Polyunsaturated fatty acids (PUFAs) as Potential Anticarcinogenic Agents Against U937 Non-Hodgkin's Lymphoma Cancer Cells

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Introduction

Non-Hodgkin's Lymphoma (NHL) is one of the most common cancers in the United States. American Cancer Society predicts that approximately 80,550 people will be diagnosed with Non-Hodgkin's Lymphoma, 20,180 of which would result in death, accounting for 3.3% of all death in 2023, making it the 9th deadliest cancer in the US. Treatments for NHL include chemotherapy, radiation, and immunotherapy, etc. These therapies are effective, but cannot provide complete cure, and can sometimes pose serious side effects ranging from hair loss to mental illnesses. Therefore, more effective, and safer alternative treatments for NHL are still an unmet medical need for the cancer patients.

Long-chain (LC) n-3 polyunsaturated fatty acids (n-3 PUFAs), also referred to as omega-3 LCPUFA, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), as well as conjugated linoleic acid (CLA), have been extensively studied for their effects in human nutrition and health. CLAs are found in meat and dairy products as well as nuts and seeds[1], while EPAs and DHAs can be found in fish and marine products. CLA, EPA and DHA have been marketed as dietary supplements due to their health benefit in anti-cardiac, anti-obesogenic effects in adults as well as in children [2]. More importantly, CLA has been shown to regulate cell growth and survival in different leukemia cell types [3]. The EPA and DHA intake from fish oil supplements has been investigated and shown to be associated with reduced risk of additional breast cancer events and all-cause mortality [4]. Zorica Cvetkovic, et al showed in their study that serum levels of n-3 PUFAs such as EPA and DHA were significantly lower in newly diagnosed, untreated clinical stage IV NHL patients compared to healthy subjects, indicating the PUFAs benefits in preventing NHL [5]. The precise mechanism of these health benefits and anti-

cancer effects of PUFAs were not well documented. The current study aims to examine the anti-cancer effects of these PUFA compounds on the NHL cell functions.

Materials and Methods

Materials

U937 Lymphoma cell line (Sigma-Aldrich) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 37ºC incubator (NAPCO). CLA, EPA, and DHA (Sigma-Aldrich) solutions were prepared at different concentrations as needed. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) purchased from Fisher Scientific was used for MTT assay. Chemokine (C-X-C motif) ligand 10 (CXCL10) purchased from R&D Systems was used for CXCL10 ELISA assay. Phorbol-12-Myristate-13-Acetate (TPA) purchased from Sigma-Aldrich.

Methods

Cell Adhesion Assay:

U937 lymphoma cells were placed in a 24-well plate with these PUFAs and Phorbol-12-Myristate-13-Acetate (TPA). After a 24-hour incubation period, 300 µL of a fixative solution was pipetted into each well, and the wells were rinsed thoroughly to remove excess waste material. An OMAX 40X-2000X Digital Binocular Biological Compound Microscope connected to a computer with ImageJ software was used to take pictures of the wells. The quantity of cells was measured using the ITCN cell counter software to examine the effects of PUFAs on the proliferation of the cancer cells.

Caspase Assay:

A six-well plate containing U937 cancer cells was prepared with control, and 10 µL solutions of CLA, EPA, and DHA. After 24-hours incubation, well contents were centrifuged and stored at

-20°C for 24-hours. The cells were lysed and transferred to a 96-well plate, where a 5-µL caspase substrate was added. The resulting solution was read using a microplate reader at 415-nm in 30-, 60-, and 80-minute intervals.

MTT Assay:

U937 cell cultures were treated with CLA, EPA, and DHA at concentrations of 1M, 0.1M, 0.01M, and 0.001M. After 24-hours of incubation, 10µL of MTT was added, the assay was further incubated for 3-hours. A Student's t-test was performed on the average of 16-well samples and controls.

