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Investigation of the effect of free fatty acids on cell growth and Cytokine secretion

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Highlights

- **The fat intake has an effect on the human immune system.**
- **In vitro, the free fatty acid DHA had the ability to inhibit the release of cytokine IL-8.**
- **The differences concentrations of the free fatty acid DHA effect on anti-inflammatory effects, where the higher concentrations of DHA was inhibited cell proliferation of THP-1 cells.**

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ABSTRACT

Interactions between immune and inflammatory cells are mediated primarily through the interaction of proteins, termed interleukins (IL) that are able to promote cell growth, differentiation as well as functional activation. TNF-α, IL-1 and IL-6 are the most important cytokines produced by monocytes. The aim of this study was to investigate the effect of docosahexaenoic acid (DAH), on immune cell proliferation along with cytokine production. This was tested using the THP-1 monocytic cell line, which in turn allowed observing how surface protein cytokine expression was affected by docosahexaenoic acid treatment and whether the effect of incubation would reduce the stimulus-induced production of pro-inflammatory cytokines. The experimental method employed Linked Immunosorbent Assays, while both Cell Titer 96® AQueous One Solution Cell Proliferation Assay and Titer 96 AQueous One Solution were used to determine cell proliferation. THP-1 cells induce growth-promoting activity and, the concentration of DHA toxic effect began at around 100 μM and reached maximum toxicity at around 200 μM. The results demonstrate that DHA inhibited endogenous IL-8 release when compared to a control group where the inhibition was maintained for 3 days. The study revealed the effect of docosahexaenoic acid (DHA), on immune cell proliferation and cytokine production.

1. Introduction

Human diet along with any associated fat intake can have a noticeable influence upon a body's ability to regulate both homeostasis and the immune system. In addition, an increase in fat intake can also contribute to a variety of disorders, which include cardiovascular disease (CVD), Type 2 diabetes, and metabolic health (Hill *et al*., 2007). Diet-derived fatty acids are essential sources of energy and fundamental structural components of cells. They also play important roles in the modulation of immune responses in health and disease. Saturated and unsaturated FAs influence the effector and regulatory functions of innate and adaptive immune cells, (Radzikowska *et al.,* 2019). Therefore, an understanding of how fat intake can affect the immune system could in turn reduce the incidence of these ailments in contemporary society. Therefore, the purpose of this study is to observe how the intake of fat can affect the human immune system by means of the THP-1 monocytic cell line and whether or not lipopolysaccharide is stimulated by cytokine secretion when exposed to Docosahexaenoic Acid (DHA). The first evidence that related dietary omega-3 polyunsaturated fatty acids (PUFAs) in inflammation came from observations of the prevalence of autoimmune and inflammatory disorders such as type-1 diabetes within Greenland Eskimo populations compared to similar social groups in Denmark during the 1970's (Kromann and Green, 1980). During the 1980's several independent lines of evidence suggested that native Greenland Eskimos (Dyerberg and Bang, 1979) as well as a significant percentage of the Japanese (Hirai *et al.,* 1982) consume a significant dietary intake made up of long-chain omega-3 PUFA from seafood while low incidences of myocardial infarction, chronic inflammatory and autoimmune disorders observed. As omega-3 fatty acids are essential for normal growth and development, there consumption may play an important role in the treatment of a variety of coronary, inflammatory or autoimmune disorders.

The interactions between immune and inflammatory cells are mediated primarily through the interaction of proteins, termed interleukins (IL) that are able to promote cell growth, differentiation as well as functional activation. TNF-α, IL-1, and IL-6 are the most important cytokines produced by monocytes along with macrophages which are involved in a variety of pathways associated with inflammation that include those associated with endothelial cells (Waugh and Wilson, 2008; Castell *et al.,* 1989; Laurikkala, *et al.,* 2002). Also, a variety of related, but different pathological responses associated with inflammation are due to the overproduction of IL1, IL8, and most noticeably TNF as these interleukins can be harmful to a person's health (Wang and Lin, 2008; Mege *et al.,* 2006).

