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Virgin Coconut Oil and Folic Acid Effects on mTOR and Growth in Rotenone-Induced Stunted Zebrafish Larvae

Farica Emiliana[1], Hanida Aisyah Ardiana[1], Syahana Aini[1*], Nurdiana[2], Ni Luh Putu Herli Mastuti[3], Ariani[4]

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

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Corresponding Author:
Syahana Aini
Master Program of Midwifery
Faculty of Medicine
Brawijaya University
Malang
Indonesia
E-mail:
syahana@student.ub.ac.id

ABSTRACT

Stunting is characterized by impaired linear growth and is associated with dysregulation of the mechanistic target of rapamycin (mTOR) pathway. Rotenone-induced mitochondrial dysfunction suppresses mTOR signaling through oxidative stress and ATP depletion. This study evaluated the effects of Virgin Coconut Oil (VCO), folic acid, and their combination on mTOR expression and body length in rotenone-induced stunted zebrafish larvae. Zebrafish were divided into five groups (n = 3 biological replicates per group, 30 larvae per replicate): negative control, positive control (rotenone 12.5 ppb), VCO (6.25%), folic acid (70 µM), and combination treatment. mTOR expression at 9 days post-fertilization was analyzed using RT-qPCR (ΔCt for statistical analysis; $2^{-\Delta\Delta Ct}$ for fold change presentation), and body length was measured at 3, 6, and 9 dpf. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post hoc test. Rotenone significantly reduced mTOR expression and body length ($p < 0.001$). VCO and folic acid alone significantly increased mTOR expression and improved linear growth, particularly at 6–9 dpf, whereas the combination did not produce a superior effect, suggesting a dose-ratio dependent response. In conclusion, VCO and folic acid individually increase mTOR expression and support growth in rotenone-induced stunted zebrafish, while their combination at the tested doses does not provide additional benefit beyond single treatment.

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ABSTRAK

Stunting ditandai dengan gangguan pertumbuhan linear dan berhubungan dengan disregulasi jalur *mechanistic target of rapamycin* (mTOR). Disfungsi mitokondria yang diinduksi rotenon dapat menekan pensinyalan mTOR melalui peningkatan stres oksidatif dan penurunan produksi ATP. Penelitian ini bertujuan untuk mengevaluasi efek *Virgin Coconut Oil* (VCO), asam folat, dan kombinasinya terhadap ekspresi mTOR dan panjang badan pada larva zebrafish model stunting terinduksi rotenon. Zebrafish dibagi menjadi lima kelompok, masing-masing terdiri atas 3 replikasi biologis dengan 30 larva per replikasi, yaitu kontrol negatif, kontrol positif dengan rotenon 12,5 ppb, VCO 6,25%, asam folat 70 µM, dan kelompok kombinasi. Ekspresi mTOR pada 9 hari pascafertilisasi dianalisis menggunakan RT-qPCR, dengan ΔCt digunakan untuk analisis statistik dan $2^{-\Delta\Delta Ct}$ untuk penyajian *fold change*. Panjang badan larva diukur pada 3, 6, dan 9 hari pascafertilisasi. Analisis statistik dilakukan menggunakan *one-way ANOVA* yang dilanjutkan dengan uji *post hoc* Tukey HSD. Rotenon secara signifikan menurunkan ekspresi mTOR dan panjang badan larva zebrafish ($p < 0,001$). Pemberian VCO dan asam folat secara tunggal meningkatkan ekspresi mTOR hingga mendekati kondisi normal serta memperbaiki pertumbuhan linear, terutama pada 6–9 hari pascafertilisasi. Namun, kombinasi VCO dan asam folat tidak menghasilkan efek yang lebih unggul dibandingkan pemberian tunggal, yang menunjukkan adanya kemungkinan respons yang bergantung pada rasio dosis. Dengan demikian, VCO dan asam folat secara individual berpotensi mendukung ekspresi mTOR dan pertumbuhan pada larva zebrafish model stunting terinduksi rotenon, sedangkan kombinasi keduanya pada dosis yang diuji belum memberikan manfaat tambahan dibandingkan terapi tunggal.

Kata Kunci: *Virgin Coconut Oil*; Asam folat; Stunting; Larva zebrafish; mTOR; Panjang badan; Rotenon.

1. Introduction

Stunting is a condition characterized by impaired growth and development in children caused by chronic malnutrition and recurrent infections, indicated by a height-for-age measurement below -2 Standard Deviations (SD) on the WHO growth chart [1]. According to the 2024 WHO report, 150.2 million children under five years old experienced stunting [2].

