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RESEARCH ARTICLE

Effect on Adipose Tissue of Diabetic Mice Supplemented with *n-3* Fatty Acids Extracted from Microalgae

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ARTICLE HISTORY

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Abstract: ***Background:*** Type 2 Diabetes Mellitus (T2DM) is considered a chronic noncommunicable disease in which oxidative stress is expected as a result of hyperglycaemia. One of the most recent approaches is the study of microalgae fatty acids and their possible antioxidant effect.

Objective: This study aimed to analyse the effect of supplementation with *n-3* fatty acids extracted from microalgae on the total antioxidant capacity (TAC) and lipid peroxidation of adipose tissue and plasma from diabetic (db/db) and healthy (CD1) mice.

Methods: Mice were supplemented with lyophilized *n-3* fatty acids extracted from microalgae or added to the diet, from week 8 to 16. TAC assay and Thiobarbituric Acid Reactive Substances assay (TBARS) were performed on adipose tissue and plasma samples.

Results: The supplementation of lyophilized *n-3* fatty acids from microalgae increased the total antioxidant capacity in adipose tissue of diabetic mice (615.67µM Trolox equivalents vs 405.02µM Trolox equivalents from control mice, $p<0.01$) and in the plasma of healthy mice (1132.97±85.75µM Trolox equivalents vs 930.64±32µM Trolox equivalents from modified diet mice, $p<0.01$). There was no significant effect on lipid peroxidation on both strains.

Conclusion: The use of *n-3* fatty acids extracted from microalgae could be a useful strategy to improve total antioxidant capacity in T2DM.

Keywords: *n-3* fatty acids, microalgae, antioxidant, lipid peroxidation, diabetes, adipose tissue.

1. INTRODUCTION

Diabetes mellitus is considered a noncommunicable chronic disease and the leading cause of many health-related complications and premature deaths [1, 2]. According to the World Health Organization, in 2014, 422 million adults worldwide had diabetes [3]. In Mexico, the prevalence of diabetes mellitus amounts to 9.4% in the adult population according to data from the Mexico National Survey of Health and Nutrition Mid-way 2016 (ENSANUT 2016 MC for its acronym in Spanish) [4]. This disease is characterized by a state of chronic oxidative stress due to the continuous condition of hyperglycaemia [5-7].

Oxidative reactions are essential in most cellular metabolic processes [8]. Oxidative stress is the excessive formation or insufficient elimination of highly reactive molecules called reactive oxygen species (ROS) and reactive nitrogen species (RNS) [9]. In diabetes, the accumulation of advanced glycation end products (AGES), the activation of the sorbitol and hexosamine pathways, as well as various pathways mediated by protein kinases C, result in increased oxidative stress [10-12]. This oxidative stress imbalance may lead to cellular damage on several macromolecules such as lipids, proteins and DNA [13, 14]. Lipids are the main target of free radicals, causing lipid peroxidation; this occurs when free radicals attack fatty acids that contain carbon double bonds, especially polyunsaturated fatty acids (PUFAs) [13, 15]. The damage lies in the modification of the physical and chemical properties of the cell membrane, resulting in the alteration of function, oedema and cell death [14, 16, 17]. The most studied sub-product of lipid peroxidation is Malondialdehyde (MDA) [18, 19]. However, on a regular basis, enzymatic and non-enzymatic antioxidant mechanisms are capable of minimizing the damage caused by oxidative stress [20, 21].

N-3 fatty acids are known because of their wide range of biological effects, among which are benefits to lipoprotein metabolism, platelet, endothelial and vascular function, as well as their anti-inflammatory and antioxidant potential [22-25]. Therefore, the consumption of *n-3* fatty acids, especially EPA

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and DHA, has been shown to have beneficial effects against chronic diseases such as diabetes mellitus in which oxidative stress is present [26]. Traditionally, the main source of these fatty acids has been fish oil [27]; however, among the disadvantages of its use, we find that exploitation in the fishing industry has reached the sustainable limits. In addition, the contamination by heavy metals and pesticides has become a major problem [28, 29]. Other disadvantages of the use of these sources are undesirable nutritional and organoleptic effects, such as oxidation (due to their high polyunsaturation) and the characteristic odor of the product [30]. A new focus on biotechnological advances is the use of microalgae as an alternative source of polyunsaturated fatty acids. Microalgae are an evolutionarily microscopic diverse eukaryotic group of unicellular and predominantly aquatic photosynthetic organisms [31]. They are the primary natural producers of EPA and DHA, because they have the biosynthetic machinery to sequentially alternate between desaturation and elongation in their carbon chains [32]. However, there are fewer studies that describe their antioxidant effects as those already described for fatty acids of animal origin [31, 33, 34].

