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Effects of Virgin Coconut Oil and Folic Acid on Glutathione Peroxidase and Locomotor Performance in a Zebrafish Model of Stunting

Hanida Aisyah Ardiana[1], Farica Emiliana[1*], Syahana Aini[1], Nurdiana[2], Brigitta Ida Resita Vebrianti Corebima[3], Ni Luh Putu Herli Mastuti[4]

[1] *Master Program of Midwifery, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.*
[2] *Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.*
[3] *Department of Pediatrics, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.*
[4] *Department of Midwifery, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.*
[*]*E-mail: farica_emiliana@student.ub.ac.id*

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Corresponding Author:
Farica Emiliana
Master Program of Midwifery
Faculty of Medicine
University Brawijaya
Malang
Indonesia
E-mail:
farica_emiliana@student.ub.ac.id

ABSTRACT

Stunting is a global health problem with multidimensional consequences, not only limited to impaired physical growth but also affecting cognitive development. One of the key mechanisms underlying stunting pathology is oxidative stress, which disrupts cellular homeostasis and developmental processes. The primary antioxidant defense system, such as glutathione peroxidase (GPx), plays a crucial role in neutralizing free radicals and protecting cells from oxidative damage. Virgin coconut oil is known to contain medium-chain fatty acids and bioactive compounds with antioxidant and anti-inflammatory properties. Meanwhile, folic acid plays an essential role in cellular metabolism, DNA synthesis, and maintaining redox balance. This study aimed to investigate the effect of VCO and folic acid on GPx gene expression and locomotor activity in zebrafish larvae using a rotenone-induced stunting model. Each group consisted of 30 larvae, divided into five groups; negative control (CN), positive control exposed to 12.5 ppb rotenone (PC), rotenone + VCO 6.25% (P1), rotenone + 70 µM folic acid (P2), and a combination of both (P3). Locomotor activity was assessed at 3, 6, and 9 dpf using EthoVision software, while GPx expression was analyzed at 9 dpf using the RT-qPCR method. The results showed that group P1 (6.25% VCO) exhibited the highest GPx expression, with a fold change of 10.41 ± 0.35 and significantly increased compared with the negative control ($p < 0.05$), followed by the group P2 (70 µM folic acid) and P3 (combination). The highest locomotor activity was also observed in group P1, with an average total distance traveled of 85.9 ± 2.7 cm over a 10-minute observation period. These findings suggest that VCO has the potential to enhance antioxidant enzyme capacity (GPx) and improve locomotor performance in zebrafish larvae, though further studies are needed to confirm its underlying mechanisms and clinical applicability.

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ABSTRAK

Stunting merupakan masalah kesehatan global dengan konsekuensi multidimensional, tidak hanya terbatas pada gangguan pertumbuhan fisik tetapi juga memengaruhi perkembangan kognitif. Salah satu mekanisme kunci yang mendasari patologi stunting adalah stres oksidatif, yang mengganggu homeostasis seluler dan proses perkembangan. Sistem pertahanan antioksidan utama, seperti glutathione peroksidase (GPx), memainkan peran penting dalam menetralkan radikal bebas dan melindungi sel dari kerusakan oksidatif. *Virgin coconut oil* diketahui mengandung asam lemak rantai menengah dan senyawa bioaktif dengan sifat antioksidan dan antiinflamasi. Sementara itu, asam folat memainkan peran penting dalam metabolisme seluler, sintesis DNA, dan menjaga keseimbangan redoks. Penelitian ini bertujuan untuk menyelidiki pengaruh VCO dan asam folat terhadap ekspresi gen GPx dan aktivitas lokomotor pada larva ikan zebra menggunakan model stunting yang diinduksi rotenon. Setiap kelompok terdiri dari 30 larva, dibagi menjadi lima kelompok; Kontrol negatif (KN), kontrol positif yang terpapar 12,5 ppb rotenon (KP), rotenon + VCO 6,25% (P1), rotenon + 70 µM asam folat (P2), dan kombinasi keduanya (P3). Aktivitas lokomotor dinilai pada 3, 6, dan 9 dpf menggunakan *Software EthoVision*, sedangkan ekspresi GPx dianalisis pada 9 dpf menggunakan metode RT-qPCR. Hasil menunjukkan bahwa kelompok P1 (6,25% VCO) menunjukkan ekspresi GPx tertinggi, dengan perubahan lipatan 10,41 ± 0,35 dan meningkat secara signifikan dibandingkan dengan kontrol negatif ($p < 0,05$), diikuti oleh kelompok P2 (70 µM asam folat) dan P3 (kombinasi). Aktivitas lokomotor tertinggi juga diamati pada kelompok P1, dengan jarak rata-rata yang ditempuh sebesar 85,9 ± 2,7 cm dalam 10 menit observasi. Temuan ini menunjukkan bahwa VCO berpotensi meningkatkan kapasitas enzim antioksidan (GPx) dan memperbaiki kinerja lokomotor pada larva *zebrafish*, meskipun penelitian lebih lanjut diperlukan untuk mengonfirmasi mekanisme yang mendasarinya dan penerapan klinis.

