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Research Article | Araştırma Makalesi

THE EFFECTS OF OMEGA-3 AND VITAMIN D ON HT-22 HIPPOCAMPAL NEURONAL CELL METABOLIC ACTIVITY AND VIABILITY: A CONCENTRATION AND TIME-DEPENDENT STUDY

OMEGA-3 VE D VİTAMİNİNİN HT-22 HİPOKAMPAL NÖRONAL HÜCRE METABOLİK AKTİVİTESİ VE CANLILIĞI ÜZERİNDEKİ ETKİLERİ: KONSANTRASYON VE ZAMANA BAĞLI BİR ÇALIŞMA

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ABSTRACT

Objective: Neurodegenerative diseases are closely associated with oxidative stress and neuronal cell death. Nutritional factors such as omega-3 fatty acids and vitamin D have been reported to influence neuronal health. This study aimed to investigate the effects of omega-3 fatty acids, vitamin D, and their combinations on the proliferation of HT-22 hippocampal neuronal cells. **Methods:** HT-22 cells were treated with different concentrations of omega-3 (10–50 µM), vitamin D (50–500 µM), and their combinations for 24 and 48 hours. Cell viability was assessed using the MTT assay. **Results:** Low concentrations of omega-3 (10–20 µM) and vitamin D (50–100 µM), particularly in combination, significantly increased HT-22 cell viability. The most notable proliferative effects were observed at 24 hours with vitamin D (100 nM) + omega-3 (20 nM) and at 48 hours with vitamin D (50 µM) + omega-3 (20 µM). In contrast, high-dose combinations, especially those involving 500 µM vitamin D, decreased viability. **Conclusion:** These findings suggest that omega-3 and vitamin D, at optimal low concentrations, may synergistically enhance neuronal proliferation and could hold therapeutic potential for neurodegenerative disorders.

Keywords: Omega-3, Vitamin D, HT-22 cells, MTT assay, neuroproliferation, combination therapy

ÖZ

Amaç: Nörodejeneratif hastalıklar oksidatif stres ve nöronal hücre ölümü ile yakından ilişkilidir. Omega-3 yağ asitleri ve D vitamini gibi beslenme faktörlerinin nöronal sağlığı etkilediği bildirilmiştir. Bu çalışma, omega-3 yağ asitleri, D vitamini ve bunların kombinasyonlarının HT-22 hipokampal nöronal hücrelerinin proliferasyonu üzerindeki etkilerini araştırmayı amaçlamıştır.

Yöntem: HT-22 hücreleri, farklı konsantrasyonlarda omega-3 (10-50 µM), D vitamini (50-500 µM) ve bunların kombinasyonları ile 24 ve 48 saat boyunca muamele edilmiştir. Hücre canlılığı MTT testi kullanılarak değerlendirilmiştir.

Bulgular: Düşük konsantrasyonlarda omega-3 (10-20 µM) ve D vitamini (50-100 µM), özellikle kombinasyon halinde, HT-22 hücre canlılığını önemli ölçüde artırmıştır. En belirgin proliferatif etkiler, D vitamini (100 µM) + omega-3 (20 µM) ile 24. saatte ve D vitamini (50 µM) + omega-3 (20 µM) ile 48. saatte gözlendi. Buna karşılık, özellikle 500 µM D vitamini içeren yüksek doz kombinasyonları, canlılığı azalttı.

Sonuç: Bu bulgular, optimum düşük konsantrasyonlarda omega-3 ve D vitamininin nöronal proliferasyonu sinerjik olarak artırabileceğini ve nörodejeneratif bozukluklar için terapötik potansiyel taşıyabileceğini düşündürmektedir.