Stunting is closely related to linear growth. mTOR (mechanistic target of rapamycin) is a serine/threonine protein kinase that plays a central role in regulating cellular metabolism, proliferation, and differentiation, including those of bone and cartilage cells. In the context of linear growth, mTOR acts as a key integrator of signals derived from nutrients, energy status, hormones, and growth factors, particularly Growth Hormone (GH) and Insulin-like Growth Factor-1 (IGF-1). Activation of the IGF-1 receptor (IGF-1R) initiates the phosphoinositide 3-kinase (PI3K)–Akt signaling pathway, which subsequently leads to the activation of two distinct mTOR complexes, mTORC1 and mTORC2. Among these, mTORC1 is the primary regulator of linear growth, as it controls protein translation, collagen synthesis, and the differentiation of osteoblasts and chondrocytes [3].

Stunting, as a consequence of disrupted growth processes, not only reflects impaired physical development in children but also poses long-term effects on health

outcomes and overall quality of life. Therefore, alternatives are needed to support growth and mitigate the impact of stunting. One promising candidate is Virgin Coconut Oil (VCO), known to contain medium-chain fatty acids such as lauric acid and to possess antioxidant and anti-inflammatory properties [4]. These bioactive compounds have been shown to reduce the formation of reactive oxygen species (ROS), thereby helping to maintain cellular redox balance. The inhibition of ROS may enhance Growth Hormone expression, thereby preventing impairment of the PI3K/Akt signaling pathway, which plays a crucial role in the activation of mechanistic target of rapamycin (mTOR), an essential regulator of protein synthesis, cell proliferation, and tissue growth [5].

In addition, folic acid is a micronutrient that plays a crucial role in growth. Folic acid plays a central role in one-carbon metabolism, leading to the production of 5-methyl-tetrahydrofolate (5-methyl-THF) as a methyl donor for methionine synthesis. Methionine subsequently reacts with ATP in a reaction catalyzed by methionine adenosyltransferase (MAT) to form S-adenosylmethionine (SAM) [6]. SAM functions not only as a universal methyl donor but also as a signal of nutrient sufficiency that regulates the mechanistic target of rapamycin (mTOR) pathway. Increased SAM levels facilitate the release of upstream inhibition of mTOR through a SAMTOR-mediated nutrient-sensing mechanism, thereby promoting mTOR activation and enhancing the phosphorylation of downstream targets involved in cell growth and protein synthesis. Therefore, adequate folate status may increase SAM availability and support mTOR activation [6].

The aim of this study was to evaluate the effects of Virgin Coconut Oil (VCO), folic acid, and their combination on mTOR (mechanistic target of rapamycin) expression and body length in rotenone-induced stunted zebrafish larvae. Both Virgin Coconut Oil and folic acid are expected to mitigate the effects of stunting and provide a scientific basis for the development of natural therapeutic approaches for stunting prevention. Although current interventions primarily focus on nutritional supplementation or pharmacological strategies, no in vivo study using a rotenone-induced zebrafish model of stunting has directly compared the effects of VCO, folic acid, and their combination on mTOR expression and linear growth. Therefore, this study offers novel insights into a more accessible, natural therapeutic strategy targeting oxidative stress–associated mechanisms of stunting, thereby contributing to the advancement of alternative growth-promoting interventions.

2. Methods

This study was a true experimental laboratory research employing a post-test only controlled group design using zebrafish (*Danio rerio*) from the embryonic to the larval stage. Samples were randomly assigned into several treatment groups and compared with a control group after receiving treatments based on the specified parameters. Zebrafish larvae at 2 hours post-fertilization were randomly allocated into five groups: a negative control group, a positive control group, Treatment 1 (VCO only), Treatment 2 (folic acid only), and Treatment 3 (a combination of VCO and folic acid). Randomization was performed by manually selecting embryos from a common pool and randomly distributing them into well plates to minimize selection bias.

All experimental procedures, including treatment administration, media changes, and observation schedules, were conducted under standardized laboratory conditions to maintain research reproducibility. This controlled experimental design ensured valid comparisons between groups and enabled reliable replication in future studies.

Materials

Virgin Coconut Oil (VCO) used in this study was VCO Palm 7, commercially produced from fresh coconut meat without heating or chemical additives and containing medium-chain fatty acids (MCFAs). Meanwhile, the folic acid used in this study was Sigma F7876, supplied as a pale yellow crystalline powder that is stable under conditions of heat and humidity. Preparation and administration of VCO and folic acid were performed using micropipettes, glass beakers, 6-well and 48-well plates for larval maintenance and treatment. RT-qPCR measurements were conducted using RNA extraction kits, PCR tubes, sterile consumables, and a qPCR machine (CFX Opus 96). Personal protective equipment, including gloves and face masks, was used throughout all stages to maintain sterility and ensure laboratory safety. Equipment for clinical growth measurement included an optilab microscope for observing zebrafish larvae and Image Raster software for measuring body length.

