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The effect of omega fatty acids oral administration on apoptotic regulatory proteins in patients with gastric adenocarcinoma under chemotherapy: A double- blind clinical trial study

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Abstract

***Background & Aims*:** The use of some active factors in diet is regarded as an attractive approach to prevent and to treat certain types of cancers. Accordingly, the main objective of this study was to evaluate the effect of PUFAs oral administration along with chemotherapeutic agent on the level of cellular apoptotic regulatory proteins in cancer cells of individuals with gastric cancer in order to identify the apoptotic changes.

Materials & Methods: This study was a Clinical Trial in which the target group consisted of the patients with gastric cancer who were recognized for the first time and cured under chemotherapy. Thirty-four patients were chosen and categorized randomly into two groups. Case group includes the patients taking PUFAs along with the chemotherapeutic agents. In control group, individuals were under the same chemotherapy protocol without taking PUFAs. Biopsy samples of tumor were taken from the patients before and after chemotherapy. The Bcl-2, Bcl-$_{XL}$ Bid, and Bad gene expression were determined by Real-Time PCR. Also, those proteins upon biopsy samples were surveyed by Frozen Section method.

***Results*:** In case group, Bcl-2 and Bcl$_{XL}$ gene expression and protein levels decreased significantly in comparison with those of the control group. While Bid and Bad gene expression and protein levels increased significantly in comparison with those of the control group.

***Conclusion*:** The results of this study showed that use of PUFAs as supplement with *Cis*-platinum may be useful to stimulate more pro-apoptotic proteins in gastric cancer cells. Consequently, this offers an effective treatment to patients with gastric cancer to respond to chemotherapy.

***Keywords*:** Pro-apoptotic Proteins, Anti-Apoptotic Proteins, Poly Unsaturated Fatty Acids, Gastric Cancer, Chemoresistance

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Introduction

The use of some active factors in diet is regarded as an attractive approach to prevent and to treat certain types of cancers. Epidemiologic and experimental studies carried out in recent years have suggested that the diets with unsaturated fatty acids may protect human against cancer, and these diets may be used in cancer treatment (1-3). Gastric cancer is the fourth most prevalent cancer, and is ranked the second leading cause of death from cancer worldwide (4). Its risk factor has been linked to a poor prognosis within the overall 5-year rate, and only less than 35% of cases with this type of cancer are expected to survive (5). It is unfortunate that in more than 60% of the cases the disease is detected when it is at advanced stages (6). Therefore, chemotherapy remains the most effective cancer treatment option to remove the offending and abnormal growth (7). Platinum compounds, either as a single agent or in combination with triple-drug compounds with fluoropyrimidine plus taxanes or anthracyclines are among the drugs used for chemotherapy in gastric cancer are (8). However, the response to the treatment in these patients varies, and some types of tumors demonstrate to be completely resistant to chemotherapy (9, 10).

Therefore, one of the main obstacles in the successful chemotherapeutic treatment of cancer is considered the rising occurrence of resistance to this type of chemotherapy (11). Based on the results reported in recent years in different countries, it has turned out that gastric cancer is of relative sensitivity to chemotherapy (12). And, tumor growth is halted in only 30 to 40% of the cases (13). Besides, overall survival and median life expectancy after recurrence remain low. Due to chemotherapy resistance, this treatment would be in vain in a number of patients, and would force them to endure very high costs and serious side effects (14).

The results of the studies in recent years have elaborated that omega fatty acids including omega-3, omega-6 and omega-9 fatty acids, along with their metabolites, inhibit the growth of cancer cells both in-vitro and in-vivo, and increase the production of prostaglandins, leukotrienes, and thromboxanes (15). Although several candidate mechanisms have been proposed for their anticancer effects, their exact mechanisms are not fully recognized (16). However, the most critical molecular mechanism is the apoptosis induced by the development of endoplasmic reticulum stress, and subsequently, the increased ionized Calcium in cancer cell cytoplasm. This change in ionized Calcium homeostasis inside cells stimulates apoptotic pathway (17).

Apoptosis, the process of programmed cell death, is considered a vital component to maintain the tissue homeostasis and to prevent cancerous growth of cells (18). It may be induced through various pathways, the two of which have been studied in more detail: extrinsic pathway (death message) and intrinsic pathway (mitochondrial pathway). The later apoptotic mechanism arises from relative concentration of Bcl-2-like proteins in mitochondrial outer membrane. Bcl-2-like proteins control the certain protein permeability of the inner mitochondrial membrane into the cytosol (19).

