

Omega-3 Fatty Acids Decrease the Proliferation of Rhabdomyosarcoma (RD) and Vero Cell Lines

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Abstract

The effect of omega-3 fatty acids, EPA and DHA, and combinations thereof on the viability of Rhabdomyosarcoma (RD) and Vero Cell Lines was investigated. Various concentrations of these chemicals ranging from 0 to 80 μM were tested using 96-well microtitration plates. Each well contained 0.2 ml of treated cell suspension containing 10^5 cells/ml. A micro-ELISA reader was used to measure the changes in cell viability. All tested individual concentrations and combinations reduced viability and this reduction was concentration dependent. Higher values of reduction were seen at 40 μM followed by lesser inhibition at higher concentrations. Data were compared using Student T-test (P value of ≤ 0.05). Mechanisms of effect of these drugs and their combinations are discussed. The present data could be used as a base line for further *in vivo* investigations and possible clinical considerations.

Keywords: Omega -3; EPA; DHA; Cancer cells; Transformed cell line; Cancer treatment; Apoptosis

Introduction

Knowledge about the importance of omega-3 polyunsaturated fatty acids (N-3 or n-3 PUFAs) in human health and diseases has been increasing constantly during recent years. Omega-3 fatty acids (popularly referred to as ω -3 fatty acids or omega-3 fatty acids) are essential unsaturated fatty acids with a double bond (C=C) starting after the third carbon atom from the end of the carbon chain [1]. They are "essential" fatty acids because they are vital for normal metabolism and cannot be synthesized by the human body [2]. Nutritionally important n-3 fatty acids include α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), all of which are polyunsaturated. More precisely, ALA cannot be synthesized by the human body at all, and the other n-3 fatty acids can only be synthesized from ALA [3]. Many studies showed a positive role of n-3 fatty acids in the treatment of cardiovascular diseases [4], various mental illnesses [5], and cancer [6]. In cancer therapy, n-3 fatty acids are used to suppress cancer-associated cachexia and to improve the quality of life [7]. Other experimental studies on different cell cultures and animal models have determined the antitumor effect of n-3 fatty acids both *in vitro* [8,9] and *in vivo* [10,11]. An important aspect of the effects of n-3 fatty acids is that they cause little or no damage to normal cells [12].

Several studies reported possible anti-cancer effects of n-3 fatty acids, in particular, breast, colon, and prostate cancer [13-15]. Omega-3 fatty acids reduced prostate tumor growth, slowed histopathological progression, and increased survival in mice [16]. Nevertheless, neither long (DHA) chain nor short-chain (EPA) forms of n-3 fatty acids were consistently associated with breast cancer risk; but high levels of DHA, the most abundant n-3 fatty acids in erythrocyte membranes, were associated with a reduced risk of breast cancer [17].

A review of literature covering cohorts from many countries with a wide variety of demographics concluded that there was no link between n-3 fatty acids and cancer [18]. Similar findings failed to find clear effects of long and shorter chain n-3 fatty acids on total mortality, combined cardiovascular events and cancer [19]. This contradicts with other findings in that EPA can enhance the radiosensitivity of different human tumor cell lines [20].

A systematic review on Omega-3 fatty acids and cachexia found evidence that oral n-3 fatty acids supplements benefit cancer patients,

improving appetite, weight, and quality of life [21]. A recent trial found that a supplement of EPA helped cancer patients retain muscle mass [22].

The present study aimed at testing the ability of omega-3 polyunsaturated fatty acid (EPA and DHA) to inhibit tumor cells *in vitro*.

Materials and Methods

Cell lines

All cell lines were kindly provided by Iraqi Center for Cancer and Medical Genetics Research.

Rhabdomyosarcoma (RD) cell line

This human cell line was derived from a biopsy specimen obtained from a pelvic rhabdomyosarcoma of a 7-year-old Caucasian girl [23]. It was used throughout this study and RPMI-1640 (Sigma, U.S.A) was used in maintaining the cells.

