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Original article

CO-ADMINISTRATION OF OLEIC AND DOCOSAHEXAENOIC ACIDS ENHANCES GLUCOSE UPTAKE RATHER THAN LIPOLYSIS IN MATURE 3T3-L1 ADIPOCYTES CELL CULTURE

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Summary

Grigorova, N., Zh. Ivanova, E. Vachkova, T. Tacheva & I. Penchev Georgiev, 2022. Co-administration of oleic and docosahexaenoic acids enhances glucose uptake rather than lipolysis in mature 3T3-L1 adipocytes cell culture. *Bulg. J. Vet. Med.,* **25**, No 3, 411–425.

This study investigated the effect of different types of long-chain fatty acids and their combination on the triglyceride accumulation, glucose utilisation, and lipolysis in already obese adipocytes. 3T3-L1 MBX cells were first differentiated into mature adipocytes using adipogenic inducers (3-isobutyl-1-methylxanthine, dexamethasone, indomethacin, insulin, and high glucose), then 100 μM 0.1% ethanol extracts of palmitic (PA), oleic (OA), or docosahexaenoic acid (DHA) were applied for nine days. Unsaturated fatty acids decreased the intracellular lipid accumulation while maintaining glucose utilisation levels. However, unlike OA, self-administration of DHA only intensified lipolysis by 25% vs induced untreated control (IC), which may have a direct detrimental impact on the whole body's metabolic state. DHA applied in equal proportion with PA elevated triglyceride accumulation by 10% compared to IC, but applied with OA, enhanced glucose uptake without any significant changes in the lipogenic drive and the lipolytic rate, suggesting that this unsaturated fatty acids combination may offer a considerable advantage in amelioration of obesity-related disorders.

Key words: 3T3-L1, glucose uptake, lipid accumulation, lipolysis, unsaturated fatty acids

INTRODUCTION

Obesity, as the main component of metabolic syndrome, is a global health problem in pets and humans and a major predisposing factor for several disorders, including insulin resistance, dyslipidaemia, type II diabetes, hypertension, hepatic lipidosis (Zoran, 2010; Martínez-Fernández *et al.*, 2015; Kahn *et al.*, 2019). In this regard, white adipose tissue plays a pivotal role (Jackson *et al.*, 2017). Along with keeping energy balance, this tissue secretes a large variety of hormone-like substances and cytokines called adipokines that modulate the whole-body homeostasis (Martínez-

Fernández *et al.*, 2015). During the onset of obesity, lipid droplets size increases, adipocytes expand, neutralising all excess of energy-dense foods. At some point, however, the overabundance of nutrients exceeds the adipocytes storage capacity, and adipose tissue dysfunction occurs due to the development of inflammation, hypoxia, and the impaired mitochondrial function (Paul *et al.*, 2018; Hammarstedt *et al.*, 2018; Ghaben & Scherer, 2019). Consequently, glucose transporter type 4 (GLUT4) protein levels and its translocation to the plasma membrane are reduced because of the impaired insulin receptor substrate (IRS) - phosphoinositide 3-kinase (PI3K)-Akt pathway. Thus, subsequent insulin-stimulated glucose and free fatty acids uptake (Hammarstedt *et al.*, 2018), as well as *de novo* lipogenesis and accumulation of neutral lipids, are severely suppressed (Jackson *et al.*, 2017). The reduced ability of adipocytes to utilise excess nutrients and to convert the incoming glucose into triglyceride (TG), along with the enhanced lipolysis in hypertrophied adipocytes, results in increased FFAs release and blood glucose level, which is associated with the development of whole-body insulin resistance and metabolic syndrome (Ghorbani & Abedinzade, 2013; Girousse *et al.*, 2013; Bodis & Roden, 2018).

Several dietary and environmental factors such as macro- and microelements availability, diet composition, physical activity, stress, chronic fatigue, hormonal status, and others have a significant effect on adipose tissue expandability (Rosen & Spiegelman, 2006; Frühbeck *et al.*, 2014; Booth *et al.*, 2016). Some food compounds directly impact lipolysis regulation (Frühbeck *et al.*, 2014; Vachkova *et al.*, 2019). Others adjust the process of adipose hypertrophy and hyperplasia by changing the expression of some critical genes encoding adipogenic factors (Murali *et al.*, 2014) or directly affecting the extracellular matrix (Nicolai *et al.*, 2017). Modulation of these processes is an important target to avoid or alleviate adipose tissue dysfunction and reduce comorbidities associated with obesity. In this aspect, fatty acid signalling has been widely studied. It has been found that they are not just energy sources but also a significant modulator of the physiological status of adipose tissue, especially during hypercaloric intake (Todorčević & Hodson, 2016). Administered even in small quantities, they substantially influence lipogenesis, lipolysis, and glucose utilisation in mature adipocytes, as their effects are directly dependent on both the level of fatty acid saturation and their combination in the diet (Shaw *et al.*, 2013).

