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Investigating the Exposition Rate of Regulatory Genes of Cell Cycle Phase G2/M in Patients With Stomach Adenocarcinoma by the Meal Prescription of Polyunsaturated Fatty Acids Before and After Chemotherapy

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Abstract

Objectives: Cancer is a hyperactive disorder which can cause uncontrolled propagation of the cells. Several reports indicated that omega polyunsaturated fatty acids can prove their own antitumor effects on different cancerous cells by stopping the operation of the cellular cycle. Therefore, this study mainly aimed to investigate the impact of omega polyunsaturated fatty acids (PUFAs) on the exposition rate of genes CDK1, CyclinB1, and the cellular cycle of the stomach cancerous tissue in under-chemotherapy patients with stomach adenocarcinoma.

Materials and Methods: The present study was a double-blind clinical trial in which neither the oncologist nor the patients were aware of the treatment before and after the intervention. The patients with stomach adenocarcinoma were first recognized and subjected to chemotherapy. A total of 24 patients were randomly selected and included in the case and control groups. The patients in the control group were treated with cisplatin and placebo while those of the case group first received cisplatin with the complementary capsule of fatty acids, namely, Natural Factors Ultimate Omega Factors with a dose of 1200 mg and 3600 mg daily, encompassing three 1200 mg medicines each course for 3 weeks. Three samples of the stomach biopsy were taken from all patients before and after the chemotherapy. All mRNA tissues were extracted out of the biopsy samples. Then, DNA was synthesized based on these samples and the exposition rate of the desired genes was measured using the real-time polymerase chain reaction (PCR) method.

Results: There was a significant decrease in the mean of exposition of the genes in the case group ($P = 0.021$) compared to the control group ($P = 0.001$).

Conclusions: Generally, the results revealed that using omega fatty acids 3, 6, and 9 along with cisplatin medicine can be effective in stopping the cell cycle phase G2 in cancerous cells of the stomach tissue.

Keywords: Stomach adenocarcinoma, PUFAs, G2/M phase of the cellular cycle, Cyclin, cyclin B1, CDK1

Introduction

Cancer is a hyperactive disorder which includes the evolution of morphological cells, disorder in regulating the apoptosis, as well as, uncontrolled cellular propagation, aggression, angiogenesis, and metastasis (1). In addition, cancer can originate from each part. Prognosis and precaution for survival in individuals with cancer depend on the location from which cancer is rooted, as well as the clinical symptoms, and treatment choices of patient status. Further, stomach adenocarcinoma is the second most common cancer in the world and its prevalence varies in different countries. This kind of cancer is relatively rare in North America, however, a large number of deaths are reported from cancer (2-5). According to recent reports in North America, gastric cancer after colorectal and pancreatic cancers is the third reported malignant disease and the third malignancy which leads to death (4). Despite a decline in its global incidence, stomach adenocarcinoma accounts for 3% to 10% of the total cancer deaths. In countries like Japan, the survival rate for gastric cancer steadily improved while no improvement was observed in North America. Although advances were made in surgery and chemotherapy, there is significant mortality related to gastric cancer. Ninety percent of the stomach cancer cases are malignant and stomach adenocarcinoma includes 95% of all these cancers. Unfortunately, the total survival rate

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of 5 years in patients with gastric adenocarcinoma is only 10% to 30% (8-10).

Generally, 3 methods of surgery, chemotherapy, and radiotherapy are utilized to treat or alleviate the severity of this cancer out of which surgery is the most common way in this respect. However, it has some limitations since surgery is possible when the lesions are accessible to the surgeons and if cancer has not spread to other organs. This procedure is successful in decreasing 40% of the cancer cases and in its more advanced cases, it is just considered as a supportive treatment (9,10). Chemotherapy implies using medicine to kill the cancerous cells. These medicines are often prescribed in venous or in the meal form. Based on the studies, resistance to the medicines is observed in the area of all effective and new medicines. Hence, the ability to predict and overcome the medicine resistance may be helpful in improving the chemotherapy (11-13).

