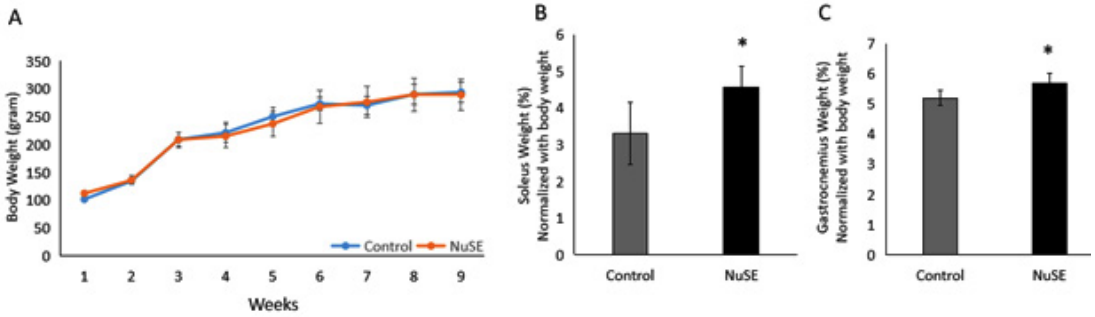

Fig. 1. Body weight and skeletal muscle mass. A body weight per week, B soleus muscle mass and C gastrocnemius muscle mass. Data presented as mean ±SD. Asteriks indicated significance difference, *p<0.05

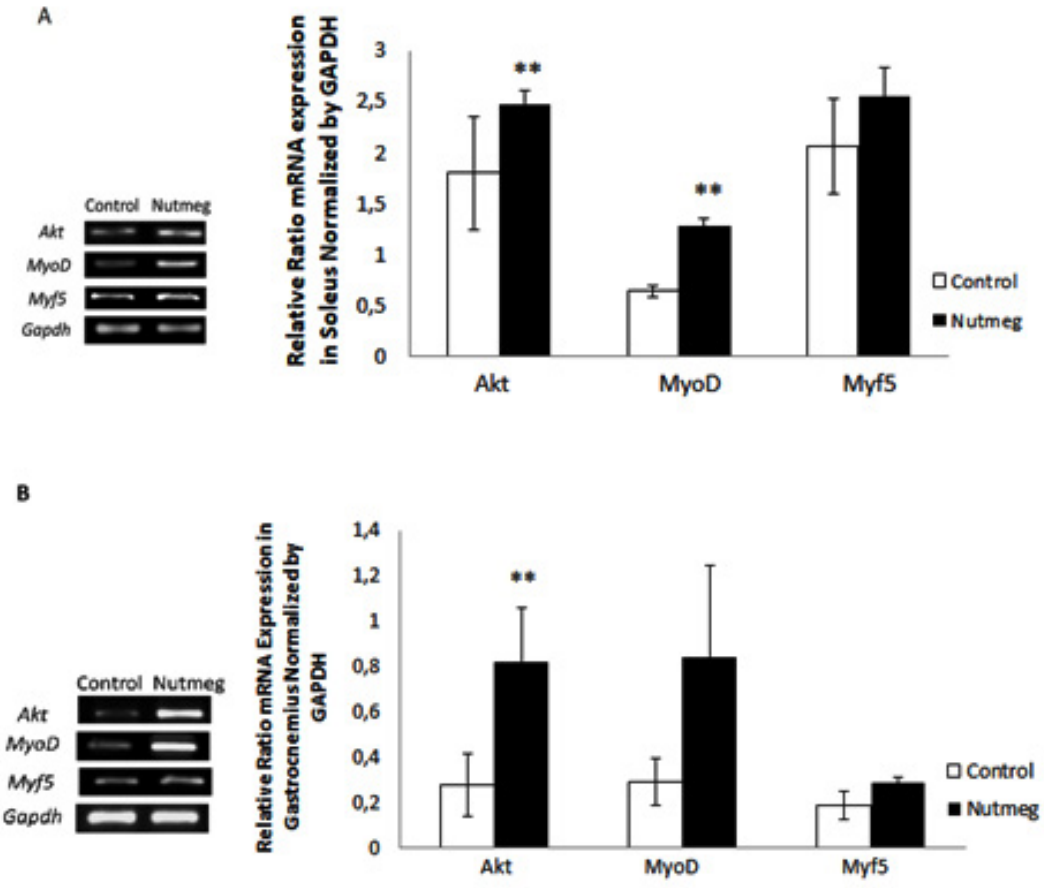

Fig. 2. Nutmeg increase Akt and *MyoD* signaling in Soleus Muscle (A). Nutmeg induce Akt expression in Gastrocnemius muscle (B). Graph were normalized with Gapdh and presented as mean ± SD. Asteriks indicated significance difference **p<0.01

muscle were removed, weighed, snap frozed in liquid nitrogen and stored at -80°C.