CXCL10 Assay:

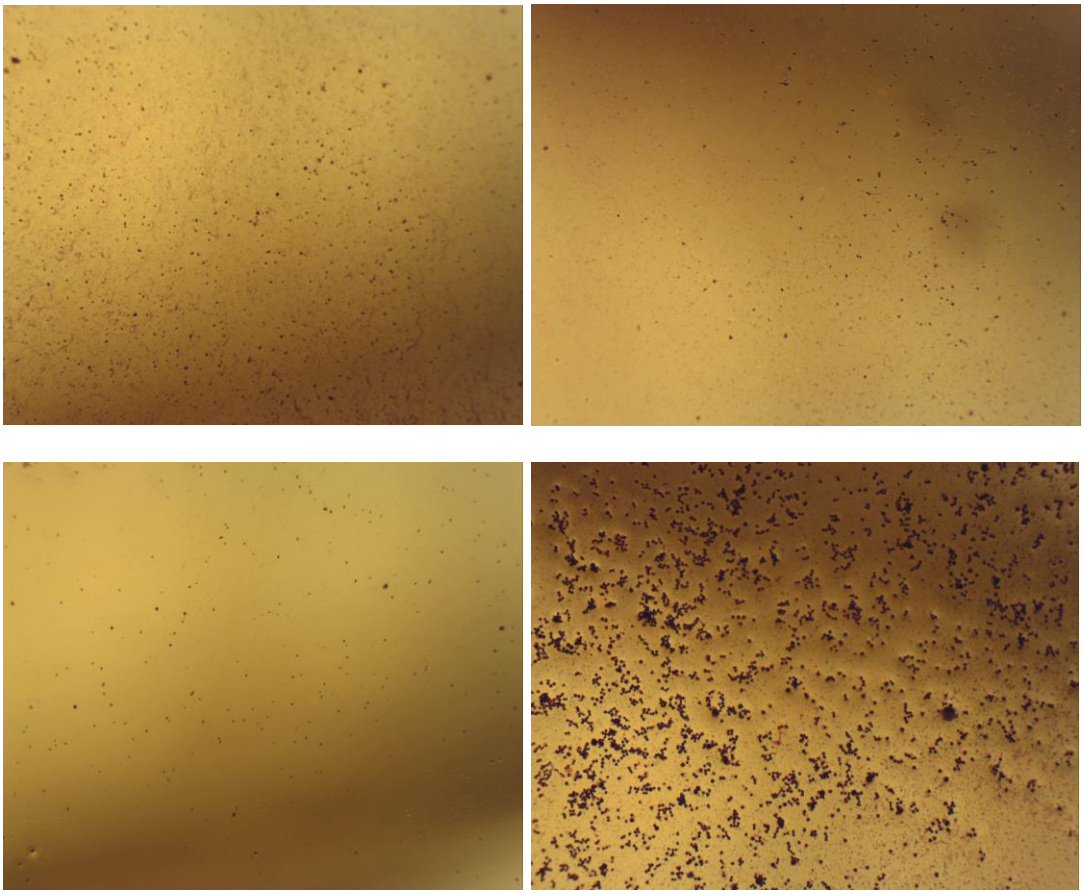
U937 NHL cells were incubated with control and 10 µL solutions of CLA, EPA, and DHA at 0.1 concentration for 24 hours. The resulting samples were centrifuged and the CXCL10 protein in the supernatant were measured by ELISA assay following the protocol supplied by manufacturer (R&D Systems, Minneapolis, MN). A Student's t-test was performed on the results of the 6 timepoints of the sample and controls.

Results

A cellular adhesion assay was conducted on U937 lymphoma cells to determine whether CLA, EPA, and DHA could inhibit TPA, a chemical known to support the proliferation of cancer cells. Greater adhesion leads to a greater spread of cancer cells. Consequently, the images shown in Figure 1 revealed that 0.1M solution of PUFAs were able to reduce cancer cell attachment compared with the control.