Anahtar Kelimeler: Omega-3, D vitamini, HT-22 hücreleri, MTT testi, nöroproliferasyon, kombinasyon tedavisi

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Introduction

Nerve cells and the connections between them are lost in the brains of patients with diseases like Parkinson's and Alzheimer's, and fewer new brain cells are produced. Chronic swelling, oxygen damage, and issues with the energy components of cells all play significant roles in the development of these illnesses. Finding compounds that support the growth and survival of nerve cells is very important. Omega-3 fats, a type of polyunsaturated fat, are particularly important for keeping cell walls strong and helping to lower swelling, as well as for brain growth. On the other hand, Vitamin D was also identified as a neurosteroid because it is associated with calcium homeostasis, neuronal differentiation, and anti-oxidative defense.[1,2] Emerging evidence indicates there may be synergistic effects of omega-3 and vitamin D on neuroprotection, potentially, the cognition state of becoming.[3] Progressive neuronal loss and cognitive decline are prominent features of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD), making them a major public health burden worldwide.[4,5] Neuronal survival and proliferation are essential for the function and plasticity of the central nervous system (CNS). Therefore, the improvement of neuroprotection and induction of neuronal regeneration have been the important goals in therapeutic strategies. Of all the potential candidates shown to be neuroprotectors, omega-3 polyunsaturated fatty acids (PUFAs) and vitamin D have attracted growing scientific interest, as they exert anti-inflammatory, antioxidant, and neurotrophic actions.[1,6] Neuronal membranes contain high levels of omega-3 FA, predominantly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have been demonstrated to regulate membrane plasticity, signal transduction, and gene expression for the survival of neurons.[7] Experimental studies have indicated that omega-3 fatty acids may stimulate neurogenisis, inhibit apoptosis, and diminish oxidative stress in various neural cell models.[8] In addition, vitamin D (a secosteroid hormone), as with AAs, affects neural development and activity by controlling calcium homeostasis, neurotrophic signalling, and brain immune responses.[1,9] Low omega-3 fatty acids or vitamin D status has been associated with a higher risk of neuropsychiatric and neurodegenerative diseases.[10]

In spite of its numerous *in vivo* and clinical studies, there is little data from *in vitro* experiments investigating the direct action of omega-3 and vitamin D on hippocampal neuronal proliferations. The HT-22 cell line, which is of mouse hippocampal neuron origin, is a well-established cell model to investigate the responses to oxidative insult, cell growth, and neurotoxicity in the CNS.[11]

Exploring the effects of these bioactive compounds on HT-22 cell proliferation might provide further insights into their neuroprotective potential for the treatment of neurodegenerative diseases. Thus, the present study was designed to investigate the dose- and time-response effects of the combination exposure to omega-3 fatty acids and vitamin D on the viability of HT-22 hippocampal neuronal cells using the MTT assay. By identifying the most effective concentrations and durations of exposure, the aim of the present study is to clarify the potential benefit of these compounds on maintaining neuronal health and robustness revived.

Methods

Reagents and Treatment

Omega-3 and vitamin D3 (cholecalciferol) were dissolved in DMSO and diluted to working concentrations (10, 20, 40, 50 µM for omega-3[12,13]; 50, 100, 500 µM for vitamin D.[14] Cells were seeded in 96-well plates and treated with individual compounds or their combinations for 24 or 48 hours. Control groups received vehicle only (0.1% DMSO).

Cell Culture

The HT-22 mouse hippocampal neuronal cell line (ATCC, CRL-2260) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Capricorn Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin (Capricorn Scientific). Cells were maintained at 37°C in a 5% CO_2 incubator. At 80–90% confluence, cells were trypsinized with 0.25% trypsin and seeded into 96-well plates for cell viability.

MTT Assay

Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, USA). HT-22 cells (2×10^4 cells/well) were treated with omega-3 (10 µM, 20 µM, 40 µM, 50 µM), vitamin D (50 µM, 100 µM, 500 µM), or their combinations for 24 or 48 h. After incubation, 20 µL of 5 mg/mL MTT solution was added to each well and incubated for 4 h. Formazan crystals were dissolved in 100 µL DMSO, and absorbance was measured at 570 nm using a microplate reader (Enspire, Perkin Elmer, USA). Experiments were performed in quadruplicate, and viability was expressed as a percentage of control.

Statistical Analysis

Data were presented as mean ± SD from three independent experiments. Comparisons were made using one-way ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered statistically significant.

