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Anti-inflammatory effects of n-3 polyunsaturated fatty acids in THP-1 macrophages: promising in-vitro insights

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ABSTRACT

Objectives: Uncontrolled inflammation is a one route to the pathogenesis and development of inflammatory diseases. The scientific literature has reported many evidences supporting the notion that polyunsaturated fatty acids (PUFAs) belonging to the family of n-3 including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have an anti-inflammatory function. Although much has been learned about EPA and DHA, so many questions remain unanswered, including the differential effects on health of DHA and EPA as well as the dose-response effect on clinical outcomes. The present study is aimed to investigate the effect of the PUFAs; EPA and DHA in the inflammatory responses in LPS-stimulated THP-1 macrophages.

Materials and Methods: Cells were incubated for 24 and 48 hours with EPA and DHA. Cell viability test were used to determine the viability of cells during and after incubation. Doses concentrations of 0.09 and 0.45 mM for both EPA and DHA were utilized to study the expression levels of inflammatory cytokines that were measured by ELISA test. All data were presented as SEM and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via one-way ANOVA test.

Results: Our study revealed interesting findings that are in a significant agreement to other studies in the literature. DHA illustrated a decrease on the levels of IL-6 in LPS-stimulated THP-1 cells treated with 0.09 mM, and a greater reduction with 0.45 mM DHA concentration ($P<0.001$). Moreover, DHA in our study, achieved no statistically significant difference in TNF-alpha inflammatory cytokines compared to cells alone ($P<0.001$). On the other hand, LPS-stimulated THP-1 cells, when subjected to EPA, it showed a significant decline in both IL-6 and TNF-alpha in the higher dose only and failed to express a statistically significant difference in 0.09 mM ($P<0.001$).

Conclusion: In conclusion, our data support the notion that PUFAs represented in EPA and DHA, are capable to reduce the expression of inflammatory cytokines. DHA stands out as a more potent anti-inflammatory agent which is a suggestive for a valuable marker to fight chronic diseases. Both in-vivo animals and human trials are urgently demanded to validate our current data.

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1. Introduction

Chen L and colleagues in 2018,[1] provided a detailed explanation on inflammation that can be defined as the host immune response to a foreign agent which could be a pathogen such as bacteria, viruses or fungi, or could be an external injury including scrapes or injury by foreign objects like a thorn, or might be chemicals and radiation. Inflammation is divided into acute and chronic inflammation. On the one hand, acute inflammation is characterized by high vascular permeability and blood flow along with a fluid accumulation, and release of inflammatory mediators such as cytokines and chemokines. Worth mentioning that pain, heat, loss of function, swelling and redness are the key signs of acute inflammation. On the other hand, a chronic inflammation may persist for months or years and it is characterized by humoral immune response which is mediated by the secretion of macromolecules like the production of antibodies, complement proteins, and particular antimicrobial peptides and the cellular immune responses to the pathogens which are present in the site of tissue injury. Additionally, a chronic inflammation can be due to a failure of preventing the agent causing an acute inflammation which could be infectious organisms including protozoa, fungi, Mycobacterium tuberculosis and parasites that can remain in the tissue for long time and resist host defenses. Not to mention the frequent episodes of acute inflammation, inflammatory and biochemical inducers which lead to oxidative stress and mitochondrial dysfunction.

Of significance, a chronic inflammation can result in different diseases for example cardiovascular disease (CVD), allergies, diabetes, rheumatoid arthritis, cancer, chronic obstructive pulmonary disease (COPD), and psoriasis. During both chronic and acute inflammatory processes, various soluble factors are participating in recruitment of leukocyte through increased expression of chemoattraction and cellular adhesion molecules. Some types of soluble mediators regulate the activation of the newly recruited inflammatory cells including lymphocytes, monocytes, eosinophils and eosinophils, and the resident cells (e.g., endothelial cells and tissue macrophages, fibroblasts and mast cells).[2]

The hall mark of an inflammation is characterized by an incline of the level of circulating cytokines. One of the function of cytokines to drive inflammation through interleukins, interferons, and TNF-α (tumor necrosis factor-alpha).[3] Additionally, there are two types of inflammatory cytokines; anti-inflammatory cytokines and pro-inflammatory cytokines. Pro-inflammatory cytokines take part in inflammatory reactions, for example tissues damaged by trauma, bacteria, or any other cause and normally produced by activated macrophages and are considered participants in the up-regulation of inflammatory reactions.[4] Anti-inflammatory cytokines control or regulate the pro- inflammatory cytokines response, thereby, inappropriate production or Overproduction of certain cytokines can progress and develop a disease. For instance, TNF-α, IL-6 (interleukin-6), and IL-1 (interleukin-1) produced in large quantity in rheumatoid arthritis (RA), where they are involved in tissue destruction and inflammation.[5]