Figure 1

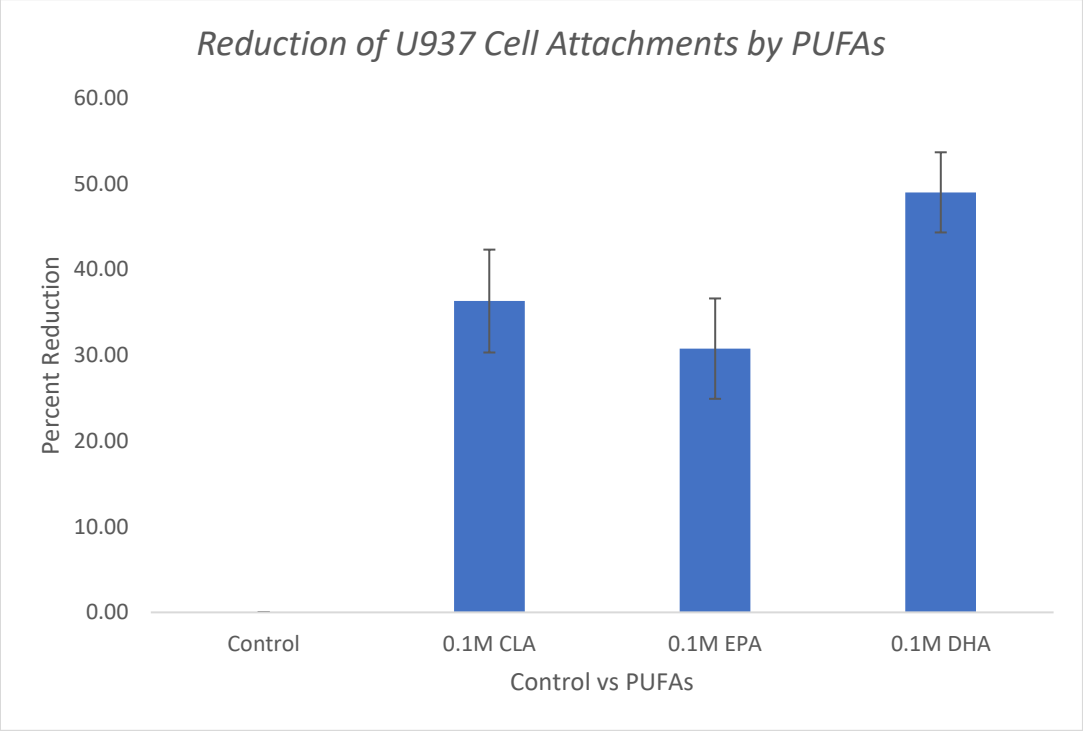
Images of Cell Adhesion to Sample Wells



Note. NHL U937 cells attachment image in the sample wells from top, left to right: 0.1M CLA, 0.1M EPA, from the bottom, left to right, 0.1 DHA and the control.

The ITCN cell counter results showed that when treated with 0.1M CLA, EPA, and DHA, the U937 cell attachments were reduced by 36.15%, 30.77%, and 49.00%, respectively, compared to the control (Figure 2).

