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Effect of some fatty acids on apoptosis related genes in human breast cancer

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Breast cancer, the second most common cancer after lung cancer, is the most common cancer type diagnosed in women. No definitive treatment has been established for breast cancer yet, but essential fatty acids offer a promising option. Omega fatty acids are classified in the essential fatty acids that the body cannot produce and, therefore, must be taken through the foods of animal or plant origin. Although in the literature the omega fatty acids have been shown to exhibit significant positive effects in inhibiting various tumor types, their mechanism of action, the apoptotic pathways they employ, and the genes they control have not been clarified yet. In this study, various doses and combinations of omega-3 [Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA)] and omega-6 [Linoleic acid (LA)] fatty acids were administered to human breast cancer MCF7 cell line for 24 h, and using the enzyme-linked immunosorbent assay (ELISA) method, the protein expression levels of the following apoptosis-related genes were determined: phospho-p53 (Ser15), p53, Bad, phospho-Bad (Ser112), cleaved Caspase-3 (Asp175), and cleaved PARP (Asp214). Even though there was no significant difference observed in the expressions of phospho-p53 (Ser15) and p53 at all doses, other protein expressions were found to increase significantly, suggesting that Omega-3 and -6 can mediate apoptotic pathway to induce cell death in breast cancer cells.

Keywords: Apoptosis, Docosahexaenoic acid, Eicosapentaenoic acid, Linoleic acid, MCF7, Omega-3, Omega-6, Protein expression

With the developing world, industrialization, and increasing population, the prevalence of most diseases, especially cancer, has increased. Cancer is defined as the tissue cluster that grows and develops very rapidly, divides in an uncontrolled fashion, and does not comply with the normal functioning mechanism of the body¹. Breast cancer, the second most common cancer type after lung cancer, is the most commonly diagnosed cancer in women². The common feature in all these breast cancer types is that they are all associated with lipid structure.

There are also some studies indicating that polyunsaturated fatty acids (PUFAs) have an inhibitory role in cancer development³⁻⁵. Fats take part in metabolic activities by means of combining with proteins to form lipoproteins^{6,7}. Human body cannot produce essential fatty acids (EFAs), so they must be taken through foods. EFAs are polyunsaturated fatty acids. There are two types of EFA in the body: n-3 and n-6⁸.

To date no consensus has been reached on how much n-6 and n-3 fatty acids should be consumed.

The World Health Organization recommends a ratio of 5:1-10:1. However, as a matter of fact, the healthiest ratio is reported to be between 1:1 and 4:1. The n-6/n-3 ratio is 1.5:1 in case 650 g of EPA+DHA and 2.22 g of ALA are taken daily as n-3 fatty acid, and 4.44 g of LA as n-6 fatty acid⁹.

Apoptosis is a homeostatic mechanism that takes place during development and aging to maintain cell population in tissues^{10,11}. It also manifests itself as a defense mechanism like in immune reactions or as a type of programmed cell death that allows cells to be destroyed without damaging their environment and causing inflammation when they are damaged by a noxious agent and diseases. Cells in apoptosis fragment into small particles called "apoptotic bodies" and are destroyed by other cells with phagocytosis¹²⁻¹⁶. Caspases are central to the apoptosis mechanism because they are both initiators and practitioners of this process¹⁷. The p53 gene encodes a tumor suppressor protein that contains DNA binding, transcriptional activation, and oligomerization domains. Mutations in this gene are associated with a variety of human cancers, including the hereditary ones¹⁸.

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The protein encoded by the *Bad* gene is a member of the BCL-2 family. BCL-2 family members are the regulators of programmed cell death¹⁹⁻²¹. PARP-1 is a nuclear protein with a great of physiological and pathological functions. Cleavage of PARP-1 by caspases is considered a specific feature of apoptosis²².

In this study, the effect of low and high n-6/n-3 fatty acid (FA) ratios (1:2.5, 1:4, 1:5, 1:10) of Linoleic acid (LA), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) on the survival and growth of MCF7 was examined. Then, using the most effective FA ratios, the expressions of phospho-p53 (Ser15), p53, Bad (BCL2-associated cell death agonist), phospho-Bad (Ser112), cleaved Caspase-3 (cysteinyl aspartate protease-3) (Asp175), and cleaved PARP (Asp214) were analyzed.

Material and Methods

Cell culture

Estrogen receptor-positive breast cancer cell line MCF7 was cultured in a DMEM medium supplemented with 10% FBS and 1% penicillinstreptomycin at a temperature of 37° C and a humidity of 95% and in an atmosphere of 5% CO₂. The medium was changed under sterile conditions in a laminar flow cabinet every 2 days until the cells reached a density of about 70%. The cells with a density of more than 70% were passaged for proliferation.

