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Original Research Article

Effect of Myristica fragrans on PGC1α and *Synaptophysin* Expression in Male Wistar Rats Hippocampus

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Abstract

Background: Nutmeg is an indigenous plant from Indonesia that has been used extensively in herbal treatment. Nutmeg seed extract (NuSE) contains the active compound macelignan, which exhibits pharmacological activities. A previous study stated that NuSE is PPARγ or peroxisome proliferator-activated receptor gamma agonist that potentially enhances synaptic signal modulation. PPARγ activation can activate PGC1α or peroxisome proliferator-activated receptor gamma coactivator-1 alpha as the primary regulator of mitochondrial biogenesis. Mitochondria are involved in synaptic transmission. Increased modulation of signals at synapses can increase neuroplasticity, potentially improving the brain's cognitive function, as seen by the amount of *Synaptophysin* in the synaptic vesicle membrane for evaluating synaptogenesis.

Objective: This research demonstrates how nutmeg seed extract (NuSE) affects PGC1α and *synaptophysin* expression compared with DHA or docosahexaenoic acid, which has been evidenced to promote neurite growth.

Methods: Twenty-four Wistar male rats aged eight weeks were divided into four groups (control, PGA group, NuSE group, and DHA group). The treatment group was administered for 12 weeks using a gavage. After that, the rats were sacrificed, and the hippocampus neurons were collected. The PGC1α and *Synaptophysin* mRNA expression was measured using semiquantitative reversed PCR, visualized with electrophoresis, and then quantified with ImageJ. The analysis used in this study was a one-way ANOVA test to measure differences between groups using SPSS 26.0. If the test leads to significant results, a post hoc test is used to to confirm the differences between groups statistically.

Results: This study showed that NuSE increased *synaptophysin* and PGC1α mRNA expressions compared to the control group with significance statistic ($p=0.017$, $p<0,05$) in *synaptophysin* expression but did not increase PGC1α expression significantly ($p=0.364$, $p>0,05$).

Conclusion: In conclusion, nutmeg seed extract (NuSE) impacts synaptogenesis in *synaptophysin* expression to modulate synaptic transmission.

Keywords: *Hippocampus; PGC-1a; NuSE; Synaptophysin*
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INTRODUCTION

Neuroplasticity is a process of structural and functional adaptive changes.[1] Plasticity in neuronal morphology and electrical responsiveness occurs with regular changes in the nervous system.[2] Other factors that affect neuroplasticity are changes in the strength and number of synapses and the reorganization of neural circuits.

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An increase or decrease in synaptic activity results in a continual increase or decrease in synaptic strength so that experience (environment) directly affects the brain's physical and functional properties. Neuroplasticity is essential for learning and memory processes because it is the central nervous system rearranging neural networks to respond to environmental stimuli. Structurally, most brain areas are related to the function of processing memory, namely the hippocampus, fornix, temporal lobe, and cerebellum, which are interconnected to and between other brain regions.[3] The process of neuroplasticity as brain development is influenced by various factors, one of which is PPARγ or peroxisome proliferator-activated receptor gamma. PPARγ is an activated ligand for transcription factors bound to the nuclear membrane.[4] Studies suggest that PPARγ has the potential to promote neuron development.[5] PPARγ activation can activate PGC1α or peroxisome proliferator-activated receptor gamma coactivator-1 alpha as the primary regulator of mitochondrial biogenesis. Mitochondrial biogenesis is a complex biological process that aims to maintain cell homeostasis by forming new mitochondria and destroying damaged mitochondria.[6,7] Substances that can increase the activity of PPARγ from natural substances, one of which is using docosahexaenoic acid (DHA), which is a group of omega-3 or omega-3 polyunsaturated fatty acids (n-3 PUFAs) for consumption by pregnant and lactating women.[8] Apart from DHA, the use of other natural substances that have the potential to increase PPARγ activity is nutmeg seed extract.