Vero cell line

This is a transformed cell line. It was initiated from the kidney of a normal adult African green monkey in 1962 by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan [24]. Cells were maintained in Minimum Essential Medium (MEM) [Gibco, Scotland].

Biochemical reagents

DHA [22:6 (n-3)] and EPA [20:5 (n-3)] were purchased from Sigma-Aldrich, U.S.A. All other chemicals, solvents and reagents were supplied by BDH (UK) unless otherwise stated.

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Detection of the effect of DHA and EPA on cell lines

The growth medium of confluent monolayer plates was decanted off and the cell sheet washed twice with PBS and trypsinized using trypsin-versene (Gibco, Canada) [25]. Cells were then re-suspended in fresh growth medium to give 10⁵ cells/ml. Aliquots of 0.2 ml were transferred to each well of sterile 96-well microtitration plates. Plates were incubated until cells reaching exponential phase. Medium was removed and EPA and DHA at varying concentrations (20, 40, 60 and 80 μM each) in serum free medium (SFM) plus DMSO (1 μl /ml) [26] were added. Similarly, combinations of these two chemicals of 10, 20, 30 and 40 μM each were employed. SFM was used to avoid any interference of the serum constituents, such as growth hormones and other components, on the actual effect of the drugs. All experiments were replicated five times. Twenty wells were used as control, i.e., cells treated with SFM plus DMSO only [25]. Plates were reincubated at 37°C for 24 hrs. Medium was replaced by 50 μl of 0.01% crystal violet dye for 20 minutes, then washed gently and allowed to dry [25]. Optical density of each well was read by using a micro-ELISA reader (OrganonTeknika, Austria) at 492 nm transmitting wavelength [25,27]. The percentage of inhibition was calculated according to the following formula [28].

$$\text{Inhibition (\%)} = \left[100 - \frac{\text{optical density of test wells}}{\text{optical density of control wells}} \right] \times 100$$

Data were analyzed using Student's t-test against untreated cells. P value of ≤ 0.05 was considered statistically significant. Uncertainty (confidence limits) was represented in the figures by error bars.

Results and Discussion

Present findings indicate that Omega-3 fatty acids, EPA and DHA, can reduce viability of RD and Vero cell lines and such effects are concentration dependent, as given in figures 1 and 2, respectively.

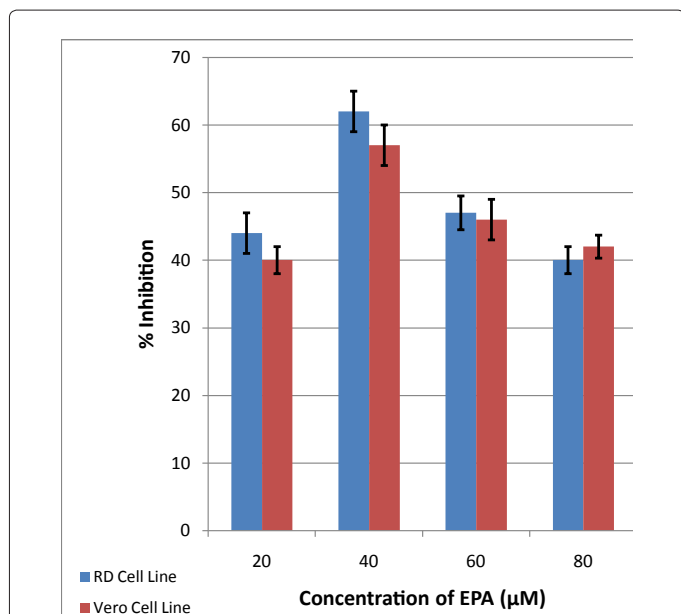


Figure 1: Effects of different concentrations of EPA on viability of RD and Vero cells ($P \leq 0.05$). Data was compared, using Student's T-test, with those obtained from control samples where cell viability was studied without adding omega-3 fatty acids. Error bars represent confidence limits at 95% confidence level.

