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# Docosahexaenoic and Oleic Acid Dissimilarly Modulate Lipid Metabolism of Immature and Already Mature Adipocytes in Vitro

Zhenya IVANOVA[1], Natalia GRIGOROVA[1], Ekaterina VACHKOVA[1], Tanya TACHEVA[2], Ivan Penchev GEORGIEV[1]

[1]Department of Animal Physiology, Trakia University, Faculty of Veterinary Medicine, Stara Zagora, Bulgaria
[2]Department of Chemistry and Biochemistry, Trakia University, Faculty of Medicine, Stara Zagora, Bulgaria

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***ORCID IDs of the authors: Z.I. 0000-0003-4053-515X, N.G. 0000-0001-5749-7773, E.V. 0000-0002-9291-332X, T.T. 0000-0002-2946-9641, I.P.G. 0000-0001-8047-9928.***

## Abstract

The study aimed to compare the effect of 100 μM docosahexaenoic acid and oleic acid alone, and as a combination in immature and already mature 3T3-L1. The cells were subjected to adipogenic induction for 2 days and then were maintained in a high glucose culture medium supplemented with 10 μg/mL of insulin for additional 16 days. The treatment with docosahexaenoic acid and oleic acid lasted 9 days in total, as half of the cells were treated during induction (from day 1 to 9), and the rest—after reaching maturity (day 9–18). The palmitic fatty acid was used as a positive control. Applied in immature adipocytes, docosahexaenoic acid prevented intracellular neutral lipid deposition and reduced lipolysis upon well-preserved glucose utilization. At the same time, oleic acid alone or combined with docosahexaenoic acid increased lipid accumulation and slightly attenuated lipolysis only related to the palmitic fatty acid-treated group. However, free fatty acids application in mature adipocytes did not cause any beneficial effect. They elevated lipolysis rate compared to the induced control, which in vivo might be a predisposing factor for ectopic fat accumulation. The results emphasized that free fatty acids' impact on adipocyte metabolism was highly affected by their maturation stage and should be considered in anti-obesity strategies based on free fatty acids signaling modulations.

***Keywords:*** 3T3-L1 cells, adipogenesis, fatty acids, glucose, lipolysis, unsaturated

## Introduction

Fatty acid signaling has been an extensively investigated field, and its supplementation in the diet is a significant modulator of the physiological status of the organism (Picklo et al., 2017; Todorčević & Hodson, 2015). Increasing the quantity of unsaturated fatty acids in the diet has been considered an essential component of healthy nutrition (Alabdulkarim et al., 2012; Nagy & Tiuca, 2017; Picklo et al., 2017).

Numerous studies prove the beneficial health effects of unsaturated fatty acids in contrast to saturated fatty acids on physiological processes. Their intake is thought to reduce the risk of metabolic syndrome and related disorders by lowering plasma triglyceride levels and improving dyslipidemia and insulin sensitivity (Martínez-González et al., 2015; Murali et al., 2012; Poudyal et al., 2011). According to the available literature, oleic acid (OA) and docosahexaenoic fatty acid (DHA) favor the health status even in obesity (López-Gómez et al., 2020; Palomer et al., 2018; Thota et al., 2020). Evidence suggests that OA has a protective effect against palmitic fatty acid (PA)-induced mitochondrial dysfunction and insulin resistance as a crucial role in amplifying insulin signaling (López-Gómez et al., 2020; Palomer et al., 2018; Polley et al., 2018). Similarly, DHA has been thought to potentiate healthy expansion in adipose tissue by stimulating adiponectin excretion and insulin-dependent glucose uptake (Bagley et al., 2013; Cao et al., 2022; Song et al., 2017). However, other authors claim that DHA-induced metabolic changes are associated with suppressed adipogenesis, increased lipolysis, and enhanced mitochondrial biogenesis in the whole body (Barber et al., 2013; Kim et al., 2006).