Nutmeg, or *Myristica fragrans*, is a spice plant native to Indonesia with high economic value. This plant has long been used in various foods and has medical benefits.[9] Based on the previous research, NuSE contains an active compound commonly known as macelignan, which has been extensively characterized for its antioxidant and anti-inflammatory properties through the activation of PPAR-γ.[4] Previous research stated that the content of nutmeg seed extract (NuSE) has the potential as a PPARγ agonist that could potentially increase signal modulation at synapses. PPARγ, as a receptor in the nucleus, can increase the process of mitochondrial biogenesis, which causes mitochondrial function also to increase. Mitochondria are involved in synaptic transmission in long-term potentiation that is essential in the learning and memory process.[10] Increased modulation of signals at synapses can increase neuroplasticity, potentially improving the brain's cognitive function, as seen by the amount of *synaptophysin* mRNA expression. *Synaptophysin* is the main integral glycoprotein forming the synaptic vesicle membrane, which delivers neurotransmitters into the synaptic cleft and transmits signals between neurons after fusion with the plasma membrane.[11]

The content of PPARγ agonists has the potential for neuroplasticity. However, until now, there has been no research about the potential neurostimulant impact of the PPAR-γ agonist from NuSE on the gene expression of PGC1α and synaptophysin in the hippocampus. This study explores the use of NuSE on PGC-1α's role as a regulator in the biogenesis of mitochondria and synaptophysin, aiming to elucidate synaptophysin's role as a marker for synaptic plasticity using rat hippocampal tissue.

MATERIALS AND METHODS

This study was conducted in according to the guidelines set by the Animal Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. The treatment procedures for the animals were approved by the Research Ethics Committee of Universitas Padjadjaran. According to our previous protocol, the NuSE in this study was extracted from the Glucopala caplet. Glucopala is a natural patent product obtained from the Faculty of Pharmacy, Universitas Padjadjaran (batch number FP08.A1604.001). The NuSE was dissolved in distilled water containing pulvis gum arabicum immediately before administration.[12]

Male Wistar rats, aged eight weeks, were obtained from Biofarma Laboratories and allowed to acclimate to the research facility for at least seven days before any experimental procedures. The rats were housed in groups of four per cage, maintained under a 12:12-hour light-dark cycle, and provided with a low-stress environment with a temperature of 22°C, 50% humidity, and minimal noise. Food and water were provided ad libitum. The rats were randomly assigned to four groups that consisted of 6 rats each: control group and treatment groups divided into three groups. The treatment groups in this study consisted of the PGA (pulvis gummi arabicum) group, NuSE (nutmeg seed extract) group, and DHA (docosahexanoenoic acid) group. According to a previous journal, PGA or pulvis gum arabicum was used as a control in this study because it is found in distilled water as a solvent for Glucopala caplet.[12] NuSE group receiving 0,945mg/day of NuSE while the DHA group receiving 0,835mg/day via gavage. The treatment period lasted for 12 weeks.

Following a 12-week treatment period, all rats underwent anesthesia using isoflurane at a flow rate sufficient to achieve a 5% or higher concentration. They were then euthanized through cervical translocation. Whole brains were carefully excised, washed with ice-cold phosphate-buffered saline (PBS), and stored at -80ºC until further analysis. The extracted brain samples used in this study are from the hippocampus area. RNA extraction from the hippocampus tissue was performed using 200 µl of TRIzol Reagent (Qiagen). The Transcriptor First Strand cDNA Synthesis kit (Takara Bio) was employed to synthesize cDNA from 500 ng of total RNA, using oligo dT and random primers. For the semi-quantitative Conventional RT-PCR, a reaction mixture was prepared containing 2.5 µl of reverse-transcribed cDNA, 0.5 µM of both sense and antisense primers, 200 µM of dNTPs, and 0.125 µl of Taq polymerase (Roche), resulting in a final volume of 25 µl. The optimal number of PCR cycles was determined to ensure amplification within the linear range. The reverse transcription step lasted 30 minutes at 50°C, followed by an initial activation step of 15 minutes at 95°C. Subsequently, denaturation was performed at 94°C for 40 seconds, followed by an annealing/extension step and repeated cycles specific to the mRNA targets (PGC1α and *synaptophysin*) as indicated in Table 1.[12]

Table 1. Table Primer PCR and Cycles

Name of Primer	Primer Sequence	Product Size Anneling (bp)	Anneling (℃)	Cycle
β-actin	F 5'-TGG AGA AGA TTT GGC ACC A-3' R 5'-CCA GAG GCA TAC AGG GAC AA-3'	193	60	37
Synap-tophysin	F 5'-GTG TAC TTT GAT GCA CCC TC-3' R 5'-TCT GCA GGA AGA TGT AGG TG-3'	177	55	37
PGC-1 alpha	F CGC ACA ACT CAG CAA GTC CTC R CCT TGC TGG CCT CCA AAG TCT C	263	62	37