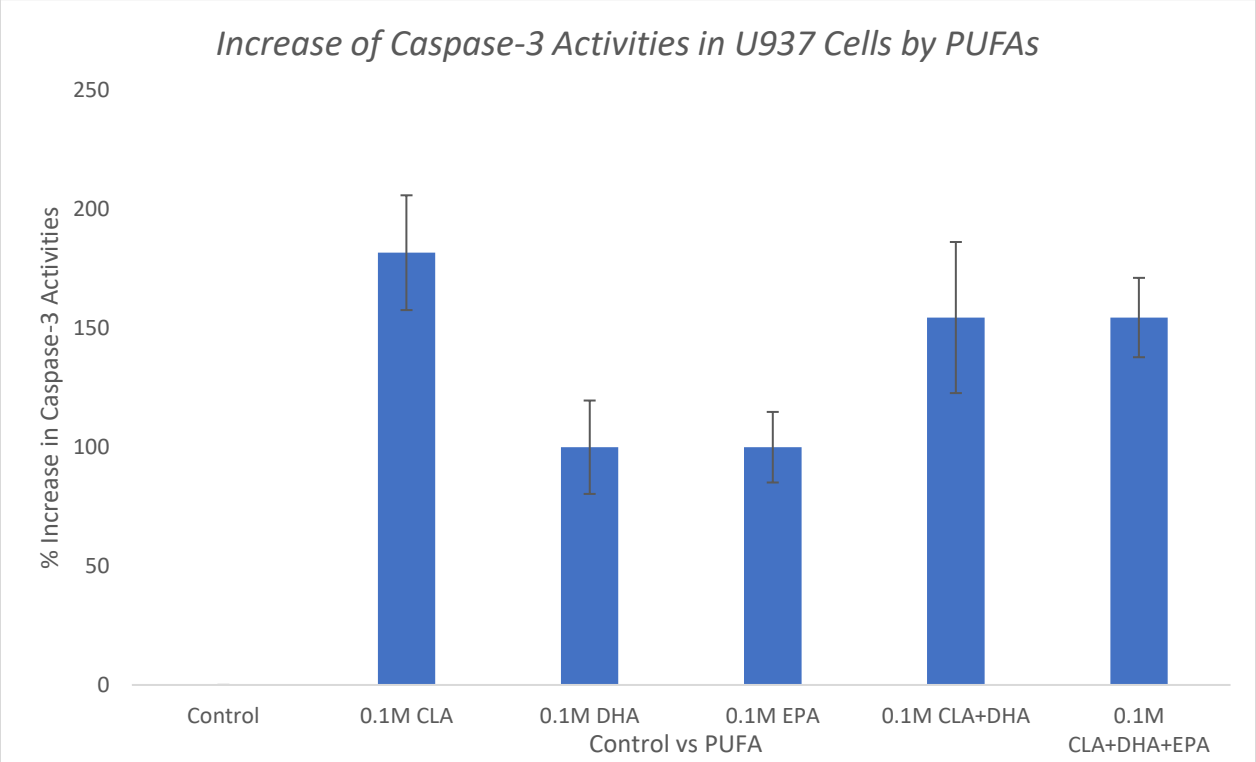
Figure 2



Note. NHL U937 cells attachments reduction by 0.1M CLA, EPA and DHA

To quantify effects of PUFAs on the proteolytic phase of apoptosis, caspase-3 activity was assessed in U937 NHL cancer cells. Caspase-3 is considered a key protease activated during early stages of apoptosis. Caspase-3 activity was increased in 0.1M CLA, 0.1M EPA, and 0.1M DHA treated NHL cells by 181.8%, 100.0%, and 100.0%, respectively, compared to the control (Figure 3). The combined PUFA solution of 0.1M CLA+DHA and 01.M CLA+DHA+EPA showed an increase of 154.5%, indicating that these combined PUFAs remain effective in activating Caspase-3, but do not have synergistic effects.

