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Modulation of HSP60 Expression by Virgin Coconut Oil and Folic Acid in a Rotenone-Induced Zebrafish Stunting Model

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Abstract

Stunting is characterized by impaired linear growth resulting from long term nutritional inadequacy and exposure to adverse environmental conditions. Mitochondrial dysfunction and disrupted cellular stress responses contribute to stunting. Heat Shock Protein 60 (HSP60) is a mitochondrial chaperone involved in proteostasis and reflects alterations in cellular stress regulation. This study evaluated the effects of VCO and folic acid on HSP60 expression in a rotenone-induced zebrafish model of stunting. A post-test only control group experiment included 450 zebrafish embryos assigned to five groups: NC, rotenone control, VCO, folic acid, and combination treatment. Treatments were administered from 2-72 hpf. HSP60 expression was quantified by RT-qPCR at 9 dpf using the 2^-ΔΔCt method. Group differences were analyzed using one-way ANOVA with Tukey's HSD as the post hoc test. Significant differences in HSP60 expression were observed among groups ($p<0.001$). Compared with NC, the rotenone group showed lower HSP60 expression, while all interventions further reduced expression, with the combination group showing the lowest level (0.13±0.04). These findings indicate that rotenone affected HSP60 expression, which may reflect changes in mitochondrial stress adaptation. VCO and folic acid were associated with additional modulation of Hsp60 expression, which may reflect altered cellular stress adaptation mechanisms.

Keywords: folic acid, Hsp60, mitochondrial, stunting, virgin coconut oil, zebrafish

Modulasi Ekspresi Hsp60 oleh Virgin Coconut Oil (VCO) dan Asam Folat pada Model Stunting Zebrafish yang diinduksi Rotenon

Abstrak

Stunting merupakan gangguan pertumbuhan kronis yang ditandai dengan terhambatnya pertumbuhan linier akibat kekurangan gizi jangka panjang dan paparan faktor lingkungan. Disfungsi mitokondria serta gangguan regulasi respons stres seluler diketahui berkontribusi terhadap proses tersebut. Heat Shock Protein 60 (HSP60) merupakan chaperone mitokondria yang berperan dalam menjaga homeostasis protein selama kondisi stres. Penelitian ini bertujuan untuk mengevaluasi pengaruh Virgin Coconut Oil (VCO) dan asam folat terhadap ekspresi HSP60 pada model stunting zebrafish yang diinduksi rotenone. Penelitian eksperimental dengan pendekatan post-test only control group menggunakan 450 embrio zebrafish yang dibagi menjadi lima kelompok: kontrol negatif, kontrol positif (rotenon 12,5 ppb), VCO (6,25%), asam folat (70 µM), dan kombinasi keduanya. Perlakuan diberikan pada 2-72 jam post fertilization. Ekspresi HSP60 dianalisis pada hari ke-9 menggunakan RT-qPCR dengan metode ΔΔCt. Data analisis menggunakan One-Way ANOVA dilanjutkan uji Tukey HSD. Terdapat perbedaan bermakna ekspresi HSP60 antar kelompok ($p<0,001$). Kelompok rotenon menunjukkan penurunan ekspresi dibandingkan kontrol negatif, sedangkan seluruh kelompok perlakuan mengalami penurunan lebih lanjut dengan nilai terendah pada kelompok kombinasi. Temuan ini menunjukkan bahwa rotenon memengaruhi regulasi Hsp60, sedangkan VCO dan asam folat berpotensi memodulasi respons adaptasi stress mitokondria.

Kata Kunci: asam folat, Hsp60, mitokondria, stunting, virgin coconut oil, zebrafish

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1. Introduction

Stunting continues to be one of the leading public health challenges worldwide, particularly among children younger than five years. The World Health Organization reported that approximately 22.3% of children under five were affected by stunting globally in in 2022.[1] In Indonesia, the prevalence has declined in recent years from 24.4% in 2021 to 21.6% in 2022 and further to 19.8% in 2024, it still remains above the national target of 14%, indicating the need for more effective interventions.[2] Stunting is characterized by impaired linear growth caused by persistent nutritional inadequacy together with recurrent infections, especially during the first 1,000 days of life.[3]
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Beyond nutritional and socioeconomic determinants, increasing evidence suggests that biological mechanisms, particularly oxidative stress and mitochondrial dysfunction, play a crucial role in stunting pathophysiology.[4] Environmental exposure to toxic substances such as pesticides is one of the contributing factors. Rotenone, a naturally occurring mitochondrial complex I inhibitor, disrupts electron transport, decreases ATP production, and promotes excessive ROS formation.[5] This condition leads to mitochondrial dysfunction and impaired cellular metabolism, which may interfere with normal growth and development.