DHA affects signal transduction, transcription as well as defense, and repair, they also affect the expression of cytokines. This hypothesis tests by using the human monocytic cell line THP-1 cells through the investigation of the effect of DHA, on the surface protein cytokine expression in monocytes and whether the effect of incubation reduce stimulus-induced production of pro-inflammatory cytokines. This study will discuss the effect of docosahexaenoic acid (DAH), on immune cell proliferation along with cytokine production.

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2. Methods

The human monocytic cell lines THP-1 were obtained from American Type Culture Collection (ATCC). THP-1 cells were seeded at a density of 0.3 X 10^6 cells/ml and passage when their growth was at 80% confluence or approximately 0.3-1.0 X 10^6 cells / ml. Cell proliferation assays were taken to determine if the treatment/concentration used affected the cells. The CellTiter 96® AQUEOUS One Solution Cell Proliferation Assay was used to check the response of the cells to each agent. The effect of DHA on basal and LPS-stimulated cytokine production with treated THP-1 was cultured in the absence or presence of LPS for 4 hours TNF-α and 8 hours for IL-8, then quantified by commercial ELISA. Levels of IL-8 in the cell culture supernatants were measured using ELISA. Ninety- six well ELISA plates were coated overnight at room temperature with the related diluted capture antibody, then washed 3 times by wash buffer between 0.05 % or 20 in PBS and blocked with 0.1% BSA in PBS for 2 hours at room temperature. After washing, the corresponding biotinylated detection antibody was added then incubated for 2 hours at room temperature. After washing, streptavidin-horseradish peroxidase was added, incubated for 20 minutes at room temperature, and washed again. The substrate solution color development system was added and incubated a further 5-10 minutes, before development was stopped with 2N H_2SO_4 solution. The plates then were analyzed using a microplate reader at 450 nm and adjusted reference of 570 nm.

3. Results

In order to establish that selected DHA had no toxicity on the THP-1 cells, it was necessary to monitor the effect DHA on the cells proliferation of THP-1 cells through the use of the MTT assay system. This experiment was used to determine an appropriate concentration of DHA, which could be used without deletion's effect on THP-1 cell growth. Fig. 1 shows that DHA has an adverse effect on the proliferation of THP-1 cells in comparison with the control group. Data suggest that the appropriate concentration of DHA was used in subsequent experiments to be 50 μM.

Fig. 2 represents the experiment was used to determine an appropriate concentration of LPS, LPS-1 E-coli smooth grade, which could be used without deletions effect to THP-1 cell growth. Cells were incubated at 37°C and 5% after each time point cells proliferation was measured with MTT assay.

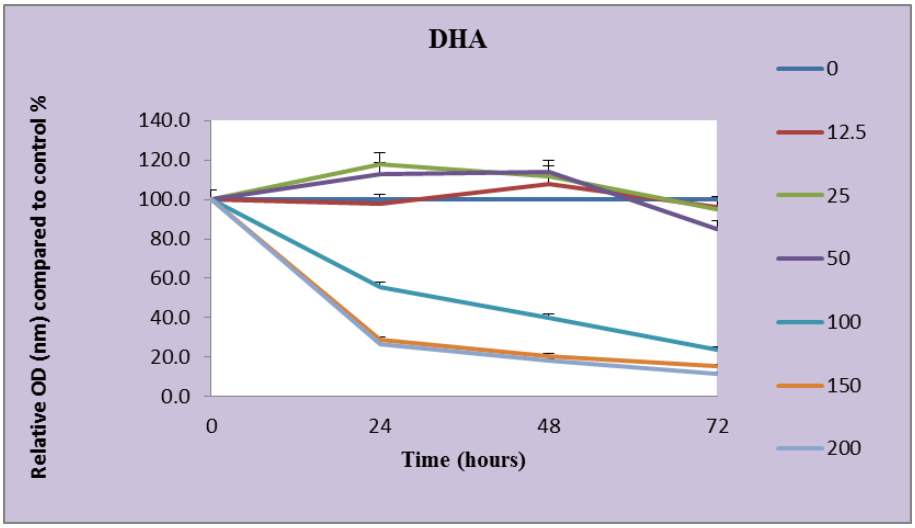

Fig. 1. Cell proliferation assay: Appropriate amount of the THP-1 cells were seeded into 96-wells as indicated and containing DHA of various concentrations. After each time point, cell proliferation was measured with MTT assay as methods, and the date generated graphically represented as means ±SD. The effect of DHA depends on the concentration used with an increasingly toxic effect starting at 100 μM before reaching maximum toxicity at 200 μM measured using MTT.

