Research Article

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DOCOSAHEXAENOIC ACID INDUCES APOPTOSIS IN COLON CANCER CELL LINE, Caco-2

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ABSTRACT:

OBJECTIVES: *The present study sought to further investigate the in vitro anticancer effects of a representative omega-3 fatty acid, docosahexaenoic acid (DHA), with a focus on assessing the induction of apoptosis as an important mechanism for its anticancer actions.* ***METHODS:*** *Caco-2, cells were incubated with DHA at concentrations, 200µM, 400µM, 600µM and 800µM for 72h at 37°C and 5% CO2. MTT assay DNA fragmentation assay, cytological and immunocytochemical investigations were investigated.* ***RESULTS:*** *DHA strongly reduces the viability and DNA synthesis of Caco-2 human colorectal cancer cells in culture, and also promotes cell death via apoptosis. MTT assay was used to test DHA on the effect of the proliferation of the colorectal cancer cell line, Caco-2. IC50 DHA inhibited the proliferation of Caco-2 cells and induced Caco-2 cell death in a dose-dependent manner. Treated cells showed typical characteristics of apoptosis including inhibited the viability and proliferation of treated Caco-2 cells in vitro even by DNA fragmentation, cytological alterations and downregulation of Bcl-2 activity.* ***CONCLUSIONS:*** *Results from this study show that DHA has antiproliferative effects against cancer cells. fish oil as a dietary supplement can help to maintain good health and protect the body against disease. Future study will may deal with further investigations of fish oil as a dietary supplement possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types especially colorectal cancer type.*

KEYWORDS:

DHA, Caco-2 Cells, Bcl-2, antiproliferative effects.

INTRODUCTION

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer deaths in both men and women around the world. Alarmingly, increasing numbers of reported cases of colon cancer in recent years has made this form of cancer a major health concern[1]. The current treatment for colorectal cancer is generally surgical resection combined with chemotherapy by cytotoxic drugs and radiation. However, this therapy is just moderately successful especially for late stage cancers; therefore new approaches to the treatment of colorectal cancer are required. In recent years, interest has increased in using natural products for pharmacological purposes, as a form of complementary or replacement therapy. It is known that the risk of colorectal cancer increases with dietary habits like high animal fat intake[2]. Epidemiological and prospective studies have reported several beneficial effects of bioactive compounds on human health, particularly in protecting against chronic degenerative diseases, such as cardiovascular disease, diabetes mellitus and cancer.

Omega-3 fatty acids are long-chain polyunsaturated. The principal dietary source of eicosapentaenoic acid (EPA, 20:5n- 3) and docosahexaenoic acid (DHA, 22:6n-3) is from oily coldwater fish[3]. Epidemiological studies have suggested that an increased fish oil intake is associated with a reduced breast cancer incidence in humans[4]. Consistent with this epidemiological observation, laboratory studies have also shown that omega-3 can suppress the formation and growth of breast cancer in animal models[5]. A number of mechanisms have been proposed for the anticancer actions of omega-3, including suppression of neoplastic transformation, inhibition of cell

proliferation, enhancement of apoptosis, and antiangiogenicity[6].

Mitochondria are involved in a variety of key events, including release of caspase-3 activators, changes in electron transport, loss of mitochondrial membrane potential, and participation of both pro- and anti-apoptotic Bcl-2 protein[7]. Alterations in mitochondrial structure and function have been shown to play a crucial role in caspase-3-dependent apoptosis and Bcl-2 expression[8]. Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis. Bcl-2 itself is an antiapoptotic gene that prevents initiation steps of apoptosis and programmed cell death[9]. The aim of this study was to evaluate the effect of DHA on the inhibition of proliferation of the colorectal cancer cell line, Caco-2.

MATERIALS AND METHODS

Chemical reagents: DHA, MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx & E) stain, primary monoclonal antibody against Bcl-2 and biotinylated immunoglobulin secondary antibody and Tween 20 were purchased from Sigma-Aldrich, Egypt.

Cell line and cell culture: Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as mono-layer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5% CO_2. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

Methods: 1.Cell Viability Assay: In vitro evaluation of antiproliferation effect: growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans[10]. Viable cell number/well is directly proportional to formazans production. 8.25×10^3 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing DHA at concentrations: 200µM/L, 400µM/L, 600µM/L and 800µM/L for 72h at 37ºC in an incubator with 5% CO_2. After incubation, DHA modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5% CO_2). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage=[1–(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against DHA concentrations.

2. Determination of DNA fragmentation by DNA laddering assay: cells were seeded in 60-mm petri dishes at density $4x10^5$ cells/plate (treated cells by IC_{50} concentration of DHA or positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, Triton-X100 0.5%) and kept at 4ºC/10min then, lysate was centrifuged at 25.000×g/20min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15min. After drying, DNA

was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH 8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide[11].

3.Cytological changes investigation: detached and trypsinized cells (IC_{50} concentration of DHA treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 10µL of the suspension were smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5min before proceeding by Hx&E stain and examined under light microscope[12].