White adipose tissue plays a crucial role in realizing these beneficial effects as it is deeply involved in regulating systemic energy balance and lipid and carbohydrate homeostasis (Booth et al., 2016). Notably, its physiological condition and fatty acid composition highly determine the metabolic outcomes of fatty acid supplementation, which could explain the abovementioned controversial statements.

**Corresponding Author:** Natalia GRIGOROVA • **E-mail:** n.gr@abv.bg

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Among all cell-based models adapted for studying obesity, including 3T3-F422A, LS14, LiSa-2, and HPB-AML-I, the 3T3-L1 murine fibroblasts are the best characterized, widely accepted as the most appropriate cell line (Ghorbani & Abedinzade, 2013; Green & Kehinde, 1974). These cells possess many of the mammalian white adipocyte's features—similar manner of adipogenic cell differentiation and lipid accumulation (Ghorbani & Abedinzade, 2013). They are very susceptible to lipolytic and antilipolytic agents (Li et al., 2012; Zhou et al., 2011). Therefore, we considered this cell model to be very suitable for achieving the goal of this study.

It is noteworthy that modern therapeutic strategies mainly focus on treating already existing obesity rather than preventing the main mechanisms involved in the process. Hence, the current study compared the anti-adipogenic potential of DHA and OA, alone or in a combination, of immature and already mature 3T3-L1 adipocytes.

## Method

The Ethics Committee at the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria, observed and approved the experimental procedures (Date: April 10, 2018, approval number: 14).

### Cell Differentiation and Experimental Design

3T3-L1 MBX—mouse embryonic fibroblasts (ATCC® CRL-3242™), purchased from the American Type Culture Collection (ATCC, Va, USA), were seeded in 24-well plates at a density of $10^4$ cells/mL, cultured with basal media (BM) consisting of Dulbecco's Modified Eagle's Medium (4500 mg/L glucose), 10% (v/v) fetal bovine serum, L-glutamine, antibiotic solution (penicillin G, streptomycin, and amphotericin B), incubated at 5% $CO_2$ and 37°C. In general, 24 hours after reaching 100% confluence (growth arrest), the cells were treated with adipogenic inducing media (AIM), composed of BM supplemented with 10 µg/mL insulin, 0.05 mM indomethacin, 0.1 mM 3-iso butyl-1-methylxanthine, and 1 µM dexamethasone for 48 hours. Then, they were kept in adipogenic maintenance media (AMM—BM with 10 µg/mL insulin) until the end of the trial (day18). An additional non-induced control group (NC) was kept in BM during the whole trial to assess the baseline levels of all tested parameters. To compare the effect of free fatty acids (FFAs) on 3T3-L1 adipogenesis in different stages, 100 µM PA (Cat. No. P5585), OA (Cat. No. O1383), DHA (Cat. No.D2534), or a combination of both unsaturated FFAs were added to the culture media at the beginning of induction (day 1)—immature adipocyte (AIM-PA; AIM-OA; AIM-DHA; AIM-OA + DHA, 50 µg each) or on already mature adipocytes (day 9 after induction) (AMM-PA; AMM-OA; AMM-DHA; AMM-OA + DHA, 50 µg each) for 9 days. A non-treated, adipogenic-induced group (AIM-C) served as a positive control. Each group consisted of six biological replicates. All reagents were provided by Sigma-Aldrich, Chemie, GmbH.

It has been reported that a 100 µM concentration of PA, OA, and DHA is safe when 3T3-L1 cells are handled and does not influence the impact of adipocyte differentiation, lipid accumulation, and lipolysis (Malodobra-Mazur et al., 2019; Manickam et al., 2010; Song et al., 2017; Wójcik et al., 2014). One of the methods of FFAs application is stepwise dissolution in the culture medium to the desired concentration after their preliminary dissolving in 100% ethanol (Manickam et al., 2010; Wójcik et al., 2014). Therefore, to determine the most appropriate final solvent concentration that does not affect the viability of 3T3-L1 MBX cells and to confirm the lack of cytotoxic effect of 100 µM concentration of the tested FFAs, we performed a cell viability assay described previously by Grigorova et al. (2020).