Furthermore, one of the goals of chemotherapy in controlling the cancers is ceasing the cell cycle. Cell cycle assures copying the chromosomes and cellular division. Wasting cell cycle regulation normally results in inactivating the apoptosis and eliminating the uncommon cells from the body. However, cells are altered to the cancerous types by overcoming the control mechanisms in some cases (14). Control mechanisms exist in the cyclin-CDK cell cycle. Therefore, it is thought that changing the exposition level of the cyclin-CDK complexes leads to some malformation of the cellular division such as cell proliferation in malignancies. Moreover, several reports demonstrated that the polyunsaturated fatty acids (PUFAs) can prove their own anticancer effects on various cancerous cells by ceasing the cell cycle (15,16).

Necessary PUFAs are the major biological compounds with structural and functional roles in natural and malign cells (17). PUFAs play a vital role in all levels of organisms. Additionally, researchers indicate that PUFAs compounds, especially the ratio of n-3 PUFAs to n-6 PUFAs in diets are more important (11). Omega-3 unsaturated fatty acids refer to high chain fatty acids which their first double bond in carbon number 3 is the methyl end (18). Typical types of omega-3 unsaturated fatty acids include alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). ALA naturally occurs in the vegetable oils while DHA and EPA are mainly found in fatty fish such as salmon, sturgeon, and sardines (19). In addition, ALA should be fed from the diet since is not synthesized in humans while DHA and EPA are synthesized from the ALA (20). Various studies reported that n-3 PUFAs are needed for the normal functioning of the growing tissues, natural maturity, and a wide range of physiological processes (21).

The n-6 PUFAs are essential for normal growth, development, and essential health. Thus, one should be very careful before deciding on whether or not it is harmful. The need for n-6 PUFAs refers to their physiological function in cellular membranes, eicosanoid production, and regulating cholesterol metabolism. The results of various studies indicate that high intake of ALA may increase the insulin resistance and hyperinsulinemia. Further, it is a substrate for lipid peroxidation and the formation of free radicals. Accordingly, diets that contain high levels of n-6 PUFAs have several serious side effects in the long term such as hyperinsulinemia, atherosclerosis, and carcinogenesis (22).

Furthermore, PUFAs among n-3 PUFAs categories, especially EPA and DHA have prohibitive effects on metastasis and cancerous cell proliferation (23,24). Moreover, the anti-inflation and anticancer effects of n-3 PUFAs were experimentally studied using in vitro systems (25,26). Additionally, it was observed that using omega fatty acids, especially DHA and EPA fatty acids resulted in increasing the vulnerability to chemotherapy and radiotherapy and decreasing their side effects. In addition, it may reduce the rate of metastasis of the cancerous cells (27,28).

Therefore, the present study mainly aimed at investigating the effects of prescribing omega 3, 6, and 9 unsaturated fatty acids with cisplatin on the exposition rate of the cyclin B1 and CDK1 in patients with stomach adenocarcinoma in order to examine the effect of omega fatty acids on ceasing the cell cycle.

Materials and Methods

Type of the Study and Sample Size

This study was a double-blind clinical trial in which neither the ecologist nor the patient was aware of the treatment. Accordingly, possible errors were inhibited. Twenty-four people out of all those patients with the probable diagnosis of stomach cancer who referred to endoscopy clinic of the Medical Sciences University of Tabriz were selected and categorized into 2 groups of 12 people based on the goals and conditions of the present thesis. The following formula was used to compute the sample size regarding the study type (29):

$N \geq S2\ (\Sigma\alpha/2+\Sigma\beta/\delta2)$

where the attained average for the exposition rate of the cyclin-B1 was considered given the reliability coefficient of 95% and the power of 85% and in accord with previous studies.

Sampling Method

Following certain diagnosis of the stomach cancer, 3 biopsy samples were taken from the stomach cancer tissue, and then transferred to the nitrogen tank. Then, these patients were referred to the oncologist and their chemotherapy initiated in 2 selected groups. The treatment was performed by the cisplatin with placebo in the control group while in the case group it was implemented by the cisplatin along with the complementary capsule of fatty acids, namely, Natural Factors Ultimate Omega Factors with a dose of 1200 mg made by the America and 3600 mg daily including three 1200 mg medicines

for 3 courses each lasting 3 weeks (30). Next, along with treatment compulsory follow-up, endoscopy was again implemented on these people and tumor treatment was persecuted by removing the stomach biopsy. Finally, the obtained samples from patients were again transferred to the nitrogen tank.