4. Immunocytochemical investigations: by detection of Bcl-2 by immunocyto chemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC_{50} concentration of DHA treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with primary monoclonal antibody against Bcl-2 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used[13]. The slides were then mounted and examined under light microscope.

5. Statistical analysis: results were presented as mean±standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at $P<0.05$ and high significance was set at $P\leq 0.01$[14].

RESULTS

1.Cell viability assay: In vitro evaluation of antiproliferation effect.

Cytotoxic effect of different concentrations of DHA (200µM, 400µM, 600µM and 800µM) for 72h on Caco-2 cell line was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with DHA concentrations in a dose dependent manner. All concentrations were found to be high significantly different ($P\leq 0.01$) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with DHA concentration increasing and 100% of cell inhibition was observed when cells were treated with 800µM/72h. Cell proliferation reduced about 30% and 45% when cells were treated with 200µM and 400µM for 72h, respectively. Cells proliferation decreased to 60% when treated with concentration of 600µM/72h.

2. Determination of DNA fragmentation by DNA laddering assay.

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with DHA concentration at 600µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that DHA induced DNA fragmentation which was caused by apoptosis.

3. Cytological changes investigation.

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by DHA concentration at 600µM/72h, apoptotic cells were identified by a series morphological changes as an important experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

4. Immunocytochemical investigation.

After Coca-2 cells treatment by DHA concentration at 600µM/72h and regarding to the positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 55% of cells had nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane brown staining, with slight intensity degrading in the same field (Figures 3e). After Coca-2 cells treatment by DHA concentration at 600µM/72h, those fields that had necrotic or apoptotic nucleus as sign for DHA apoptotic effect with Bcl-2 negative reaction (faint to non-brown stain) (Figure 3f).

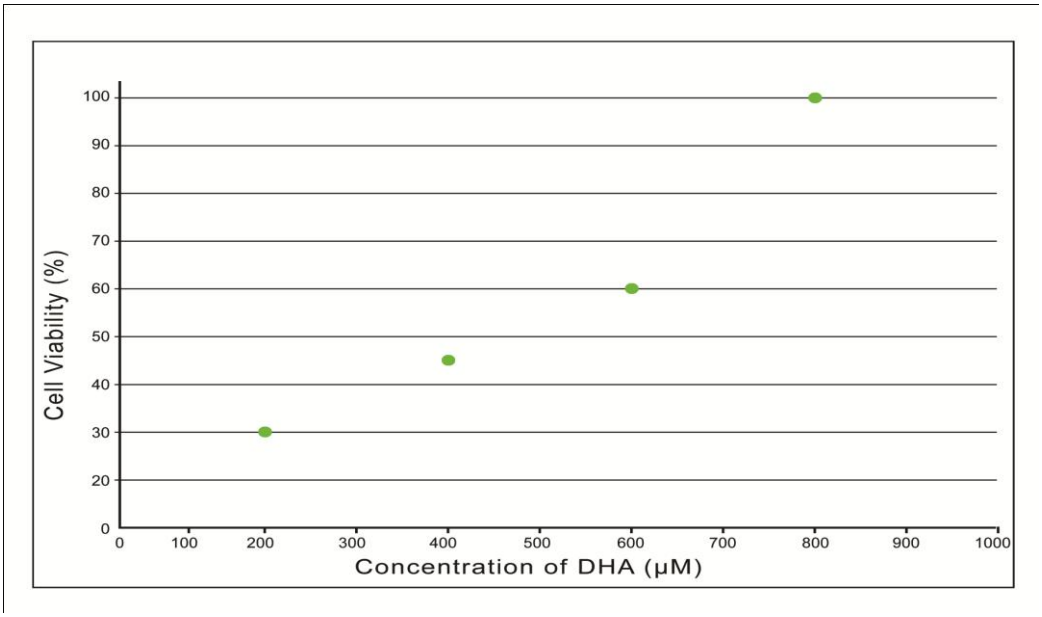

Figure 1: Effect of DHA with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean ± SD, n (for each concentration) =4].

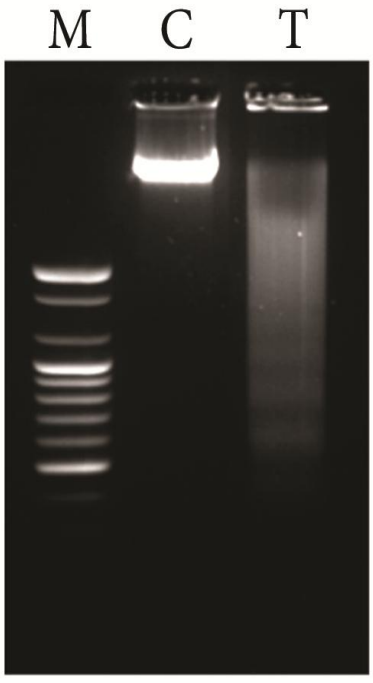

Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from DHA treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker.