To normalize the PCR results, the mRNA levels of β-actin were measured as an internal control. The experiments were repeated three times to validate the consistency of the results. All experimental parameters and conditions remained constant throughout the study. The resulting images were saved in TIFF format and subjected to digital image analysis using ImageJ software version 1.4.3u. The relative quantities of RNA from PGC1α and *synaptophysin* were determined by comparing the kinetic amplification of β-actin, which served as an endogenous control.

The collected data were quantified using ImageJ, illustrated in Figure 1A and Figure 2A. After quantification, the expression level of protein expression is then divided towards β-actin level as internal control. The data was analyzed using SPSS V.13. Data analysis was first tested for normality using the Shapiro-Wilk and homogeneity tests using the Levene Statistics tests. Differences in gene expression between these groups were analyzed with a One-Way Analysis of Variance (ANOVA) test showing the mean ± minimum standard error (SEM). A significance level of less than 0.05 ($p < 0.05$) was utilized to determine the statistical significance of the findings in this study.

RESULTS

After giving different treatments, we used semiquantitative PCR to analyze PGC1α and synaptophysin mRNA expression. The unit of protein expression data used in this study is arbitrary. The PGC1α mRNA expression is illustrated in Figure 1A. Compared to the control (1.055 ± 0.088 arbitrary unit), the NuSE group (0.968 ± 0.057) tended to increase PGC1α expression to the control group; however, this difference was not statistically significant, with a p-value of 0.364 ($p>0.05$). Other treatment groups, the PGA group (0.932 ± 0.035) and the DHA group (1.099 ± 0.091) did not increase the PGC1α expression if compared to the control group.

The results for synaptophysin mRNA expression are presented in Figure 1B. Compared to the control (1.050 ± 0.037), the NuSE group (1.238 ± 0.024) tended to increase synaptophysin expression and was statistically significant with a p-value of 0.017 ($p<0.05$) (Figure 2B). The DHA group (1.050 ± 0.037) also showed an increase significantly compared to the control group. Compared to the control, PGA (1.192 ± 0.065) did not increase the expression of PGC1α expression if compared to the control group.

DISCUSSION

Nutmeg is an indigenous plant originating from Indonesia with high economic value. It has been used extensively as a culinary and medicinal ingredient throughout history.[9] Prior investigations have indicated that nutmeg contains compounds acting as agonists for the PPARγ receptor, potentially enhancing synaptic signal modulation.[4,13] PPARγ within the cell nucleus promotes mitochondrial biogenesis, increasing mitochondrial functionality.[6] Mitochondria are involved in synaptic transmission during long-term potentiation, a vital process underlying learning and memory.[7] This study explored the effect of NuSE on PGC-1a, a marker regulator of mitochondrial biogenesis, compared with docosahexaenoic acid.

Based on the previous research, macelignan as an active compound in NuSE that has a natural ligand of PPAR γ agonist assumed contribution in increasing PGC-1α in the biogenesis of mitochondria and synaptophysin as a marker for synaptic plasticity.[4] Macelignan exhibits pharmacological activities such as antibacterial, anti-inflammatory, anticancer, and neuroprotective effects.[12] This study yielded no significant differences between the control and treatment groups administered PGA, NuSE, and DHA. The extract used in this research excluded myristicin and safrole from nutmeg seeds due to their hallucinogenic and hepatotoxic properties, respectively.[13] Phytochemical analysis revealed the presence of various secondary metabolites, including polyphenols, flavonoids, tannins, quinones, monoterpenoids, and sesquiterpenoids.[14] Other secondary metabolites may influence the outcomes of this study in the treatment group. Other research studies have reported that tannins can downregulate the mRNA expression of transcription factors in PPARγ.[15,16] Another study utilizing plants with tea leaves' phenols, polyphenols, and tannins demonstrated the ability to reduce PGC1α mRNA levels.[17] The presence of other compounds apart from macelignan in this research could potentially affect the study results.