Determining the pattern of fatty acids present in omega 3, 6, and 9 capsules was prescribed to the patients to assure the accurate percentage rate of the fatty acids in the capsules used in the present study. The pattern of fatty acids present in these capsules was determined by a gas chromatography device made by Buck Corporation, America compared to the relative standards. Figure 1 illustrates the chromatogram related to the fatty acids in the soft-gel capsule. Furthermore, Table 1 represents the percentage of PUFAs in natural factors ultimate-omega 3, 6, and 9.

Quality assessment of the extracted RNA was used for extracting the total RNA from all the biopsy specimens. Then, the concentration and quality of the extracted RNA were examined by using NanoDrop ND-1000 spectrophotometer and agarose gel electrophoresis, respectively. The 28s, 18s, and 5s bands were observed to control the integrity of the total extracted RNA. To this end, 1.5% (W/v) nucleotide-agarose gel was prepared along with the agarose gel stain. Then, RNA of the interest was loaded on the gel. Finally, as Figure 2A displays, separation of RNA of interest was observed by the electrophoresis (13). After performing the gradient polymerase chain reaction (PCR), the temperature of the gene of interest was found (i.e., 59°C for the GAPDH, 59°C for the cyclin B1, and 59°C for the CDK1). Then, 2% agarose gel was prepared for evaluating the PCR products. Finally, sharp bands including 70, 119, and 13 bp were observed under the UV light for the CDK1, GAPDH, and cyclin B1, respectively (Figure 2B).

The tissue made by the EURx Corporation in Poland was employed to extract the RNA according to the RNA extraction kit protocol. The first strand synthesis hyper script produced by the commercial Gene All company was applied to manufacture the cDNA. The primers for cyclin B1 and CDK1 were designed and blasted. Eventually, the GAPDH gene was utilized as an internal control and relative measurement of the mRNA exposition was calculated by $2^{-\Delta\Delta\Delta ct}$.

Designing and Selecting the Primer

In the above-mentioned cases, the understudied gene primers were designed by primer 3 software and then checked by Oligo7 software. They were tested in terms of proprietary evolution in the NCBI/primer blast. Then, the designed sequences were sent to Sinaclon Company for the synthesis (Table 2).

In this research, the primers synthesized by Sinaclon Company was used to do the real-time PCR. The diagnosis compound of the SYBR-Green was also used.

The average of the results was computed by the SPSS software, version 22 in each group since it was

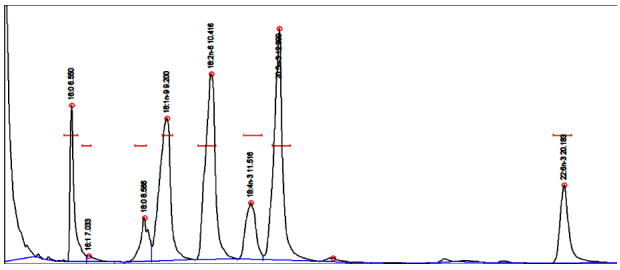

Figure 1. Chromatogram of the Fatty Acids in the Soft-Gel Capsule.

Table 1. The Percentage of Polyunsaturated Fatty Acids in Natural Factors Ultimate-Omega 3, 6, and 9

Unsaturated Fatty Acids	% Of Total
16:0	8.35
16:1n-7	0.65
18:0	4.49
18:1n-9	20.61
18:2n-6	23.88
18:4n-3	7.37
20:5n-3	25.70
22:6n-3	8.95