Heat Shock Protein 60 (HSP60) as a mitochondrial molecular chaperone responsible for maintaining protein folding, mitochondrial integrity, and cellular adaptation during stress conditions.[6] During cellular stress, HSP60 supports mitochondrial protein quality control by facilitating proper protein folding and assembly.[7] However, dysregulation of HSP60 expression may indicate mitochondrial stress and impaired cellular adaptation, which are associated with growth disturbances.

Hsp60 is an important component of the mitochondrial unfolded protein response (UPRmt), a protective pathway that preserves mitochondrial function under proteotoxic stress.[8] Previous studies have shown that under moderate mitochondrial stress, increase Hsp60 expression may occur as a compensatory mechanism to preserve protein folding and mitochondrial function. However, under severe or prolonged stress, mitochondrial dysfunction may impair adaptive responses, resulting in reduced Hsp60 expression.[9] Therefore, Hsp60 may serve as a potential marker for evaluating mitochondrial stress adaptation in stunting related conditions.[6,10]

Various antioxidant based strategies have been investigated to counteract oxidative stress associated with mitochondrial dysfunction. Virgin Coconut Oil (VCO), contains medium chain fatty acids and phenolic compounds, that exhibit antioxidant and support mitochondrial stability by reducing oxidative damage.[11] Folic acid, an essential micronutrient, plays a key role in DNA synthesis and cellular growth, and contributes to antioxidant defense through one-carbon metabolism and redox regulation.[12] However, the combined effects of VCO and folic acid on mitochondrial stress markers, particularly HSP60 expression, remain limited. The zebrafish (Danio rerio) has become a well-established vertebrate model because of its genetic similarity to humans and its suitability for developmental and molecular studies.[13] Rotenone-induced zebrafish models have been shown to mimic oxidative stress and growth impairment, making them suitable for studying the molecular mechanisms of stunting.

Most previous studies using rotenone induced zebrafish models have primarily focused on morphological outcomes, such as body length and developmental abnormalities with limited attention to molecular markers associated with mitochondrial stress.[14,15] Previous studies have evaluated HSP60 expression in rotenone-induced zebrafish models and demonstrated that antioxidant intervention may modulate HSP60 levels.[16] However, the effects of Virgin Coconut Oil (VCO) and folic acid, either individually or in combination, on HSP60 expression in a rotenone-induced zebrafish stunting model have not been investigated. Furthermore, the biological response of HSP60 to combined antioxidant interventions remains poorly understood. Therefore, this study aims to evaluate the modulatory effects of Virgin Coconut Oil and folic acid on HSP60 expression in a rotenone-induced zebrafish stunting model, providing insight into cellular stress adaptation associated with growth impairment.

2. Materials and Method

2.1. Experimental Design

Zebrafish (Danio rerio) embryos of the wild type strain were used from the embryonic stages of aged 0-2 hours post fertilization (hpf) until 9 days post fertilization (dpf), representing the period before sexual differentiation. Therefore, sex related hormonal influences were considered negligible in this study. Embryos with a transparent appearance, normal morphology, round in shape, and not moldy, were selected from the Laboratory of Fish Reproduction, Faculty of Fisheries and Marine Sciences, Brawijaya University. A total of 450 embryos were randomly selected to five experimental groups. Each group consisted of three independent biological replicates, with 30 embryos per replicated (90 embryos per group). Replicates were