Seeding cells in plates

The counted cells were seeded in 96-well plates for MTT test (10,000 cells in 100 μ L medium) and in 6-well plates for ELISA test (300,000 cells in 3 mL medium)²³.

Cell viability test (MTT assay)

After the cells were counted and seeded in 96-well plates, they were incubated for 24 h. Then, they were treated with Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and Linoleic acid (LA) at the concentrations of 40, 80, 120, 160, 200, 240, 280 and 320 μ M and their combinations with different

ratios (LA:EPA+DHA; 1:1, 1:2.5, 1:4, 1:5, 1:10, 2.5: 1, 4: 1, 5:1 and 10:1). During incubation, the mitochondrial dehydrogenases in metabolically active cells formed purple MTT-formazan crystals by breaking down the tetrazolium ring. Crystals were dissolved in DMSO, and the optical densities (ODs) were read at 570 nm (630 nm, reference) using a microplate reader²³.

Protein isolation

The MCF7 cells seeded in 6-well plates were treated for 24 h with 40 μ M and 280 μ M of DHA, 80 μ M and 240 μ M of EPA, 80 μ M and 320 μ M of LA, and LA/EPA+DHA at the ratios of 1:1 and 10:1, which were determined based on the MTT results. Protein isolation was carried out using M-PER as per the kit protocol, and the isolated proteins were stored at -80° C for the next steps of the study. The concentration of the isolated proteins was determined by following the protocol of Thermo Fisher Protein Assay kit²³.

PathScan apoptosis ELISA application

The expression levels of the cellular proteins were determined as per the kit protocol (PathScan Apoptosis Multi-Target Sandwich ELISA Kit) using the antibodies specific to Phospho-p53 (Ser15), p53, Cleaved Caspase3 (Asp175), Cleaved PARP (Asp214), Phospho-Bad (SER112), and BAD proteins.

Results

Effects of n-6 and n-3 fatty acids on the viability of MCF7 cells

EPA, DHA, and LA at the concentrations of 40 μ M, 80 μ M, 120 μ M, 160 μ M, 200 μ M, 240 μ M, 280 μ M, 320 μ M and 360 μ M and their combinations were administered to MCF7 cells, and the MTT results were shown in Table 1.

Fig. 1 shows the minimum and maximum values for the cell viability and death in different concentrations of each fatty acid at the end of 24 hincubation. The concentrations at which the maximum cell viability and death values were observed were 240 μ M and 320 μ M for EPA (Fig. 1A), 80 μ M and 280 μ M for DHA (Fig. 1B), 40 μ M and 320 μ M for

Table 1 — Concentrations of n-6 (LA) and n-3 (EPA, DHA) fatty acids used in different proportions at total fatty acid concentration of 280 μ M

	Low n-6/n-3 Ratios					High n-6/n-3 Ratios			
Fatty acids	1:1	1:2.5	1:4	1:5	1:10	2.5:1	4:1	5:1	10:1
Linoleic acid (LA)	140	80	56	46.67	25.45	200	224	233.33	254.55
Eicosapentaenoic acid (EPA)	84	120	134.4	140	152.73	48	33.6	28	15.27
Docosahexaenoic acid (DHA)	56	80	89.6	93.33	101.82	32	22.4	18.67	10.18



Fig. 1 — Effect of concentrations of (A) EPA; (B) DHA; (C) LA; and (D) LA/EPA+DHA on viability of MCF7 cells after 24 h incubation



Fig. 2 — ELISA results of Phospho-p53 (Ser15), p53, Cleaved Caspase3 (Asp175), Cleaved PARP (Asp214), Phospho-Bad (Ser112) and BAD protein expressions

LA (Fig. 1C), and the ratios of 10:1 and 1:1 for LA/EPA+DHA (Fig. 1D).

These concentrations were used as a reference for the next steps of the study, and based on these concentrations, the cell treatments were carried out in the ELISA test.

Apoptotic effects of the fatty acids on MCF7 cells

Based on the data obtained from the viability tests, ELISA study was carried out using the most effective doses of 80 μ M and 240 μ M for EPA, 40 μ M and 280 μ M for DHA, 80 μ M and 320 μ M for LA, and 10:1 and 1:1 for LA/EPA+DHA. Fig. 2 shows the expressions of Phospho-p53 (Ser15), p53, Cleaved Caspase 3 (Asp175), Cleaved PARP (Asp214), Phospho-Bad (Ser112), and BAD protein.