Historically, n-3 PUFAs are considered to be anti-inflammatory, whereas n-6 PUFAs such as AA (arachidonic acid) and LA (linoleic acid) are considered proinflammatory.[6] Polyunsaturated fatty acids (PUFAs) contain more than one double bonds in their dynamic structures. The essential PUFAs are further subdivided into n-3 PUFAs and n-6 PUFAs. Needless to say, polyunsaturated fatty acids names are derived and inspired from their molecular structure[7] which described as follow: n-6 PUFAs family contains the first double-bond on the sixth carbon counting from the terminal methyl end of the fatty acid, while n-3 PUFAs family having the first double-bond on the third carbon counting from the terminal methyl end of the fatty acids. Long-chain derivatives of those essential polyunsaturated fatty acids (PUFAs), for example n-3 PUFAs eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) as well as n-6 PUFAs arachidonic acid (AA; C20:4) and γ-linolenic acid (GLA; C18:3) are considered very vital for different physiological functions at all stage of human life. Although they are manufactured in the body, they must be delivered from the food, hence they are called conditionally essential fatty acids.[8] The most predominant n-3 FA is ALA is found in very high quantities in flaxseed oil (approximately 55%) and in canola (roughly 10%) and nearly 7% in un hydrogenated soybean oil. A very small quantities of ingested ALA (1 - 5%) is converted into EPA, and even less than that amount is converted into the DHA. DHA and EPA are found in fish oil, which is extract from the gut and tissues of oily fish such as salmon, tuna, sardine, mackerel and herring.[8]

Hansolaf Bang and Jørn Dyerberg indicated a significant contribution for highlighting an earlier effort on the positive impacts of PUFAs in promoting a good health. They have studied the effects of diet in different populations including: Greenland, and Dane's residents where Greenland diets were characterized by a richer unsaturated fatty acid content, hence induced a health betterment measured by a reduction in pre-β-lipoproteins, cholesterol and triglycerides in plasma.[9,10] A thorough investigation of the scientific literature highlighted several relevant and positive evidences in favor of the anti- inflammatory feature possessed by n-3 PUFAs. In support of that, in an in-vivo study design, fish oil nutrition decreases the production of IL-1b, IL-6 and TNF-a by rodent

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macrophages.[11] In addition, a cell culture study showed that DHA and EPA can reduce the production of tumor necrosis factor (TNF)-a and IL-1b by monocytes, and the production of IL-8 and IL-6 by venous endothelial cells, where Docosahexaenoic and eicosapentaenoic acids produced anti-inflammatory eicosanoids that help resolve inflammation.[12] Moreover, according to in vitro studies, both DHA and EPA may affect the function of immune cells by reducing proinflammatory mediator production.[13] However, extensive research has been carried out on the effects of PUFAs in reducing inflammation, gaps still exist rendering better understanding.

Thus, it comes essential to further understand the potential anti-inflammatory effects of dietary poly unsaturated fatty acids (EPA and DHA) on THP-1 cell lines. Therefore, the current study aimed to provide significant insights into the potential anti-inflammatory effects of dietary polyunsaturated fatty acids on THP-1 cell lines and to compare the individual effects of EPA with DHA on inflammatory cytokine by preparing different concentrations of DHA and EPA. Subsequently, selection of the best concentration based on cell viability. With the presence of lipopolysaccharide (LPS), which will stimulate an inflammatory response in the cells, DHA and EPA will be introduced to THP-1 cell lines demonstrate an effect on inflammatory cytokine release by adding diluent detection antibody IL-6 and TNF alpha then measured them by using ELISA.

2. Materials and Methods

2.1. Materials

2.1.1. Fatty acids

Eicosapentaenoic acid: Eicosapentaenoic acid (EPA) was obtained as a solution in ethanol from Cayman chemical, and must be stored at -20°C.

Docosahexaenoic acid: Docosahexaenoic acid (DHA) was obtained as a solution in ethanol from Cayman chemical, and must be stored at -20°C.

2.1.2. Inflammation inducer

Lipopolysaccharides: Lipopolysaccharides (LPS) extracted from Escherichia coli lipopolysaccharide (LPS, 0111: B4) was obtained from Sigma – Aldrich and stored at 2 – 8 °C.

2.1.3. Cell titer-blue cell viability assay (rozrin)

The cell titer-blue cell viability assay (rozrin) was obtained from Promega and must be stored frozen at –20°C protected from light. The product is stable for at least 10 freeze- thaw cycles. For repeated use, the product can be stored tightly capped at 4°C or at temperature (22–25°C) for 6–8 weeks and protect from light.

2.1.4. Human interleukin-6 (IL-6) detection antibody

IL-6 detection Ab's was obtained from DuoSet® ELISA DEVELOPMENT SYSTEM and must be Stored the unopened kit at 2-8 °C.

2.1.5. Human tumor necrosis factor alpha (TNF-α) detection antibody

TNF-α detection Ab's was obtained from DuoSet® ELISA DEVELOPMENT SYSTEM and must be Stored the unopened kit at 2-8 °C.