maintained in separate well and treated independently throughout the experiment. Embryos were cultured in six-well plates according to their assigned treatment groups. The experimental groups a Negative Control group (NC), Positive Control group (PC; 12,5 ppb), Treatment 1 (T1; rotenone 12,5 ppb + VCO 6,25%), Treatment 2 (T2; rotenone 12,5 ppb + Folic acid 70μM) and Treatment 3 (T3; rotenone 12,5 ppb + VCO 6,25% + Folic acid 70μM). All treatments were initiated at 2 hpf and maintained continuously until 72 hpf.[17] Treatment solutions were prepared using calibrated micropipettes and freshly diluted to achieve the desired final concentrations. The solutions were thoroughly homogenized prior to administration and distributed equally into each well. To maintain concentration stability throughout the exposure period, the treatment media were renewed every 24 hours to maintain concentration stability and minimize potential losses due to degradation, adsorption, or evaporation.

2.1.1. Place and Time of Research

This study was carried out at the Pharmacology Laboratory and Integrated Biomedical Laboratory, Faculty of medicine, Brawijaya University, Malang, Indonesia (GPS coordinate: 7°57'13.7"S 112°36'47.6"E) from November 2025 to February 2026. All procedures were approved by the Health Research Ethics Committee of the Faculty of Medicine, Brawijaya University, with ethics ID number 523/EC/KEPK-S2/12/2025.

2.2. Methods

2.2.1. Preparation of Embryonic Medium

A 10-fold stock solution of 0,25 g Cacl, 0,15 g KCl, 5 g NaCl, and 0,815 g MgSO4 was made by dissolving in distilled water and made up to 500 mL. A 10-fold dilution of the stock solution was made by mixing one part of stock solution with nine parts of distilled water.[18]

2.2.2. Zebrafish Model of Stunting

The zebrafish model of stunting was induced by exposure of zebrafish embryo (2 hpf to 72 hpf) to a 12,5 ppb of rotenone (Product No. R8875-IG) and it was dissolved in dimethylsulfoxide (DMSO) as the solvent. This stunting model aligns with the developmental equivalence of zebrafish embryo age 0-72 hpf to the fetal stage in utero. This temporal adaptation underscores the suitability of zebrafish as a model organism for studying stunting conditions.[5,19]

2.2.3. Preparation of VCO

The Virgin coconut oil (VCO) used for the study was obtained from VCO Palm 7 (Cemoro Kandang, Malang, Indonesia; P-IRT 2063573011924-29) was food grade VCO produced by the cold press method from fresh coconut meat (Cocos Nucifera L.). The oil was stored at room temperature (25 °C) in tightly closed container until use. For the preparation of stock solution, 4 mL of VCO was added 80 μL of Dimethyl sulfoxide (DMSO) as the solvent and 8 μL rotenone, followed by addition of distilled water to a final volume of 64 mL to produce a solution with concentration of 6,25%. The solution was mixed thoroughly before use. The selected concentration (6,25%) was based on the optimal dose identified in our previous study using a rotenone induced zebrafish stunting model, in which VCO demonstrated beneficial biological effects, including improved locomotor performance and reduced bax expression. Preliminary observations also indicated acceptable culture media conditions at these concentrations. Therefore, 6.25% was selected for the present study.[17]

2.2.4. Preparation of Folic Acid

Folic Acid solution was prepared using folic acid powder (Sigma F7876, Sigma Aldrich, USA). Sodium Carbonate (Na_2CO_3) was used as a solvent to enhance solubility, and distilled water was used for dilution. The equipment included 64 mL Falcon Tubes, a digital analytical balance (Mattler Toledo), and standart laboratory mixing tools. A stock solution of folic acid ($5x10^3$ μM) was prepared prior to dilution. The working solution was prepared to obtain a final concentration of 70 μM in a total volume of 64 mL. The appropriate volume of stock solution was transferred into a Falcon tube and diluted with distilled water to reach the final volume. Based on previous literature, 70 μM was selected as the optimal concentration for this study.[20]

2.2.5. Preparation of VCO and Folic Acid Combination

The combination treatment was prepared based on the optimal doses identified in preliminary study. The selected concentrations were 6,25% for virgin coconut oil (VCO) and 70 μM for folic acid. The mixture consisted of 4 ml VCO, 0,896 mL folic acid solution (70 μM), 8 μL rotenone, and 80 μL of 1% dimethyl sulfoxide (DMSO). Distilled water was added to reach a final volume of 64 mL. The solution was mixed thoroughly prior to administration. The total volume used for each zebrafish treatment group was 64 mL.