Results

Effects of Omega-3 and Vitamin D on HT-22 Cell Viability

The effects of omega-3, vitamin D, and their combination on the viability of HT-22 hippocampal neuronal cells (Figure 1) were evaluated using the MTT assay at 24 h and 48 h (Figures 2 and 3). Cell viability was expressed as a percentage relative to the control group (100%). At 24 h, treatment with omega-3 (10 µM) and vitamin D (100 µM) individually resulted in a moderate increase in cell viability (102% and 104%, respectively), indicating a mild

proliferative effect. However, higher doses of either agent alone or in combination, particularly vitamin D (100 µM) with omega-3 (10 µM), led to a marked reduction in cell viability (as low as respectively at 24h and 48h 18%, 29%), suggesting potential cytotoxicity at elevated concentrations. Notably, the combination of vitamin D (100 µM) and omega-3 (20 µM) induced a significant increase in cell proliferation, reaching 101%, suggesting a possible synergistic effect. However, higher doses of either agent alone or in combination, particularly vitamin D (500 µM) with omega-3 (10-50 µM), led to a marked reduction in cell viability (as low as 13%), suggesting potential cytotoxicity at elevated concentrations. Table 1 summarizes the effects of individual and combined treatments on HT-22 cells at 24 and 48 hours. The most significant proliferative effects were observed in the vitamin D (100 µM) + omega-3 (20 µM) group at 24 hours, and in the vitamin D (50 µM) + omega-3 (20 µM) group at 48 hours.

Similarly, at 48 h, a comparable trend was observed. Omega-3 (20 µM) and vitamin D (50 µM) slightly increased proliferation (98% and 82%, respectively), while the combination of vitamin D (50 nM) with omega-3 (20 µM) resulted in a significant increase in viability (74%). In contrast, combinations involving vitamin D (500 µM) demonstrated a concentration-dependent cytotoxic effect, particularly when combined with omega-3 (10-50 µM), reducing viability to as low as 11%. These results indicate that low concentrations of omega-3 and vitamin D, especially in combination, promote HT-22 cell proliferation, whereas higher doses may induce cytotoxicity. The most significant proliferative response was observed with vitamin D (100 µM) + omega-3 (20 µM) at 24 h and with vitamin D (50 µM) + omega-3 (20 µM) at 48 h (Figures 2 and 3).

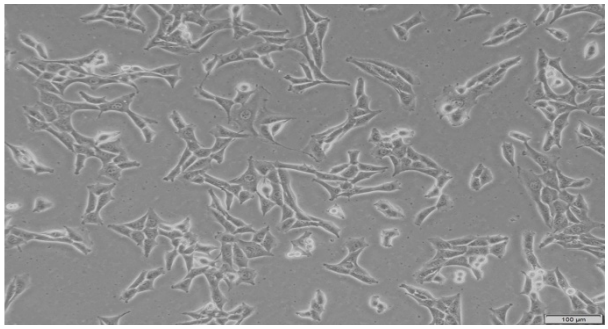

Figure 1. Morphological Alterations in HT-22 Cells Following Treatment with Omega-3 and Vitamin D. Representative phase-contrast microscopy images of HT-22 cells after 24-hour treatment with omega-3 and vitamin D. Compared to untreated control cells, treated cells exhibited marked morphological alterations, including reduced cell confluency, cell shrinkage, loss of neurite-like extensions, and increased cellular granularity. These changes suggest potential cytotoxic or antiproliferative effects of the treatments. Scale bar=100 µm

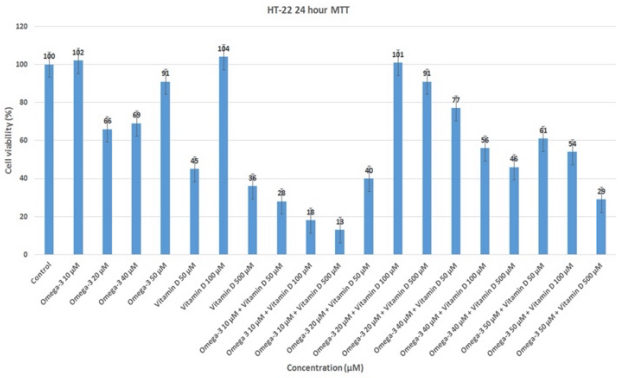

Figure 2. Effects of different concentrations of omega-3, vitamin D, and their combinations on HT-22 cell viability after 24 h treatment as determined by the MTT assay. Data are presented as percentage of control (100%).