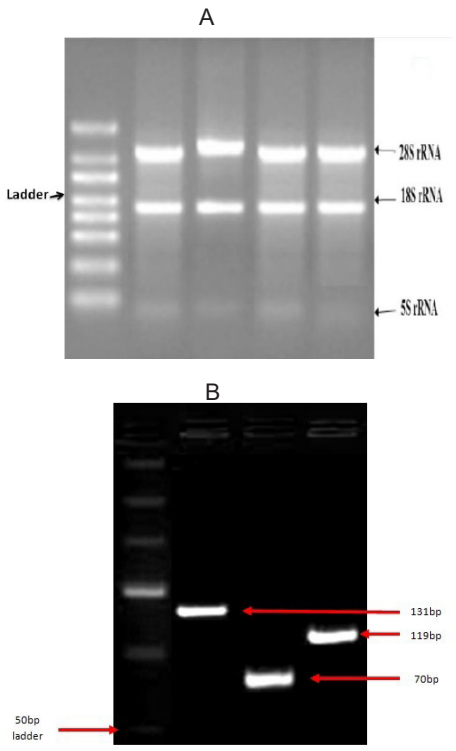

Figure 2. (A) 18s and 28s electrophoresed bands 1 of the RNA samples; (B) Sharp bands for the GAPDH, cyclin B1, and CDK1 (Gradient PCR, TM = 60˚C, 58˚C, & 58˚C).

Table 2. Information of Cyclin B1, CDK1, and GAPDH Primers

Primer	Sequence (5′→3′)	NCBI Ref. Sequence
CDK-1		
Forward Revers	ATGGAAACCAGGAAGCCTAGC TTTCGAGAGCAAATCCAAGCC	NM_001320918.1
Cyclin-B1		
Forward Revers	TATGCAGCACCTGGCTAAGA CCAGGCATTTGCAGGCATTAAG	NC_000005.10
GAPDH		
Forward Revers	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	NC_000012.12

independent of the groups under investigation. Moreover, the normality distribution of the results was then studied by the Shapiro-Wilks test. Next, the results of normal and non-normal distributions were compared between the two groups by independent sample *t* test and Mann-Whitney nonparametric test.

Results

Comparing the Demographic Data on Patients in the Groups Under Investigation

Demographic data of the patients in both groups are provided in Table 1. These data were statistically compared to each other. Based on the table, the study groups were well equalized in terms of the age, gender, tumor spot, tumor degree, and other information with each other (Table 3).

Comparing the Exposition Rate of the Genes (Cyclin B1 CDK1) in the Study Groups

Exposition rates of the cyclin B1 gene in both study groups were compared. Based on the results, the exposition rate of the cyclin B1 gene which was performed following the chemotherapy with the complementary omega fatty acids in case group demonstrated a significant decrease compared to the control group including the patients with chemotherapy without receiving the complementary fatty acids. Using Mann-Whitney test, it was found that the

Table 3. Demographic Information of the Patients

Clinical and Pathologic Factors	Groups		*P* Value
	Control (n=12)	Case (n=12)	
Age (y) (Means± SD)	67.5±11.21	71.25±9.81	0.235
Gender			0.695
Male (n=15)	7	8	
Female (n=9)	5	4	
Tumor size			0.759
<4 cm (n=11)	4	7	
>4 cm (n=13)	8	5	
Tumor primary location			
Upper (n=7)	3	4	0.714
Median (n=11)	5	6	0.790
Lower (n=6)	4	2	0.452
Stage classification of malignant tumors (TNM)			0.089
I (n=4)	2	2	
II (n=7)	4	3	
II (n=5)	2	3	
IV (n=8)	4	4	
Systolic blood pressure (mm Hg)	131.1±9.2	128.8±10.2	0.235
Diastolic blood pressure (mm Hg)	85.1±7.1	79.2±7.9	0.985
Cigarette smoking			
Current Smoking (n=10)	5	5	1
Non-Smoker (n=7)	4	3	0.714
Ex-Smoker (n=7)	3	4	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			0.73
Yes (n=13)	7	6	
No (n=11)	5	6	