Growing evidence exist about the beneficial effect of monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) in adverse metabolic syndrome manifestation (Todorčević & Hodson, 2016; Picklo *et al.*, 2017; Yang *et al.*, 2017; Malodobra-Mazur *et al.*, 2019; Tutunchi *et al.*, 2020). However, the intimate mechanism by which fatty acids affect adipocyte metabolism is still unclear, and the scientific findings are quite controversial. Some authors have speculated that unsaturated fatty acids potentiate the adipose tissue expansion by direct activation of the insulin-signalling pathway and correspondingly increase glucose uptake into adipocytes pathway while inhibiting basal lipolysis in adipocytes (Tsuchiya *et al.*, 2014; Morley *et al.*, 2015; Huang *et al.*, 2017; Yang *et al.*, 2017). Others have described completely different effects (Manickam *et al.*, 2010; Vachkova *et al.*, 2019; Tutunchi *et al.*, 2020).

Therefore, the present study investigated the effects of palmitic acid (PA), OA, and DHA administered alone and in combination son triglyceride accumulation, lipolysis rate, and glucose uptake in mature 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials and chemical reagents

In the current trial, 3T3-L1 MBX-Mouse Embryotic Fibroblast (ATCC® CRL-3242™) and Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) were used, purchased from American Type Culture Collection, Virginia. DMEM - high glucose (4500 mg/L), together with the following chemical reagents: foetal bovine serum (FBS), L-glutamine, antibiotic solution (penicillin G, streptomycin, amphotericin B), insulin, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, palmitic acid (C10:0), oleic acid (C18:1) and docosahexaenoic acid (C22:6) suitable for cell culture, Oil Red O powder, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide powder (MTT) were purchased from Sigma-Aldrich Chemie GmbH.

Culture media composition

The 3T3-L1 MBX cell line was cultured in the media with different compositions: *Basal medium (BM)* – the cells were grown in a culture medium composed of DMEM **(**ATCC), 10% (v/v) FBS, and 1% antibiotic solution (penicillin G, streptomycin, amphotericin B); *Adipocyte inducing medium (AIM)* – adipogenic differentiation of 3T3-L1 was induced via medium consisting of DMEM (high glucose), supplemented with: 10% (v/v) FBS, 2% L-glutamine, 0.1 mM IBMX, 0.05 mM indomethacin, 1 μM dexamethasone, 10 μg/mL insulin and 1% antibiotic solution (penicillin G, streptomycin, amphotericin B); *Adipocyte maintenance medium (AMM)* – after adipogenic differentiation, the cells were cultured in DMEM (high glucose), supplemented with 10% (v/v) FBS, 2% L-glutamine, 10 μg/mL insulin, and 1% antibiotic solution (penicillin G, streptomycin, amphotericin B).

Preparation of fatty acid-maintenance medium (PA-AMM, OA-AMM, DHA-AMM)

PA, OA, or DHA were first dissolved in 100% sterile, cell-culture-tested ethanol (stock solutions), and then were diluted *ex tempore* in AMM to a final concentration of 100 μM FFA and 0.1% ethanol. Stock solutions were kept at –20 ºC until use.

Pre-experimental procedure: Cell viability assay

To determine the appropriate ethanol concentration required to dissolve FFAs, the cells were subjected to cell viability assay before the actual experiment. 3T3-L1 MBX mouse fibroblasts were seeded in 24 well plates at a density of 1×10^4 cells per 1 mL culture medium. Upon reaching 100% confluence, the cells were divided into four groups of 6 wells each and were tested with 0.05%, 0.1%, 0.5%, and 1% ethanol concentration, respectively, for 48 h. Cell viability was further determined calorimetrically using an MTT assay (Yang *et al.*, 2007). Briefly, the tested cells' supernatants were removed and replaced with 60 μL MTT solution (5 mg/mL in BM), followed by 3 hours incubation at 37 ºC. Then the MTT-BM solution was aspirated, and 0.04 N HCl/isopropanol solution was added for 10 minutes to solve the formazan crystals forming in live cells. Formazan solutions were replaced in 96 well plates, and absorbance was measured at 570 nm (against OD at

630 nm) (Kim *et al.*, 2010). The solution from each vial was measured three times, and an average value was included in the final calculation. Cell viability in percentage was estimated according to the following equation (Park *et al.*, 2003):

$$\text{Cell viability (\%)} = [OD_{570}\text{ (sample)}/OD_{570}\text{ (control)}]\times 100$$

After that, PA, OA, and DHA, dissolved in 0.1% ethanol-BM at a dose of 100 μM were further tested for cytotoxicity effect in confluent 3T3-L1 MBX mouse fibroblasts using the MTT assay as described above.