2.2.6. Measurement of Heat Shock Protein 60 expression by Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

The Hsp60 expression was measured by reverse transcriptase polymerase chain reaction (RT-qPCR)

method to evaluate mitochondrial stress adaptation and cellular stress responses in zebrafish larvae. For gene expression analysis, larvae form each biological replicate (30 larvae for each replicate) were pooled into a single sample prior to RNA extraction. Consequently, three pooled biological samples were analyzed for each experimental group. Zebrafish larvae aged 9 days post fertilization (dpf) were euthanized by rapid cooling, and then frozen in a -80oC freezer. The total RNA of the zebrafish larvae was extracted according to the protocol of the Total RNA Mini Kit (Geneaid, catalog number RT100, New Taipei City, Taiwan). RNA concentration and purity were assessed using a Nano Drop spectrophotometer, and samples with an A260/A280 ratio between 1.84 and 1.96 were considered suitable for downstream RT-qPCR analysis. Reverse transcription to convert RNA into cDNA template, Isolated RNA following the protocol of the ReverTra Ace qPCR RT Mastermix with gDNA Remover kit (TOYOBO catalog number FSQ-301, Osaka, Japan).

RT-qPCR analysis was performed using three independent biological replicates per group. No additional technical replicates were performed. The resulting expression values from the biological replicates were used for statistical analysis. The reaction process was carried out using a Real Time PCR instrument (CFX Opus 96) with total volume of 20 µL, consisting of 10 µL of 2×SensiFAST™ SYBR® No-ROX One-Step Mix, 0.8 µL of forward primer, 0.8 µL of reverse primer, and 1 µL of cDNA template, with the remaining volume consisting of RNase-free water.[14] No-template controls (NTCs) were included for each primer set to monitor potential contamination during amplification. Melt curve analysis was performed at the end of each RT-qPCR run to verify amplification specificity. A single melting peak was observed for each target, indicating the absence of non-specific amplification and primer-dimer formation. The base sequence of the primers is listed in Table 1 and the RT-PCR programs used is shown in Table 2, respectively.[15] β-actin was used as the housekeeping gene for normalization because it shows relatively stable expression and it has been previously applied in zebrafish rotenone induce stunting models and demonstrated suitability for RT-qPCR based gene expression analysis.[17]

Table 1. Primer Base Sequence of Heat Shock Protein 60 (Hsp60)

	Forward Primer
Hsp60	5'-CGCTGTGGAGGAAGGAATTG-3'
β-Actin	5'-CGA GCA GGA GAT GGG AAC-3'
	Reverse Primer
Hsp60	5'-CAGCGCAGTAAAGCACATCC-3'
β-Actin	5'-CAA CGG AAA CGC TCA TTG C-3'

2.2.7. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 27. Data distribution was evaluated using the Shapiro Wilk test, whereas homogeneity of variance was examined with Levene's test. Differences among experimental groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD for multiple pairwise comparisons. Statistical significance was established at $p < 0.05$.

Statistical analyses were based on ΔCq values generated from RT-qPCR because these data provide a more appropriate basis for parametric statistical testing. Normality, homogeneity, one-way ANOVA, and Tukey's HSD analyses were therefore performed using ΔCq values. Relative gene expression was subsequently calculated using the 2^-ΔΔCq (Livak) method and presented as fold change for data visualization. Statistical analyses were based on three independent biological replicates for each experimental group (n = 3 per group).

3. Result

Relative HSP60 expression in zebrafish larvae was quantified as fold-change values using the 2^−ΔΔCq (Livak) method across all experimental groups. The negative control (NC) exhibited the highest mean expression (1.05 ± 0.37), representing normal physiological conditions. In comparison, larvae exposed to rotenone (positive control, PC) showed a lower HSP60 expression level (0.40 ± 0.14) (Table 3).