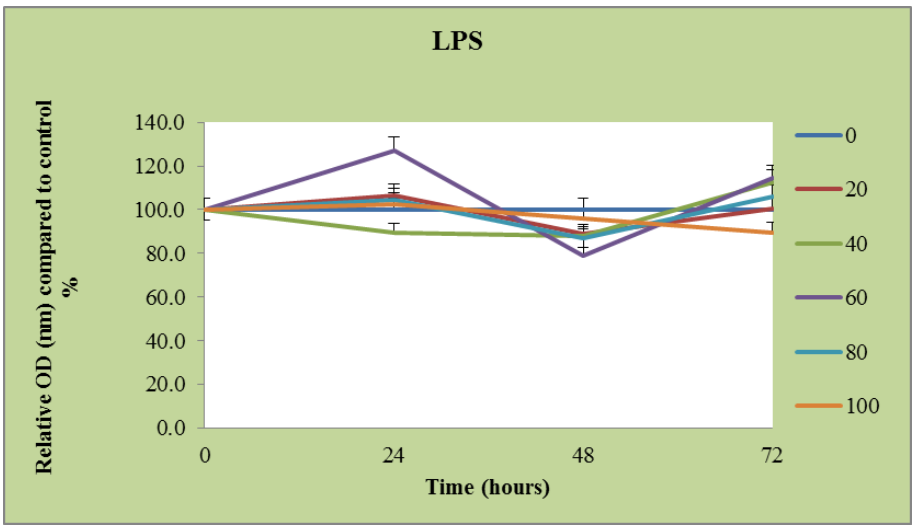

Fig. 2. Cell proliferation assay shows appropriate amount of the THP-1 cells were seeded into 96-wells that were containing LPS of various concentrations. After each time point cells proliferation was measured with MTT assay as methods and data generated graphically represented as means ±SD. Range of concentrations 20-100 μM and time intervals employed 24-72 hours related a lack of effect of LPS on THP-1 cell growth when compared to control-treated cells.

Range of concentrations 20-100 µM and time intervals employed 24-72 hours related a lack of effect of LPS on THP-1 cell growth when compared to control cells. Therefore, it was appropriate to use a concentration of 20 µM LPS in all subsequent experiments. The THP-1 cells were exposed to DHA, which was used at the 50 µM concentrations at the same time each day for 0, 24, 48 and 72 hours. The levels of this pro-inflammatory cytokine Interleukin-8, (IL-8) pg/ml, were measured in the culture supernatants by using ELISA. The control cells without treatment presented a basal line concentration of free fatty acid DHA throughout the 72 hours, while IL-8 cell production was the same for subsequent groups for the initial 24 hours. However, the effect of DHA on cytokine production monitor from the results that DHA inhibited endogenous IL-8 release by 50% compared to control and inhibition was maintained for 3 days. These investigations using the *in vitro* THP-1 human monocyte cell model indicate that the tested fatty acid DHA had the ability to inhibit cytokine IL-8 release, Fig. 3.