Experimental procedure

3T3-L1 MBX cells were expanded to a third passage and then were cultured in 12 well plates at a density of 1×10^4 cells/mL, in BM, humidified 5% CO_2 condition at 37 ºC. After reaching confluence, the cells were left additional 24 hours for growth arrest. The adipogenic differentiation was performed by double-induction within nine days (three days in AIM + three days in AMM + three days in AIM). To mimic chronic energy excess, the already mature 3T3-L1 MBX was treated with 100 μM FFAs for another nine days, while maintaining the high glucose and insulin concentration in the culture medium (AMM).

Further, the cells were randomly divided into the following groups: *IC* - double-induced 3T3-L1, then cultured in AMM; *PA* - double-induced 3T3-L1, then cultured in AMM supplemented with 100 μM PA; *OA* - double-induced 3T3-L1, then cultured in AMM supplemented with 100 μM OA; *DHA* - double-induced 3T3-L, then cultured in AMM supplemented with 100 μM DHA; *DHA+PA* - double-induced 3T3-L1, then cultured in AMM supplemented with 50 μM DHA and 50 μM PA (100 μM FFAs in total) in a one-to-one ratio; *DHA+OA* - double-induced 3T3-L1, then cultured in AMM supplemented with 50 μM DHA and 50 μM OA (100 μM FFAs in total); *NC* - an additional non-induced control group was included to estimate basal levels of all studied parameters. After reaching confluence and growth arrest, the cells from this group were cultured only in BM.

At the end of the experiment (day 18), the supernatants were collected, and glucose and glycerol concentration were established. The cells were stained following the Oil Red O protocol, and isopropanol lipid extraction was performed for intracellular lipid accumulation measurement.

Oil red O staining and assessment of intracellular lipid accumulation

Intracellular lipid accumulation in all cell groups was visualised and measured by Oil Red O staining and subsequent isopropanol lipid extraction. The supernatants were removed, and cells were fixed with 10% (v/v) neutral buffered formalin for ten minutes at room temperature. Further, the cells were treated with a freshly prepared working solution of 0.5% Oil Red O stock solution in isopropanol with dd H_2O, mixed for 10 min at room temperature (Yang *et al.*, 2011). After the fixing step, cells were washed twice with PBS and once with 60% isopropanol. The staining was performed by adding 300 μL/well working solution of Oil Red O. After 30 minutes incubation at room temperature, the Oil Red O dye was discarded, and stained cells were washed three times with dd H_2O. Microscopic images for intracellular lipid accumulation observation were captured by an inverted Leica Inverted Microscope for Cell and Tissue Culture, equipped with a 5-megapixel resolution DMi1 camera version. The accumulated Oil Red O was

further extracted with 100% isopropanol for 10 min, and absorbance was measured at 490 nm (Biochrom Anthos Zenyth 200 rt microplate reader, Biochrom Ltd, Cambridge, UK). Then, the data were expressed relatively to the double-induced control group (IC) after excluding spontaneous adipogenesis established in non-induced control group (NC).

Percentage of glucose utilisation

The media used during the experiment were changed every three days. On day 18, the extracellular glucose concentration in supernatants (EG) was evaluated using Mindray BS-120 Automatic Biochemical Analyzer and Glucose GOD-PAD reagent (Biolabo SAS, France). Cell-free culture medium from each group was also maintained at the same conditions and analysed in parallel with the experimental supernatants. Its glucose concentration was accepted as an initial (IG) and was further used for calculation according to the equation of Diaz *et al.* (2020):

$$\text{Glucose uptake} = IG - EG.$$

The results were calculated either in the absence (basal, non-induced glucose uptake – BG_{NC}) or in the presence of 10 µg/mL insulin (stimulated glucose uptake in all induced groups – SG_i) in the culture medium. Final glucose utilisation by adipocytes in percents was quantified by subtracting the basal glucose uptake (BG_{NC}) from the calculated glucose uptake in each induced group, and the results were expressed relative to the IC (SG_{IC}).