Preparation of Virgin Coconut Oil Concentrations

The concentration of Virgin Coconut Oil (VCO) used in this study was based on the findings of previous studies, which reported that a 6.25% concentration exerted the most optimal effect [7]. The VCO solution was prepared by mixing 4 mL of VCO with 80 µL of Dimethyl Sulfoxide (DMSO), followed by the addition of distilled water to achieve a final volume of 64 mL. The mixture was subsequently sonicated to ensure proper homogenization, resulting in a 6.25% VCO solution [7]. Based on this preparation, the concentration of DMSO used to solubilize VCO (80 µL in 64 mL) was 0.125%. In addition, the solution contained rotenone at a concentration of 12.5 ppb, which contributed an additional 0.0124% DMSO. Therefore, the total DMSO concentration in the final solution was 0.1374%.

Preparation of Folic Acid Concentrations

The concentration of folic acid used in this study was based on the findings of previous studies, which reported that a concentration of 70 µM produced the most optimal effect. To obtain a final concentration of 70 µM, a 5 mM (5000 µM) folic acid stock solution was first prepared by dissolving 22.07 mg of folic acid powder together with 7 mg of Na_2CO_3, followed by the addition of distilled water to reach a total volume of 10 mL. Subsequently, 0.896 mL of the stock solution was taken and further diluted with distilled water to a final volume of 64 mL to achieve the desired 70 µM concentration. Based on this preparation, the final concentration of Na_2CO_3 used to solubilize folic acid was 92 µM. In addition, the solution contained rotenone at a concentration of 12.5 ppb, which contributed an additional 0.0124% DMSO.

Preparation of Combined VCO and Folic Acid Concentrations

The preparation of the combined Virgin Coconut Oil (VCO) and folic acid solution was based on the optimal concentrations of each compound as reported in previous studies, with VCO at 6.25% [7] and folic acid at 70 µM. The combination solution was prepared by mixing 4 mL of VCO with 0.896 mL of folic acid solution, followed by the addition of distilled water to achieve a final volume of 64 mL. The mixture was subsequently sonicated to ensure proper homogenization. In addition, the solution contained rotenone at a concentration of 12.5 ppb. The DMSO used to solubilize VCO contributed 0.125%, while the rotenone solution contributed an additional 0.0124% DMSO, resulting in a total DMSO concentration of 0.1374%. Furthermore, Na_2CO_3 from the folic acid preparation was present at a final concentration of 92 µM.

Stunting Model Induced by Rotenone

Rotenone was used to induce stunting in zebrafish larvae. It was dissolved in dimethyl sulfoxide (DMSO) as a solvent to prepare a working solution at a concentration of 12.5 ppb, which contained 0.0124% DMSO [8]. The positive control group was exposed to 12.5 ppb rotenone, but the negative control group received neither rotenone, VCO, nor folic acid and was maintained in embryonic medium. All treatment groups were also exposed to the same concentration of rotenone. Treatment 1 received rotenone in combination with VCO, Treatment 2 received rotenone with folic acid, and Treatment 3 received rotenone together with the combination of VCO and folic acid. In the treatment groups, VCO and folic acid were administered simultaneously with rotenone as co-exposure. Co-exposure was initiated at 2 hpf and continued until 3 dpf, with daily medium renewal to maintain stable exposure conditions. From 4 dpf to 9 dpf, larvae were transferred to distilled water, and the medium was renewed every two days to maintain media quality and prevent microbial contamination, including fungal growth, in the well plates.

Embryonic Medium

To prepare the zebrafish embryonic medium, calcium chloride ($CaCl_2$), potassium chloride (KCl), sodium chloride (NaCl), and magnesium sulfate ($MgSO_4$) were utilized. The stock solution was diluted with distilled water as needed at a ratio of 1:9 between the embryonic medium stock and distilled water before use [8].

Animal Model

This study was approved by the Ethics Committee for Research and Innovation of Universitas Brawijaya with approval number 490/EC/KEPK-S2/12/2025, and all procedures involving laboratory animals complied with standard animal research guidelines to ensure welfare and humane handling throughout the experiment. This study utilized zebrafish larvae (*Danio rerio*) as an experimental animal model. Zebrafish embryos at 2 hours post-fertilization were used as the research model, and larvae were observed from 0 to 9 days post-fertilization. Zebrafish were divided into five groups (n = 3 biological replicates per group, 30 larvae per replicate): negative control, positive control (rotenone 12.5 ppb), VCO (6.25%), folic acid (70 µM), and combination treatment. Zebrafish were selected due to their physiological similarity to humans and their advantages as a model for stunting research, including rapid and transparent embryonic development, as well as ease of dietary and environmental manipulation [9]. The developmental stage of zebrafish larvae at 3 days post-fertilization (dpf) corresponds to that of a human newborn, larvae at 6 dpf are comparable to a 2-year-old human child, and larvae at 9 dpf resemble the developmental stage of an approximately 8-year-old human child [7].