2.1.6. THP-1 cell line

THP-1 cells were obtained from European Collection of Authenticated Cell Cultures (ECACC), Public Hea lth England. (https://www.phe- culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId= 88081201 & coll ection= ecacc_gc). The medium used for routine culture is RPMI1640 with L-glutamine and sodium bicarbonate (Gibco brand, ThermoFisher Scientific UK) supplemented with 100 nM Non-essential amino acids (Gibco brand, ThermoFisher Scientific UK), 1 mM Sodium pyruvate (Gibco brand, ThermoFisher Scientific UK), 10% (v/v) heat inactivated, low endotoxin Foetal Bovine Serum (FBS) (Pan Biotech, UK) and 100 units/mL of penicillin and 100 μg/mL streptomycin (i.e. 1 X working concentration of Pen-Strep solution, Gibco brand, ThermoFisher Scientific UK).

2.1.7. Human interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) Kit for ELISA

Enzyme-linked immunosorbent assay determined cytokine concentrations. Specific ELISA (Infinite M Plex by Tecan) measured the IL-6 and TNF-α. Human IL-6 and TNF- α Kit for ELISA tests were obtained from DuoSet® ELISA DEVELOPMENT SYSTEM.

2.1.8. Machine

Enzyme-linked immunosorbent assay (ELISA) machine which was used in the project to determine the cytokine concentrations was Infinite M Plex by Tecan.

2.2. Cells viability test

Cells viability tests was obtained for both fatty acids (EPA and DHA). EPA and DHA toxicity were determined by diluted EPA and DHA with THP-1 cells to made different concentration of EPA and DHA to see which concentration of the EPA and DHA are suitable to start the project and did not kill the cells. EPA concentration was (0.12, 0.24, 0.48, 0.95, 1.9, 3.8 and 7.6mM). DHA concentration was (0.24, 0.48, 0.95, 1.9, 3.8, 7.6, and 15.2 mM). 100 μl from the above concentrations were transfer to the 96 plate well (two different 96 plates one for EPA and another for DHA). First row of the 96 plates for the first concentration, second row for the second concentration etc. After that both plates

were incubated for 24 hours in 37°C with 5% CO2. After 24 hours, 20 μl from Cell Titer- Blue Cell Viability Assay (rozrin) was added to the first four columns to see the cells viability after 24 hours. The fluorescent was measured by Tecan F50 colorimeters machine. The same step was repeated after 48 hours but Cell Titer-Blue Cell Viability Assay (rozrin) was added to the second four column after 48 hours incubations to see the cells viability after 48 hr.

2.3. Preparation of the low and high dose of fatty acids (EPA and DHA) EPA

First of all, (1:10) dilution of EPA was prepared by adding 10 μl of EPA to 90 μl of THP- 1 cells. Then 43.5 μl was taken from the above preparation and added to 7956.5 μl of THP-1 cells to prepare the high dose of EPA (0.45 mM). After that 1.4 ml was taken from step two and added to 5.6 ml of THP-1 Cells to prepare the low dose of EPA (0.09mM). Fake control was done as well, by adding 10 μl of ethanol (0.05% volume / volume) to 90 μl of THP-1 cells. After that 43.5 μl was taken from the above preparation and added to 7956.5 μl of THP-1 cells DHA.

First of all, (1:10) dilution of DHA was prepared by adding 10 μl of DHA to 90 μl of THP-1 cells. Then 24 μl was taken from the above preparation and added to 7956.5 μl of THP-1 cells to prepare the high dose of DHA (0.45 mM). After that 1.4 ml was taken from step two and added to 5.6 ml of THP-1 Cells to prepared the low dose of DHA (0.09 mM). Fake control was done as well, by adding 10 μl of ethanol (0.05% volume / volume) to 90 μl of THP-1 cells. After that 24 μl was taken from the above preparation and added to 7956.5 μl of THP-1 cells.