Treatment with Virgin Coconut Oil (VCO) (T1) was associated with a lower Hsp60 expression level (0.27 ± 0.09)compared to the PC group and did not differ significantly from the positive control group. Similarly, folic acid treatment (T2) showed an expression level of (0.16 ± 0.03), while the combination treatment (T3) showed the lowest mean expression level among the treatment groups (0.13 ± 0.05), as shown in Figure 1.

One way ANOVA demonstrated a significant difference in HSP60 expression among the experimental groups ($p < 0.001$). Subsequent Tukey's HSD analysis indicated that the NC group differed significantly from all other

Table 2. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Protocol

Steps	Hsp60 (time/temperature)
Pre Denaturation	2'/95°C
Denaturation	5"/95°C
Annealing	30"/56.8°C
Extension	5"/65°C
Cycles	40 cycles

Table 3. Effect of Combination of VCO and Folic Acid on Hsp60 Expression in Each Group Measured by RT-qPCR

Group	NC	PC	T1	T2	T3
Biological replicates (n)	3	3	3	3	3
Mean Fold Change ± SD	1.05 ± 0.37	0.40 ± 0.14	0.27 ± 0.09	0.16 ± 0.03	0.13 ± 0.04
p-value			<0.001		

Note
Each biological replicate consisted of pooled RNA from 30 larvae.
NC: Negatif Control Group
PC: Positive Control (Rotenone 12.5 ppb)
T1: Treatment 1 (Rotenone12.5 ppb + VCO 6,25%)
T2: Treatment 2 (Rotenone 12.5 ppb + Folic Acid 70 µM)
T3: Treatment 3 (Rotenone 12.5 ppb + VCO 6,25% + Folic Acid 70 µM)

groups. Relative to the PC group, T2 and T3 showed significantly different HSP60 expression, whereas T1 did not. Furthermore, T1 differed significantly from both T2 and T3, while T2 and T3 showed comparable expression levels (p = 0.016).

4. Discussion

Our findings indicate that rotenone treatment reduced HSP60 expression in zebrafish larvae. Rotenone primarily acts by inhibiting mitochondrial complex I, leading to impaired electron transport, diminished ATP generation, and increased ROS production.[16] The resulting oxidative stress can disrupt mitochondrial homeostasis and adversely affect cellular processes involved in growth and development. Accordingly, the lower HSP60 expression detected in the positive control group may indicate impaired mitochondrial adaptive responses under rotenone-induced oxidative stress.

HSP60 serves as a key mitochondrial chaperone involved in protein quality control, ensuring proper protein folding while preserving mitochondrial integrity and function. Under normal conditions, HSP60 supports mitochondrial homeostasis, however its dysregulation may reflect altered cellular adaptation to stress.[21] In the context of this study, the reduced HSP60 expression following rotenone exposure may indicate alterations in cellular stress adaptation mechanisms.

Treatment with Virgin Coconut Oil (VCO) (T1) and folic acid (T2) was associated with lower of HSP60 expression compared to the positive control group. However, statistical significance was observed only in T2 and T3. The combination treatment (T3) exhibited the lowest mean Hsp60 expression among all groups. These findings indicate that the interventions were associated with differential modulation of Hsp60 expression under rotenone induced conditions.

The antioxidant activity of VCO is attributed to its high content of medium-chain fatty acids and phenolic compounds, which help attenuate ROS generation and maintain mitochondrial membrane integrity.[17] In addition, folic acid participates in one-carbon metabolism and supports intracellular redox homeostasis by facilitating NADPH synthesis