mean of exposition rates for the cyclin B1 gene was equal to ($2^{-\Delta\Delta\Delta ct}$) 3.99 and ($2^{-\Delta\Delta\Delta ct}$) 0.53 in the case and in control groups, respectively. The *P* value was 0.024. As Figures 3A and B depict, the minimum and maximum responses were equal to ($2^{-\Delta\Delta\Delta ct}$) 3.63 and ($2^{-\Delta\Delta\Delta ct}$) 0.5 in the case group while in the control group, these responses were ($2^{-\Delta\Delta\Delta ct}$) 0.5 and 1.73, respectively. The test coefficient was 2.3. Additionally, the exposition rates of the CDk1 gene were compared in both study groups. As the results indicate, such rate demonstrated a decline in the case group after chemotherapy with complimentary omega fatty acids compared to the control group containing patients with chemotherapy without receiving any complementary. Employing the Mann-Whitney test, the mean of exposition rates for CDK1 gene was equal to ($2^{-\Delta\Delta\Delta ct}$) 0.58 and ($2^{-\Delta\Delta\Delta ct}$) 0.7, in the case and control groups, respectively. The *P* value was 0.001. Finally, as Figures 4A and B illustrate, the minimum and maximum responses are ($2^{-\Delta\Delta\Delta ct}$) 0.7 and ($2^{-\Delta\Delta\Delta ct}$) 3.97, as well as ($2^{-\Delta\Delta\Delta ct}$) 1.14 and 14.35 for the case and control groups, respectively. Test coefficient was 13.

Discussion

The n-3 and n-6 PUFAs are regarded as the necessary cellular components with various biological properties. In addition, the n-3 PUFAs are mostly found in the seafood. Further, as regards the human health, n-3 PUFAs have more considerable effects including repressing cancer, prohibiting the cardiovascular diseases, and improving intellectual ability (31,32). Researchers demonstrated that understudied fatty acids, especially N-3 PUFAs may play

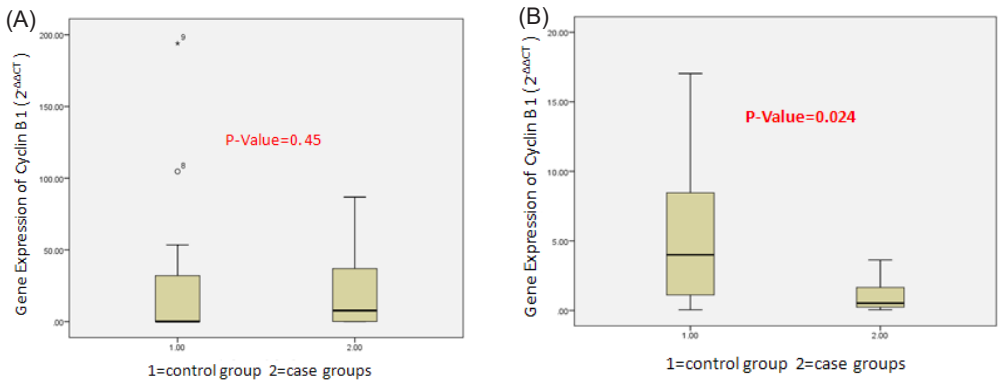

Figure 3. (A) The Expression of Cyclin B1 Genes in the Case and Control Groups Before the Chemotrophy. The median of the exposition rates of cyclin B1 gene was equal to 7.72 and 0.07 in the case and control groups, respectively and the *P*-value was 0.47. In addition, the minimum response was 0.00 while the maximum response was equal to 86.82 in the case group. However, the minimum and maximum responses were equal to 0.00 and 194.01 in the control group and (B) the median of the exposition rates of cyclin B1 gene were 3.99 and 0.53 in the case and control groups after the chemotrophy, respectively and the *P*-value was 0.024. Further, the minimum response was equal to 3.63 while the maximum response was 0.5 in the case group. Furthermore, the minimum and maximum responses were equal to 0.5 and 1.73 in the control group, respectively.