Kata Kunci: Virgin coconut oil; Folic acid; Glutathione Peroxidase; Zebrafish larvae; Rotenone; Locomotor Activity

1. Introduction

Stunting, defined as impaired linear growth in children, is a major global health problem associated with chronic malnutrition[1]. Beyond macronutrient deficiency, stunting is increasingly linked to disruptions in systemic redox homeostasis. Malnutrition promotes excessive production of reactive oxygen species (ROS), leading to oxidative stress that interferes with essential biological processes, including cell proliferation, differentiation, and energy metabolism, all of which are critical during early growth and development[2],[3].

Mitochondria play a central role in oxidative stress, acting as both the primary source and target of ROS[4]. Under physiological conditions, ROS are generated as by-products of the electron transport chain and are tightly regulated by endogenous antioxidant systems. However, under conditions such as malnutrition or toxic exposure, excessive ROS production overwhelms cellular antioxidant capacity[5]. This results in

oxidative damage to mitochondrial components, including membrane lipids, proteins, and mitochondrial DNA. Damage to the electron transport chain, particularly at complex I, leads to electron leakage, further amplifying ROS production and establishing a vicious cycle of mitochondrial dysfunction[4],[5]. Rotenone, a selective inhibitor of mitochondrial Complex I, has been widely utilized as an experimental tool to replicate this condition by inducing excessive ROS production through disruption of the electron transport chain. This rotenone-induced oxidative environment mimics the mitochondrial dysfunction observed in stunting pathology, where elevated ROS levels impair growth hormone signaling, reduce IGF-1 activity, and suppress linear growth, making it a relevant preclinical model for studying stunting-related oxidative mechanisms. Consequently, impaired mitochondrial function reduces adenosine triphosphate (ATP) production, limiting cellular energy availability required for growth. In addition, mitochondrial damage can trigger apoptotic pathways and inhibit cell proliferation and differentiation[6], thereby contributing directly to growth retardation observed in stunting.

To counteract oxidative stress, cells rely on endogenous antioxidant enzymes such as glutathione peroxidase (GPx), which reduces hydrogen peroxide and lipid peroxides into less harmful compounds[7]. Enhancing antioxidant defense systems has therefore emerged as a potential strategy to mitigate mitochondrial dysfunction. Virgin coconut oil (VCO), rich in medium-chain fatty acids and bioactive compounds, has been reported to exhibit antioxidant and metabolic regulatory effects, including the reduction of mitochondrial ROS production[8],[9]. Folic acid, on the other hand, plays a critical role in one-carbon metabolism, DNA synthesis, and redox regulation[10]. It also supports antioxidant defense through its involvement in methylation pathways and homocysteine metabolism[11], where imbalance is associated with increased oxidative stress. Despite evidence supporting the individual antioxidant roles of VCO and folic acid, their combined effects on mitochondrial dysfunction and antioxidant responses in stunting conditions remain poorly understood.

Previous studies have investigated the individual effects of VCO and folic acid on oxidative stress markers in zebrafish; however, no study has yet examined their combined effect on GPx gene expression and locomotor performance in a rotenone-induced zebrafish stunting model. This represents a significant gap in preclinical evidence, as the interaction between lipid-based antioxidants and one-carbon metabolism modulators may yield distinct outcomes compared to either agent alone. Therefore, this study aims to investigate whether the combination of VCO and folic acid provides superior antioxidant responses and locomotor recovery compared to individual supplementation, using GPx expression and locomotor activity as outcome measures.