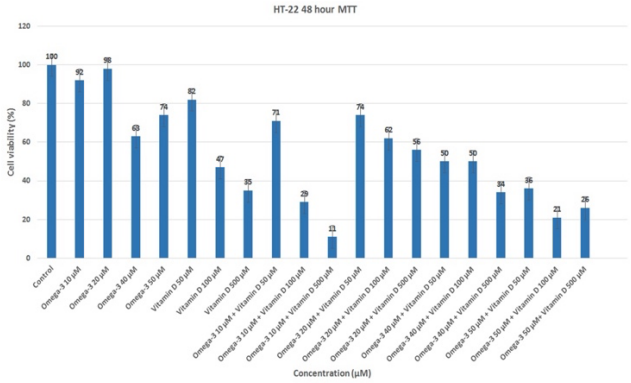

Figure 3. Effects of different concentrations of omega-3, vitamin D, and their combinations on HT-22 cell viability after 48 h treatment. Values represent cell viability relative to untreated controls. Data are presented as percentage of control (100%).

Discussion

The proliferation of HT-22 was enhanced by low dosages of omega-3 and vitamin D, while viability was decreased by larger quantities, particularly at 500 µM vitamin D. These results agree with previous studies, which indicated that the two agents in low doses support neuroprotection and neurogenesis.[1,3]

The proliferative response elicited at 24 h by 100 µM vitamin D + 20 µM omega-3 could reflect a time-dependent positive interaction maybe resulting from a modulation of cell survival signaling molecules such as PI3K/Akt or MAPK/ERK.[15] At 48 h, significant reductions in viability were also seen at exposure to 50 µM vitamin D + 20 nM omega-3, suggesting sustained protection with lower dosages. Only high-dose combinations were cytotoxic and this may be due to a larger oxidative

Table 1. Effects of Omega-3, Vitamin D, and combined treatments on HT-22 cell viability (MTT Assay)

Treatment Group	Concentration	Incubation Time	Effect on Cell Viability	p value
Omega-3	10 nM	24 hour	Increased	p<0.05
Vitamin D	100 nM	24 hour	Increased	p<0.05
Vitamin D + Omega-3	100 nM + 20 nM	24 hour	Significantly increased	p<0.01
Omega-3	20 nM	48 hour	Increased	p<0.05
Vitamin D	50 nM	48 hour	Increased	p<0.05
Vitamin D + Omega-3	50 nM + 20 nM	48 hour	Significantly increased	p<0.01

burden or changes in membrane integrity as seen with supraphysiological concentrations of vitamin D or PUFA.[2]

Collectively, these findings indicate that the optimal dosage of omega-3 and vitamin D may provide a neuroprotective avenue for treatment in the setting of oxidative stress-mediated neurodegeneration, and more studies at the molecular level are necessary to elucidate mechanisms involved. Therefore, the aim of the present study was to examine the concentration- and time-dependent effect of omega-3 fatty acids and vitamin D on the proliferation of HT-22 hippocampal neuronal cells. We found that both compounds affected cell viability, showing two different dose-response and time-response patterns. These results contribute with new evidence for potential roles of omega-3 and vitamin D as neuroprotectants in hippocampal neurons in a model of central nervous system injury.

The stimulatory effects seen at lower doses of omega-3 and vitamin D further imply that these bioactives have the potential to augment neuroprotection and neurogenesis at physiologically achievable doses. This is consistent with the previous finding that DHA and EPA, the principal bioactive omega-3 FAs, promoted neurogenesis, synaptogenesis, and mitochondrial function in neural cell lines.[7,16]