Body Length Measurement

The body length of zebrafish larvae was measured at 3, 6, and 9 days post-fertilization using a microscope and image raster software. The body length of zebrafish larvae was determined from the tip of the snout to the base of the caudal fin in millimeters. Images were captured while the larvae were positioned straight, motionless, and immersed in water on a glass slide. Body-length measurements were not performed in a blinded manner, as each larva was assigned an identification number to allow continuous monitoring of individual growth across 3, 6, and 9 dpf. Because body-length

measurements were not performed under blinded conditions, observer-related measurement bias cannot be completely excluded and is acknowledged as a limitation.

mTOR Expression Measurement by RT-qPCR

The expression of mTOR (mechanistic Target of Rapamycin) was analyzed using larvae at 9 days post-fertilization (dpf). Quantification was performed using the Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) method. Zebrafish larvae were extracted, and total RNA was isolated using the RNA Mini Kit Tissue (Genoid), followed by reverse transcription to convert RNA into cDNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). In this study, β-actin was used as the housekeeping gene based on previous validation in zebrafish studies [7]. All RT-qPCR reactions were performed in triplicate as technical replicates to improve data reliability.

Table 1. Primer base sequence of mTOR

Gene	Forward Primer	Reverse Primer
mTOR	5′-ATG GTC ACT GGC CTG AAG TG-3′	5′-GTG CAC GTG GCG TAT CAA TC-3′
β-Actin	5′-CGA GCA GGA GAT GGG AAC-3′	5′-CAA CGG AAA CGC TCA TTG C-3′

The base sequences of the primers used are listed in **Table 1**, and the RT-qPCR protocol is presented in **Table 2.**

Table 2. RT-qPCR protocol

Step	mTOR
Pre-denaturation	2 min at 95 °C
Denaturation	5 s at 95 °C
Annealing	10 s at 60 °C
Extension	20 s at 72 °C
Cycles	40 cycles

The reaction was carried out using a real-time PCR instrument (CFX Opus 96) with the following reaction mixture: 1 µL of cDNA template, 10 µL of SensiFAST SYBR, 7.4 µL of nuclease-free water, 0.8 µL of reverse primer, and 0.8 µL of forward primer.

Data Analysis

Statistical analyses were performed using SPSS statistical software version 27. One-way ANOVA followed by Tukey's HSD post hoc test was used for group comparisons. A p-value < 0.05 was considered statistically significant. Data were analyzed using ΔCt values. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and presented as fold change for the bar chart.

3. Results and Discussion

The Effect of Virgin Coconut Oil, Folic Acid, And Their Combination on mTOR Expression

The expression of mTOR in zebrafish larvae across all experimental groups was measured at 9 days post-fertilization (dpf) after larval termination using RT-qPCR.

Statistical analyses were conducted using ΔCt values. The normality test indicated that the ΔCt data were normally distributed ($p = 0.239$), and the homogeneity test showed that the data were homogeneous ($p = 0.755$). Therefore, the ΔCt values met the assumptions required for parametric analysis.

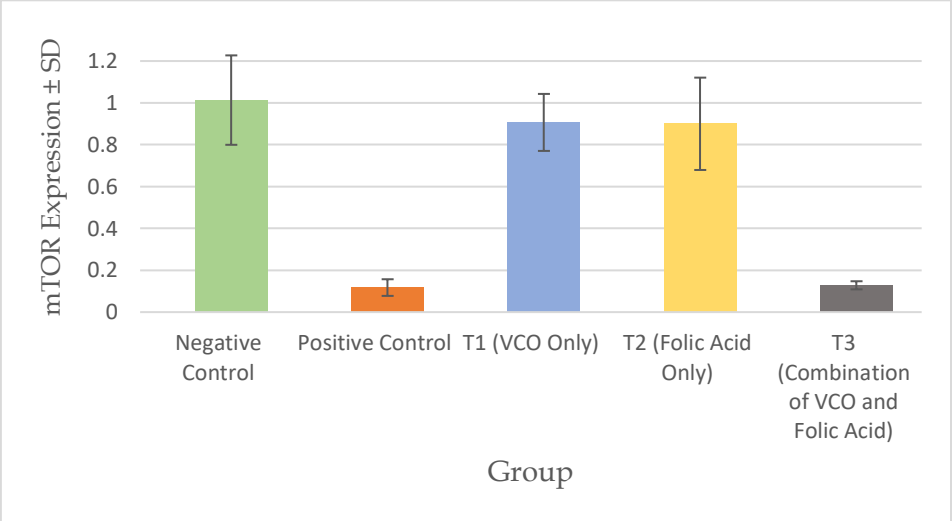

Figure 1. Effect of Virgin Coconut Oil, folic acid, and their combination on relative mTOR expression in rotenone-induced stunted zebrafish larvae.