2. Methods

Study Design

This study employed a true experimental laboratory design using a post-test-only controlled group approach. Zebrafish (Danio rerio) were used as the experimental model and were observed from the embryonic stage through the larval stage. Embryos were randomly assigned to one of five experimental groups: negative control, positive control, VCO treatment, folic acid treatment, and combined VCO and folic acid treatment. The effects of the interventions were evaluated through locomotor performance assessment at 3, 6, and 9 days post-fertilization (dpf) and glutathione

peroxidase (GPx) gene expression analysis at 9 dpf using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Animal Model

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine, University Brawijaya (approval number: 268/EC/KEPK/09/2025). The experimental model used in this study was zebrafish (*Danio rerio*) larvae. Zebrafish embryos at 2 hours post-fertilization (hpf) were obtained through natural spawning of adult wild-type male and female zebrafish. Adult zebrafish were maintained under standard laboratory conditions prior to breeding, with controlled temperature (28 ± 1^{0}C) and a 14:10 h light-dark cycle. The inclusion criteria for embryo selection were: transparent appearance, developmental stage between 0-2 hpf, intact and spherical chorion, and absence of visible signs of infection or abnormalities under a stereomicroscope.

Group Allocation

Zebrafish embryos were randomly allocated into five experimental groups: (1) negative control group, (2) positive control group, (3) VCO treatment group, (4) folic acid treatment group, and (5) combined VCO and folic acid treatment group. For gene expression analysis, each treatment group consisted of three independent biological replicates. Each biological replicate contained 30 embryos, resulting in a total of 90 embryos per group. At 9 days post-fertilization (dpf), larvae from each biological replicate were pooled and processed as a single sample for RNA extraction and subsequent RT-qPCR analysis.

A separate cohort of embryos was allocated for locomotor assessment to prevent potential interference between behavioral and molecular analyses. Larvae assigned to locomotor testing were monitored longitudinally, and the same individuals were assessed repeatedly at 3, 6, and 9 dpf.

Treatment Preparation

Embryonic Medium

The embryonic medium was prepared by dissolving 0.25 g of CaCl2, 0.15 g of KCl, 5 g of NaCl, and 0.815 g of MgSO4 in distilled water to a final volume of 500 mL. The solution was diluted with distilled water at a ratio of 1:9 prior to use[12].

Stunting Model Induced by Rotenone

Rotenone was used to induce a stunting model in zebrafish larvae at a final concentration of 12.5 ppb, following dissolution in dimethyl sulfoxide (DMSO). At 2 hours post-fertilization (hpf), larvae were randomly assigned to five groups: negative control, positive control and three treatment groups. The negative control was maintained in embryo medium without any exposure, whereas the positive control received 12.5 ppb rotenone alone. Treatment groups were exposed to the same concentration of rotenone and subsequently treated with virgin coconut oil (VCO), folic acid, or their combination from 2 hpf until 3 days post-fertilization (dpf).

Preparation Virgin coconut oil (VCO) Concentration

The VCO used in this study was a commercially available product (VCO Palm 7; production permit number P-IRT 2063573011924-29). To prepare a 6.25% VCO solution, 1 mL of VCO was mixed with 20 µL of dimethyl sulfoxide (DMSO), 2 µL of rotenone

(stock solution, 100.000 ppb; final concentration 12.5 ppb), and diluted with distilled water to a final volume of 16 mL[13].

Preparation of Folic Acid Concentrations

The folic acid used in this study was Folic Acid No. HY-16637 (MedChemExpress). Folic acid stock solution (5 mM) was prepared by dissolving 22.07 mg of folic acid powder in 10 mL of distilled water with the addition of 7 mg sodium carbonate (Na_2CO_3) to enhance solubility. A working solution of 70 µM was then prepared by diluting 224 µL of the stock solution with distilled water to a final volume of 16 mL.

Combination of VCO and Folic Acid

A combined solution of VCO and folic acid was prepared using predetermined concentrations. Briefly, 1 mL of VCO was mixed with 20 µL of dimethyl sulfoxide (DMSO), followed by the addition of 2 µL of rotenone (stock solution, 100.000 ppb; final concentration 12.5 ppb) and 224 µL of folic acid stock solution (5 mM; final concentration 70 µM). The mixture was then diluted with distilled water to a final volume of 16 mL. This formulation yielded a working solution containing 6.25% VCO and 70 µM folic acid.