### Oil Red O Staining

On day 18 of the experiment, intracellular lipid accumulation was visualized by Oil Red O staining and assessed using subsequent extraction with isopropanol. The entire Oil Red O staining procedure has been previously described (Vachkova et al., 2016; Yang et al., 2011). Microscopic images were obtained via Leica cell and tissue culture inverted microscope equipped with a 5-megapixel resolution DMi1 camera version. The optical density (OD) of isopropanol extracted Oil Red O was spectrophotometrically determined at a wavelength of 490 nm (Biochrom Anthos Zenyth 200 rt microplate reader, Biochrom Ltd, Cambridge, UK). All data were calculated relative to the induced control (AIM-C) after excluding spontaneous adipogenesis established in NC, and the data were presented as a percentage of AIM-C.

### Glucose Utilization Rate (%)

On day 18, the supernatants from all wells were collected, and the extracellular glucose (EG) concentration was determined using an automatic biochemical analyzer Mindray BS-120 and Glucose GOD-PAD reagent (Biolabo S.A.S, Maizy, France). Each group's cell-free culture media was kept under the same conditions and analyzed with the experimental supernatants. This glucose concentration was accepted as initial (IG) and was further used for net glucose uptake calculation in all groups as follows: net glucose uptake (NGU) (mg/L) = IG − EG (Rivera Diaz et al., 2020). Finally, to express the net effect of glucose utilization graphically in percentages, the following calculation was performed: glucose utilization (%) = $(NGU_i - NGU_{NC})/NGU_{AIM\text{-}C} \times 100$, where $NGU_i$ is the net glucose uptake (mg/L) from each experimental group; $NGU_{NC}$ is the glucose uptake (mg/L) in non-treated, non-stimulated control (baseline uptake), $NGU_{AIM\text{-}C}$ is the glucose uptake (mg/L) in the non-treated adipogenic induced group.

### Adipolysis Assay

At the end of the experiment, glycerol concentration in the collected supernatants was measured using the MAK313 Adipolysis assay kit (Sigma-Aldrich). The OD was measured at a wavelength of 570 nm (Biochrom Anthos Zenyth 200 rt microplate reader; Biochrom Ltd, Cambridge, UK) with a correction of 620 nm. The glycerol concentration was calculated in µg/mL according to the OD of the standards and the standard curve. The percentage of lipolysis was further determined, relative to AIM-C after excluding the basal lipolysis found in NC.

### Statistical Analysis

The statistical analyses were performed using Statistica version 10 for Windows (StatSoft, Inc., Tusla, Oklahoma, USA (2011)). Descriptive data analyses were applied to assess the mean, standard error of the mean, and the significance of the differences between the groups. The analysis of variance test evaluated the difference between means of groups in tested parameters. Further, the exact differences between groups were determined by Fisher's Least Significant Difference multiple pairwise comparisons test of the post hoc procedure (Fisher, 1936). The $p$ values $\leq .05$ were assumed as statistically significant.