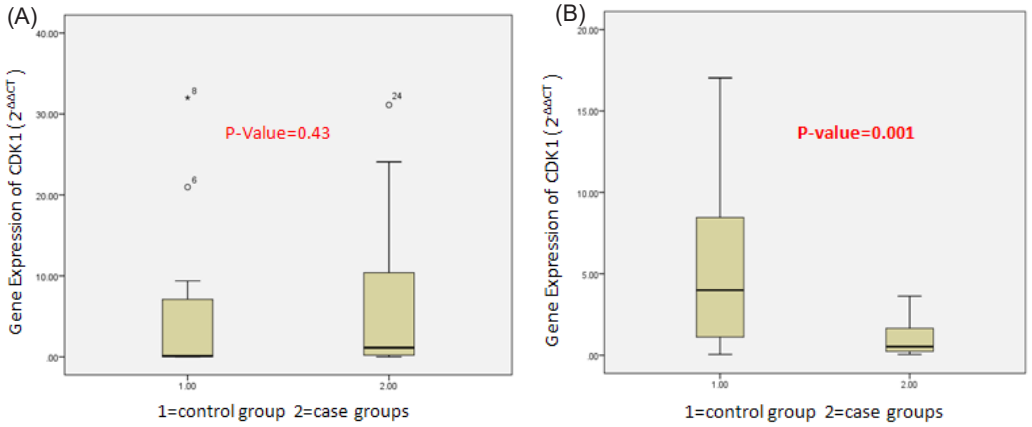

Figure 4. (A) The expression of CDK1 genes in case and control groups before chemotrophy. The median of the exposition rates of CDK1 gene was equal to 1.13 and 0.1 in the case and control groups before the chemotrophy, respectively and P-value was 0.44. Moreover, the minimum and maximum response of the case group were 0.00 and 31 while those of the control group was equal to 0.00 and 32. (B) The median of the exposition rates of CDK1 gene was equal to 0.58 and 2.77 in the case and control groups after the chemotrophy, respectively and P-value was 0.001. Finally, the minimum and maximum response of the case group were 0.7 and 3.97 whereas those of the control group were 1.14 and 14.35, respectively.

a major role in altering cancer (33). Furthermore, many other reports imply that great consumption of these fatty acids is correlated with decreasing the danger of catching stomach cancer in animal and human being models. Specifically, recent studies indicate that n-3 PUFAs lead to a delay in the progression of the colon and stomach cancers. Moreover, their decrease may be the main strategy for stomach cancer prevention in patients with a high risk by various procedures of cancer prevention using the n-3 PUFA. Additionally, using the n-3 PUFA along with other factors (medicines) with antitumor complementary can improve its effectiveness in cancer prevention (18). In addition, n-6 PUFAs is highly abundant in the daily diet. Some studies demonstrated that excessive consumption of 5 of n-6 PUFAs may be dangerous and increase the risk of suffering from breast, prostate, and colon cancers (34-36). However, evidence indicates that some n-6 PUFAs include anticancer effects (31). Research by the Kian group demonstrates that n-6 PUFAs exert their own anticancer effects by affecting the exposition rate of the genes and proteins. Accordingly, they may prevent cell cycle progression and create apoptosis. For example, in rat's carcinoma cells (LLC-WRC256), gamma-linolenic acid caused the dispersion of cytochrome C in relation to mitochondria metabolism variation and increased the activity of caspase 3. Therefore, it caused cell apoptosis (37). Several researchers reported that free radical derivatives of dihomo-γ-linolenic acid may halt the cell growth and cycle and apoptosis in cancerous cells of the human colon cancer from the category 29HCA-7 (38).