Furthermore, the anti-apoptotic effects of omega-3 by Bcl-2 family proteins modulation and caspase activation inhibition have been revealed[8], which could also be implicated in the augmented cellular viability noticed in our experiment. Moreover, the neurotrophic effects of vitamin D at moderate doses align with its established function in the control of neuronal calcium homeostasis, decreasing oxidative stress, and upregulating neurotrophic factors, such as NGF and BDNF.[1,9] HT-22 cell line, which does not express NMDA-Rs and is highly susceptible to oxidative stress, may be an apropos system to investigate how vitamin D anti-oxidative and anti-inflammatory actions stimulate neuron protection.[11]

In addition, the potential synergistic or additive effects of omega-3 and vitamin D conjugation should be taken into account. While not directly investigated here, other evidence also suggests that these nutrients may act synergistically on neuronal cells through common pathways that include inflammation, oxidative stress, and neurotrophin regulation.[10] The combined effects of these substances in *in vitro* or *in vivo* models of neurodegeneration require further research. Notably, with increasing doses and prolonged exposure (72 h), omega-3 and vitamin D revealed a plateau phase or even a decrease in proliferation at higher concentrations, indicative for potential cytotoxic action or feedback inhibition at supraphysiological concentrations. This biphasic effect had also been demonstrated in other works when excess of DHA or vitamin D caused higher peroxidation of lipids or calcium overload, which finally impaired the survival of the cells.[17,18] These results emphasize the necessity of dose optimization for therapeutic settings of these compounds.

The present findings collectively highlight the potential of both natural and synthetic bioactive compounds to attenuate oxidative stress– and inflammation-induced neurotoxicity through converging molecular mechanisms. Spirulina maxima 70% ethanol extract (SM70EE) effectively protected neuronal cells against trimethyltin-induced toxicity by suppressing ROS generation, enhancing BDNF/CREB signalling, and reducing acetylcholinesterase activity, which correlated with improved cognitive performance *in vivo*.[19] Similarly, the multifunctional anti-Alzheimer's dimer tacrine-3-caffeic acid (T3CA) demonstrated robust neuroprotection in HT-22 cells by mitigating glutamate-induced oxidative stress, preserving mitochondrial membrane potential, and activating the Nrf2/ARE/HO-1 antioxidant pathway.[20] In parallel, Auricularia polytricha hexane extract and its active component ergosterol attenuated TNF-α-induced neuronal injury via upregulation of antioxidant defenses (including SOD-1), activation of Akt phosphorylation, and modulation of NMDA receptor subunit expression through EGR-1–dependent transcriptional control.[21] Together, these data suggest that targeting oxidative stress and neuroinflammation through Nrf2, BDNF/CREB, and Akt/EGR-1 signalling axes constitutes a promising therapeutic strategy for neurodegenerative conditions such as Alzheimer's disease. Moreover, the complementary mechanisms observed across natural extracts and synthetic analogues indicate potential for combinatorial or multi-target approaches to restore neuronal homeostasis and preserve cognitive function under neurotoxic stress.

The limitations of the current study are that only a single cell line was utilized, and no mechanistical assays ie, gene and protein expressions, were performed. Furthermore, the MTT assay does not measure cell growth or apoptosis despite being a reliable indicator of metabolic activity. Further validation of these results and extension of the findings, for example using flow cytometry, real-time PCR and immunoblotting, would be desirable.

To summarize briefly, our findings indicate that omega-3 fatty acids and vitamin D have the capability to improve

the survival rate of hippocampal nerve cells in a dosage- and time-dependent manner, with the best results being seen at moderate concentrations and shorter exposure durations. The present study not only underscores their role as neuroprotective agents but also opens the door for further research in the development of nutritional or pharmaceutical interventions in the treatment of neurodegenerative disorders.

In conclusion, low concentrations of omega-3 and vitamin D significantly enhance HT-22 neuronal cell viability in a concentration- and time-dependent manner, while high doses are cytotoxic. These results support their potential role as neuroprotective agents and highlight the importance of dose optimization. To confirm their therapeutic use in neurodegenerative diseases, more mechanistic and *in vivo* research is necessary.

Compliance with Ethical Standards

The research does not involve any human or animal subjects; therefore, ethical approval is not required.

Conflict of Interest

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

Author Contributions

IC, EA; Idea and design, IC, EA; Data collection and processing, IC, EA; Analysis and interpretation of data, IC; Writing of significant parts of the article.

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