Apoptosis may be activated by cytochrome C exit from the pores created by Bcl-2, Bak and Bax pro-apoptotic proteins in the mitochondrial outer membrane (20). Each cluster is characterized by its structural characteristics and associates with withdrawal of cytochrome C from mitochondria. The pores through which cytochrome C passes are created by a series of Bax and Bak (dimers or polymers) (1, 18-20).

The cytoplasmic sets which are functionally similar to the DISC of death receptor pathway are called apoptosome. Apoptosome facilitates the autocatalytic activation of caspase-9. As a consequence, the activated caspase-9 may trigger procaspase-3 and procaspase-7 activation. Then, the activation of caspase-3, AIF and

EndoG mitochondrial proteins could enter the nucleus and cleave DNA. (21).

Accordingly, the main objective of this study was to evaluate the effect of oral administration of omega-3, 6, and 9 along with *Cis-Platin* drugs on the level of cellular apoptotic regulatory proteins and Bcl-2, Bcl-X_L, Bid and Bad gene expressions in cancer cells of individuals with gastric cancer in order to identify the apoptotic changes in those patients.

Materials and Methods

Type of the study and determining the sample size: The present study was a double-blind before and after clinical trial (intervention) in which neither the oncologist nor the patients were aware of the treatment; thus many of the potential errors were prevented. Among the patients with a presumptive diagnosis of gastric cancer referred to the endoscopy clinic of Tabriz University of Medical Science, 34 were selected and divided into two groups consisting of 17 cases each, with respect to the objectives and conditions of this study.

Criteria for excluding patients from the study:

In this study, these cases were excluded from the research due to their safety issues in complementary medicine consumption, and due to avoid interference with the results:

- The patients who could not take the medicine due to their cardiac obstruction,
- The patients who could not take the medicine due to their pyloric obstruction,
- diabetics,
- The patients with renal impairment,
- The patients who were taking supplements of omega until three months before participating in the study,
- The patients who had underlying inflammatory diseases.

Ethical Considerations: Ethical code number 92213 was issued for the study by the Ethics Committee of Tabriz University of Medical Sciences. The study was registered in Iran clinical trials registry center under No. IRCT2014031016922N1.

Sampling: After the definite diagnosis of gastric cancer, 3 biopsies were obtained from the tumor tissues of gastric cancer, distal third, and transferred to a nitrogen tank. A total of 34 patients were chosen and randomly assigned to two groups, each included 17 cases. Then, they were referred to an oncologist to begin treatment with chemotherapy. The treatment was performed by giving the first group (control group) *Cis-Platin* medication without supplements and the second group (intervention group) *Cis-Platin* with Natural Factors Ultimate-Omega 1200 mg capsules. Patients in the intervention group took three capsules daily (3600 mg total per day) for three courses; each course was three weeks long. Previously, the results of many studies showed that PUFAs could be selectively cytotoxic to various tumor cells in vitro and in vivo (22, 23). This was followed, under the required medical follow-up, by performing endoscopy on the patient for the second time, and the cancer treatment was completed by taking stomach biopsy. The biopsy specimens taken from patients were immediately transferred to the nitrogen tank.

Identifying fatty acids pattern in Omega-3, 6 and 9 capsules prescribed to the patients: To ensure the exact amount of fatty acids in capsules, the "Natural Factors Ultimate-Omega Factors" brand name was administered to the patients in the study, the pattern of fatty acids in the capsules was determined by applying the protocol of Bligh & Dyer (24).

Determining the amount of apoptotic regulatory proteins gene expression involved in apoptosis using Real-Time PCR: The mRNA expression of apoptotic

regulatory proteins was determined by RT-PCR and Real-Time PCR as described before (25). The obtained CT values for each gene were calculated by the $2^{-\Delta\Delta CT}$ formula (26). The sequences of our primers for Bcl-2, Bcl-X_L, Bid and Bad genes and housekeeping GAPDH gene were designed (Table-1). The results were displayed as Mean ± SD from two independent experiments.