Generally, both drugs reduced cell viability in all tested concentrations. EPA at 20 μM produced inhibitions of 44% and 40% for RD and Vero, respectively (Figure 1). Increasing the concentration of EPA increased viability inhibition. Highest values of inhibition of 62% for RD and 57% for Vero cell lines were seen at 40 μM, respectively. Further increase in concentration caused a continuous reduction in value of inhibition. At 60 μM 47% for RD and 46% for Vero were achieved. While using 80 μM of EPA produced the lower percentage of inhibition (40% for RD and 42% for Vero).

The picture is almost the same when different concentrations of DHA were tested. All tested concentrations produced profound reduction in viability of both RD and Vero cells (Figure 2). At 20 μM inhibitions of 50% for RD and 46% for Vero were produced. The highest inhibition was seen at 40 μM, giving values of 74% and 69% for RD and Vero, respectively. Further increase in concentration (60 and 80 μM), produced continuous reductions in the inhibition of 56% for RD and 55% for Vero, and 44% for RD and 42% for Vero, respectively.

Study of the effects of combinations of EPA and HAD on the viability of the two cell lines is shown in (Figure 3). Inhibitions of 60% for Rd and 56% for Vero were seen when 10 μM of each chemical were employed. Increasing the concentrations to 20 μM of each of the two chemicals produced the highest percentage of inhibition to give values of inhibition of 80% and 74% inhibitions for RD and Vero, respectively. Further increase in concentration produced a continuous reduction in inhibition. When 30 μM of each of the agents were tested values of inhibition of 65% for RD and 62% for Vero was observed. Additional reduction was observed when 40 μM of each agent giving viability inhibitions of 52% and 50% for RD and Vero, respectively.

Careful look at the combination data reveals that the two chemical acted additively at the low concentrations, particularly at 40 μM each (where the values of reduction are around 45% compared to that of

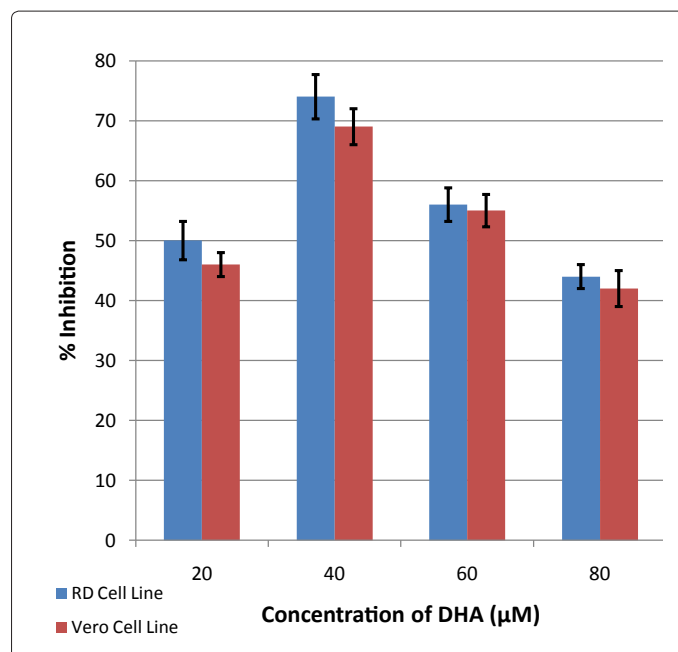


Figure 2: Effects of different concentrations of DHA on viability of RD and Vero cells ($P \leq 0.05$). Data was compared, using Student's T-test, with those obtained from control samples where cell viability was studied without adding omega-3 fatty acids. Error bars represent confidence limits at 95% confidence level.