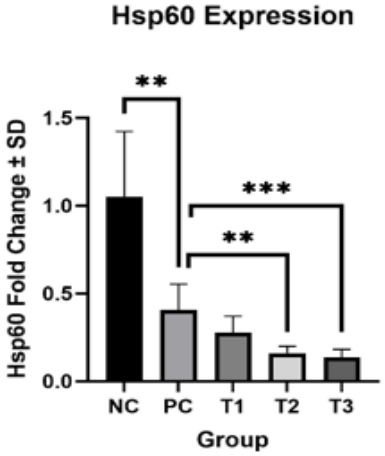

Figure 1. Relative expression of HSP60 in zebrafish larvae across experimental groups.
NC = Negative Control;
PC = Positive Control (rotenone 12.5 ppb);
T1 = rotenone 12.5 ppb + VCO 6.25%;
T2 = rotenone 12.5 ppb + folic acid 70 µM;
T3 = rotenone 12.5 ppb + VCO 6.25% + folic acid 70 µM.
Data are presented as mean ± SD. Statistical significance is indicated as **p < 0.01; ***p < 0.001; ****p < 0.0001, and ns = not significant. Exact p-values for significant comparisons were NC vs PC (p = 0.003), PC vs T2 (p = 0.001), PC vs T3 (p < 0.001), and T1 vs T3 (p = 0.016).

and endogenous antioxidant defense systems.[12] Collectively, these biological actions may account for the modulation of HSP60 expression detected following treatment.

The markedly lower HSP60 expression observed in the combination treatment group (T3) indicates that the combined intervention was associated with a different pattern of HSP60 regulation compared to the single-treatment groups. One possible explanation for this finding is altered redox signaling. Physiological levels of ROS are known to function as signaling molecules involved in mitochondrial adaptive responses through redox-sensitive pathways, including mitochondrial unfolded protein response (UPRmt)-related signaling. These pathways regulate the expression of mitochondrial chaperones such as HSP60 and may therefore influence HSP60 regulation under conditions of altered cellular redox homeostasis.[8,9] However, the biological significance of the observed reduction in HSP60 expression remains unclear because HSP60 expression alone cannot determine whether the response reflects improved mitochondrial function, altered cellular stress adaptation, or other regulatory processes. Furthermore, ROS levels, mitochondrial function, and UPRmt-related pathways were not directly measured in this study. Therefore, the proposed mechanism remains speculative and requires further investigation using additional physiological and molecular parameters. Previous studies have reported variable responses of heat shock proteins under oxidative stress conditions, depending on the intensity and duration of exposure. Some studies have demonstrated increased HSP expression as a compensatory response to moderate stress, while others have reported decreased expression under severe or prolonged stress conditions.[16] These findings suggest that HSP60 regulation may vary according to the cellular stress environment and experimental conditions.

Previous studies have established the contribution of oxidative stress and mitochondrial dysfunction to growth impairment, and the present findings support this evidence.[4] However, this study extends current knowledge by demonstrating alteration in HSP60 expression following VCO and folic acid intervention in a rotenone induced zebrafish stunting model. In addition, the lower HSP60 expression observed in the combination treatment highlights the complexity of cellular stress responses under these experimental conditions.

Several limitations should be acknowledged. This study focused on HSP60 expression as a marker of cellular stress adaptation, while other relevant parameters, such as ROS levels, ATP production, and additional molecular pathways, were not directly measured. Furthermore, the potential dose-dependent effects and interactions between VCO and folic acid require further investigation. Future studies are needed to explore these mechanisms in greater detail and to better understand the role of cellular stress adaptation in stunting pathophysiology.

Another limitation of this study is the absence of DMSO- only solvent control group. DMSO was used as a vehicle for rotenone and VCO preparation, and its concentration may have differed slightly among treatment groups. Although the concentrations used were low and commonly applied in zebrafish experiments, potential effects of DMSO on embryonic development and gene expression cannot be completely excluded. Therefore, future studies should include an appropriate solvent control group to distinguish treatment specific effects from potential vehicle related influences.

5. Conclusion

This study demonstrated that rotenone-induced stunting was associated with reduced HSP60 expression in zebrafish larvae. Treatment with Virgin Coconut Oil (VCO) and folic acid was associated with further modulated HSP60 expression, with the combination treatment showing the lowest expression level among all groups.

These findings suggest that antioxidant interventions may be associated with changes in Hsp60 expression under rotenone induce conditions. However, the biological significance and underlying mechanisms of these changes remain unclear and require further investigation. Future studies incorporating additional physiological and molecular markers are needed to clarify the role of HSP60 in stunting-related cellular stress responses.

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Conflict of Interest

The authors declare no conflicts of interest.

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