The analysis of mTOR ΔCt values using one-way ANOVA revealed a significant difference among the groups ($p < 0.001$). For presentation purposes, relative expression levels are presented as fold change ($2^{-\Delta\Delta Ct}$) in **Figure 1**. The fold change values were calculated from the ΔCt data used for statistical analysis. Based on the results, the positive control group exhibited the lowest mean fold change (0.1174 ± 0.03977), which was lower than that of the negative control group. Among the treatment groups, the group receiving VCO only (T1) demonstrated the greatest increase in mTOR expression, with a mean fold change of 0.9067 ± 0.13614. However, the increase observed in the T1 group showed only a relatively small difference compared with the T2 group, which had a mean fold change of 0.9000 ± 0.22068. Statistical analysis based on ΔCt values showed that T1 and T2 were not significantly different from the negative control, suggesting that mTOR expression levels approached normal levels. Meanwhile, the T3 group exhibited a lower increase compared with T1 and T2, with a mean fold change of 0.1283 ± 0.01939. Overall, mTOR expression increased following the administration of VCO or folic acid alone. However, the combination of both treatments (T3) did not result in a greater increase in mTOR expression compared with their individual administration. This finding suggests that the mTOR response may be dose-ratio dependent and not necessarily synergistic, and may also be influenced by factors such as mixture stability.

The Tukey HSD post hoc test revealed significant differences in mTOR ΔCt values across several group comparisons. The positive control group showed significant differences compared with the negative control group, the T1 and T2 groups. In addition, the negative control group differed significantly from the T3 group ($p < 0.001$). Significant differences were also observed between the T1 and T3 groups ($p < 0.001$), as well as between the T2 and T3 groups ($p < 0.001$). However, no significant differences were found between the negative control and the T1 group ($p = 0.982$), between the

negative control and the T2 group (p = 0.962), or between the T1 and T2 groups (p = 1.000).

These findings indicate that VCO or folic acid increased mTOR expression to levels approaching the negative control condition in rotenone-induced stunted zebrafish larvae. Rotenone is a potent inhibitor of complex I in the mitochondrial electron transport chain. This inhibition reduces ATP production and stimulates the generation of reactive oxygen species (ROS), which disrupt the balance between oxidant and antioxidant systems, ultimately leading to oxidative stress and chronic inflammation that decrease mTOR expression, as observed in the positive control group. The reduction in mTOR expression indicates that rotenone impairs cellular growth and development [10].

Overall, VCO and folic acid as single treatments increased mTOR expression compared with the rotenone-induced stunted condition, whereas the combination treatment did not produce a comparable improvement.

The primary components of VCO, medium-chain fatty acids (MCFAs), are rapidly absorbed and oxidized in the mitochondria without requiring the carnitine transport system, thereby serving as a readily available energy source [11]. β-oxidation of MCFAs generates NADH and $FADH_2$, which contribute to the electron transport chain for ATP synthesis. Under conditions of complex I inhibition induced by rotenone, the contribution of $FADH_2$ via complex II may still permit partial oxidative phosphorylation. The resulting increase in ATP availability may attenuate AMP-activated protein kinase (AMPK) activation and subsequently promote mTOR activation [12].

In addition, MCFAs exhibit potent antioxidant and anti-inflammatory properties. These bioactive components have been shown to reduce the generation of reactive oxygen species (ROS), thereby preserving cellular redox homeostasis. The suppression of ROS may enhance GH expression, thereby preventing impairment of the PI3K/Akt signaling pathway, which plays a critical role in mTOR activation. mTOR functions as a key regulator of protein synthesis, cell proliferation, and tissue growth [5].

In addition, the results of this study demonstrated that the group receiving folic acid alone (T2) exhibited an increase in mTOR expression approaching that of the normal control group. This effect is presumed to be associated with the central role of folic acid in one-carbon metabolism, which generates 5-methyltetrahydrofolate (5-methyl-THF) as a methyl donor in the remethylation of homocysteine to methionine. Methionine subsequently reacts with ATP in a reaction catalyzed by methionine adenosyltransferase (MAT) to produce S-adenosylmethionine (SAM) [6].