Table-1: Information of Bcl-2, Bcl-X_L, Bid, Bad and GAPDH primers

Bcl-2 primer	**Sequence (5'->3')**	**NCBI Reference Sequence**
Forward primer	ATGTGTGTGGAGAGCGTCAA	NM_000633.2
Reverse primer	TCTTCAGAGACAGCCAGGAGA	
Bcl-X_L primer		
Forward primer	GCCACTTACCTGAATGACCAC	NP_612815.1
Reverse primer	CTGAAGAGTGAGCCCAGCA	
Bid primer		
Forward primer	AGTGGGAGGGCTACGATGAG	NP_001187.1
Reverse primer	ATGCTACGGTCCATGCTGTC	
Bad primer		
Forward primer	CGGAGGATGAGTGACGAG	NP_004313.1
Reverse primer	AAGTTCCGATCCCACCAG	
GAPDH primer		
Forward primer	GAAGGTGAAGGTCGGAGTC	XM_005253678.1
Reverse primer	GAAGATGGTGATGGGATTTC	

Immunohistochemistry staining of frozen sections for the semi-quantitative study of apoptotic regulatory proteins: Frozen section staining was applied to confirm the changes in protein expression of Bcl-2, Bcl-X_L, Bid and Bad [26]. Rabbit polyclonal antibodies for Bcl-2, Bcl-X_L, Bid and Bad (Sigma-Aldrich Co. LLC-UK) were used.

The method of analyzing data (statistical methods):

Data obtained from the two independent experiments were analyzed and expressed as Mean ± SD. Statistical analysis was performed by Mann Whitney-U Test and Wilcoxon Matched-Pairs Signed-Ranks Test. These tests were considered significant when the p-value was less than 0.05. All authors had access to the study data and reviewed and approved the final manuscript.

Results

Comparison of the demographic data in the study groups: In Table-2, the demographic data showed that both patient groups were compared statistically, and as can be seen, the groups were well homogenized in terms of age, gender, tumor location, tumor grade and other information (in all cases, $p>0.05$) (2).

Table 2: Demographic data of the patients in the study group (2)

Groups / **Clinical and Pathologic Factors**	**Control (n=17)**	**Case (n=17)**	**p-Value**
Age(Years)(means±SD)	67.5±11.21	71.25±9.81	0.235
Gender			
Male (n=19)	9	10	0.695
Female (n=15)	8	7	
Tumor Size			
<4 cm (n=16)	7	9	0.759
>4 cm (n=18)	10	8	
Tumor Primary Location			
Upper (n=11)	5	6	0.714
Median (n=13)	6	7	0.790
Lower (n=10)	6	4	0.452
Stage Classification of Malignant Tumors (*TNM*)			
I (n=7)	4	3	
II (n=11)	6	5	0.089
II (n=9)	4	5	
IV (n=7)	3	4	
Systolic Blood Pressure (mmHg)	131.1±9.2	128.8±10.2	0.235
Diastolic Blood Pressure (mmHg)	85.1±7.1	79.2±7.9	0.985
Cigarette Smoking			
Current Smoking (n=12)	7	7	1
Non-Smoker (n=11)	6	5	0.714
Ex-Smoker (n=11)	4	5	0.697
Fasting Blood Sugar (mg/dl)	98.54±15.25	102.85±18.65	0.235
Cholesterol (mg/dl)	148.98±21.56	151.25±25.65	0.125
Triglyceride (mg/dl)	87.25±18.25	78.25±15.65	0.256
History of Family			
Yes (n=19)	10	9	0.73
No (n=15)	7	8	

Determination of fatty acids pattern in Omega-3, 6 and 9 capsules prescribed to patients: As shown in Figure-1, the percent of fatty acids pattern in "Natural Factors Ultimate-Omega Factors" capsules has been turned out. The percent of Omega-3 fatty acids equaled 22.76%, percent of Omega-6 fatty acids equaled to 28.71% and percent of Omega-9 fatty acids equaled to 16.04%.

Comparison of Bcl-2, Bcl-X_L, Bid and Bad expression amounts in the study groups: As shown in Figures-2, the gene expression of Bcl-2, Bcl-X_L, Bid and Bad in the two groups were compared. In the case group, Bcl-2 and Bcl-X_L anti-apoptotic proteins gene expression amount, after being treated with chemotherapy supplemented with fatty acids, decreased significantly in comparison with that of the control group who received chemotherapy without supplementary fatty acids (p=0.007 and p=0.006, respectively). In case group, Bid and Bad pro-apoptotic proteins gene expression amount increased significantly after chemotherapy in comparison with that of the control one (p=0.001and p=0.002, respectively).