RNA Extraction and Semi-Quantitative PCR

The RNA isolated from ± 2 mg muscles tissue with Trizol reagent (from Invitrogen, USA). Polymerase Chain Reaction (PCR) using One Step PCR kit (Bioline, USA). Primers for AKT, *MyoD*, *Myf5* and GAPDH were presented in Table 1.The gene expression results were normalized with GAPDH.

Electrophoresis

Analysis of the results of RT-PCR was carried out using 2% agarose electrophoresis gel. The PCR band were visualized using BluePAD (Biohelix, Taiwan) and band image were quantified using ImageJ software (NIH, USA).

Statistical Analysis

The Data were analyzed by comparing the mean with Independent t Test using SPSS v.25 with significant value if $p<0.05$. Data were presented as mean ± standard deviation (SD).

RESULTS

Body weightand skeletal muscle mass

During the treatment period, body weight were measured weekly to analyze whether skeletal muscle weight increase due to difference body weight. Rats body weight were increase in both groups (Figure 1A). But there was no difference of body weight among groups. By the end of treatment period, rats were euthanized and skeletal muscle were isolated. Soleus and Gastrocnemius muscle were isolated and measured. Muscle weight were normalized with rats' body weight. Soleus muscle and gastrocnemius muscle mass were higher in Nutmeg group compared with control, $p<0.05$ (Figure 1B and 1C).

Nutmeg Extract Stimulate Satellite Cells Proliferation through Akt Pathway

The Akt pathway is an important pathways to increase the protein synthesis. Moreover, the Akt involved in the cells survivals, differentiation and proliferation. In this study we observed the soleus and gastrocnimeus Akt expression in the treatment group was increase significantly $p<0.01$ (Figure 2). Akt expression were increased 1.37 fold in Soleus and 2.9 fold in Gastrocnemius.

Interestingly, *MyoD* expression were increase only in soleus of the Nutmeg group (2 fold, $p<0.01$, Figure 2A). While in gastrocnemius muscle, no difference of *MyoD* expression were found in control and Nutmeg group ($p=0.43$). *Myf5* is the earliest Myf that expressed in early myogenesis. We measured *Myf5* gene expression in Soleus and Gastrocnemius muscle. We found no changes of *Myf5* mRNA expression in both muscle (Figure 2A,B).

Nutmeg Extract Inhibit Autophagy in Soleus Muscle

The autophagy is involved in the balance of protein synthesis and degradation through the AKT-mTOR pathways. The activation of AKT blocks the FoxO and inhibit protein degradation.

Table 1. Primer Sequences

Primer	Sequence	Tm (°C)	Length (bp)
GAPDH	F: 5' TGGAGAAGATTTGGCACCA 3' R: 5' CCAGAGGCATACAGGGACAA 3'	61	177
Myf5	F: 5' ACGTCCCCAATGAGATTAGCA 3' R:5' GGGCTTCACTTACTGGGCAT 3'	60	189
Myo D[33]	F: 5' AGCACTACAGTGGCGACTCA3' R: 5' GGCCGCTGTAATCCATCA 3'	56	212
AKT	F: 5' -CTAACTTGAGCCGCAGGAAC 3' R: 5' -GCTTGCTCAGTTTGCTACCC-3'	57	165
mTOR[38]	F: CTGATGTCATTTATTGGCACAAA R: CAGGGACTCAGAACACAAATGC	57	170
LC3[38]	F:GGTCCAGTTGTGCCTTTATTGA R: GTGTGTGGGTTGTGTACGTCG	59,5	153
P62[38]	F: CTAGGCATCGAGGTTGACATT R:CTTGGCTGAGTACCACTCTTATC	56	116

The previous study showed the mTOR inhibits autophagy by increase the autophagy mediator which is known to indicate inhibition of autophagy [39].

As shown in Figure 3A,Nutmeg extract increase mTOR 1.25 fold and P62 gene 1.3 fold compared to control ($p<0.05$) but did not increase the Igf-1 in soleus muscles. While in gastrocnemius muscle,no significant difference on autophagy pathway were observed between nutmeg and control group ($p>0.05$; Figure 3B).