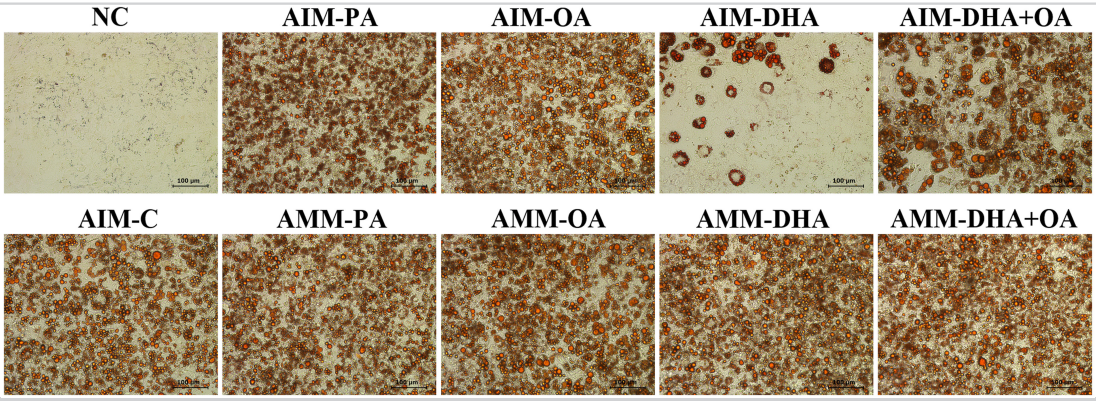


**Figure 1.**
*Effect of FFAs on Lipid Droplets Accumulation in 3T3-L1 MBX Adipocytes. Oil Red O Stained Microscopic Images Were Taken on Day 18 After the Initial Induction of Preadipocytes (Magnification 20×; Bar Scale, 100 μM). AIM-C = Non-treated, induced control; AIM-DHA = Induced, treated with DHA at the beginning of adipogenic induction; AIM-OA = Induced, treated with OA at the beginning of adipogenic induction; AIM-OA + DHA = Induced, treated with DHA and OA at the beginning of adipogenic induction; AIM-PA = Induced, treated with PA at the beginning of adipogenic induction; AMM-DHA = Induced, treated with DHA after adipogenic induction; AMM-OA = Induced, treated with OA after adipogenic induction; AMM-OA + DHA = Induced, treated with DHA and OA after adipogenic induction; AMM-PA = Induced, treated with PA after adipogenic induction; NC = Non-treated, non-induced control; FFA = Free fatty acid.*

## Results

### Effect of Free Fatty Acids on Lipid Droplets Accumulation

To compare the intracellular accumulation of lipid droplets among the experimental groups, we took microscopic images after each group's Oil Red O staining (Figure 1). The pictures depicted that treatment of 3T3-L1 with 100 μM DHA during the induction period of adipogenesis (from day 1 to day 9) inhibited preadipocytes' adipogenic differentiation to a great extent, as the cell monolayers were with well-preserved integrity. Most of the cells remained poorly differentiated to the end of the trial, and the visible small lipid droplets in them resembled the spontaneous (basal) induction found in the NC. Less in number were the mature adipocytes, situated in clusters, which seem to be more prominent in their size than the differentiated adipocytes from the other induced groups.