Macelignan not only impacts the increase in PPARγ activity due to its dual agonist nature for PPARα and PPARγ.[13] Another study suggests that macelignan demonstrated a higher level of effectiveness as an agonist for PPARα when compared to PPARγ. PPARα plays a critical role in regulating the synthesis of neuroprotective proteins and controlling synaptic signaling.[18] In addition to PPARα, another study indicates that nutmeg significantly reduces acetylcholinesterase activity, which affects the cholinergic pathways that play a prominent role in learning and memory processes.[19]

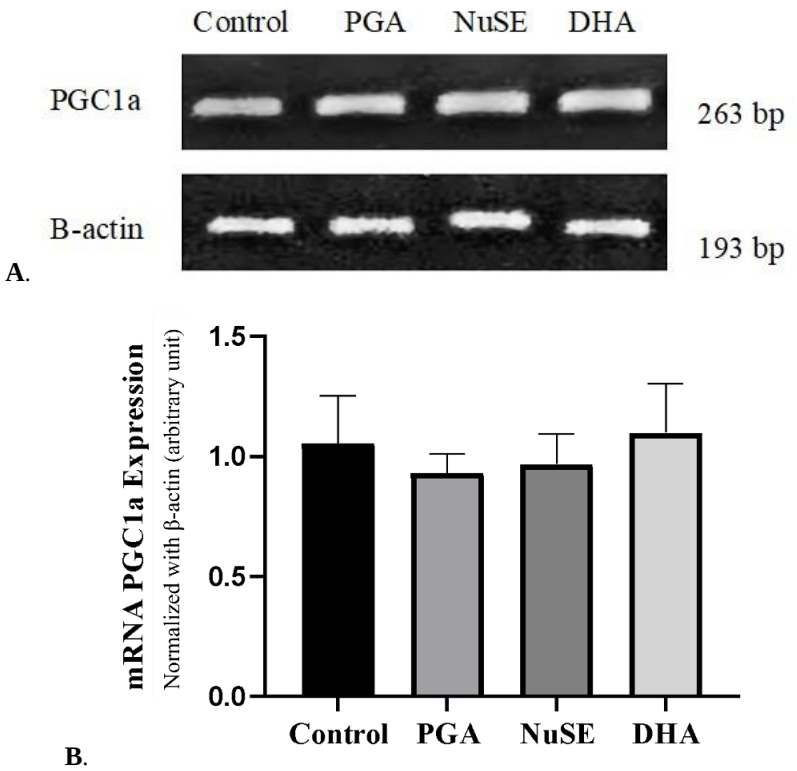

Figure 1. PGC 1 alpha mRNA expression, (A) mRNA band visualization, (B) quantification of mRNA band density (normalized with b actin). Data were presented as Mean ± SEM