Beyond functioning as a universal methyl donor, SAM also serves as a signal of nutrient sufficiency that regulates the mechanistic target of rapamycin (mTOR) pathway. Previous studies have reported that elevated intracellular SAM levels facilitate the release of upstream inhibition of mTOR through a SAMTOR-based nutrient-sensing mechanism, thereby promoting mTOR activation and enhancing the phosphorylation of downstream targets involved in cell growth and protein synthesis [6]. Therefore, adequate folate status may increase SAM availability and support mTOR activation, which could explain the increased mTOR expression observed in the folic acid-treated group in this study.

Moreover, folate has been demonstrated to function as a positive regulator of the mechanistic target of rapamycin (mTOR) pathway through a mechanism that acts as an in vivo "folate sensor." Previous studies have shown that folate deficiency leads to a reduction in both mTORC1 and mTORC2 activities, as evidenced by decreased

phosphorylation of mTOR, S6K1, S6, and 4E-BP1 as readouts of mTORC1 activity, as well as reduced Akt phosphorylation as an indicator of mTORC2 activity. Folate deficiency has also been reported to impair mTOR translocation to the lysosome and inhibit amino acid transport, thereby adversely affecting tissue growth [13].

Therefore, adequate folate status plays a critical role as a positive regulator of the mTOR pathway. In the context of the rotenone-induced model used in this study, restoration of folate status may have contributed to the recovery of mTOR activity through improvements in cellular metabolism and nutrient availability, ultimately supporting larval growth and development.

However, only the groups receiving VCO or folic acid as single treatments exhibited expression levels that more closely approached the normal condition, whereas the combination of both agents at their respective optimal doses did not result in a greater increase in mTOR expression compared with each treatment administered alone.

Meanwhile, the combination treatment at the tested doses did not produce a superior effect compared with single-agent administration. This finding suggests that the mTOR response may be dose-ratio dependent and not necessarily synergistic, possibly due to feedback regulation, mixture stability, bioavailability competition, or vehicle-related effects. The literature indicates that excessive or imbalanced nutrient stimulation can trigger feedback regulatory mechanisms that reduce pathway sensitivity, thereby preventing the anabolic response from increasing proportionally with dose escalation. This pattern is consistent with the concept of a biphasic dose–response, or hormesis, in which physiological effects reach an optimal level within a specific range but may decline when exposure exceeds cellular regulatory capacity. Therefore, the combination of two agents that individually demonstrate optimal effects does not necessarily result in a superior outcome when administered together, as mTOR responds to integrated nutrient balance rather than to the simple additive effect of the absolute doses of each component [14].

Body Length of Zebrafish Larvae

The body length of zebrafish larvae in all groups was measured at 3, 6, and 9 days post fertilization (dpf).

At 3 dpf (**Table 3**), the negative control group exhibited the highest mean body length (3.51 mm), representing normal growth conditions. In contrast, the positive control group showed the lowest mean body length (3.01 mm), indicating growth impairment following stunting induction. Among the treatment groups, T1 demonstrated the highest mean body length (3.42 mm), followed by T2 (3.24 mm) and T3 (3.11 mm).

Table 3. Average body length of zebrafish larvae at 3 dpf

Age	3 days post fertilization				
Group	Negative Control	Positive Control	T1 (VCO Only)	T2 (Folic Acid Only)	T3 (Combination of VCO and Folic Acid)
Picture					
Mean (mm) ± SD	3.51 ± 0.11	3.01 ± 0.49	3.42 ± 0.08	3.24 ± 0.03	3.11 ± 0.14
P Value	< 0.001				

Note: Data are presented as mean ± SD. VCO, Virgin Coconut Oil; dpf, days post-fertilization. The p-value was obtained using one-way ANOVA.

One-way ANOVA revealed a significant difference in body length among groups at 3 dpf ($p < 0.001$). Post hoc Tukey analysis demonstrated that the positive control group differed significantly from the negative control group ($p < 0.001$), and the T1 group ($p = 0.002$), but did not differ significantly from T2 ($p = 0.060$), and T3 ($p = 0.670$). The negative control group differed significantly from T2 ($p = 0.026$) and T3 ($p = 0.002$), but not from T1 ($p = 0.693$). Furthermore, T1 did not differ significantly from T2 ($p = 0.191$), but differed significantly from T3 ($p = 0.012$).

Table 4 shows that at 6 dpf, the increase in larval body length becomes more apparent. The positive control group continued to exhibit the lowest mean body length (3.40 mm), whereas the negative control group continued to show the highest mean body length (4.03 mm). Among the treatment groups, T1 again demonstrated the highest mean body length (3.91 mm) compared to T2 (3.82 mm) and T3 (3.50 mm).