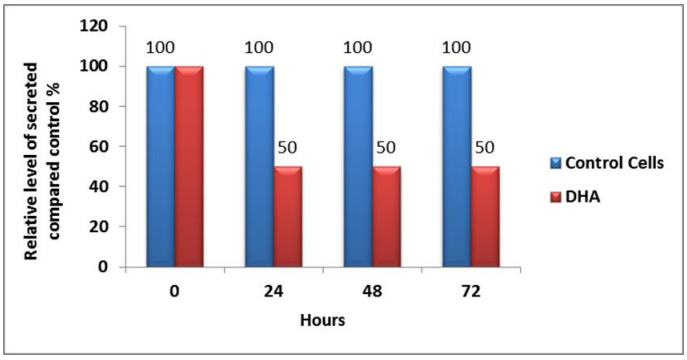

Fig. 3. Production of IL-8 by THP-1 cells exposed to fatty acids alone. THP-1 cells (500,000 cells/ml) were treated with DHA 50 µM over a variety of the time points: 0–24–48-72 hours and secreted cytokine specifically IL-8, measured in the culture supernatants by using ELISA.

DHA was used at the 50 µM concentrations and stimulated with 20 µg/ml LPS concentrations at the same time each day for 0, 24, 48, and 72 hours. The levels of these pro-inflammatory cytokines were measured in the culture supernatants by using ELISA. The LPS induced dose-response effects of DHA on THP-1 cytokine secretion for 8 hours with IL-8. The control cells without treatment presented a basal line concentration of DHA throughout the 72 hours; IL-8 cell production was the same for subsequent groups for the initial 24 hours. However, data generated indicate that THP-1 cells previously exposed to DHA 50µM revealed that had the capacity to inhibit cytokine IL-8 release from LPS stimulated THP-1 cells at 24 hours (90%) and 48 hours (75%), however, the 72 hours DHA retained an inhibitory function (75%) compared to control cells, Fig. 4.

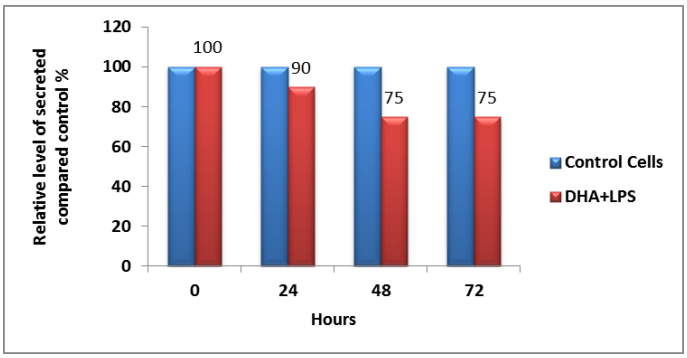

Fig. 4. IL-8 production by stimulated THP-1 cells exposed to LPS in media supplemented with fatty acids. THP-1 cells (500,000 cells/ml) were treated with DHA 50µM over a variety of the time points 24-72 hours and secreted cytokine specifically IL-8, stimulated with 20 µg/ml LPS which measured in the culture supernatants by using ELISA.