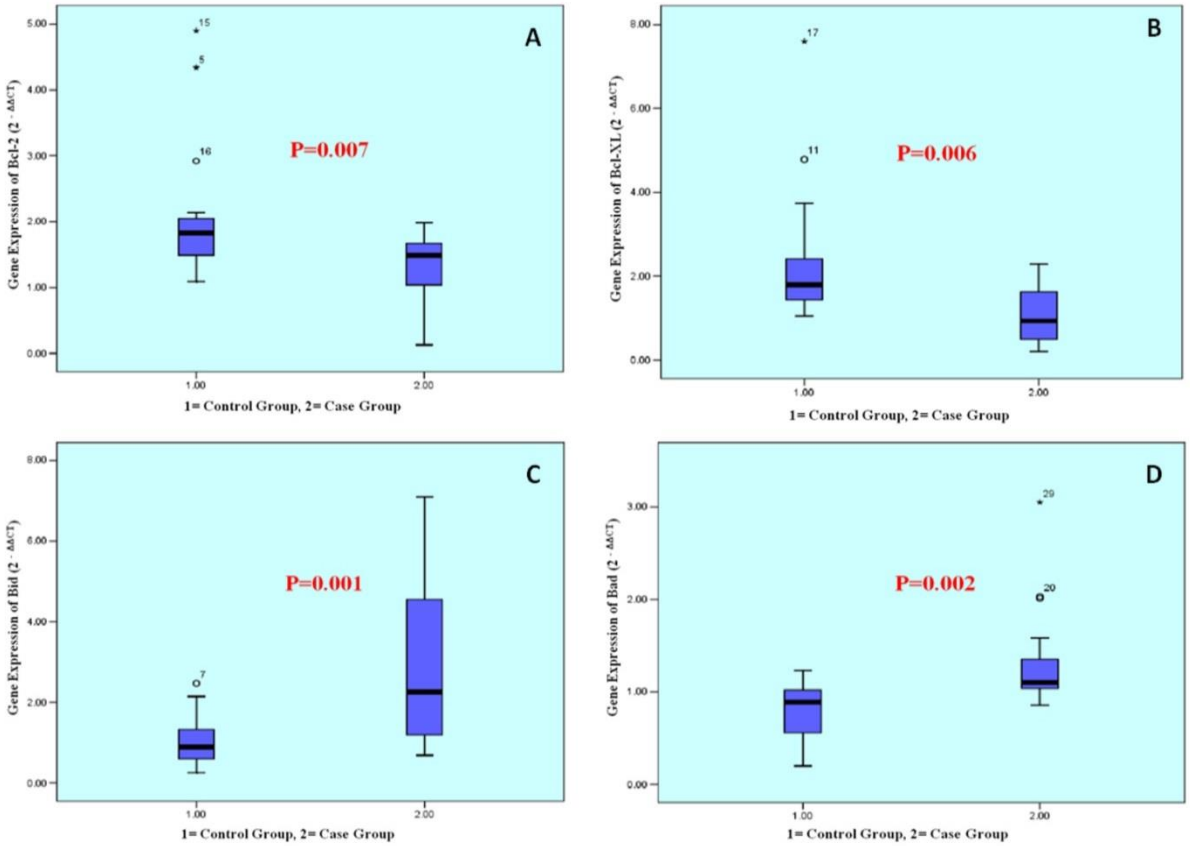

Figure-1:**A.** Comparison of g*ene's expression* level of *Bcl-2* in Control and Case *groups.* ***B.*** Comparison of g*ene's expression* level of *Bcl-*X_L in Control and Case *groups.* ***C.*** Comparison of g*ene's expression* level of *Bid* in Control and Case *groups.* ***D.*** Comparison of g*ene's expression* level of *Bad* in Control and Case *groups.*

Comparison of Bcl-2, Bcl-X_L, Bid and Bad proteins in gastric cancer tissues in the study Groups: The results of Bcl-2, Bcl-X_L, Bid and Bad proteins were reported from Negative (-) to +4 (++++ Positive). As demonstrated in Table-3, the semi-quantitative amount of Bcl-2, Bcl-X_L, Bid and Bad protein after chemotherapy with fatty acid supplement, in the treatment group showed a significant variation compared to that of the control group who received only *Cis*-Platin. The results of the above-mentioned level of changes in the proteins can confirm the level of changes in gene expression of these proteins.