It was found that phospho-p53 expression did not differ in the DHA concentration of 40 μM and LA

concentration of 80 μ M, and the slight increases in other doses did not yield a significant difference. Quite slight increases in the p53 expression were not significant either. Although there was a marked increase in the expression of the Cleaved Caspase 3 for all doses, the increases in the treatments of LA-80 μ M, LA-320 μ M, and LA/EPA+DHA-1:1 were found to be higher than other doses applied. These three doses significantly increased the expression of Cleaved Caspase 3. All the treatments significantly increased the Phospho-Bad (Ser112) and BAD expressions. The increase in the expression of BAD was more pronounced than the phospho-Bad.

Discussion

Uncontrolled proliferation of cells due to losing regulation of apoptosis pathways causes the development of cancer²⁴⁻²⁶. In today's industry and

technology world, studies still continue to develop traditional cytotoxic drugs for the treatment of cancer, one of the most common and deadly diseases. However, although very important steps have been taken in this field, no definitive therapy has been set against cancer. In recent years, more and more emphasis has been put on the alternative methods for the treatment and prevention of cancer. The multifactorial nature of cancer types, including breast cancer, have highlighted the supportive aspects of dietary factors for cancer treatment. Today, omega fatty acids, which are among the dietary factors for breast cancer, attract much attention. Various studies have shown that Omega-3 fatty acids of fish origin play an important role as a protective factor in the nutritional etiology of breast cancer^{9,27-32}.

n-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and n-6 fatty acid (linoleic acid) were used in the present study. The main reason for choosing these fatty acids was that eicosapentaenoic acid and docosahexaenoic acid, which are naturally found in coldwater fish, are highly promising in preventing carcinogenesis and reducing the risk of breast cancer^{29,33}. The roles played by omega-6 fatty acids in many pathological processes, including cancer development, have been mostly attributed to arachidonic acid. Although gamma linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA) produced from linoleic acid have been shown to have anti-cancer activities³⁴, there is not enough data on the activity of linoleic acid. The reason why we used the MCF7 cell line in this study was because breast cancer is the second most common cancer type worldwide and expresses estrogen receptors (ER) and progesterone receptors (PR) which are responsive to lipids.

In this study, the effects of triple combinations of fatty acids on the expressions of phospho-p53 (Ser15), p53, Cleaved Caspase 3 (Asp175), Cleaved PARP (Asp214), phospho-Bad (Ser112), and BAD, which are the key signal proteins in pathways controlling survival and apoptosis, were examined in comparison with the control samples.

When we examined the effect of eicosapentaenoic acid (EPA) on the cell viability, it was found that the 24-hour treatment caused a small increase in viability at the concentrations of 40, 80, and 120 μ M, and an increasing toxicity at the concentrations of 160, 200, 240, 280, 320 and 360 μ M. Our viability test results

for EPA were in line with the results reported by Mansara & colleagues³⁵. In their study examining the effect of AA/EPA+DHA fatty acids on MDA-MB-231 and MCF-7 breast cancer cell lines and MCF-10A non-cancerous breast cell line, they reported that the low n6/n3 ratio reduced the viability and growth of cancer cells compared to control cells, while the high n6/n3 ratio reduced the survivability in both cancer and control cells. In the present study, the dose at which EPA was the least effective on cell toxicity was found to be 80 μ M, and the most effective dose was 240 μ M.

In the viability test results of docosahexaenoic acid (DHA), almost the same low levels of toxicity were observed at the concentrations of 40, 120, 160 and 200 µM, while there was a marked increase in the toxicity at the concentration of 80 µM. When the concentration of DHA was increased to 240 µM and 280 µM, the toxicity gradually increased, but it decreased at the concentrations of 320 μ M and 360 μ M. The role of DHA in preventing breast cancer has been proven in epidemiological studies, dietary studies in mice and humans, and cell culture studies, but its mechanisms have not been elucidated yet. The results found in our study and those reported by Larsson and colleagues³⁶ are consistent with these data. The least and most effective concentrations on vitality were found to be 40 µM and 280 µM, respectively.

It was observed that all the doses of linoleic acid had positive effects on cell viability, and these effects increased or decreased in a dose-dependent manner. It was found that the concentrations of 320 μ M and 80 μ M had the least and most effect on the cells, respectively. Since there is no similar study on the effect of linoleic acid on the viability of MCF7 cells, our data can serve as a reference in this regard.