$$\text{Glucose utilisation (\%)} = [(SG_i - BG_{NC})] / SG_{IC}\ ] \times 100$$

Lipolysis assay

The quantification of glycerol concentration was performed using Adipolysis Assay Kit for cell culture supernatants (Sigma-Aldrich, St. Louis, USA, cat No MAK313). Collected cell culture supernatants were assayed immediately. All samples were run in duplicates. In each well, 10 µL of sample/standard was pipetted. A working reagent, containing assay buffer, enzyme mix, ATP, and dye reagent, was transferred into each well (100 µL per well) followed by 20 minutes incubation at room temperature.

The results were expressed as optical density (OD) at 570 nm (Biochrom Anthos Zenyth 200 rt microplate reader, Biochrom Ltd, Cambridge, UK), and the glycerol concentration was calculated in µg/mL according to the OD of the standards and standard curve. The percentage of the lipolysis was evaluated by comparing it to the IC, after basal lipolysis rate deduction, established in NC.

Statistical analysis

All data were analysed via software system – Statistica version 10 (StatSoft, Inc. (2011). The descriptive statistical tests were performed according to the standard methods. Final results are presented as mean and the standard error of the mean. The significance of the differences between groups was determined using the Least Significant Difference (LSD) test of the PostHoc procedure. The statistical significance of differences was set at $P \leq 0.05$.

RESULTS

Cell viability (MTT assay; pre-experimental procedure) of 3T3-L1 MBX

The MTT test showed that the vitality of 3T3-L1 MBX cells cultured with an excess of ethanol was unchanged up to a concentration of 0.1%. In concentrations higher than 0.1% (0.5% and 1%), cell viability decreased by 20% on the average

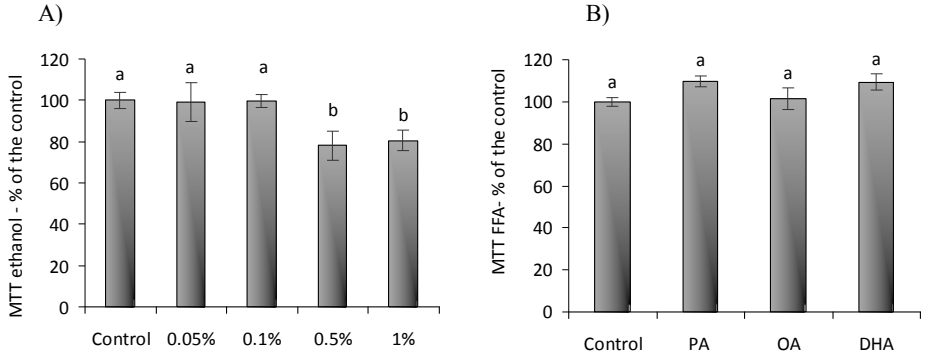

Fig. 1. Cell viability (MTT assay; pre-experimental procedure) of 3T3-L1 MBX after 48 h of incubation with 0.05%, 0.1%, 0.5%, and 1% ethanol concentration, respectively (**A**) and 100 µM palmitic acid (PA), oleic acid (OA) or docosahexaenoic acid (DHA) dissolved in 0.1% ethanol – basal medium (**B**). The results (mean±SEM, n=4) are normalised to the untreated group (control). The different superscript letters indicate significant differences between groups (P≤0.05).

(Fig. 1A). Therefore, for further experiments, fatty acid dilution with 0.1% ethanol was chosen.

Based on previous studies (Manickam *et al.*, 2010; Song *et al.*, 2017; Malodobra-Mazur *et al.*, 2019), 100 µM of PA, OA, and DHA has been accepted as a safe concentration when 3T3-L1 cells were handled, but to assess that this concentration did not influence 3T3-L1 MBX cells viability, MTT test was performed after 48 hours of FFAs exposure. As shown on Fig. 1B, no significant differences were established among PA-, OA-, DHA-treated, and control groups. Therefore, a concentration of 100 µM FFA was used in the current experiment and it was assumed that the non-specific cytotoxic effect did not influence the impact of adipocyte differentiation, lipid accumulation, and lipolysis.

Effect of FFAs on intracellular lipid accumulation in induced 3T3-L1 MBX

At the end of the experiment (day 18), Oil red O stained, microscopic images (40×) of lipid droplets (LD) formation were obtained (Fig. 2). All induced groups showed abundant intracellular lipid accumulation compared to NC (P<0.001). 3T3-L1 adipocytes exposed to DHA or OA after induction resulted in a 10% reduction of intracellular lipid accumulation (Fig. 3) (P<0.05). The highest OD values among all groups were found in DHA+PA (P≤0.05), where a 10% elevation in triglyceride accumulation compared to IC was observed (P<0.05).