DISCUSSION

Nutmeg seeds have various active components, including antioxidants, antibacterial, analgesic and antidiabetic effects. Nutmeg seed has potential role as antidiabetic agent by increasing insulin sensitivity through PPARγ agonists. PPARγ agonist increasing insulin sensitivity and involves in phosphorylation of insulin receptors (IRS-1) to activate the PI3K/AKT pathway cascade [31,40].

The molecular mechanism of muscle mass regulation involves various signal transduction pathways. Increasing skeletal muscle mass in the post-natal period is related to the process of hypertrophy of muscle fibers through the regulatory pathway between protein synthesis and degradation which are carried out through the PI3K/AKT pathway [10]. Akt pathway activate satellite cells differentiation through MRFs expression. MRFs including *MyoD* and *Myf5* induce in satellite cell differentiation and proliferation.

In this study, we found that Nutmeg extract increased soleus and gastrocnemius muscle mass supported by increased of AKT gene expression. It suggest that nutmeg extract increased skeletal muscle mass through activation Akt signaling. Activation of the PI3K/Akt pathway causes phosphorylation of mTOR and GSK3â which are important regulators of the protein translation process. mTOR has two different complexes that work through regulation of protein metabolism and autophagy [16]. Increased expression of Akt

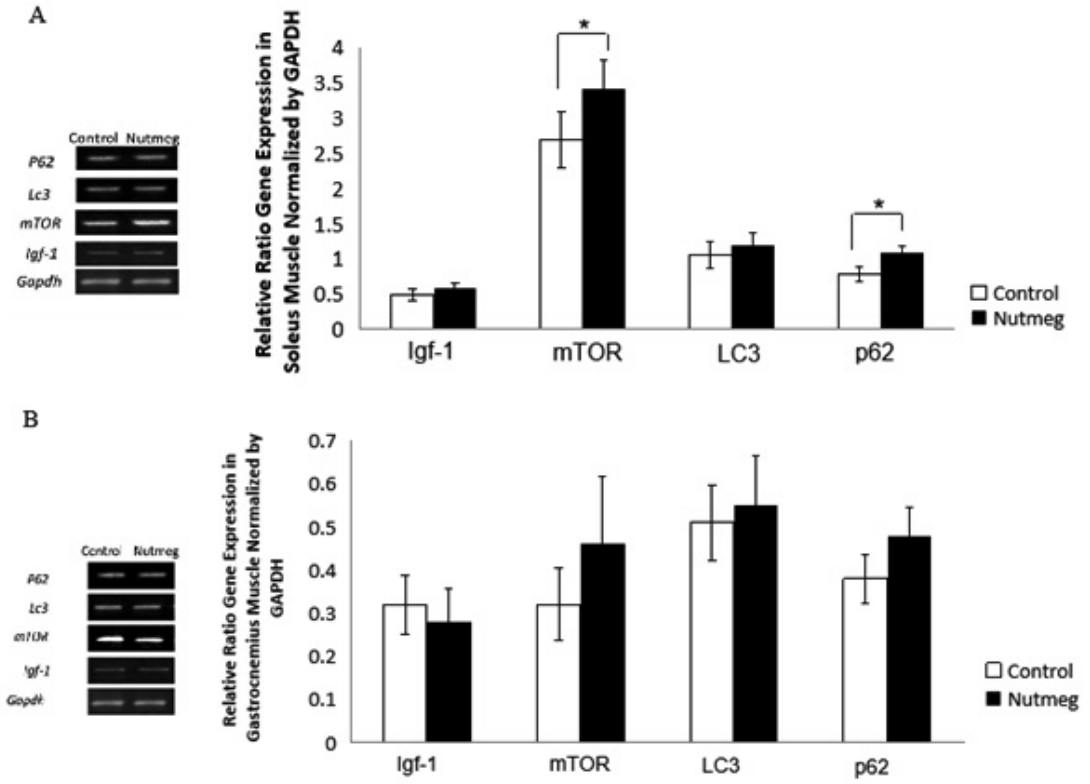

Fig. 3. A-B Autophagy gene expression in Soleus muscle, normalized by GAPDH. Autophagy gene expression in Gastrocnemius muscle normalized by GAPDH. Data represent mean ± SD. Asterisk indicated significant difference to the control group, * $p<0.05$

was observed in our previous study using old mice that show the effect of Nutmeg on increasing soleus muscle mass, through the IGF-AKT-mTOR pathway thereby increasing protein synthesis and inhibiting autophagy in aging [33]. This study showed similar results, Akt-mTOR pathway were increasing while autophagy process were inhibited in soleus muscle. However, in young rat, we observed no significant change in gastrocnemius mTOR expression. Possibly, this phenomenon was due to activation of FoxOs as the balancer of mTOR and Akt pathway[51].