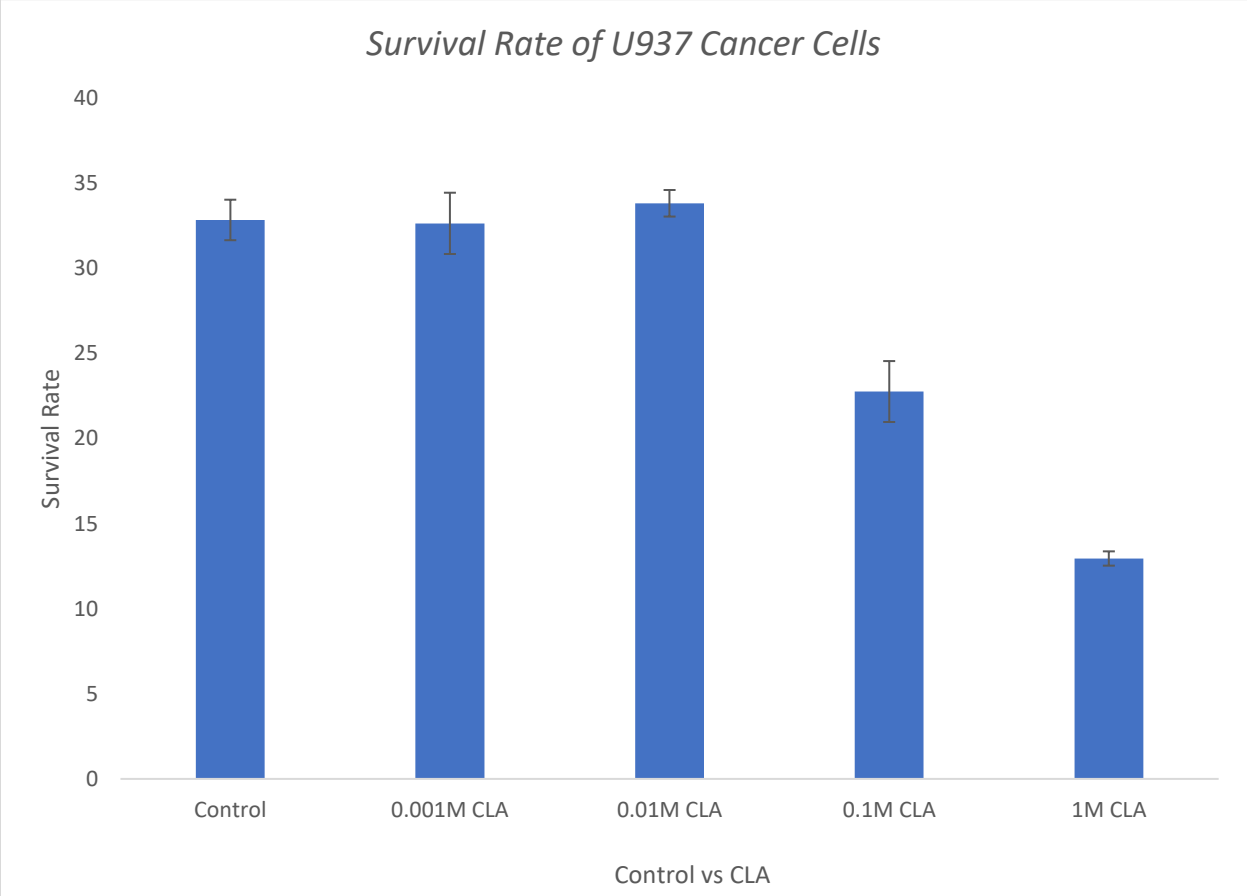
Figure 3.



Note. U937 lymphoma cell's caspase-3 activation with the addition of PUFAs.

In MTT assay, the survival rate of cancer cells was determined by analyzing the amount of purple formazan present. The average cell survival rates of U937 samples treated with CLA at 0.001M, 0.01M, 0.1M, and 1M concentrations were 32.64%, 33.82%, 22.76%, and 12.95%, respectively, with survival rate of 32.84% for the control (Figure 4). Student T-test performed for samples of 0.1M and 1M concentrations showed a significant p-value of <0.0001, compared to the control samples. While the p-value for 0.01M and 0.001M samples showed no significant difference with the control sample.

Figure 4.



Note. Survival rate of U937 cancer cells treated with CLA as measured in MTT assay.

EPA and DHA produced similar survival rates in U937 cancer cells (Table 1). This result indicated a dose dependent outcome of the PUFAs on the NHL cancer cell survival. The 0.1M and 1M samples exhibit the most significant anti-cancer effects, while the 0.001M and 0.01M dilution samples had no impact on the cancer survival.

Table 1.

MTT Assay Results

Samples	Average Survival Rate	% Decrease	T-test p-value
Control	0.3284		
0.001M CLA	0.3264	0.5995	0.9280
0.01M CLA	0.3382	-2.978	0.4962
0.1M CLA	0.2276	30.71	<0.0001
1M CLA	0.1295	60.57	<0.0001
0.01M EPA	0.3489	-6.252	0.2292
0.1M EPA	0.2376	27.64	<0.0001
0.01M DHA	0.3388	-3.150	0.7108
0.1M DHA	0.2723	17.10	0.0013

Note. This table shows the average survival rate of 16 samples over 24 hours incubation with PUFAs. P-value is calculated using student T-test.

The effect of PUFAs on CXCL10 protein stimulation in the U937 cell sample was determined through the ELISA assay. The CLA, EPA and DHA treated samples showed increase in average concentrations of CXCL10 over 30-, 60-, 80-, 120-, and 160-minuites intervals. The percentage increases of CXCL10 protein concentration in U937 cancer cells with 0.1M of CLA, EPA and DHA were 32.36%, 4.16% and 11.76%, respectively, compared to the control, with the 0.1M CLA exhibiting the statistically significant stimulating effect (Table 2). Combining CLA and DHA or CLA and DHA and EPA do not seem to have synergistic effect.