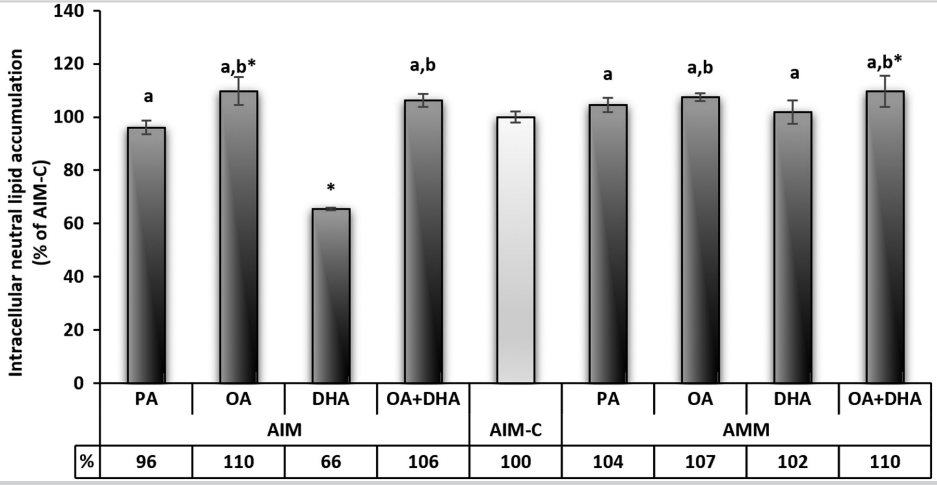


**Figure 2.**
*Effect of FFAs on Intracellular Neutral Lipid Accumulation in 3T3-L1 MBX Adipocytes. The Results Have Been Relative to the AIM-C After Excluding the Baseline Values Established in Non-Induced, Non-Treated Control. Data Are Shown as Mean ± SEM, n = 6 Biological Replicates per Group. Bars Denoted With an Asterisk Are Statistically Significant From AIM-C. The Letter "a" or "b" Indicates a Significant Difference Between Each Experimental Group and AIM-DHA or AIM-PA, Respectively ($p \leq .05$). AIM-C = Non-treated, induced control; AIM-DHA = Induced, treated with DHA at the beginning of adipogenic induction; AIM-OA = Induced, treated with OA at the beginning of adipogenic induction; AIM-OA + DHA = Induced, treated with DHA and OA at the beginning of adipogenic induction; AIM-PA = Induced, treated with PA at the beginning of adipogenic induction; AMM-DHA = Induced, treated with DHA after adipogenic induction; AMM-OA = Induced, treated with OA after adipogenic induction; AMM-OA + DHA = Induced, treated with DHA and OA after adipogenic induction; AMM-PA = Induced, treated with PA after adipogenic induction; FFA = Free fatty acid; SEM = Standard error of mean.*

**Effect of Free Fatty Acids on Intracellular Lipid Deposition**

The neutral lipids deposition was quantified by isopropanol extraction of already stained Oil Red O intracellular lipid droplets in each group. The measured OD values have been relatively expressed as a percentage of the induced control group (AIM-C) in Figure 2. We measured more than a 30% decrease in intracellular neutral lipid accumulation in 3T3-L1 adipocytes differentiated in the presence of 100 μM DHA compared to all other induced groups ($p < .001$) (Figure 2), which is well-visualized in the Oil Red O stained images (Figure 1). Oleic acid at the same concentration and time of application provoked a significant increase in adipocyte lipid deposition compared to AIM-C ($p < .05$), AIM-PA, and AIM-DHA ($p < .01$). The self-administration of PA, OA, and DHA in mature adipocytes did not affect intracellular fat accumulation compared to AIM-C. Still, in AMM-OA + DHA, a 10% increase in the studied parameter was established ($p < .05$).

**Effect of Free Fatty Acids on Glucose Utilization**

To examine the cellular insulin response, we calculated the difference between the culture medium input and the residual amount of glucose. The values are presented as a percentage of the AIM-C in Figure 3. The glucose uptake in the non-treated non-induced group (baseline uptake) was 49% of AIM-C, and a statistically significant increase in the percentage of glucose utilization was observed in all adipogenic-induced groups ($p < .001$). Docosahexaenoic acid treatment slightly attenuated this effect in both immature and mature adipocytes, as the lowest value among all induced groups was found in AMM-DHA ($p < .01$).

**Effect of Free Fatty Acids on Lipolysis Rate**

To investigate the effect of FFAs supplementation in immature and mature 3T3-L1 adipocytes, we evaluated the lipolysis rate as a percentage of the induced control group (AIM-C). The basal lipolysis established in non-differentiated 3T3-L1 was 6% of AIM-C, and as expected, the value was significantly increased in all induced groups ($p < .001$). However, the intensity of lipolysis stimulation, when compared to the AIM-C, was significantly affected by the type of FFA added and the maturity of the adipocytes, as shown in Figure 4. Palmitic fatty acid and OA supplementation enhanced lipolysis by a minimum of 19% compared to AIM-C, regardless of adipocyte maturation ($p < .05$). In contrast, DHA alone or in combination with OA increased lipolysis only when applied to already mature adipocytes ($p < .001$), while in preadipocytes, DHA reduced this parameter by 45% ($p < .001$) and upon OA + DHA treatment, the lipolysis rate in 3T3-L1 stayed unchanged (Figure 4).