The aim of this study was to analyse the effect of the consumption of *n-3* fatty acids extracted from two families of microalgae (*Chlorophyceae* and *Eustigamatophyceae*) provided either as a supplement or incorporated to the diet, on the total antioxidant capacity and lipid peroxidation in adipose tissue of diabetic mice.

2. METHODS

2.1. Animals

The present study is an experimental, prospective, controlled and randomized study. Forty-eight 8-week old male mice from two different strains were used: 24 diabetic mice (db/db mice BKS.Cg-+Leprdb+LeprdbOlaHsd Harlan®) and 24 healthy mice (CD1 mice. Crl: CD1 [ICR] Universidad Autónoma del Estado de México, UAEMex). Animal care and experimental procedures were carried out in accordance with the standards of the guidelines of the Mexican Ministry of Health for the Production and Care of Laboratory Animals (NOM-062-ZOO-1999 Ministry of Agriculture, Mexico City, Mexico) and the Internal Regulations for the Use of Laboratory Animals and the Committee of Ethics in Research of the UAEMex.

Animals were housed in acrylic cages, three mice per cage. food and water were offered *ad libitum* during the entire experiment and all animals were maintained on a 12/12 h light/dark cycle.

2.2. Experimental Groups

For both strains, mice were randomly assigned to one of the five study groups: 1) A Rodent Chow group (RC); 2) a group supplemented with lyophilized microalgae *n-3* fatty acids (LY); 3) a group supplemented with coconut oil group (CO); 4) a modified diet group with *n-3* fatty acids from microalgae incorporated in the chow (MD). Groups 1, 2, and 3 were fed a standard normal diet (Rodent Laboratory Chow 5001 from Purina® [12.63 kJ/g]). The experimental study was conducted from the 8th to the 16th week of life.

2.3. Supplementation

a) The LY group was fed with a standard rodent diet and supplemented with lyophilized powder containing EPA+DHA extracted from microalgae (*Chlorophyceae* and *Eustigamatophyceae* families) at a dosage of 1mg/g of mouse weight; the powder was reconstituted in 10uL of distilled water and administered by direct oral gavage with a micropipette at 8:00 am every day.

b) The CO group was also fed with a standard rodent diet and supplemented with coconut oil (C1758-500 Sigma Aldrich®) at a dosage of 1mg/g of mouse weight administered by direct oral gavage with a micropipette at 8:00 am every day.

c) The MD group was fed with a rodent chow enriched with microalgae EPA+DHA for a total content of 2.0% n-3 fatty acid meaning 10x of the original content. Chow was administered ad libitum.

The EPA and DHA contents of the diet are shown in Supplementary Table **1**.

2.4. Collection of Biological Samples

The sacrifice of animals was conducted in the 16th week of life of all treatment groups. Anaesthesia was given using an ether chamber; afterward, animals were bled by direct cardiac puncture to obtain 1mL of blood and then sacrificed by cervical dislocation. Blood was centrifuged at 2500rpm for 15min, plasma was collected and stored in 1.5mL microtubes at -80°C until used. 200mg of the mesenteric adipose tissue was removed and stored into 1.5mL microtubes at -80° C until used.

2.5. Sample Preparation

Plasma samples were assayed directly. The adipose tissue samples were homogenized with 1xPBS at 12,000 rpm for 1 minute per sample. The homogenization equipment was a rotary Dragon Lab brand(R) homogenizer used for the preparation of samples. Afterward, samples were centrifuged at 14,000rpm for 10 minutes to form pellets and remove any debris. Finally, the supernatant was used for the determination of antioxidant capacity and lipid peroxidation.

2.6. Total Antioxidant Capacity Determination (TAC)

One common way to calculate the total antioxidant capacity (TAC) of an organism is through assays based on the reduction of metals such as copper and iron. For this study, a quantitative colorimetric determination of total antioxidant capacity assay kit was used (QuantiChrom™ Antioxidant Assay Kit), the procedure was performed following manufacturer´s instructions and samples were read in a spectrophotometer at an optical density (OD) of 570nm.

2.7. Lipid Peroxidation Determination

A common method for the detection of MDA is the thiobarbituric acid reactive substances fluorometric assay (TBARS) in which MDA reacts with thiobarbituric acid to give colorful products that can be read in a colorimetric plate

reader. For this purpose, a commercial kit (QuantiChrom™ TBARS Assay Kit) was used and the manufacturer's specifications were followed according to the colorimetric test instructions. Finally, the samples were read in a spectrophotometer at an OD of 535nm.

2.8. Blood Glucose, Body Mass Index, Food and Water Intake

Blood glucose was measured with a Bayer Contour TS glucometer through tail puncture at the beginning and end of the study.

The body mass index (BMI) was calculated weekly, using the weight and length of each mouse. Mice were weighed weekly until sacrifice, on a Triple Beam 700/800 Series mouse scale (Ohaus Cat. No. 2,729,439). The length was measured under anaesthesia (0.1 mL of sodium pentobarbital at 1%) with a tape from the nose to the anus. BMI was calculated with the following formula: BMI = Weight (g)/length (cm2) [35].