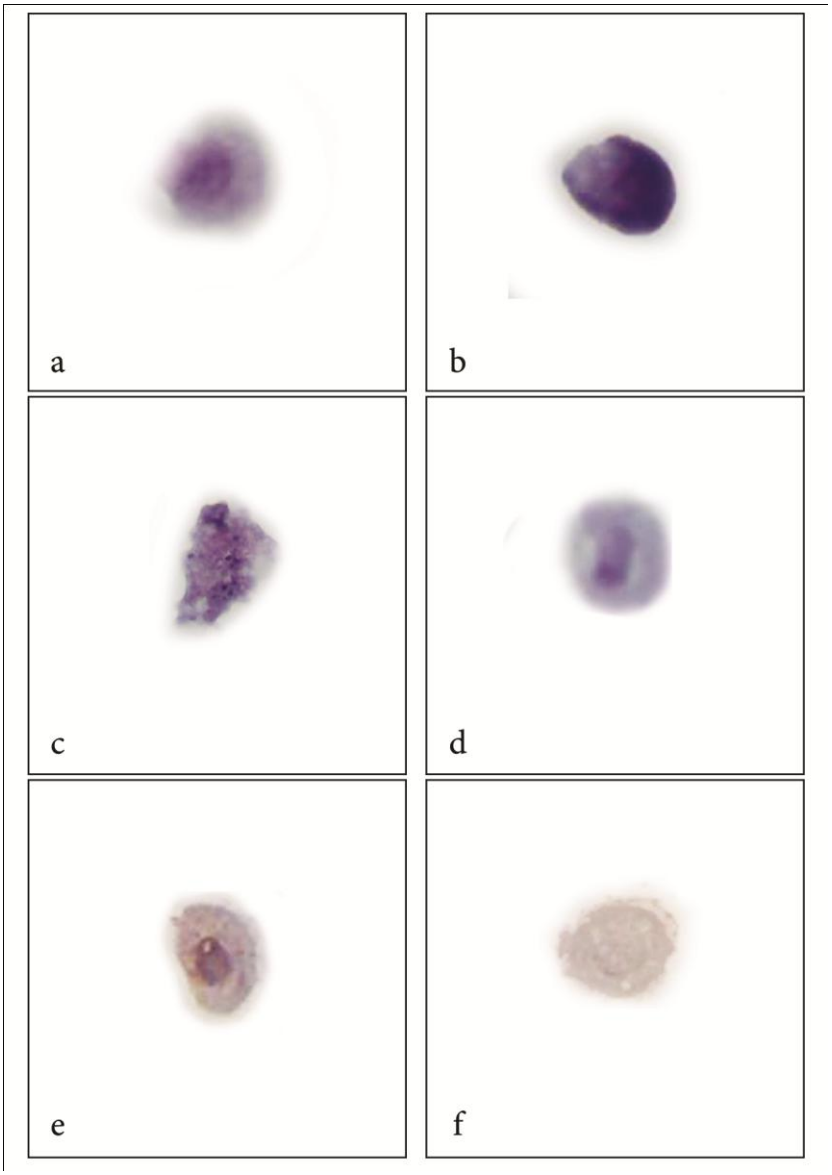

Figure 3: Cells in different apoptosis stages in treated cells are easily distinguishable. Cell with normal morphology (a). Complete apoptotic cell (b). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (c). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Immunocytochemistry of Bcl-2 protein as control positive cell showing Bcl-2 protein nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane showing brownish positive reaction (e). Treated cell showing negative reaction of apoptotic cell (f).

DISSCUTION

The results of our present study showed that DHA, an omega-3, has a strong anticancer activity in cultured Caco-2, human colorectal cancer cells through a combination of multiple actions, including inhibition of DNA synthesis, suppression of cell viability, and induction of apoptotic cell death. It was reported earlier that while omega-3 could selectively inhibit tumor cell proliferation, they were significantly less cytotoxic in normal cells[15]. The results of our present study showed that the anticancer effect of DHA

Apoptosis, as programmed cell death, is a highly organized cell death process characterized by an early obvious condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of nucleases, enzymatic cleavage of DNA into

oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies[16]. DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor[17]. Apoptosis has specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process[18]. That was agreed with the results of recent study after treatment by DHA. DHA decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells.

Therefore, we may presume that as primary mechanism involved in DHA growth-inhibitory effects as it considered main apoptotic signals. Caco-2 cells which were treated with DHA exhibited down regulated levels of Bcl-2 expression at concentration of 600µM/72h, which suggested that Bcl-2 involved in DHA-induced Caco-2 cell death as mitochondrial pathway was involved in DHA-induced Caco-2 cell death[19].

CONCLUSION

In this study, we have demonstrated that fish oil as a dietary supplement inhibited proliferation and induced apoptosis in colon cancer (Caco-2) cells which depended on down-regulation of Bcl-2 protein. Future in vitro and in vivo study will may deal with further investigations of the possible usages of fish oil as a dietary supplement as a new alternative chemotherapeutic agent but in limit doses for human colorectal cancer suggested treatment and other types of cancer.

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