## Discussion

The obtained results revealed a different effect of investigated unsaturated fatty acids on 3T3-L1 adipogenesis, depending on the treatment time and the degree of fatty acid saturation. Applied from day 1 of adipogenesis, the DHA had an inhibiting impact on adipogenesis, despite the highly predisposing factors in the culture medium. At the same time, OA added alone or combined with DHA potentiated the intracellular lipid deposition and slightly reduced lipolysis compared to AIM-PA upon a well-preserved glucose uptake.

The differentiation of immature into mature fat cells is a process of consequent activation of several groups of transcription factors, initiating with the increased expression of CCAAT/enhancing -linked proteins γ and δ in the onset of adipogenesis, followed by the subsequent stimulation of CCAAT/enhancing-linked proteins α (C/EBPα) and peroxisome proliferator factor γ (PPARγ) expression

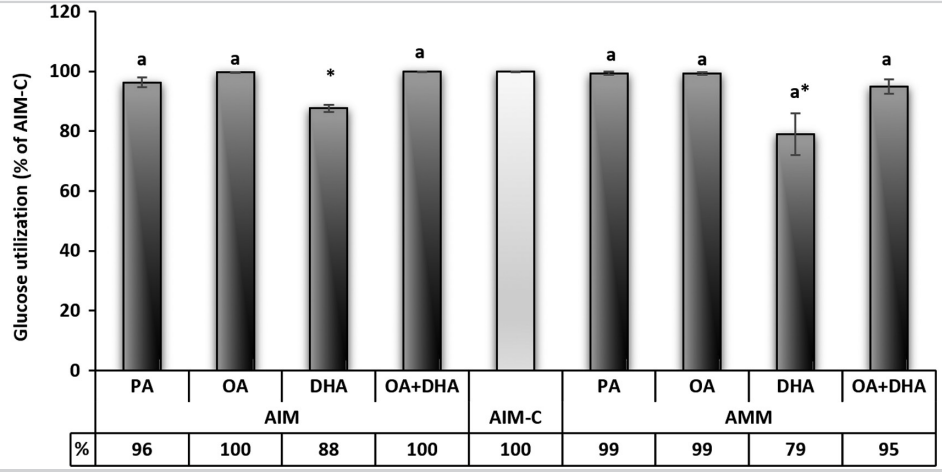


**Figure 3.**

*Effect of FFAs on Glucose Utilization in 3T3-L1 MBX Adipocytes. The Results Have Been Relative to the AIM-C After Excluding the Baseline Values Established in Non-Induced, Non-Treated Control. Data Are Shown as Mean ± SEM,* n = *6 Biological Replicates per Group. Bars Denoted With an Asterisk Are Statistically Significant From AIM-C. The Letter "a" or "b" Indicates a Significant Difference Between Each Experimental Group and AIM-DHA or AIM-PA, Respectively* ($p \leq .05$). *AIM-C = Non-treated, induced control; AIM-DHA = Induced, treated with DHA at the beginning of adipogenic induction; AIM-OA = Induced, treated with OA at the beginning of adipogenic induction, AIM-OA + DHA = Induced, treated with DHA and OA at the beginning of adipogenic induction; AIM-PA = Induced, treated with PA at the beginning of adipogenic induction; AMM-DHA = Induced, treated with DHA after adipogenic induction; AMM-OA = Induced, treated with OA after adipogenic induction; AMM-OA + DHA = Induced, treated with DHA and OA after adipogenic induction; AMM-PA = Induced, treated with PA after adipogenic induction; FFA = Free fatty acid; SEM = Standard error of mean.*