One-way ANOVA at 6 dpf revealed a significant difference among groups ($p < 0.001$). Furthermore, post hoc Tukey analysis indicated that the positive control group differed significantly from several other groups, including the negative control ($p < 0.001$), T1 ($p = 0.002$), and T2 ($p = 0.007$), but not from T3 ($p = 0.816$). Meanwhile, the negative control group did not differ significantly from T1 ($p = 0.720$) and T2 ($p = 0.223$), but differed significantly from T3 ($p = 0.001$). In addition, no significant differences were observed between T1 and T2 ($p = 0.833$). However, significant differences were found between T1 and T3 ($p = 0.007$) and between T2 and T3 ($p = 0.033$).

Table 4. Average body length of zebrafish larvae at 6 dpf

Age	6 days post fertilization				
Group	Negative Control	Positive Control	T1 (VCO Only)	T2 (Folic Acid Only)	T3 (Combination of VCO and Folic Acid)
Picture					
Mean (mm) ± SD	4.03 ± 0.11	3.40 ± 0.07	3.91 ± 0.11	3.82 ± 0.08	3.50 ± 0.16
P Value	< 0.001				

Note: Data are presented as mean ± SD. VCO, Virgin Coconut Oil; dpf, days post-fertilization. The p-value was obtained using one-way ANOVA.

In table 5, it is shown that at 9 dpf, the pattern of larval body length growth was similar to that observed at 6 dpf, although with higher mean values. The positive control group continued to exhibit the lowest mean body length (3.60 mm), whereas the negative control group showed the highest mean body length (4.24 mm). Among the treatment groups, T1 again demonstrated the highest mean body length (4.17 mm).

Table 5. Average body length of zebrafish larvae at 9 dpf

Age	9 days post fertilization				
Group	Negative Control	Positive Control	T1 (VCO Only)	T2 (Folic Acid Only)	T3 (Combination of VCO and Folic Acid)
Picture					
Mean (mm) ± SD	4.24 ± 0.07	3.60 ± 0.03	4.17 ± 0.07	4.08 ± 0.06	3.82 ± 0.18
P Value	< 0.001				

Note: Data are presented as mean ± SD. VCO, Virgin Coconut Oil; dpf, days post-fertilization. The p-value was obtained using one-way ANOVA.

One-way ANOVA at 9 dpf revealed a significant difference among groups ($p < 0.001$). Post hoc Tukey analysis showed that the positive control group differed significantly from the negative control ($p < 0.001$), T1 ($p < 0.001$), and T2 ($p = 0.001$), but

not from T3 (p = 0.131). The negative control group did not differ significantly from T1 (p = 0.896) or T2 (p = 0.342), but differed significantly from T3 (p = 0.003). Furthermore, no significant difference was observed between T1 and T2 (p = 0.807) as well as between T2 and T3 (p = 0.052). However, significant differences were observed between T1 and T3 (p = 0.010).

Overall, the clinical parameter of body length in this study demonstrated a pattern consistent with mTOR expression across all treatment groups. This finding can be biologically explained by the role of mTOR as a central regulator of cell growth, protein synthesis, and anabolic metabolism. Previous studies have reported that the mechanistic target of rapamycin (mTOR) pathway serves as a key regulator of organismal growth by integrating nutrient signals, energy status, and growth factors to control cellular anabolic processes. Activation of mTOR promotes protein synthesis, ribosome biogenesis, and the regulation of gene expression that supports biosynthesis and cell proliferation. Conversely, when mTOR activity is suppressed due to nutrient limitation or metabolic disturbances, cellular anabolic capacity declines, resulting in impaired overall organismal growth. Therefore, mTOR functions as a primary metabolic sensor that determines whether cells enter an active growth phase or maintain a conservative state, such that its activity is directly correlated with growth rate and capacity [15]. Accordingly, alterations in mTOR expression or activity may influence phenotypic growth outcomes through modulation of biosynthetic and proliferative processes.

However, at the 3-day post-fertilization (3 dpf) observation, the body length pattern did not fully correspond to the mTOR expression profile as observed at 6 and 9 dpf. At this early stage, several treatment groups exhibited significant differences that were not yet entirely consistent with the molecular profile. This condition may be attributed to the very early developmental phase of the larvae, during which somatic growth is still largely supported by nutrient reserves from the yolk sac and not yet fully dependent on anabolic regulation through the mTOR pathway. In addition, the metabolic disruption induced by rotenone exposure is progressive in nature; therefore, the phenotypic manifestation in the form of impaired linear growth may not have been fully apparent at 3 dpf.