Effect of FFAs on the percentage of utilised glucose by induced 3T3-L1 MBX

A noticeable, almost complete glucose consumption in all induced adipocytes (P<0.001) was found compared to NC (Table 1). Even further increase was established when differentiated 3T3-L1 were exposed to DHA+OA combination (P<0.001). (Fig. 4).

Effect of FFAs on the percentage of lipolysis rate in induced 3T3-L1 MBX

Regardless of fatty acid supplementation, at the end of the current experiment, more than sixteen-fold lipolysis rate (percentage) elevation was established in differ-

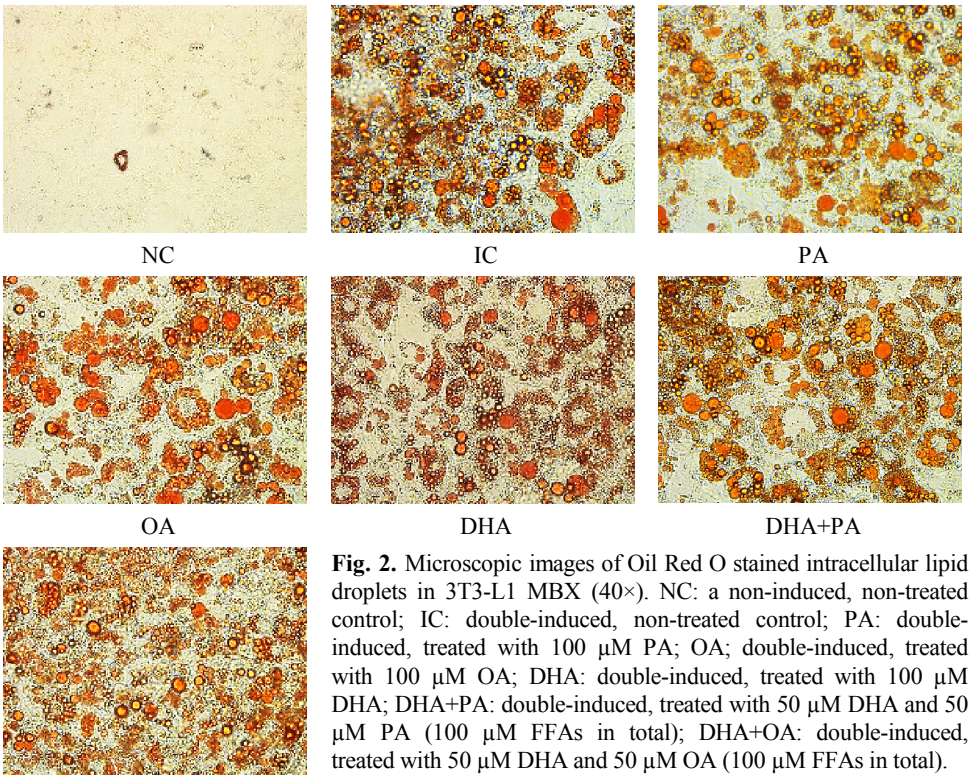

Fig. 2. Microscopic images of Oil Red O stained intracellular lipid droplets in 3T3-L1 MBX (40×). NC: a non-induced, non-treated control; IC: double-induced, non-treated control; PA: double-induced, treated with 100 µM PA; OA; double-induced, treated with 100 µM OA; DHA: double-induced, treated with 100 µM DHA; DHA+PA: double-induced, treated with 50 µM DHA and 50 µM PA (100 µM FFAs in total); DHA+OA: double-induced, treated with 50 µM DHA and 50 µM OA (100 µM FFAs in total).