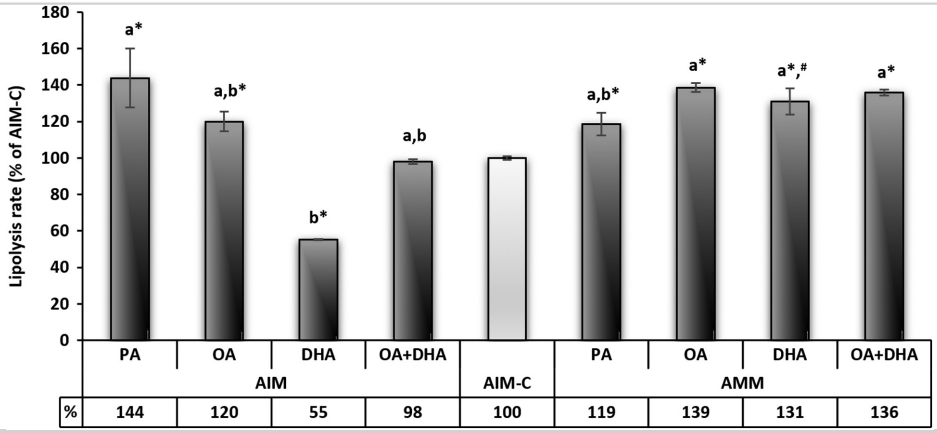


**Figure 4.**

*Effect of FFAs on Lipolysis Rate in 3T3-L1 MBX Adipocytes. The Results Have Been Relative to the AIM-C After Excluding the Baseline Values Established in Non-Induced, Non-Treated Control. Data Are Shown as Mean ± SEM,* n = *6 Biological Replicates per Group. Bars Denoted With An Asterisk Are Statistically Significant From AIM-C. The Letter "a" or "b" Indicates a Significant Difference Between Each Experimental Group and AIM-DHA or AIM-PA, Respectively (*$p \leq .05$*). AIM-C = Non-treated, induced control; AIM-DHA = Induced, treated with DHA at the beginning of adipogenic induction; AIM-OA = Induced, treated with OA at the beginning of adipogenic induction, AIM-OA + DHA = Induced, treated with DHA and OA at the beginning of adipogenic induction; AIM-PA = Induced, treated with PA at the beginning of adipogenic induction; AMM-DHA = Induced, treated with DHA after adipogenic induction; AMM-OA = Induced, treated with OA after adipogenic induction; AMM-OA + DHA = Induced, treated with DHA and OA after adipogenic induction; AMM-PA = Induced, treated with PA after adipogenic induction; FFA = Free fatty acid; SEM = Standard error of mean.*

(Grygiel-Górniak, 2014). CCAAT/enhancing-linked proteins α and PPARγ are pivotal triggers that induce the expression of adipocyte-specific genes such as glucose transporter type 4, fatty acid synthase, fatty acid-binding protein, fatty acid transport protein, and fatty acid translocase, resulting in increased cell supply of energy, a specific adipocytes phenotype appearance, and facilitating the intracellular lipid accumulation (Grygiel-Górniak, 2014; Li et al., 2017).

It should be emphasized that at the very beginning, the PPARγ alone is considered sufficient and absolutely necessary to activate the adipogenic cascade, and unsaturated fatty acids are known to be natural ligands of PPARγ, which directly affect its activity (Grygiel-Górniak, 2014; Niemelä et al., 2008; Song et al., 2017; Yanting et al., 2018). Most studies reported the agonist effect of mono- and poly-unsaturated fatty acids on PPARγ, potentiating healthy adipose tissue expansion (Holland et al., 2011; Madsen et al., 2005). The increased insulin sensitivity and adiponectin expression, in this case, prevent the whole body from lipotoxicity (Booth et al., 2016; Palomer et al., 2018; Yang et al., 2017). This statement followed our observation concerning OA supplementation at the beginning of the induction, but it contradicted the results obtained for DHA at the same time of treatment.

Numerous research demonstrates antiadipogenic, antiproliferative, and even apoptotic effects of omega-3 polyunsaturated fatty acids (omega-3 PUFAs) and PPARγ interaction in adipose tissue (Li et al., 2017; Logan & Spriet, 2015; Ortuño Sahagún et al., 2012; Song et al., 2017), but not all of them reported PPARγ downregulation (Ortuño Sahagún et al., 2012; Song et al., 2017). It seems that PPARγ post-translational phosphorylation contributed to its degradation, which plays a critical role in omega-3 PUFAs influence on adipose tissue. This process depends directly on the magnitude of PPARγ activation and adipocyte maturation stage (Song et al., 2017). Moreover, omega-3 PUFAs have been thought to increase the expression of genes associated with the induction of mitochondrial biogenesis in adipocytes, thus contributing to the lipid-lowering process in the whole body (Kim et al., 2006; Nagy & Tiuca, 2017).