Table-3: The information of the semi-quantitative amount of Bcl-2, Bcl-X_L, Bid and Bad proteins in Control and Case Groups

Patients No. / Protein (Groups)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Bcl-2	Control	+/-	+/-	+	+/-	+/-	-	-	+/-	+	-	-	-	+	-	-	-	+/-
	Case	+	-	+/-	-	+/-	-	+/-	-	-	+/-	-	-	+/-	+/-	-	-	-
Bcl-XL	Control	+/-	+/-	+	+/-	+/-	-	-	+/-	+	-	-	-	+	-	-	-	+/-

	Case	+/-	-	+	-	+	+/-	-	+/-	-	+/-	+/-	-	+	+/-	+	-	-
Bid	**Control**	2+	2+	+	+	+	2+	2+	+	+	2+	2+	+	2+	+	+	3+	2+
	Case	2+	3+	+	+	+	3+	3+	2+	2+	3+	4+	2+	3+	3+	2+	3+	2+
Bad	**Control**	+	2+	2+	+	2+	2+	2+	2+	2+	2+	2+	+	2+	2+	2+	3+	+
	Case	+	2+	2+	+	2+	3+	2+	3+	3+	2+	2+	+	3+	3+	2+	3+	+

The semi-quantitative results were converted to percentage and with non-parametric tests 2-Independent Samples were compared with each other. As can be seen in Figure-4, the percentage of Bcl-2, Bcl-X_L, Bid and Bad proteins in the two groups were compared. In Case group, Bcl-2 and Bcl-X_L proteins percentage after being treated with chemotherapy supplemented with fatty acids, reduced significantly in comparison with that of the control group who received chemotherapy without supplementary fatty acids. The percentage of the Bcl-2 protein was p= 0.001, and the percentage of the Bcl-X_L protein was p=0.024. This is while the percentage of Bid and Bad proteins in case group showed a significant increase in comparison with that of the control group (p = 0.008 and p=0.04, respectively).