The concentrations of VCO (6.25%) and folic acid (70 µM) were selected based on previously published studies demonstrating their efficacy in modulating oxidative stress in zebrafish models [12], [13]. These concentrations were further considered based on their complementary mechanisms of action; VCO primarily supports mitochondrial energy metabolism through its medium-chain fatty acids, while folic acid contributes to ROS reduction via homocysteine remethylation and the transsulfuration pathway. The combination of these two agents at the selected concentrations was therefore expected to provide a more optimal protective effect against stunting-related oxidative stress.

Locomotor Assessment

Locomotor performance was evaluated by measuring the distance traveled by larvae at 3, 6, and 9 days post-fertilization (dpf). The same larvae were followed longitudinally throughout the study and assessed repeatedly at 3, 6, and 9 dpf. Sample size was calculated using the Federer formula for experimental designs with five treatment groups, yielding a minimum of seven larvae per group. To account for potential dropout during the experimental period, the sample size was adjusted to 12 larvae per group. Recordings were obtained using smartphone-based video capture under standardized and consistent lighting conditions. The recorded videos were subsequently analyzed using EthoVision XT 15 (Noldus Information Technology, Wageningen, Netherlands) with a calibrated tracking system. Measurements were conducted using a 6-well plate format, with one larva placed in each well. Locomotor performance was recorded for a duration of 10 minutes per session under controlled environmental conditions. The primary parameter assessed was total distance moved, which was used as an indicator of locomotor performance.

Gene Expression Analysis

Glutathione peroxidase (GPx) expression was analyzed in zebrafish larvae at 9 days post-fertilization (dpf) using Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) at the Biomedical Laboratory, Faculty of Medicine, Brawijaya University (**Table 1**). Gene expression analysis was performed using three independent biological replicates per treatment group. Each biological replicate consisted of a pooled sample of 30 larvae, resulting in a total of 90 larvae per treatment group. The larvae used

for RT-qPCR analysis were independent from those used for locomotor assessment. Total RNA was isolated from each pooled sample using TRIzol reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by taking 2 µL. Subsequently, 100-200 ng of total RNA was reverse-transcribed into complementary DNA (cDNA) using the ReverTra Ace™ qPCR RT Master Mix (Toyobo, FSQ-3010). A thermal cycler was used to amplify RNA segments through the Polymerase Chain Reaction (PCR) technique.

RT-qPCR was performed in a total reaction volume of 20 µL, consisting of 10 µL master mix, 7.4 µL RNase-free water, 0.8 µL forward primer, 0.8 µL reverse primer, and 1 µL cDNA template (GPx) (**Table 2**). Amplification was carried out using a Thermal Cycler to amplify RNA segments through the Polymerase Chain Reaction (PCR) technique. Gene expression levels were normalized to the housekeeping gene β-actin as an internal control. Relative gene expression was calculated using the comparative Ct (2^−ΔΔCt) method. Briefly, the threshold cycle (Ct) values of GPx were normalized to β-actin to obtain ΔCt (ΔCt = Ct_target − Ct_reference). The ΔΔCt value was then determined by comparing each treatment group to the control group (ΔΔCt = ΔCt_treatment − ΔCt_control). Fold changes in gene expression were expressed as 2^−ΔΔCt.

Table 1. Primer Base Sequence of Glutathione Peroxidase (GPx)

	Forward Primer
GPx	5'- GAGGCACAACAGTCAGGGATT -3'
β-Actin	5' CGAGCAGGAGATGGGAACC-3'
	Reverse Primer
GPx	5'-CTTCATTCTTGCAGTTCTCCTGGT -3'
β-Actin	5'- CAACGGAAACGCTCATTGC-3'

Source : Mirna Velki, 2017

Table 2. RT-qPCR Protocol for Glutathione Peroxidase (GPx)

Step	GPx
Pre-denaturation (time/temperature)	5'/94°C
Denaturation (time/temperature)	30"/94°C
Annealing (time/temperature)	30"/56°C
Extension (time/temperature)	60"/72 °C
Cycles	40 cycles