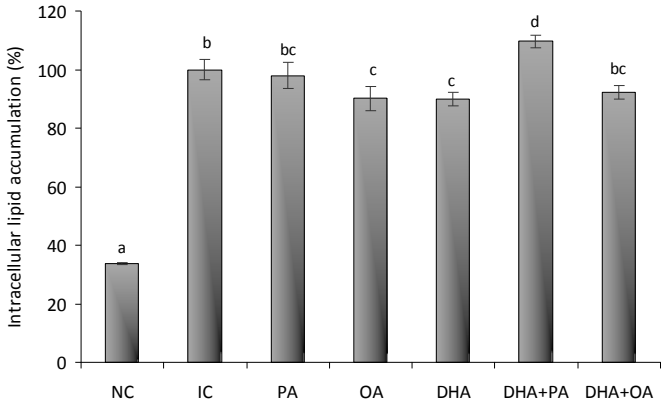

Fig. 3. The amount of intracellular lipid accumulation (%) in 3T3-L1 MBX. NC: a non-induced, non-treated control; IC: double-induced, non-treated control; PA: double-induced, treated with 100 µM PA; OA; double-induced, treated with 100 µM OA; DHA: double-induced, treated with 100 µM DHA; DHA+PA: double-induced, treated with 50 µM DHA and 50 µM PA (100 µM FFAs in total); DHA+OA: double-induced, treated with 50 µM DHA and 50 µM OA (100 µM FFAs in total). The results are presented relative to IC after the exclusion of spontaneous adipogenesis established in NC (mean±SEM; n=4). The different superscript letters indicate significant differences between groups (P≤0.05).

Table 1. Glucose uptake (mg/l) by 3T3-L1 MBX. NC: a non-induced, non-treated control; IC: double-induced, non-treated control; PA: double-induced, treated with 100 μM PA; OA; double-induced, treated with 100 μM OA; DHA: double-induced, treated with 100 μM DHA; DHA+PA: double-induced, treated with 50 μM DHA and 50 μM PA (100 μM FFAs in total); DHA+OA: double-induced, treated with 50 μM DHA and 50 μM OA (100 μM FFAs in total). The data are shown as mean ± SEM (n=4); * P≤0.001 between non-induced, non-treated, and all induced groups.

Groups	Glucose uptake (mg/L)
NC	2132.63 ± 111.82
IC	4456.53 ± 0.45*
PA	4457.88 ± 0.52*
OA	4458.20 ± 0.43*
DHA	4456.53 ± 0.45*
DHA+PA	4458.78 ± 0.74*
DHA+OA	4463.74 ± 1.71*

entiated adipocytes compared to NC. In contrast to our expectations, the lowest level of lipolysis was found in the PA group, with significant differences only compared to OA, DHA, and DHA+OA groups (P<0.05). DHA supplementation in 3T3-L1 led to more than 20% enhancement of basal lipolysis rate related to all induced groups (P<0.05) (Fig. 5).

DISCUSSION

This work highlights the importance of long-chain FFAs as crucial modulators of adipocyte metabolism. Already differentiated 3T3-L1 MBX cells were exposed to different FFAs, administered for nine days while maintaining high glucose and insulin concentration in the culture medium. Adipocytes are the most insulin-sensitive cells, and the presence of insulin and intact insulin signalling are critical factors for adequate adipogenesis (Bodis & Roden, 2018). Insulin promotes adipose tissue expansion by activating a range of intracellular pathways responsible for glucose uptake, synthesis, and TG storage

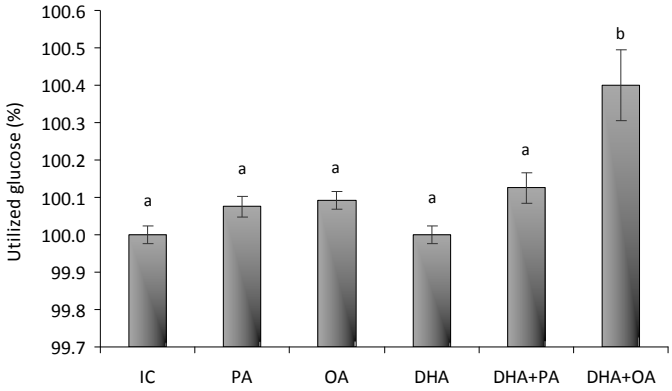

Fig. 4. Utilised glucose (%) by 3T3-L1 MBX. NC: a non-induced, non-treated control; IC: double-induced, non-treated control; PA: double-induced, treated with 100 μM PA; OA; double-induced, treated with 100 μM OA; DHA: double-induced, treated with 100 μM DHA; DHA+PA: double-induced, treated with 50 μM DHA and 50 μM PA (100 μM FFAs in total); DHA+OA: double-induced, treated with 50 μM DHA and 50 μM OA (100 μM FFAs in total). Basal (non-stimulated) glucose uptake was first subtracted from each induced group's values, and then all results are presented relatively as a percentage of the IC (mean±SEM; n=4). The different superscript letters indicate significant differences between groups (P≤0.05).