2.4. ELISA (IL-6 & TNF-α)

First of all, (1:100) dilution of Capture Antibody (IL-6 and TNF-α) was prepared in phosphate buffer saline (PBS). Then, 100 μl of diluted Capture Antibody reagent from both IL-6 and TNF-α was added to each well in 96 plates (one plate for IL-6 and other plate for TNF-α). After that both plates were sealed and incubated at room temperature (RT) overnight. Then, the both plates were washed three times with washing buffer (0.05% Tween® 20 in PBS), by using a squirt bottle. After the third wash, both plates were inverted against clean paper tissue to make sure all washing buffer were removed. Then, 300 μl of blocking buffer (1% BSA in PBS, 1:20) was added to the each well in both plates, and the plates were incubated for one hour at room temperature. After the incubation, both plates were washed three times with ELISA washing solution. After that, 1:400 dilutions of IL-6 and TNF-α were prepared, and the serial dilution was prepared according to DuoSet® ELISA manufactures instruction. Then, 100 μl from both samples (IL-6 and TNF-α) and standards were added to the selected wells in the 96 plates (one plate for IL-6 and another plate for TNF-α) and the plates were covered with an adhesive strip and incubated for 2 hours at room temperature. Next, both plates were washed three times with washing solution. Then, 100 μl of the diluted Detection Antibody (IL-6 and TNF-α) were added to each well (IL-6 diluted detection Antibody to IL-6 plate and TNF-α diluted detection Antibody to TNF-α plate) and the both plates were covered with the adhesive strip and incubate two hours at room temperature. After that the both plates were washed three times with washing buffer. After the third wash, the both plates were inverted against clean paper tissue to make sure all washing buffer were removed. Then 100 μl of the working dilution of Streptavidin-HRP were added to each well in the both plates and covered. Then both plates were incubated for 20 minutes at room temperature in dark room. Then, the washing steps were repeated. After that, 100 μl of Substrate Solution was added to each well of the both plates and incubated for 20 minutes in the dark room. Finally, 50 μl of Stop Solution was added to each well of the both plates and gently taped, and the plate was measured at 450 nm1 by using Tecan F50 ELISA.

2.5. Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism 9 software (GraphPad Software). Data represent the mean and error bars represent standard error of the mean (SEM). All Data underwent for normality tests using Anderson and Pearson tests before being analyzed. Statistical significance was determined using two-Way ANOVA- Tukey's multiple comparisons test. Differences between samples were considered significant based on the p-value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

2.6. Ethical considerations

Study was carried out with cautious and adherence to research ethics. All relevant ethical considerations have been covered in accordance to sacred principles of the Helsinki declaration of 1964 principles (Reference number of ethical approvals: UG-4738).

3. Results

3.1. Cells viability test

The effect of EPA and DHA on THP-1 macrophage viability was performed using the cells viability test assay. THP-1 cells were incubated with 0.12, 0.24, 0.48, 0.95, 1.9, 3.8- and 7.6-mM EPA concentrations for 24 and 48 h. Also, THP-1 cells were incubated with 0.24, 0.48, 0.95, 1.9, 3.8, 7.6 and 15.2 mM of DHA concentrations for 24 and 48 h. After the incubation with EPA, we found there were no effects of 0.12, 0.24- and 0.48-mM EPA concentrations on the cell's viability comparing each individual concentration

to the cells only (Not significant). On the other hand, there were significant effects of 0.95, 1.9, 3.8- and 7.6-mM EPA concentrations on the cell's viability comparing each individual concentration to the cells only ($P < 0.001$) (Figure 1). Moreover, After the incubation with DHA, we found there were no effects of 0.24- and 0.48-mM DHA concentrations on the cell's viability comparing each individual concentration to the cells only (Not significant). While, there were significant effects of 0.95, 1.9, 3.8, 7.6- and 15.2-mM DHA concentrations on the cell's viability comparing each individual concentration to the cells only ($P<0.001$) (Figure 2).

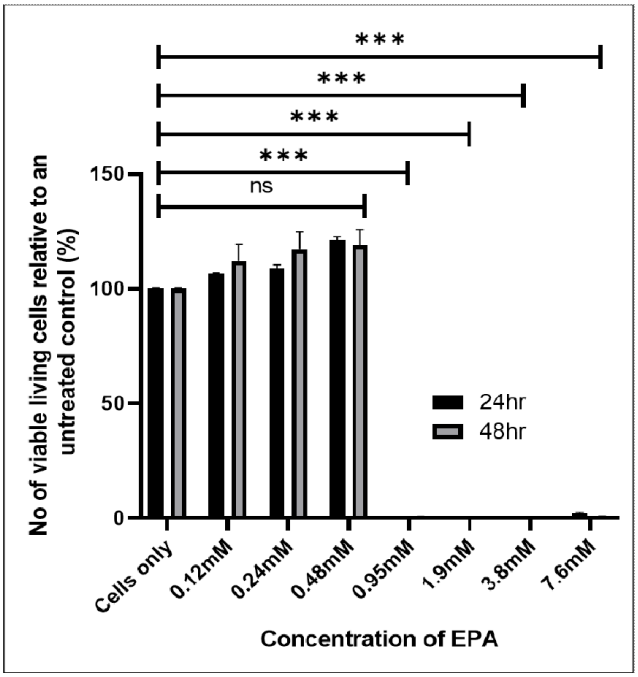

Fig. 1: Cells viability response to EPA after incubation in 37°C with 5%CO_2 for 24h and 48h. The figure illustrates no significance on the cell's viability with 0.12, 0.24- and 0.48- mM EPA concentrations after 24 and 48-hours incubation comparing to cells only (THP- 1 cells). A significant difference was observed with 0.95, 1.9, 3.8- and 7.6-mM EPA concentrations on the cell's viability comparing to cells only (THP-1 cells). All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.2. *Anti-inflammatory impact of DHA in IL-6*