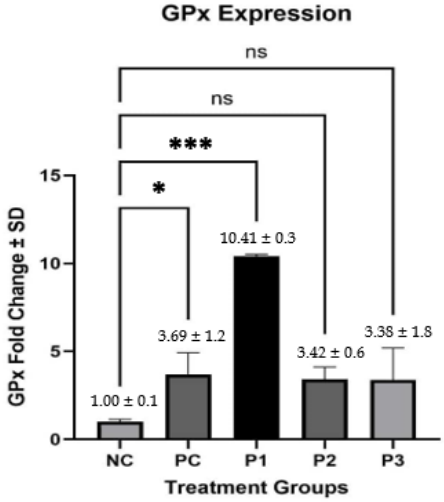
Data Analysis

All data, including locomotor performance and gene expression levels, were presented as mean ± standard deviation (mean ± SD). Data normality was assessed using the Shapiro-Wilk test, while homogeneity of variance was evaluated using Levene's test. A p-value greater than 0.05 ($p > 0.05$) indicates that the data were normally distributed and homogeneous. GPx expression data were analyzed using one-way ANOVA, while locomotor data were analyzed using two-way ANOVA with treatment group and larval age as fixed factors. Statistical significance was defined as $p < 0.05$. When significant differences were observed, post hoc comparisons were performed using the Tukey honestly significant difference (HSD) test to determine pairwise group differences. All statistical analyses were conducted using IBM SPSS Statistics, while graphical visualization was performed using GraphPad Prism.

3. Results and Discussion

GPx Expression of Zebrafish Larvae as Stunting

Glutathione peroxidase (GPx) expression, a key marker of antioxidant defense against reactive oxygen species (ROS), was evaluated in rotenone-induced stunted zebrafish larvae. Gene expression levels were quantified using RT-qPCR across treatment groups receiving virgin coconut oil (VCO), folic acid, and their combination. The results are summarized in the following figure.



*Notes : NC (Negative Control), PC (Positive Control), P1 (6.25% VCO), P2 (70 µM folic acid), P3 (Combination of VCO 6,25% and 70 µM folic acid). Statistical significance was defined as follows: ns, not significant ($p > 0.05$); * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$*

Figure 1. Graph of the Effect of VCO, Folic Acid, and the Combination on GPx Expression in Zebrafish (*Danio rerio*) Larvae in Stunting Model

As shown in **Figure 1**, one-way ANOVA analysis of GPx expression revealed a statistically significant difference among the experimental groups ($p < 0.05$). The highest relative GPx expression was observed in the P1 group (6.25% VCO), with a value of 10.41 ± 0.3, followed by the P2 group (70 µM folic acid) at 3.42 ± 0.6, and P3 groups (combination of VCO and folic acid) at 3.38 ± 1.8. The positive control group exhibited a higher mean GPx expression compared to the negative control group, with values of 3.69 ± 1.2 and 1.00 ± 0.1.

Table 3. Post Hoc Tukey's HSD pairwise comparisons of GPx expression among treatment groups

Comparison	P-value	Significance
NC vs PC	0.042	*
NC vs P1 (VCO)	0.001	***
NC vs P2 (Folic acid)	0.152	ns
NC vs P3 (Combination)	0.203	ns

Notes : Different superscript letters indicate significant differences between groups according to Tukey's HSD test ($p < 0.05$).

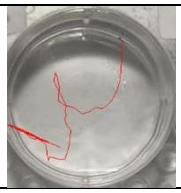
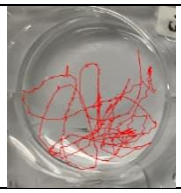
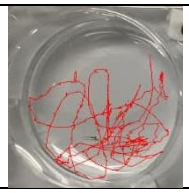
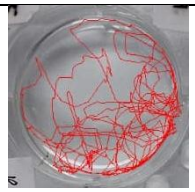
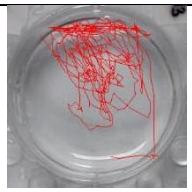
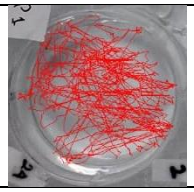
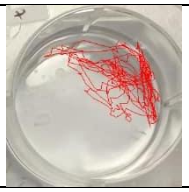
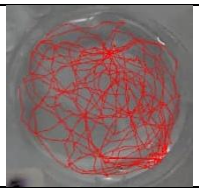
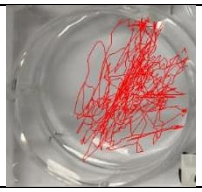
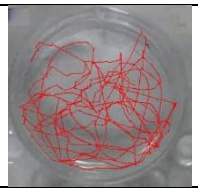
Post hoc multiple comparisons using Tukey's HSD test (**Table 3**) indicated a significant difference between the negative and positive control groups ($p = 0.042$). In the negative control (NC) group, GPx expression showed a significant difference when compared with the P1 group (6.25% of VCO), with a p-value of 0.001 ($p < 0.05$). In