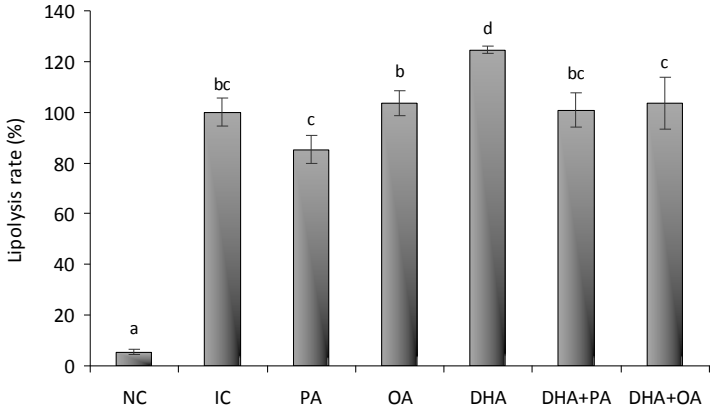

Fig. 5. Lipolysis rate (%) in 3T3-L1 MBX. NC: a non-induced, non-treated control; IC: double-induced, non-treated control; PA: double-induced, treated with 100 μM PA; OA; double-induced, treated with 100 μM OA; DHA: double-induced, treated with 100 μM DHA; DHA+PA: double-induced, treated with 50 μM DHA and 50 μM PA (100 μM FFAs in total); DHA+OA: double-induced, treated with 50 μM DHA and 50 μM OA (100 μM FFAs in total). The percentage of the lipolysis was comparatively evaluated vs IC after basal lipolysis rate deduction, established in NC (mean±SEM, n=4). The different superscript letters indicate significant differences between groups (P≤0.05).

while inhibiting the basal adipocyte lipolysis (Saponaro *et al.*, 2015, Bodis & Roden, 2018). Numerous dietary factors, including FFAs, could modify glucose adipocyte utilisation by modulating insulin sensitivity (Parillo & Riccardi, 2004). It is generally accepted that SFAs impair glucose uptake, while MUFAs and PUFAs improve glucose utilisation (Cao *et al.*, 2008). Therefore, to mimic chronic energy excess, the first group of obese 3T3-L1 was treated with PA, indicated as the most harmful SFA concerning insulin sensitivity and glucose transport (Sokolowska & Błachnio-Zabielska, 2019).

Contrary to our expectations, the PA supplementation did not affect the studied parameters compared to the IC. The calculated glucose uptake by adipocytes was similar in PA and OA groups, and although there was an enhancement of lipolysis in OA compared to the PA group, no statistically significant differences were observed in relation to IC. Sokolowska & Błachnio-Zabielska (2019) indicated that SFA's detrimental effect is a consequence of adipocyte hypertrophy and ceramide accumulation in them, which was closely associated with dysregulation of insulin-stimulated GLUT4 expression and subsequent decrease in glucose uptake. The lack of significant lipid accumulation in the PA group in our experiment could explain the absence of substantial obesity-related adverse metabolic changes in this group. Malodobra-Mazur *et al.* (2019) reported that *in vitro*, PA and OA supplementation affected adipocyte metabolism in a dose-dependent manner. When 3T3-L1 cells were exposed to 500 μM concentration of PA or OA, the insulin signalling pathway was suppressed (Malodobra-Mazur *et al.*, 2019). However, PA applied at a dose of 100 or 200 μM attenuated *de novo* lipogenesis and did not affect GLUT4 gene expression and insulin-sti-

mulated glucose uptake in adipocytes, respectively (Bolsoni-Lopes *et al.*, 2014; Malodobra-Mazur *et al.*, 2019), which could be a possible explanation for our results.

Concerning OA supplementation, we found out that intracellular lipid accumulation was decreased by 10% against the background of preserved glucose utilisation and slightly increased lipolysis compared to IC. In contrast, Tsuchiya *et al.* (2014) established that even at a dose of 1 μM, OA improved insulin receptor signaling via the PIK3/PDK1/Akt/Rac1 pathway resulting in increased GLUT4 expression and intensified translocation to the cell surface. Enhanced insulin sensitivity and subsequently improved lipid accumulation were also found at a dose of 100, 200, 300 μM *in vitro* (Kokta *et al.*, 2008; López-Gómez *et al.*, 2020). It should be emphasised that in most of the studies, OA was applied during the adipogenesis, while in our experiment, it was supplemented on already differentiated adipocytes. Therefore, we may assume that the OA effect strongly depends on the stage of adipocyte maturation. OA supplementation leads to PPARγ activation (Kotka *et al.*, 2008) and increased adiponectin expression (Palomer *et al.*, 2018), potentiating lipid accumulation in early stages of adipogenic differentiation, but some authors emphasised that they may decrease adiposity when applied on already mature adipocytes by activation of key enzymes of β-oxidation (Madsen *et al.*, 2005; Holland *et al.*, 2011), which is in line with our observation. Holland *et al.* (2011) explained that the most likely reason for enhanced β-oxidation was increased ceramide degradation and 5' AMP-activated protein kinase, provoked by adiponectin expression elevation. Increased fat oxidation after increased OA consumption has also been established *in vivo* by Polley *et al.* (2018).