Table 2.

CXCL10 Protein Assay Results

Samples	Average	% Change	T-test p-value
Control	0.1885	0	
0.1M CLA	0.2495	32.36	0.0042
0.1M DHA	0.1963	4.156	0.6355
0.1M EPA	0.2107	11.76	0.2215
0.1M CLA+DHA	0.2097	11.23	0.1704
0.1M CLA+DHA+EPA	0.1972	4.598	0.5824

Note. This table demonstrates the concentrations of CXCL10 in U937 cell cultures over 5 time intervals in the presence of PUFAs. P-value is calculated using student T-test.

Discussion and Conclusions

This research project examined the anti-cancer properties of CLA, DHA, and EPA that are part of dietary long chain PUFAs. A cellular adhesion assay was performed to assess PUFA's ability to prevent cancer cells from attaching to the surface of the well plate. Cancer cells must first attach to physical surfaces inside the body to be able to metastasize and develop into tumors [6]. Results from the data shown in figures 1 and 2 indicate that PUFAs played a major role in inhibiting U937 cell attachments, which can prevent the growth of the cancer cells.

Caspase-3 is a cysteine–aspartic acid protease that is involved in the process of apoptosis. Once activated, caspase-3 will cleave key proteins in the cells and leads to cell death [7]. Our assay results show that the PUFAs at 0.1M concentration increased the caspase activities by at least 1-fold, with the CLA exhibiting the most activating effect. This enhanced caspase-3 activities demonstrates that PUFAs can induce cancer cell apoptosis and cell death through caspase activation.

MTT cell proliferation assay, which measured cellular metabolic activity after exposure to treatment solutions, is an effective method to determine cell survival rates. Based on a cell's ability to metabolize compounds, MTT is converted from a yellow tetrazole into a purple formazan. The amount of formazan was recorded using a microplate reader, from which cell survival rates were obtained. Using CLA at 0.001M, 0.01M, 0.1M, and 1M concentrations, the data revealed that only 32.64%, 33.82%, 22.76%, and 12.95% U937 lymphoma cells in these wells survived. In contrast, 32.84% U937 cells in control survived. Wells with the highest concentration of PUFA resulted in the least amounts of formazan, signaling that it had successfully lysed a majority of the cancer cells. These results confirmed that PUFAs possessed anticarcinogenic properties.

Existing research found that overexpression of CXCL10 protein in ovarian cancer cells can effectively enhance tumor suppression and apoptosis caused by T cells [8]. CLA, EPA and DHA at 0.1M concentration stimulated overexpression of CXCL10 in U937 cells by 32.36%, 4.16% and 11.76%, while 01.M of CLA+DHA, 01.M of CLA+DHA+EPA increased CXCL10 concentration by 11.23% and 4.598%. CLA at 0.1M showed the highest percentage increase in stimulating the overexpression of CXCL10, with a statistically significant p-value of 0.0042 calculated using the student T-test. This indicates that all PUFAs can potentially stimulate the CXCL10 expression in U937 NHL cells and facilitate the apoptosis of the cancer cells, although they do not seem to have synergistic effects when combined.

The current study revealed that long chain PUFAs could potentially act as inhibitors of cancerous cell growth, metastasis, and survival by disrupting the cancer cell attachments, activating caspase-3 protease during apoptosis, and/or stimulating the overexpression of CXCL10, thereby inducing cancer cell death. Much recent research on the dietary supplement values of these PUFAs, such as marine n-3 (also called omega-3) fatty acids intake did not result in a lower incidence than placebo of invasive cancer [9]. On the other hand, many in vitro and in vivo research have shown the benefits of these PUFAs against breast cancer [4] [10] and other types of cancers [11]. This study has proved the effectiveness of the n-3 long chain PUFAs to prevent NHL cancer cell growth. More importantly, the ultimate sources of the PUFAs are algae, which is naturally abundant and could be commercialized through biosynthesis as part of a healthy human dietary lipid consumption, in addition to fish oils [12]. The potential anti-cancer effects of PUFAs present an intriguing prospect of finding a cancer treatment with sufficient and sustainable natural supplies.

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