Nassar et al (39), Celik et al (40), and Liang et al (41) attributed the toxic effect on the cancer cells to the omega-3 and omega-6 fatty acids which induce oxidative stress and reduce the growth of cancer cells. The result of the studies and the mechanisms mentioned above were mostly reported in terms of cell culture media and on laboratory animals. As a result, the presented data provide no precise explanation regarding the mechanism of anti-cancer effects of the omega fatty acids and thus cannot be generalized to clinical samples and to the present research findings. Therefore, to the best of our knowledge, the current study is the first one to investigate the inhibition cell cycle in gastric adenocarcinoma of the omega fatty acids into the human samples. The study by Dolatkhah et al (13) implies the increase of apoptosis rate through mitochondria route due to the consumption of omega 3, 6, and 9 fatty acids. Further, a number of studies on the anticancer effects of the peroxisome proliferator-activated receptor (PPARY) ligands in several colon cancer cells represent that these ligands affect inducing the apoptosis, ceasing the cell cycle, and altering the growth and proliferation (42). Hosseinzadeh et al (43) reported that the exposition of PPARY mRNA increases considerably in the patients with cisplatin treatment using the PUFA complementary foods. Therefore, the cell cycle can be stopped increasing the apoptosis rate in the cancerous cells. In another study by Sato et al (44), it was revealed that inducing the apoptosis which ceases the cell cycle in G1 phase may be one of the mechanisms with anticoagulation effects to activate the PPARY by omega unsaturated fatty acids in humans' cancerous cells. It is partly consistent with the results obtained in the present study. As displayed in diagrams 1 and 2, PUFAs could totally halt the cyclin B1 and CDK1 genes in the case group compared to those in the control group. In the following section, this issue is explained in details.

The cyclin B1 is a member of great cyclin family and has high importance since it is believed that cyclin B1 can control the examination protocol of the G2/M phase and regulate the accurate initiation of the mitosis necessary for the DNA synthesis and cellular propagation. The obtained evidence represented that cyclin B1 is greatly observed in breast cancer, swallow squared cell carcinoma, as well as, lung and stomach cancers and other cancerous cells (45-49). Modification of this cyclin in G2 phase led to the prevention of M-phase progress in the cell cycle, stopping the cell growth and differentiation, apoptosis, and metastasis in various kinds of cancers. So far, increasing the exposition of cyclin B1 has been emphasized in the breast, prostate, swallow, lung, colon, and stomach cancers (50-53). For instance, Chae et al (54) reported that the exposition of cyclin B1 has no effect on the survival of patients with breast cancer. Furthermore, Bjorck et al (55) highlighted that patients with follicle lymphoma who are exposed to high level of cyclin B1 following chemotherapy demonstrate better results compared to patients with low cyclin B1 exposition. Moreover, Yifeng et al (56), among others, declared that cyclin B1 was heavily exposed. Additionally, cyclin B1 may cause cellular propagation and tumor growth by advancing the cellular cells and decreasing the apoptosis in colorectal cancerous cells.

Using the MHCC97L cell of human hepatocarcinoma cells, Lee et al (16) found that DHA could stop cell cycle through halting DNA synthesis and inducing the apoptosis. In addition, the number of cells in the G2/M phase in undertreatment cells with DHA was lower than that of the control cells. These results are in line with those of the present study since reducing the exposition rates of CDK1 and cyclin B1 genes inhibited the formation of CDK1-Cyclin B1 complex and increased the period of cell stopping in G2 phase. Similarly, Aurelia Barascu et al (17) concluded that docosahexaenoic (DH) and eicosapentaenoic acid (EP) increased the length of the G2/M phase of the cell cycle. Therefore, they may halt cell cycle and cellular propagation in the cancerous cells. This is in conformity with the results obtained in the current study. Evaluating the regulatory proteins of the G2/M phase represented that cyclin B1-CDK1 which are the key factors in cell entrance to the mitosis phase are simultaneously restrained. Halting the G2/M progression due to DHA and EPA demonstrates that these fatty acids can affect G2 or M-phase regulators. Further, it is implied

that EPA and DHA can reduce the phosphorylation state of the cyclin B1 and exposition of the CDC25. Finally, they may result in halting the activity of CSK-cyclin B1, therefore increasing the length of the G2 phase.

Conclusions

Generally speaking, based on the results of the present study, using PUFAs with cisplatin medicine in patients with stomach adenocarcinoma can have a role in stopping the G2/M phase of the cell cycle in stomach cancerous cells. This is regarded as a promising finding for controlling the cancerous cells in stomach adenocarcinoma.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

This study was approved by Ethics Committee of Tabriz University of Medical Sciences (ethical code of IR.TBZMED.REC.1395.677) and registered in the Iranian Registry of Clinical Trials website (identifier: IRCT2016121216922N2).

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