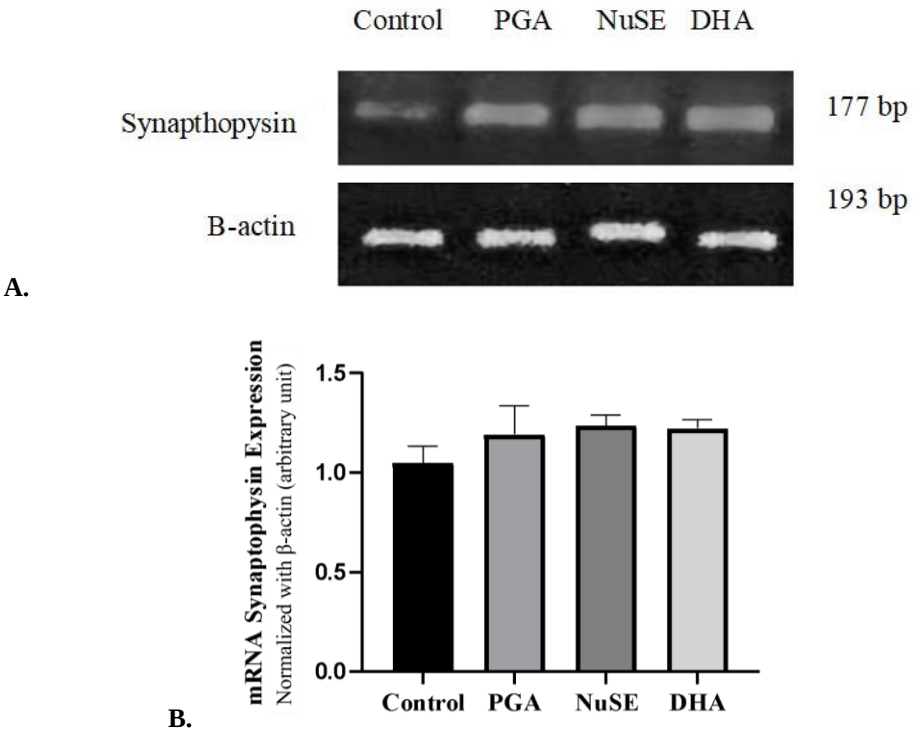

Figure 2. Synaptophysin mRNA expression, (A) Synaptophysin mRNA band visualization, (B) quantification of Synaptophysin mRNA band density (normalized with b actin). Data were presented as Mean ± SEM

Augmented signal modulation at synapses can enhance neuroplasticity, thereby improving cognitive function within the brain, as evidenced by an upregulation in *synaptophysin* mRNA expression.[11,20] *Synaptophysin*, a key integral glycoprotein composing the synaptic vesicle membrane, facilitates the release of neurotransmitters into the synaptic cleft, enabling signal transmission between neurons following fusion with the plasma membrane.[20] In prior studies, *synaptophysin* has previously served as a biomarker for evaluating

synaptogenesis in cultured hippocampal neurons.[21] A previous study reported increased *synaptophysin* expression in younger rats aged 12 weeks that were administered nutmeg seed extract.[12] We observed a similar trend in this study, where *synaptophysin* expression increased statistically significantly between the nutmeg and control groups. Statistically, the increase in DHA groups also significant compared to the control group. An increase in the DHA group also accompanied the increase in *synaptophysin* expression in the nutmeg group. This study is similar to the previous study in that DHA potentially affects *synaptophysin* expression to raised.[22]

Synaptogenesis in brain plasticity can occur through the PPARγ-PGC1α pathways and other pathways, such as the MAPK/Erk pathway. This pathway leads to the transcription of protein factors, including cAMP response element-binding protein (CREB), Myc, and ribosomal S6 kinase (RSK)[23]. Another study has reported that *Myristica fragrans* have diaryl butane-type lignan which activates the AMPK pathways.[24] Conversely, another natural substance that can enhance *synaptophysin* expression is *Centella asiatica*, which contains triterpenoids and flavonoids that can increase mitochondrial expression by upregulating NRF2 expression.[25] This study is similar to another study that stated DHA potentially activates the NRF2 pathway to upregulate brain plasticity.[26] Quercetin, the most common compound found in medicinal plants such as in NuSE, positively influenced the expression of the hippocampal FoxG1/CREB/BDNF signaling pathway. BDNF regulates phosphorylation and CREB activation, which increases sympathetic neuron survival and is an essential factor in short-term and long-term memory.[27] DHA also exhibits an enhancing effect on the CREB pathway and CREB-regulated genes. The activation of CREB has been associated with promoting of neuronal plasticity and playing a crucial role in neuronal development. BDNF, which plays a crucial role in modulating neuronal plasticity within the hippocampus, is considered one of the essential regulatory genes influenced by CREB.[28] NuSE may affect many synaptogenesis pathways that increase *synaptophysin* expression, which needs more research.

Certain limitations in this study should be considered. First, PGC1α expression is regulated by PPARγ, but since macelignan has a dual agonist effect on PPARα/γ, this study did not observe the effect on PPARα. Second, this study only examined neurons in the hippocampus that needed further studies that might have implications in another region. Third, the duration used in this study is only 12 weeks; further studies are needed to reveal long-term effect of nutmeg if compared to control and DHA.

This study concludes that NuSE increased *synaptophysin* mRNA expressions compared to the control group in significance statistics but did not increase PGC1α expression significantly. The duration, concentration of the treatment groups, other compounds contained in NuSE, or the age of the rats may contribute to this result. Further studies are needed to search for another pathway besides PPARγ that may affect neuroplasticity in NuSE.

CONCLUSION

This study concludes that NuSE increased *synaptophysin* mRNA expressions compared to the control group in significance statistics but did not increase PGC1α expression significantly. The duration, concentration of the treatment groups, other compounds contained in NuSE, or the age of the rats may contribute to this result. Further studies are needed to search for another pathway besides PPARγ that may affect neuroplasticity in NuSE.

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