These findings are consistent with previous studies reporting that during the early developmental stages of zebrafish larvae, growth remains highly dependent on the utilization of endogenous nutrient reserves stored in the yolk sac. The yolk serves as the primary source of energy and biosynthetic substrates that support tissue growth and differentiation before the digestive system and external feeding mechanisms become fully functional. Physiologically, the yolk reserves are depleted at approximately 6 dpf, at which point the larvae must rely on exogenous nutrient intake to sustain growth and survival. Therefore, at 3 dpf, the larvae are still within a period of maternal nutrient dependence, and the somatic growth observed at this stage may not yet fully reflect complex anabolic regulation that is sensitive to nutritional status, including growth-related signaling pathways [16].

As the larvae progressed to 6 and 9 dpf, the body length pattern began to demonstrate a consistent correspondence with mTOR expression. This finding suggests that molecular alterations within the mTOR pathway require time to be translated into measurable morphometric changes. Therefore, the alignment between mTOR expression and body length observed at the later stages further supports the role of mTOR as a key mediator of linear growth. In contrast, the discrepancy observed at 3 dpf likely reflects a transitional developmental phase during which molecular regulation has not yet been fully manifested at the phenotypic level.

These findings are also consistent with previous studies reporting that the linear growth phenotype in zebrafish larvae does not yet exhibit consistent differences during the early stage (3 dpf), but becomes more pronounced at 6 and 9 dpf. At 3 dpf, although a significant difference was observed between the negative control and positive control groups, several treatment groups did not yet demonstrate clear distinctions compared with the other groups. This suggests that, proportionally, growth at this stage remains in the early phase of development and is still influenced by the presence of the yolk sac [17].

In contrast, at 6 and 9 dpf, increases in body length were more evident and statistically significant among groups, reflecting further morphological maturation. These data indicate that larval body growth occurs in a progressive and cumulative manner, such that phenotypic differences in linear growth resulting from the interventions become more apparent with increasing age. Therefore, the 6–9 dpf period represents a developmental window in which the impact of interventions on linear growth can be more clearly observed compared with the 3 dpf stage, which remains within the early phase of development [17].

This study has several limitations that should be acknowledged. First, this study adopted the optimal doses of each agent based on previous research; therefore, the combination dose was derived solely from the aggregation of the two individual optimal doses without specific exploration of dose ratios or variation in combination dosing. Second, mTOR pathway activity was assessed only at the mRNA level using RT-qPCR, without protein-level confirmation (e.g., p-S6K or p-S6), which may limit the interpretation of pathway activation. Finally, DMSO was used as a solvent in the preparation of VCO and rotenone; however, the final DMSO concentration was not fully matched across all experimental groups. The VCO and combination groups contained a higher total DMSO concentration (0.1374%) than the rotenone-only positive control group (0.0124%). Therefore, potential vehicle-related confounding effects cannot be completely excluded and should be controlled in future studies by using matched vehicle concentrations across all groups.

Future studies are recommended to address these limitations by systematically exploring variations in dose and combination ratios of VCO and folic acid to identify potential synergistic, additive, or even antagonistic effects on mTOR pathway activation and linear growth. A more comprehensive dose–response approach, including the evaluation of various inter-agent ratio combinations, may provide deeper insight into the dose-ratio dependent characteristics of the response. Such an approach is expected to generate a more robust mechanistic understanding of dose-ratio dependent responses and the dynamic interaction between the two agents within the mTOR pathway, thereby strengthening the scientific basis for determining more effective combination strategies.

4. Conclusion

This study demonstrates that rotenone-induced mitochondrial dysfunction suppresses mTOR expression and impairs linear growth in rotenone-induced stunted zebrafish larvae. Administration of Virgin Coconut Oil (VCO) or folic acid alone significantly increased mTOR expression and improved body length, particularly at later developmental stages (6–9 dpf), suggesting partial normalization of growth-related molecular and phenotypic responses. However, the combination of VCO and folic acid at their respective optimal single-agent doses did not produce a greater effect than either treatment alone, suggesting a dose-ratio dependent response rather than synergistic activation of the mTOR pathway. These findings highlight the potential of VCO and folic

acid as natural growth-supporting interventions targeting mTOR regulation under conditions of metabolic stress. However, as this study was conducted in a rotenone-induced stunted zebrafish larval model, the translation of these findings to human stunting should be interpreted with caution and requires further validation. Further studies exploring dose optimization and mechanistic interactions are warranted to clarify combination strategies and enhance translational relevance for growth impairment management.

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Conflict of Interest:

The authors declare that there is no conflict of interest regarding the publication of this article.

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