According to the available literature, 100 μM DHA applied in fully differentiated 3T3-L1, or rabbit adipocytes intensified lipolysis by up-regulation of some essential lipolysis-related genes such as ATGL, HSL, and MGL, and simultaneously downregulated lipid droplets associated proteins expression such as PLIN 1 (Barber *et al.*, 2013; Vachkova *et al.*, 2019). Our results showed that DHA treatment alone provoked an increase in glycerol release by 25% and a decrease by 10% in intracellular lipid content compared to IC, which is most likely associated with a significant increase in FFAs efflux from adipocytes.

It is well known that in obesity, there is an exaggerated elevation of lipolysis in adipose tissue, accompanied by intensive FFAs release into the systemic circulation, which is also clearly distinguishable in our study. As a consequence, systemic lipotoxic pro-inflammatory adverse effects could be provoked. Therefore, when taking food supplements in the presence of obesity or along with high predisposing factors of obesity, the focus should be placed on preventing the facilitation of lipolysis and maintaining buffer capacity of adipose tissue rather than simply weight lowering. In this respect, it is questionable whether the observed DHA provoked lipolysis enhancement along with the preserved percentage of glucose utilisation, and decreased TG storage in adipocytes in our experiment could be assumed as a positive result.

Madsen *et al.* (2005) speculated that n-3 PUFA facilitated the process of lipolysis in adipocytes and simultaneously activated β-oxidation in the body. Thus the released FFAs could be metabolised without any adverse effect for the whole

organism. However, previous studies of ours have found that enhanced lipolysis as a result of a combination of n-3 PUFA supplements, and restricted diet substantially increased LDL cholesterol level, despite the decreased body mass index, intensified β-oxidation, and improved insulin sensitivity in obese rabbits (Ivanova *et al.*, 2015; Grigorova *et al.*, 2019). Therefore, a careful approach is needed when already obese individuals consume dietary supplements with a pronounced lipolytic effect because this could be detrimental for the health.

Contrary to our findings, some authors consider that PUFAs sensibilise the insulin signalling pathway and significantly increase glucose and FFAs uptake by adipocytes. The induced adipocyte hypertrophy prevented ectopic lipid deposition and further lipotoxic effects on peripheral tissues (Huang *et al.*, 2017; Yang *et al.*, 2017). PUFAs are the most potent PPARγ and adiponectin activators among all types of FFAs (Madsen *et al.*, 2005; Song *et al.*, 2017). Consequently, they modulate numerous metabolic pathways underlying the above mentioned healthy adipose tissue expansion (Puglisi *et al.*, 2011; Murali *et al.*, 2014; Todorčević & Hodson, 2016). Notably, this effect of n-3 PUFA was found mainly *in vivo* (Rossi *et al.*, 2010; Puglisi *et al.*, 2011; Wójcik *et al.*, 2014), and one of the main reasons could be that dietary fats are not composed only of one type of FFA. None of independent treatments with DHA, OA, or PA has increased adipocyte's glucose utilisation or lipid accumulation in our experiment. However, DHA applied with OA increased glucose utilisation, while co-administration of DHA and PA provoked a 10% increase in intracellular lipid accumulation. Further studies are needed to clarify the intimate mechanism involved in this event.

Taken together, our results revealed that exposing an already induced 3T3-L1 MBX to unsaturated fatty acids only attenuated the intracellular lipid accumulation while maintaining glucose utilisation rate. It should be noted that DHA intensified lipolysis by 25% related to the induced untreated control, which may exert a direct detrimental impact on the metabolic state. However, when DHA was applied with OA at an equal ratio, glucose utilisation was enhanced without any significant changes in the lipogenic and lipolytic rate, suggesting that the combination of these unsaturated fatty acids may offer a considerable advantage in improving insulin sensitivity in adipose tissue and thus, ameliorating obesity-related disorders.

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