The THP-1 cells were cultivated in two conditions. First, the macrophages were mixed with 0.09 and 0.45 mM of DHA without stimulation with LPS. In the second condition, the macrophages were mixed with 0.09 and 0.45 mM of DHA with 10 ng/ml LPS. In the presence of LPS stimulation,

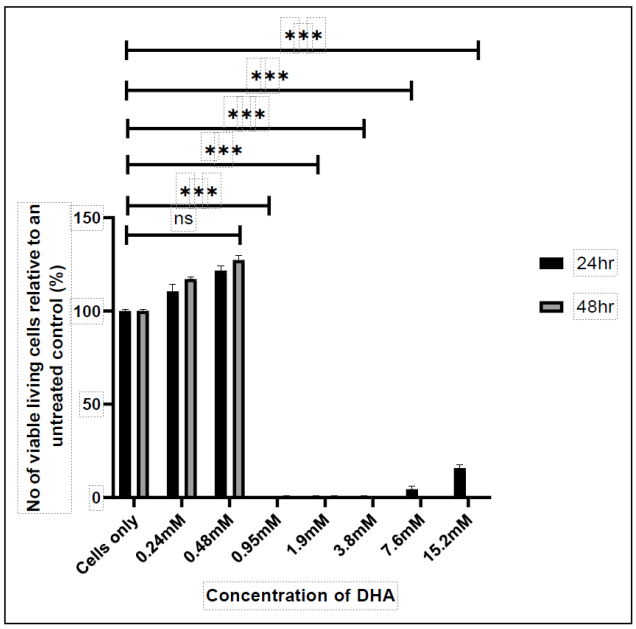

Fig. 2: Cells viability response to DHA after incubation in 37°C with 5%CO_2 for 24h and 48h. The figure presents no effects on the cell's viability with 0.24- and 0.48-mM DHA concentrations after 24 and 48-hours incubation comparing to cells only (THP-1 cells). It also highlights a statistical significance difference with 0.95, 1.9, 3.8, 7.6- and 15.2-mM DHA concentrations comparing to cells only (THP-1 cells). All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

there was a global increase of IL-6 expression levels ($P < 0.001$). With the presence of LPS and 0.09mM of DHA, there were a significant reduction of IL-6 levels compared with the cells only ($P < 0.01$). In addition, there were a greater reduction of IL-6 levels when we mix the cells with 0.45mM of DHA with the present of LPS compared with the cells only ($P < 0.001$) (Figure 3).

3.3. *Anti-inflammatory effect of EPA in IL-6*

The macrophages type THP-1, were cultivated in two conditions. Initially, the macrophages were mixed with 0.09 and 0.45 mM of EPA without stimulated with LPS. Subsequently, the macrophages were mixed with 0.09 and 0.45 mM of EPA with 10 ng/ml of LPS. In the presence of LPS stimulation, there was a remarkable incline of IL-6 expression levels ($P < 0.001$). When the cells were mixed with 0.09mM of EPA with the present of LPS, there was no significant reduction on the IL-6 levels. Interestingly, there were a significant reduction of IL-6 levels when we mix the cells with 0.45mM of EPA with the present of LPS compared with the cells only ($P < 0.01$) (Figure 4).

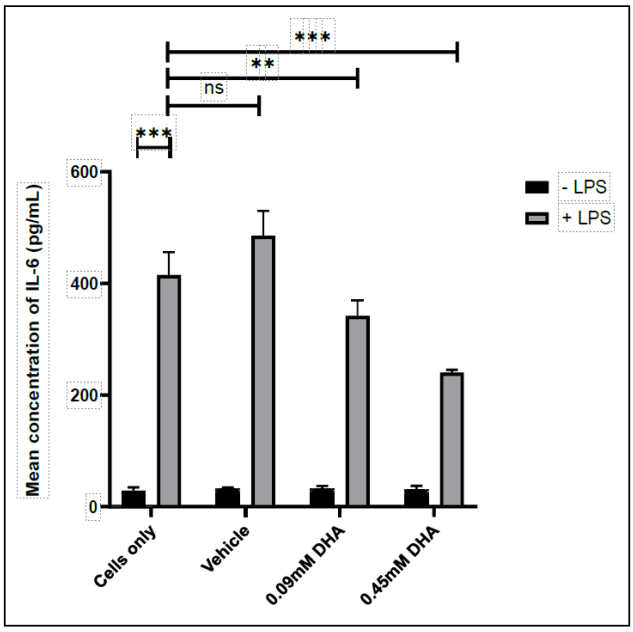

Fig. 3: Effects of DHA in IL-6. The figure indicates the production of IL-6 on LPS- stimulated and unstimulated THP-1 cells, with 10 ng/ml LPS concentration for 24 hours in RPMI1640 medium supplemented with 100 nM non-essential amino acids, FBS and 100 units/mL of penicillin and 100 μg/mL streptomycin, on pre-treated cells with 0.09- and 0.45-mM concentrations of DHA. Vehicle control consist of 0.030% ethanol (vol/vol) in medium, equivalent amount of ethanol present in 0.45mM DHA. All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