The pathway to increase protein synthesis is carried out through IGF1 / Akt / mTOR. However, in this study, the Akt and mTOR increase did not preceded by change in IGF1 expression. Upregulated of Akt / mTOR expression might be caused by the improvement of glucose uptake by PPAR agonists in Nutmeg seed extract especially in soleus muscle[31].

Correlated with Akt/mTOR gene alteration in soleus muscle, we observed significant changes in autophagy related gene in nutmeg extract treated rats. Our results showed autophagy related gene were increased in nutmeg group. Increased in p62 gene expression together with trend of LC3 upregulation (Figure 3A) indicated the inhibition of autophagy in soleus muscle. This results was consistent with our previous study in aging rats, that nutmeg extract decreased autophagy in 80 weeks rat' soleus muscle[33]. In gastrocnemius muscle, we found the similar trend of autophagy inhibition although not statistically significant (Figure 3B)[38].

Satellite cells mainly known for their contributions to regenerate the muscle cells while injury, the maintenance of muscle mass and hypertrophy.[41,42] Satellite cells suggested promoting the muscle fibres hypertrophy by mediating myonuclear addition and sustains muscle growth. *MyoD* and *Myf5* expression in adult muscles increases when satellite cells are activated. This marker shows the satellite cells differentiation, followed by myogenic determination and finally directing progenitor cells to form skeletal muscle lines. Several studies have shown the role of *MyoD* and *Myf5* in response to muscle hypertrophy, denervation, disease, and atrophy.[11,43]

Our results showed an increase in the expression of *MyoD* in the soleus muscles but not in gastrocnemius muscle. This could be relates to the type of muscle fiber, where the soleus muscle is dominated by type I muscle fibers (oxidative), while the gastrocnemius muscle is dominated by type II fibers (glycolytic). Oxidative muscle fibers are known to have a greater capacity of protein synthesis and autophagy process. This high protein turnover process, in type I muscle fibers showed higher potential for adaptation and regeneration in this type of fiber[44–47].However, both types of muscle fibers have the same sensitivity to the process of insulin phosphorylation. This statement is in line with the results of this study, the Aktas the insulin signaling downstream- expression was increased in both muscle type [48].

The increasing of *MyoD* expression was not accompanied by an increase in *Myf5*.This results might be associated with the different role of the two genes during satellite cell activation. *MyoD* and *Myf5* expression in adult muscles increases when satellite cells are activated. Several studies have shown the role of *MyoD* and *Myf5* in response to muscular hypertrophy, denervation, disease, and atrophy [43,49]. Research on transgenic mice that have been omitted by the expression of *Myf5* and *MyoD* shows failure of regeneration and differentiation. This shows the role of *Myf5* in the process of activation and differentiation of satellite cells [24]. However, other studies have shown different roles between *Myf5* and *MyoD*. *Myf5* involved in proliferation prior differentiation of myoblast while *MyoD* increased during early differentiation of satellite cells[50].

It suggest that nutmeg extract might increase skeletal muscle mass through satellite cells differentiation. This results was consistent with our previous results in elderly rats[33]. However, nutmeg crude extract that has some different effect on young and elder rat. Discrepancies in young rats might be caused by age difference between subjects (young and elderly rats) and post transcriptional modification, enzymatic reactions and hormonal modulation. In both studies, we use crude extract of nutmeg. The nutmeg extract contain another active compound that might have different effect on young and aging rats skeletal muscle.Another studies to characterized the active compound in nutmeg extract that is essential for skeletal muscle differentiation need to be conducted.

Limitation of this study was we only use gene expression levels to detect early changes

in skeletal muscle. Future study to analyze the protein levels is needed to address discrepancies of soleus and gastrocnemius response to Nutmeg administration.

CONCLUSION

Taken together, we conclude the Nutmeg extract increase skeletal muscle mass in young rats through alteration satellite cells differentiation gene and autophagy gene expression in soleus muscle. However, although gastrocnemius muscle cell mass was increased by Nutmeg extract administration, no changes was observed in *MyoD*, *Myf5* and autophagy gene expresssion. Thus, Nutmeg extract supplementation has potential effect on increasing skeletal muscle mass in young individual.

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