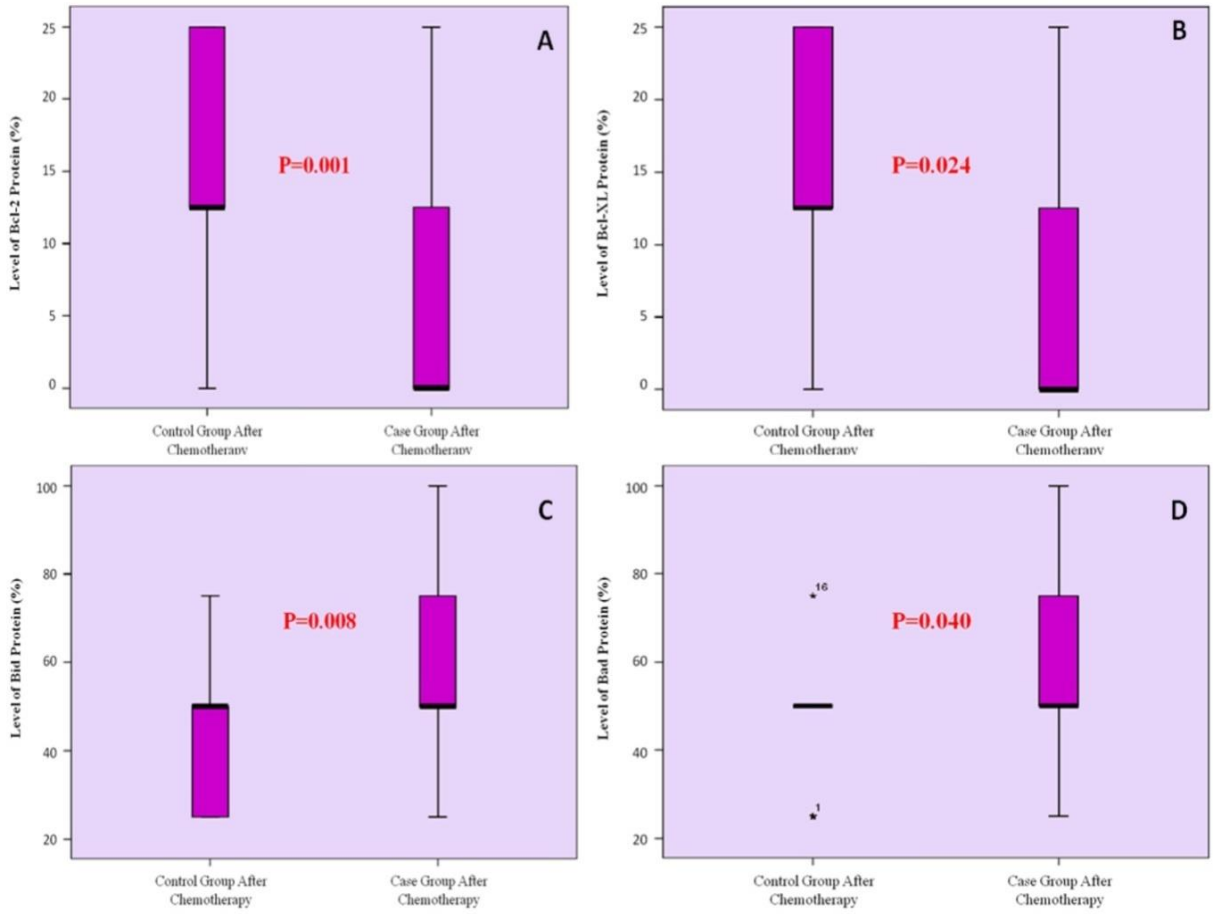

Figure-2:**A.** Comparison of percentage of *Bcl-2* protein *in Control and Case* groups. **B.** Comparison of percentage of *Bcl-X_L* protein *in Control and Case* groups. **C.** Comparison of percentage of *Bid* Protein *in Control and Case* groups. **D.** Comparison of percentage of *Bad Protein in Control and Case* groups

Discussion

The concept of using natural food chemicals, such as polyunsaturated fatty acids (PUFAs), as chemo-preventive agents in cancer therapy has gained attention (27-29). In addition, chemotherapy is counted as the main therapeutic method to fight against stomach cancer (7). However, when chemotherapy resistance occurs in cancer cells, it reduces therapeutic efficiency of chemotherapy, and consequently, cancer chemotherapy fails (30). A marked inhibition of cell apoptosis is thought to be the main reason to mediate cancer cell resistance to chemotherapy. Various studies have revealed the increased amount of anti-apoptotic proteins, such as Bcl-2 and Bcl-$_{XL}$, and the decreased amount of pro-apoptotic proteins, such as Bid and Bad in protection of cancer cells against apoptosis (31). Therefore, the novel strategies include the use of less toxic compounds which may result in stomach cancer cells to respond well to chemotherapy. Numerous studies in recent years have shown that fatty acids, especially omega-3, 6 and 9 increase chemotherapy effectiveness by triggering the activation of apoptosis (16, 17, 32).

This study was designed to evaluate the effect of oral administration of omega 3, 6 and 9 on apoptosis regulatory proteins in gastric cancer tissue in patients with gastric cancer. Apoptosis regulatory proteins, Bcl-2 protein family members, are groups of regulatory proteins engaged in apoptosis. According to the structural and functional criteria, these proteins can be classified in two independent groups: anti-apoptotic proteins, including Al/ Bfll, Bcl-2, Bcl-w, Bcl-$_{XL,}$ Boo/ Diva, Mcl, NR-13 and NRF3, and pro-apoptotic proteins including Bad, Bak m Bad, NiP3, BIK, Bim, Rambo-Bik, Bid, Bcl-xs, Bcl in mammals. These proteins are initially located in the cytoplasm of cells, and then move to the outer membrane of the mitochondria when apoptosis is activated. Following the movement, the possible oligomerization changes make these proteins enter the outer mitochondria membrane (20).