contrast, comparisons between the NC group and P2 (70 µM of folic acid) (p = 0.152) as well as P3 (combination) (p = 0.203) did not reveal statistically significant differences (p > 0.05).

Locomotor Performance of Zebrafish Larvae in the Stunting Model

Two-way ANOVA revealed that treatment group ($F(4,165) = 4046.193$, $p < 0.001$), larval age ($F(2,165) = 32228.617$, $p < 0.001$), and their interaction ($F(8,165) = 337.833$, $p < 0.001$) significantly affected the distance traveled by zebrafish larvae (**Table 4**). The model explained 99.8% of the variance in the data ($R^2 = 0.998$).

Table 4. Distance traveled (cm/10 min, Mean ± SD, n = 12) by stunting model zebrafish larvae across treatment groups and larval ages.

Age	NC	PC	P1	P2	P3	P-value
3 dpf						< 0.001
Mean	22.3 ± 0.6	5.9 ± 0.2	19.6 ± 0.4	18.1 ± 0.4	16.5 ± 1.2	
6 dpf						< 0.001
Mean	72.0 ± 1.7	33.3 ± 0.8	66.5 ± 2.5	63.7 ± 0.5	56.8 ± 1.0	
9 dpf						< 0.001
Mean	94.0 ± 2.5	41.7 ± 1.0	85.9 ± 2.7	79.4 ± 2.5	76.1 ± 2.9	
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Notes : NC: group not exposed to rotenone; PC: group exposed to rotenone; P1: rotenone + 6.25 VCO; P2: rotenone + 70 µM folic acid; P3: rotenone + 6.25% VCO + 70 µM folic acid. p-values based on two-way ANOVA with Tukey HSD post hoc test.

Post hoc Tukey HSD analysis indicated that the negative control (NC) group exhibited the greatest distance traveled across all ages, while the positive control (PC) group showed the lowest locomotor activity ($p < 0.001$ for all comparisons). Among treatment groups, P1 (rotenone + 6.25% VCO) demonstrated the highest locomotor recovery, followed by the P2 (rotenone + 70 µM folic acid) and P3 (rotenone + 6.25% VCO + 70 µM folic acid), with all pairwise comparisons yielding $p < 0.001$. Larval distance traveled also increased significantly with age, with 9 dpf larvae exhibiting greater locomotor activity than 6 dpf and 3 dpf larvae ($p < 0.001$ for all age comparisons).

GPx Expression of Zebrafish Larvae as Stunting

GPx expression varied across treatment groups, reflecting differential antioxidant responses to rotenone-induced oxidative stress. The rotenone-exposed group exhibited higher GPx expression compared to the negative control, indicating an

adaptive response to increased levels, whereas oxidative stress stimulates its upregulation as part of the cellular defense mechanism. This finding is consistent with previous reports demonstrating that oxidative stress induces upregulation of antioxidant enzymes as a compensatory cellular response[14]. Rotenone is known to inhibit mitochondrial complex I, leading to excessive ROS production, impaired ATP synthesis, and oxidative stress[15],[16]. In response, GPx utilizes glutathione (GSH) to reduce hydrogen peroxide and lipid peroxides into less harmful compounds, thereby protecting cellular integrity[17].

The highest GPx expression was observed in the VCO-treated group (P1), suggesting a strong antioxidant effect. This finding is consistent with previous studies reporting that VCO enhances endogenous antioxidant systems, possibly through activation of the Nrf2/ARE signaling pathway although this pathway was not directly assessed in the present study and warrants further investigation[13], including GPx. Bioactive compounds in VCO, such as phenolic compounds and medium-chain fatty acids, may contribute to this effect by promoting cellular antioxidant defense mechanisms[19].