Unexpectedly, the co-supplementation of DHA with OA in immature 3T3-L1 did not cause significant changes in intracellular lipid accumulation. It has been estimated that omega-3 PUFAs insufficiency in adolescent animals was provoked by OA and saturated fatty acids supplementation (Steffen et al., 2018). Picklo et al. (2017) also established decreased omega-3 PUFAs content in all internal organs when mice's obesogenic diet was enriched in OA. The same authors speculate that the main reason in this respect could be the competitive entry of fatty acids into the cell through the CD36 transporter, where OA has a significant advantage because of its lower transport Km value (FA concentration at which the velocity of uptake is one-half of the maximum). The extent to which monounsaturated fatty acids, particularly OA, influence the utilization and intracellular omega-3 PUFAs metabolism is still disputable and should be further examined.

An entirely different impact on adipogenesis was established when we administrated the FFA to mature adipocytes in the same concentration and duration of treatment. A 30% increase in lipolysis compared to AIM-C was observed in all treated groups, without changes in intracellular neutral lipid content. Enhanced lipolysis is a common finding in mature adipocytes due to their enlargement of lipid droplets and intracellular mechanical stress (Hammarstedt et al., 2018; Paul et al., 2018). Due to the increased FFA release, ectopic fat accumulation in various organs and systems is stimulated. Thus, further intensification of this process induced by different additives, as in our case, could promote weight loss but might be detrimental to the whole body's health status.

According to Prostek et al. (2016), the post-inducted 3T3-L1 cell applied with EPA or DHA for 48 hours enhanced the gene expression of critical factors of adipogenesis only in immature adipocytes. Their experiments reveal that omega-3 PUFAs desensitization of adipocytes occurs with advancing maturity. Moreover, Picklo et al. (2017) indicate that in immature adipocytes, the intracellular fatty acid composition is hardly affected by any fatty acids supplementation, even upon constant omega-3 PUFAs supply. They assumed that omega-3 PUFAs had been highly diluted in obese tissues because of the abundance of FFA due to hyper-caloric nutrition and increased lipolysis. Thus, their entry into the cell is increasingly hindered.

## Conclusion and Recommendations

The current study sheds more light on the appropriate time for unsaturated FFAs application. Our results emphasized that DHA supplementation had a well-defined prophylactic rather than therapeutic anti-adipogenic effect in 3T3-L1 adipocytes. This beneficial impact was strongly suppressed when combined in equal proportion with OA, revealing the need to be further clarified to what extent the monounsaturated fatty acids modulate the antiobesogenic effect of omega-3 PUFAs. FFAs administration in mature adipocytes potentiated lipolysis, regardless of their degree of saturation. Moreover, DHA treatment slightly reduced glucose utilization, which in vivo might be a predisposing factor for ectopic fat deposition.

Maintaining the balance between lipolysis and lipogenesis in adipose tissue, especially in a state of hypercaloric intake, is crucial in preventing metabolic diseases, so further investigation is needed to determine the most suitable mode of unsaturated fatty acids application.

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**Ethics Committee Approval:** The Ethics Committee at the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria, observed and approved the experimental procedures (Date: April 10, 2018, approval number: 14).

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept – Z.I., N.G., E.V., I.P.G.; Design – Z.I., N.G., E.V.; Supervision – Z.I., N.G., E.V., I.P.G.; Materials – T.T.; Data Collection and/or Processing – N.G., Z.I., T.T.; Analysis and/or Interpretation – N.G., E.V., T.T.; Literature Review – N.G.; Writing – N.G., Z.I.; Critical Review – T.T., I.P.G., E.V.

**Declaration of Interests:** The authors declare that they have no competing interest.

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