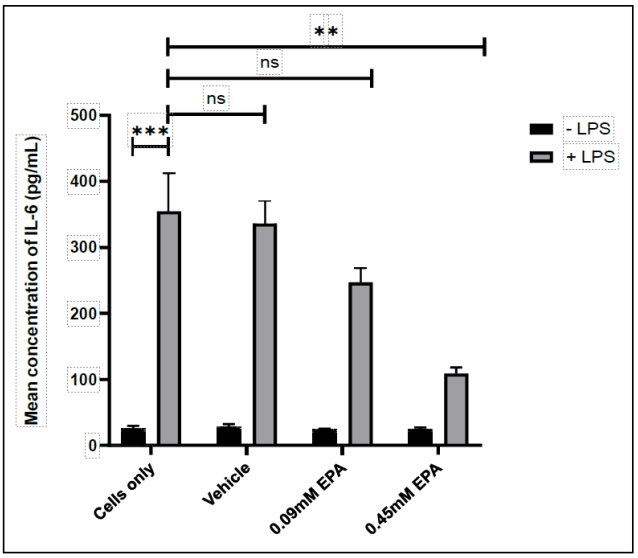

Fig. 4: Effects of EPA in IL-6. The figure presents the production of IL-6 In THP-1 macrophages which subjected to treatment with 0.09- and 0.45-mM concentrations of EPA on the presence or absence of 10 ng/ml LPS for 24 hours in RPMI1640 medium supplemented with 100 nM non-essential amino acids, FBS and 100 units/mL of penicillin and 100 μg/mL streptomycin. Vehicle control consist of 0.054% ethanol (vol/vol) in medium, equivalent amount of ethanol present in 0.45mM EPA. All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

3.4. Anti-inflammatory impact of DHA in TNF- α

The THP-1 cells were cultivated with 0.09 and 0.45 mM of DHA with/without stimulations with LPS. LPS-stimulated THP-1 showed a significant elevation of TNF- α levels (P < 0.001). However, there were no significant reduction of TNF- α levels when we mix the cells with 0.09 and 0.45 mM of DHA with the present of LPS compared with the cells only (Figure 5).

3.5. Anti-inflammatory effect of EPA in TNF- α

The THP-1 cells were mixed with 0.09 and 0.45 mM of EPA in two conditions: with and without LPS enhancement. On the one hand, with LPS stimulation, TNF- α expression levels showed a significant increase (P < 0.001). On the other hand, there were no significant reduction of TNF- α levels when we mix the cells with 0.09mM of EPA with the present of LPS compared with the cells only (Not significant). Strikingly, there were a significant reduction of TNF- α levels when we mix the cells with 0.45mM of EPA with the present of LPS compared with the cells only (P < 0.05) (Figure 6).

4. Discussion

Inflammation is a condition that plays a vital role in the reduction of a wide range of human maladies.[14,15] EPA and DHA are two major and dominant n-3 fatty acids ground in fish oil and oily fish supplements. The scientific literature is rich of evidences supporting that these fatty acids, to a certain extent have ability to prohibit various characteristic features of inflammation. Of significance, the production of eicosanoids from n-6[16] and the manufacturing of inflammatory cells.[15] Moreover, there has been substantial evidence that DHA and EPA exhibited influences that regulated by distinct mechanism of actions on the functionality of immune cells.[17] In this current study, we focused on investigating the effect of different levels of PUFAs, including low doses (e.g., 0.09 mM) and high doses (e.g., 0.45 mM) of EPA and DHA on the regulation of inflammatory markers on stimulated and unstimulated THP-1 cells.

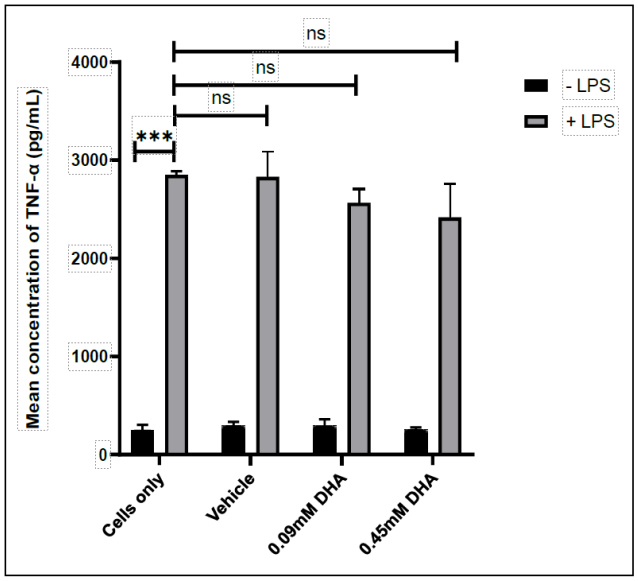

Fig. 5: Effects of DHA in TNF- α. The figure displays the expression of TNF- α. In THP-1 cells treated with 0.09- and 0.45-mM concentrations of DHA on the presence or absence of 10 ng/ml LPS for 24 hours in RPMI1640 medium supplemented with 100M non-essential amino acids, FBS and 100 units/mL of penicillin and 100 μg/mL streptomycin. Vehicle control consist of 0.030% ethanol (vol/vol) in medium, equivalent amount of ethanol present in 0.45mM DHA. All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