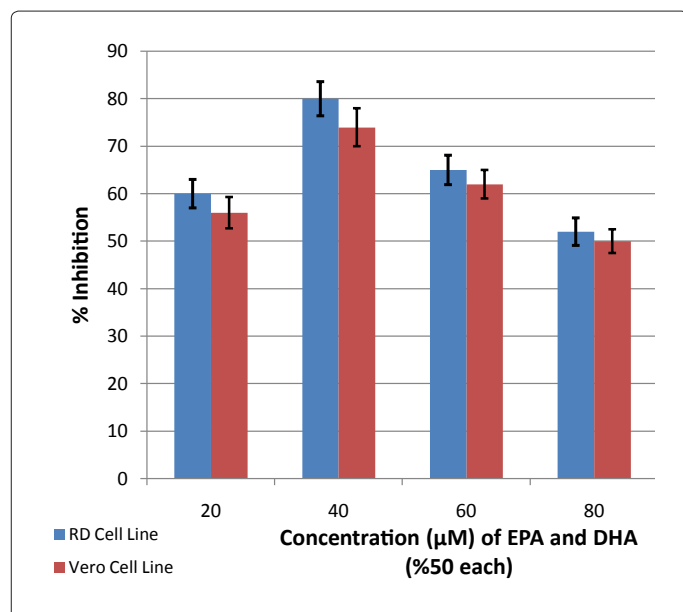


Figure 3: Effects of deferent combinations of EPA and DHA on viability of RD and Vero cells ($P \leq 0.05$). The value of concentration is the sum of the concentrations (50% each) of the two drugs. Data was compared, using Student's T-test, with those obtained from control samples where cell viability was studied without adding omega-3 fatty acids. Error bars confidence limits at 95% confidence level.

the combined drugs at 20 μM where each falls around 80%. This may implicate that their modes of action are similar. Support to this notion comes from the fact that both drugs produce lesser values of viability reduction as higher concentrations and combinations thereof.

The reduction in the viability inhibition at higher concentrations may be explained on the bases that Omega-3 fatty acids are important structural components of cell membranes. When incorporated into phospholipids, they affect cell membrane properties such as fluidity, flexibility, permeability and the activity of membrane bound enzymes [29]. The cellular mechanisms studies of omega-3 with cancer cells suggest that it induces cell cycle arrest and apoptosis by activating protein phosphatases, leading to dephosphorylation of proteins. Protein phosphatases are also involved with the protein Bcl2, which regulates the release of cytochrome c (a protein found in inner membranes of mitochondria), from mitochondria, and eventually, activation of the apoptotic enzyme caspase 3 [30]. Additionally, high omega-3 concentrations may reduce phosphorylation in various cell lines [31-33], which supports the present findings in those higher concentrations reduce inhibition. However, this action is limited because the continuous increase of Omega-3 fatty acids concentration will inhibit all components that contain phosphate and lead to reduction in ATP formation. Such reduction in ATP will reduce the incorporation of Omega-3 fatty acids into cell membrane and eventually stop the distribution of Omega-3 fatty acids through the cytoplasm. Accordingly, the effects of Omega-3 fatty acids will be reduced due to the fact that the incorporation of Omega-3 fatty acids into phospholipids could induce apoptosis [30-32] and that was clearly seen at 60 and 80 μM .

The present findings also show that DHA was a little more effective at increasing inhibition than EPA for cell lines under test. This may mainly be due to that part of the carbon chain of DHA has a higher degree of unsaturation than EPA. These double bounds can also serve

as a substrate for peroxidation reactions resulting in reactive oxygen species and a pro-oxidant environment, which have been shown to induce apoptosis [34,35].

Additional mechanistic implications is offered by the fact that omega-3 fatty acids influence inflammation through a variety of mechanisms; many of these are mediated by, or at least for the reduction associated with, changes in fatty acid composition of cell membranes. Changes in these compositions can modify membrane fluidity and cell signaling leading to altered gene expression [36]. Lipid signaling plays a critical role in the status of prostate cancer and many other human cancers [37-39]. The mitochondrial protein, cytochrome c, has a function in the intrinsic pathway of apoptosis and leads to the activation of caspase-3, which is a downstream enzyme in the apoptosis process and is involved in the execution phase of the death pathway [40]. As reported earlier [41-43] that DHA is rapidly taken up by cells and incorporated into membrane phospholipids and that both DHA and EPA activate protein phosphatases, leading to dephosphorylation of retinoblastoma protein (pRB). Accordingly, treating cells with DHA and EPA can possibly lead to the release of cytochrome c and activation of caspase-3. These two compounds induce a cell signaling pathway for apoptosis that eventually leads to the death of cancer cells.

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