4. Discussion

The free fatty acids DHA has different effects on leukocyte functions such as phagocytosis, chemotactic response and cytokine production (Gorjão *et al.,* 2009). Verification of the initial cell concentration is fundamental for optimal substrate utilization and was found to be 1.25 x 10^5 THP-1 cells/ml which was supported by Banka *et al.* (1991), where THP-1 cells were shown the models of human of those experiments, use in this study is characterized by distinct monocytic surface markers, enzymatic activities, and the ability to phagocytise (Banka *et al.,* 1991), and these cells are clearly able to secrete and react to such cytokines as IL-1, IL-6, IL-8, IL-12 and TNF-α which in turn may imply the presence of appropriate cytokine receptors on this cell line (Whiteside, 2007). THP-1 cells were plated at a low density of 1.25×10^5 cells/ml that was maintained throughout exponential growth. Interestingly, if all cells grow exponentially the populations progress through the cell cycle with the ratio of cells with respect to the phase of the cell cycle result in phase durations for G_0-G_1, S, and G_2 + M in THP-1 cells. This observation was supported by Takeuchi *et al.* (1993) and Aneja *et al.* (2006), who reported that the THP-1 cells induce growth-promoting activity. That was dependent on an initial low cell density but not promoting proliferation at high cell density. In order to determine an appropriate concentration of free fatty acid to be used their determination of the growth curves of THP-1 cells exposed to DHA was necessary. However, the concentration of DHA at lower doses between 12.5 - 50.0 µM over three consecutive days was observed to have minimal effect on the growth of THP-1 cells when compared to the control samples, while higher concentrations of DHA ≥100 µM were observed to inhibit cell proliferation of THP-1 cells. Results from Fig. 1 revealed that the toxic effect began at around 100µM and reached maximum toxicity at around 200 µM. The six double bonds of DHA make this most unsaturated of the membrane fatty acids (DHA, C22: 6, ω-3), where the macrophage cell membrane affects liquid fluidity and structure. Phospholipids, which contain DHA, may segregate into specific membrane regions there by altering the molecular structure and function of particular membrane proteins residing within the lipid domain. DHA affects interactions of IFN-α with its receptor. Therefore, inhibition of macrophages activated via DHA could provide valuable effects of fish-oil diets that include DHA with respect to autoimmune diseases. Therefore, this data clearly suggests that the concentration of DHA should be in the region of 50 µM as this has no impediment on cell growth. At this concentration, DHA has a more potent effect on the growth of THP-1 cells, supported by data from De Lime *et al.* (2007), although cells grow from 12.5 µM upwards these cells reach their maximum growth rate at 50µM, but decline thereafter, (De Lime *et al.,* 2007). In addition, DHA is a feature of membrane phospholipids found in both human brain and retina cells (Long *et al.,* 2014) and therefore to maintain human health, the modification of signal transduction across the cell membrane would be necessary.

Marion-Letellier *et al.* (2008) discovered that DHA mediates its effect through PPARγ in intestinal epithelial cells human enterocyte-like cell line (Caco-2 cells) (Marion-Letellier *et al.,* 2008). PPARγ is activated by both natural ligands, such as polyunsaturated fatty acids, or via synthetic ligands. In human dendritic cells, PPARγ ligands decrease the formation of inflammatory cytokines along with the inhibition of several other proinflammatory pathways such as inducible nitric oxide synthase (iNOS) or matrix metalloproteinase-9 in Caco-2 cells. DHA decreases the release of proinflammatory cytokines IL-6 and IL-8, and abrogated iNOS expression. The effect of DHA on LPS-induced responses was generally more potent. This observation agreed with a number of studies such as De Caterina *et al.* (1994); Khalfoun *et al.* (1997); Zhao *et al.* (2005), as their research suggested that DHA has a greater effect upon the protein levels of the NF- **k**B transcription system mechanism in which DHA and EPA down-regulate expression level of the IL-8 via venous endothelial cells (De Caterina *et al.,* 1994; Khalfoun *et al.,* 1997; Zhao, *et al.,* 2005; Pedersen *et al.,* 2011).

5. Conclusion

Endothelial and immune cells are associated with the initiation and development of inflammatory diseases via activation of free fatty acid receptors type 2 and 3, G protein-coupled receptor 109A, and inhibition of histone deacetylases (Li, Meng., *et al.,* 2018). DHA has several different anti-inflammatory effects which take place between functions which leukocytes, insulin-secreting cells, and endothelial cells (Gorjão *et al.,* 2009; De Caterina *et al.,* 1994; Calder, 2008). DHA can inhibit LPS induced IL-8 release in THP-1 cells. The effects of DHA on LPS-induced responses were generally more potent.

The finding of this study clearly demonstrates that DHA has effects on how THP-1 cells produce IL-8, where different concentrations of DHA were affected on anti-inflammatory effects. The concentration of DHA at lower doses was observed to have minimal effect on the growth of THP-1 cells, while higher concentrations of DHA ≥100 μM were inhibited cell proliferation of THP-1 cells in humans. Zhao *et al.* (2005) suggest that the response of DHA in human kidney-2 (HK-2) cells are mediated by activation of PPAR-γ which inhibited NF- kB activity through direct interactions with the p65 subunit of NF- kB or through the increase in the synthesis of the NF- kB inhibitor I kB (Calder, 2008; Blok *et al.,* 1997).

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