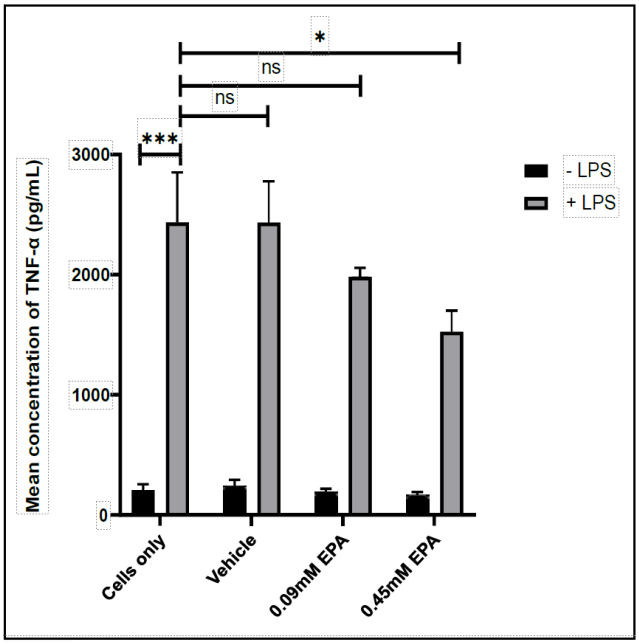

Fig. 6: Impact of EPA in TNF- α. The figure decorates the level of expression by TNF- α in THP-1 cells treated with 0.09- and 0.45-mM concentrations of EPA on the presence or absence of ng/ml LPS for 24 hours in RPMI1640 medium supplemented with 100 nM non-essential amino acids, FBS and 100 units/mL of penicillin and 100 μg/mL streptomycin. Vehicle control consist of 0.054% ethanol (vol/vol) in medium, equivalent amount of ethanol present in 0.45mM EPA All data were presented as error bars and subjected to normality test by Anderson and Pearson tests and the statistical significance difference was determined via two-way ANOVA test. Differences between samples were considered significant based on the p-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Our study findings have successfully demonstrated that the polyunsaturated fatty acids, DHA and EPA, have an inhibitory effect which remarkably were corelated with a decline in the expression of cytokines, regardless of inflammation diminish. Upregulation of genes encoding for the mediators of inflammation, i.e., pro-inflammatory cytokines, chemokines, and cell adhesion molecules has the potential to activate monocytes/macrophages endothelial adhesion and its infiltration into the vessel wall, hence initiating the development of atherosclerosis.[18] Worth to mention, the findings released from this study are similar to other findings from various studies, of particular in-vivo studies, where DHA and EPA dietary supplements from fish oil showed a decrease in the pro-inflammatory cytokines production.[19–21] In consistent with our study outcomes, studies of in-vitro and clinical design demonstrated a decline in the production of pro-inflammatory cytokines from fish oil supplementation of DHA and EPA.[22] Additionally, and in accordance to our current study findings, another study showed that there is a significant decrease in the expression of IL-6 and TNF-alpha, on macrophages cells subjected to DHA and EPA.[23] These findings highlight and suggest the DHA and EPA FAs ability in the down regulation of pro-inflammatory markers and more interestingly even with the absence of pro-inframammary stimulus, thereby provide a sound suggestion of the protective feature on THP-a macrophages inflammatory profile harmonious with documented beneficial influences to a healthy life by n-3 fatty acids.[14] Combined with data from the current study and consistent to the literature, the findings are suggestive that the anticorrelation between inflammatory mechanism and n-3 fatty acids, may be explained partly as a consequence of n-3 FAs ability in a regulating the secretion of macrophages cytokines by NF-κB pathway.