In contrast, the folic acid group (P2) demonstrated a moderate increase in GPx expression. Folic acid primarily functions in one-carbon metabolism and homocysteine regulation, thereby indirectly reducing oxidative stress[11]. This mechanism may explain its relatively lower effect on GPx expression compared to VCO, as its role is more preventive in limiting ROS formation rather than directly enhancing antioxidant enzyme activity.

Interestingly, the combination group (P3) did not exhibit a synergistic effect, with GPx expression comparable to the folic acid group. This may be attributed to a ceiling effect or differences in the underlying mechanisms of action between VCO and folic acid. While VCO directly enhances antioxidant defenses[19], folic acid acts indirectly through metabolic regulation[10], which may limit further upregulation of GPx expression when combined. Similar findings have been reported in combination studies where antioxidant effects do not necessarily produce additive outcomes[20].

Locomotor Performance of Zebrafish Larvae in the Stunting Model

Locomotor performance, assessed by distance traveled and movement speed, may reflect cellular energy status and mitochondrial function in zebrafish larvae, though mitochondrial function was not directly measured in this study. Reduced locomotor activity is commonly associated with impaired ATP production and increased oxidative stress[16]. This is consistent with previous studies reporting that locomotor parameters serve as indicators of neuromuscular dysfunction and mitochondrial impairment under oxidative stress conditions[21].

In this study, the rotenone-exposed group exhibited reduced locomotor performance, likely due to mitochondrial dysfunction. Rotenone is known to inhibit the electron transport chain, which may lead to decreased ATP production and increased ROS generation; however, these parameters were not directly measured in the present study[22].

Among the treatment groups, VCO administration resulted in the most notable improvement in locomotor performance, consistent with its ability to enhance energy metabolism and mitigate oxidative damage. This effect may be attributed to the rapid oxidation of medium-chain fatty acids, which are proposed to support mitochondrial ATP production, though ATP levels were not directly quantified in this study[13]. In contrast, folic acid showed a moderate improvement, likely through indirect reduction of oxidative stress via metabolic regulation[23]. Interestingly, the absence of a synergistic

effect in the combination group suggests that improvements in locomotor performance may depend more on direct enhancement of mitochondrial function rather than indirect metabolic pathways. This observation is consistent with the GPx expression findings, where VCO demonstrated a stronger effect compared to folic acid and the combined treatment. These findings suggest that locomotor performance may serve as an indirect indicator of mitochondrial integrity, oxidative balance, and energy metabolism in zebrafish larvae, though direct measurement of these parameters would be needed to confirm this interpretation.

This study has several limitations. First, the analysis was limited to antioxidant enzyme expression (GPx) and functional parameters, without direct measurement of oxidative stress markers (e.g., ROS) or cellular energy status (e.g., ATP), thus limiting mechanistic interpretation. Second, the use of zebrafish larvae represents an early developmental stage, which may limit the generalizability of the findings. Finally, the range of concentrations tested was relatively limited, and the interaction between VCO and folic acid was not explored at a molecular level.

4. Conclusion

This study demonstrated that VCO (6.25%), folic acid (70 µM), and their combination differentially influenced GPx gene expression and locomotor performance in a rotenone-induced zebrafish stunting model. VCO treatment (P1) produced the greatest upregulation of GPx expression and the highest locomotor recovery across all observed time points (3, 6, and 9 dpf), suggesting a stronger antioxidant effect compared to folic acid alone or the combination. However, the combination treatment did not yield a synergistic effect, indicating that the interaction between VCO and folic acid requires further elucidation. As this study did not directly measure oxidative stress markers (e.g., ROS levels, lipid peroxidation), mitochondrial function parameters (e.g., ATP production, mitochondrial membrane potential), or Nrf2/ARE pathway activation, mechanistic conclusions remain inferential. Future studies should incorporate these direct biomarkers and explore a broader range of dose combinations to better characterize the therapeutic potential of VCO and folic acid in stunting-related oxidative stress.

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Conflict of Interest:

The authors have no conflicts of interest to disclose.

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