Bcl-2 and Bcl-X_L anti-apoptotic proteins competitively bind to Bak and Bax, therefore its connection prevents the formation of membrane pores, and thereby stops cytochrome C leakage and cell death. Thus, this apoptotic pathway depends on the relative concentration of pro- and anti-apoptotic proteins. If the concentration of Bcl-2 and Bcl -$_{XL}$ anti-apoptotic proteins tend to be higher than that of Bak and Bax pro-apoptotic proteins, the collection of Bak and Bax is not formed, and cell death does not occur. Some facilitator proteins compete for Bak/ Bax ligand binding sites to Bcl-2/ Bcl-X_L on Bcl-2 or Bcl-X_L and trigger Bcl-2 and Bcl-X_L heterodimers degradation to create pores in the mitochondrial membrane. Other facilitator proteins directly bind to Bax/ Bak oligomers, providing a more active structure for these proteins. Bad, Bid, Bcl-$X_{L,}$ Bcl-2, Bax, Bak proteins are regarded as the most prominent examples of pro-apoptotic mitochondrial death pathway (33). Therefore, to detect the effects of PUFAs on the amount of regulatory proteins in apoptotic pathway, we chose four key proteins involved in this pathway: Bcl-2 and Bcl-X_L as anti-apoptotic proteins, and Bid and Bad as pro-apoptotic proteins, and consequently assessed them at both gene-level and active protein level. As can be seen in Figures 1 and 2, in the case group of patients with gastric cancer undergoing chemotherapy along with receiving PUFAs, the levels of protein and gene expression of Bcl-2 and Bcl-X_L anti-apoptotic proteins revealed a significant decrease in comparison with those of the control group of patients with gastric cancer who only received Cis-Platin. On the other hand, the levels of protein and Bid and Bad pro-apoptotic gene expression proteins increased significantly in the case group as compared to those of the control group. These results may indicate that PUFAs oral administration in combination with cis-platin can enhance apoptotic activity in gastric cancer patients, and also reduce drug resistance in them.

Omega-3, 6 and 9 unsaturated fatty acids are three main families of polyunsaturated fatty acids (PUFA) with double bonds, and as cellular components and the essential fatty acids have diverse bioactivities (34). Several studies suggest that PUFAs have cytotoxic effects on different types of cancer cells and may be used as supplements with current chemotherapy drugs. Hyde and et al. (2009) (35) suggested that PUFAs can activate

apoptosis in tumor cells by their ability to convert sphingomyelin to ceramide which induces the release of pre-apoptotic proteins, corresponding with the results of the present research. Moreover, Comba et al. (2009) (36) indicated that PUFAs can be converted to eicosanoids by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450). These compounds are counted as the very potent biological compounds which play a very important role in preventing the growth of cancer cells. Thus, the results of this study may be justified by the results of Comba et al..

Yin Y et al. (2007) (37) in studies on gastric cancer cell line have revealed that omega-3 fatty acids may inhibit cancer growth and induce apoptosis in these cells, confirming the results of this study. In the studies carried out by Jakobsen et al. (2008) (38) and Slagsvold et al. (2009-2010) (39) on human colon cancer cells, it was found that PUFAs are engaged in the expression of several genes involved in cell cycle regulation and apoptosis, especially in the creation of reticulum endoplasmic which is one of the goals of chemotherapy in cancer treatment to overcome drug resistance.

However, the results of a number of studies prove that PUFAs can present their anti-cancer effects through different mechanisms among which suppression of neoplastic transformation, cell cycle inhibition, and induction of apoptosis and also anti-angiogenesis effects have been reported in the studies conducted by Bartram et al. (1993) (40), Shirota et al. (2005) (41) and Dekoj et al. (2007) (42). Some studies have also published that the accumulation of PUFAs is accompanied by increasing lipid peroxidation and the formation of lipid hydroperoxides, and other degradation products of fat in cells that may be harmful to cancer cells (43).

Furthermore, an important feature attributed to our study was that it benefited from a clinical trial in which the anticancer effects of PUFAs have been directly reviewed on human samples. Another prominent feature of this project was to examine the changes of apoptotic regulatory proteins (Bcl-2, Bcl-$_{XL}$, Bid and Bad) at both gene expression level and protein expression level which largely represent these changes.

Conclusion

In line with the results of the present study, it can be concluded that likely use of PUFAs as supplement with platinum drugs may be useful to stimulate more apoptosis in gastric cancer cells. Consequently, this offers an effective treatment to patients with gastric cancer to respond to chemotherapy.

Acknowledgement

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