In reviewing the literature, prior studies have noted that elevated consumption of DHA and EPA has a positive impact to escalate n-3 fatty acids incorporation in the phospholipid membrane of immune cells.[24] In consistent with that, these fatty acids exhibit a major role in the regulation of different inflammatory genes expression through their interaction with many transcription factors and

nuclear receptor.[11]

The transcription factor, NFKB proven to up-regulate inflammation cytokines, adhesion molecules and cyclooxygenase gene.[25,26] Another study in human monocytes, has reported a decline in LPS-modulated activation of NFKB as consequence of fish oil intake.[27] Zhao Y et al, investigated the assumption provided by Singer et al reported in their study, suggesting that DHA and EPA may have the potential inhibition of NFKB activity during NF-κB pathway.[28] Zhao y et al,[28] build on that, and demonstrated through their experiments that EPA, DHA+EPA, but not DHA alone, have a potent feature to depress genes expression of NF-κB pathway, which suggests that cytokines being affected by n-3 FAs can cause NF-κB pathway genes to be upregulated. NF-κB signaling transduction activation, takes a paramount role in the formation of atherosclerotic lesions.[29] Particular fatty acids including oleic acid and linoleic acids, and some therapeutic drugs such as lipophilic statins, have involved in suppression of inflammatory response in the atherosclerotic lesions by inhibiting the activity of NF-κB pathway in mononuclear cells and vascular smooth muscle cells.[30,31] In the same fashion, a study by Zhao Y, et al,[28] evidenced that EPA hindered TNF-alpha expression in THP-1 macrophages cells upon LPS stimuli, by halt the activation of NF-κB. Our results corroborate with these findings of a great deal, the current study revealed a decline in the expression of the inflammatory cytokines TNF-alpha by EPA in LPS-stimulated THP-1 macrophages cells. Which is a suggestive element for the inhibitory ramifications of EPA on of NF-κB pathway activation.

An astonishing finding of the current study is that DHA showed a higher potent ability to decrease the cytokine IL-6 in both dose, 0.09 mM and 0.45 mM in LPS-stimulated THP-1 cells, whereas EPA failed to exhibit anti-inflammatory effect in both doses' concentrations, only it lowered the IL-6 levels under 0.45 mM. Interestingly, these results correspond with a significant affinity to other studies' outcomes. In other research works, DHA illustrated more potent ability to inhibit inflammation than EPA.[32–34] The study design of these studies and current study has similarities in terms of the induced inflammation, as on some studies the effect of n-3 FAs on macrophages was studied without being induced. There are several plausible explanations for the superior anti- inflammatory effects of DHA. It seems to be referred to DHA secretions of higher amount of anti-inflammatory lipids mediators, called protectins, Resolvin D, and maresins than the fatty acid EPA that secrets Resolvin E.[24,35] Thus, it provides an explanation of the reason behind the higher intensity of DHA over EPA effects on expression degree of inflammatory markers. Last but not least, consistent with these findings, our data found in accordance to other study as well, where DHA provokes a greater anti-inflammatory influence amongst n-3 fatty acids. A possible explanation is stated that the more unsaturated and longer fatty acids can present more significant anti-inflammatory effects.[36] Indeed, the more unsaturated and longer is the fatty acid, DHA.[37–39]

The current study has several strength and limitations. On the one hand, Strength of importance is that this study demonstrated an in-vitro research design that was similar to other studies research design, which stands out as a significant element for validation to certain extent. On the other hand, limitations in exist as well. The vast majority of in vitro studies showed different chemicals to induce inflammatory response including interferon gamma, LPS and TNFA in order to evaluate and study the effects of PUFAs. In this study, only LPS was used, and that's why it could explain the reason behind some points or the effects and influences of these PUFAs are not as pronounced here as in other in vitro design research works. Mullen A et al is an example of a study that have utilized interferon gamma, LPS and TNFA to induce inflammation.[33] Moreover, an in vitro study evidenced a reduction on the expression of MCP1, IL-6 and TNFA on macrophages that were treated with DHA and EPA.[40] Our data have to be evaluated and validated by future studies using different research designs, including animal trials and human trials as the effect may vary due to various variables. In a clinical randomized trial, fish oil supplements, DHA and EPA illustrated a decline in the pro-inflammatory cytokines production.[22] Further studies could focus on the dose concentration effects utilizing different doses that need to be prepared with caution.

5. Conclusion

In summary, the present study was designed to determine the anti-inflammatory effect of polyunsaturated fatty acids in regulating inflammatory cytokines. Polyunsaturated fatty acids demonstrated a significant inhibitory effect on LPS-stimulated THP-1 macrophages inflammatory responses when subjected for treatment with DHA and EPA. Our study, expresses marvelous outcomes that are in a significant agreement with former evidences, were DHA showed more favorable outcomes than EPA. Thereby, a rich diet with food sources of DHA and EPA (e.g., fish oil) is advocated as it should lead to a reduction in the pro-inflammatory cytokines, hence reducing risks of chronic inflammatory diseases such as CVD, COPD, diabetes, rheumatoid arthritis, and cancer. There is, therefore, a definite need for further studies is urgently demanded to validate our conclusions and further evaluate anti-inflammatory effects of these long unsaturated fatty acids in both, in vivo animals and human trials.

6. Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis

were performed by Hamed Sulaiyam Al Hinai, Dr. Sam Hooper, Dr. Steve Potter, Sulaiman Amur Al Alawi, Mohammed Abdullah Al Shuhoumi, Dr. Dorel Anna. The first draft of the manuscript was written by Hamed Al Hunai, Mohammed Abdullah Al Shuhoumi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.[41]

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