In the treatments of LA/EPA+DHA in various ratios combinations, although the high of LA/EPA+DHA were expected to have positive effects on the cell viability and the low LA/EPA+DHA ratios to have high toxic effects, our results did not support this expectation. The treatment ratio of 1:1 showed a toxicity that is significantly different from other doses at a quite high level. On the other hand, it was found that the treatment ratio of 10:1 had a positive effect on the cell viability. The most and least effective doses of all fatty acids on the viability of MCF7 cells were used as a reference for identifying the expressions of phospho-p53 (Ser15), p53, Cleaved Caspase 3 (Asp175), Cleaved PARP (Asp214), phospho-Bad (SER112), and BAD proteins.

The tumor suppressor protein p53 is a key factor in cell cycle arrest, DNA repair, and in induction of apoptosis in response to a stimuli. This protein is phosphorylated at serine 15 and serine 20 during DNA damage repair or at serine 46 to induce apoptosis³⁷. When we compared the results of the treated groups both among themselves and with the untreated group, no significant difference was observed in terms of the expressions of p53 and phospho-p53. Based on these results, we are of the opinion that EPA, DHA, and LA do not mediate the apoptosis in MCF7 cells via p53 dependent pathway.

The increase in cleaved Caspase-3 (Asp175) protein is one of the indicators of apoptosis. Cleaved Caspase-3 activity affects nucleases and triggers apoptosis. The increase in the expression level of Asp175 indicates the induction of apoptosis^{38,39}. In our study, the expression of Asp175 in the treated groups was compared with that in the untreated group, and it was found that it increased in all the doses of fatty acids. The most marked increase was observed to be at the doses of linoleic acid with 85%, and its both doses caused a proportional increase. The increase in the expression of Asp175 in EPA was slightly higher at the concentration of 80 µM than at 240 µM. In other words, the EPA dose of 80 µM was found to drive the cells to apoptosis through Asp175 more than the dose of 240 µM. While the treatment of DHA at the concentration of 280 µM increased the Asp175 expression by $\sim 42\%$, its treatment at the concentration of 40 µM increased the Asp175 expression by ~14%. The LA/EPA+DHA ratios of 1:1 and 10:1 were also found to significantly increase the expression of Asp175 and drive the cells to apoptosis. In a previous study on gastric cancer, it was reported that DHA and EPA reduced the viability of gastric cancer cells and induced apoptosis by activating caspase-3⁴⁰. In another study on MCF-7 breast cancer cell line, it was reported that DHA increased the levels of cleaved caspase-8, -9 and -3 and ultimately caused the apoptosis of human breast cancer cells in vitro via the death receptor and mitochondriamediated pathways^{35,41,42}.

PARP protein takes part in repairing the breaks in DNA and functions in the form of cleaved PARP (Asp214), leading cells to apoptosis. In the present

study, the treatments caused a very high increase in the Asp214 expression, and this increase was observed in all doses of fatty acids. Similarly, based on the potential of omega-3-polyunsaturated fatty acids (3-PUFAs) in the prevention and treatment of types, Kim & colleagues⁴³ various cancer administered DHA to glioblatoma (GBM) cell lines and reported that the treatment increased the number of cleaved PARP, sub-G1 cell population, and TUNEL-positive cells, which are the indicators of apoptosis in GBM cells⁴³.

BAD protein functions in active form and takes part in inducing apoptosis. Under normal conditions, it exists in the form of inactive phospho Bad (Ser112) in cell and undergoes dephosphorylation by any apoptotic signal and changes to the active form of BAD. In the present study, the increase in BAD expression as a result of the treatments with fatty acids was found to be much higher than the increase in the expression of other proteins. This showed that the most effective apoptosis pathway of EPA, DHA, LA, and their combinations in MCF7 cells was through BAD. In another study on MCF-7 cells, it was reported that only DHA significantly reduced the phosphorylation of MEK, Erk, and Bad^{44,45}.

Conclusion

Our study is significant in that it is the first study examining the effects of Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), and Linoleic acid (LA) and their combination on the expression levels of phospho-p53 (Ser15), p53, cleaved Caspase 3 (Asp175), cleaved PARP (Asp214), phospho-Bad (SER112), and BAD proteins in the MCF7 human breast cancer cell line and it is an original study that is expected to pioneer the future studies on the treatment of breast cancer. In order to better understand the effects of omega fatty acids on the apoptosis mechanism of MCF7 breast cancer cells and to obtain clearer results, the studies on other genes mediating in the apoptosis pathway should continue and the results should be supported by using various analysis methods.

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Conflict of Interest

Authors declare no competing interests.

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