Food and water intake was recorded at the beginning and end of the study.

2.9 Statistical Analysis

For parametric values, One-way ANOVA was performed for comparison between the groups from each strain (MD, LY, SAT and CO), Bonferroni post hoc was applied. For non-parametric values, the Kruskal-Wallis H test was performed. Two-way ANOVA was run to compare mice and treatment groups. Differences were considered significant at $p<0.05$. The software used to run statistical analysis was SPSS v.23 for Windows.

3. RESULTS

3.1. Food and Water Intake, BMI and Glucose Values

With respect to food intake, all Db/db mice consumed less than the CD1 groups. The MD was the lowest food consuming group and the LY was the highest ($p<0.05$) in the Db/db mice. Food intake in CD1 mice was only found to be increased in the CO group. Water intake was higher in the MD Db/db and in the LY and CO CD1 groups.

In the Db/db mice, the MD group had the highest BMI, their blood glucose levels were all higher than the CD1 mice with the highest value in the CO group. BMI in the CD1 mice was found to be significantly ($p<0.05$) increased in the LY and CO groups. Blood glucose was lower in the CO group, with no significant differences between treatment groups (Table **1**).

When analysing both CD1 and Db/db groups by two-way ANOVA, we found differences between treatment groups in BMI (F1=.028, p=0.384, Bonferroni=NS); blood glucose (F=0.179, p=0.910, Bonferroni=NS); food intake (F=0.3003, p=0.035, Bonferroni: CL vs MD= 0.007, CL vs CO= 0.031, LY vs MD=0.000, co VS MD= 0.000), and water intake (F=5.944, p=0.001, Bonferroni: MD vs LY= 0.024, MD vs CO= 0.027).

3.2. Total Antioxidant Capacity in Adipose Tissue and Plasma of Diabetic Mice

In adipose tissue, the LY group showed a higher antioxidant capacity than the RC and CO group. On the other hand, the CO group had a significantly lower antioxidant capacity compared to LY and RC (post hoc $p<0.05$). In plasma, LY group showed higher antioxidant capacity than the CO

Table 1. Food and water intake, BMI and glucose of Db/db and CD1 mice during the study.

	RC	MD	LY	CO	F (p)	Bonferroni (p<0.05)
	n=6	n=6	n=6	n=6		
Db/db mice						
Food intake, *g/week*	32.92±0.34	27.68±0.97	37.50±0.77	36.30±2.41	31.166 (**0.000**)	RC vs MD, LY vs MD, CO vs MD
Water intake, *mL/week*	78.25±7.18	81.39±10.40	79.61±1.81	67.23±3.41	1.305 (0.285)	NS
BMI, g/cm^2	61.27±2.46	65.05±1.67	62.22±1.52	59.66±2.34	0.768 (0.518)	NS
Glucose, *mg/dL*	551.83±83.66	505.00±73.95	525.50±50.93	580.67±22.01*	0.122 (0.947)	NS
CD1 mice						
Food intake, *g/week*	58.34±5.60	42.75±4.98	60.96±1.91	73.3±20.81	7.415 (**0.000**)	LY vs MD, CO vs MD
Water intake, *mL/week*	67.28±3.23	53.91±1.32	89.78±6.10	93.25±4.77	9.367 (**0.000**)	CO vs MD, LY vs MD
BMI, g/cm^2	31.40±1.95	32.18±3.09	34.44±4.11	34.27±2.41	0.728 (0.541)	NS
Glucose, *mg/dL*	119.33±12.97	127.50±18.68	109.67±13.31	106.83±12.40	0.891 (0.891)	NS

RC: Rodent Chow; MD: RC+EPA & DHA in chow; LY: RC + lyophilized EPA & DHA.
Data are presented as means ± SD. One-way ANOVA for comparison of differences between BL and all groups.
*p value was significant at <0.05. NS: Non-signficant.

group. Finally, the CO group showed a lower antioxidant capacity than the RC group (post hoc p <0.05) (Table **2**).

3.3. Lipid Peroxidation in Adipose Tissue and Plasma of Diabetic Mice

Lipid peroxidation in both tissues we studied showed no significant differences between treatments in the Db/db mice.

3.4. Total Antioxidant Capacity in Adipose Tissue and Plasma of Healthy Mice

In adipose tissue, the CO group had the lowest antioxidant capacity, while the LY showed the highest values, although these differences were not significant. Plasma total antioxidant capacity showed a similar but statistically significant pattern, with the highest values in the LY group (Table **2**).

3.5. Lipid Peroxidation in Adipose Tissue and Plasma of Healthy Mice

Although lipid peroxidation in adipose tissue and plasma in the MD group showed a tendency to be higher than the other groups, no significant differences were found between treatment groups (Table **2**).

When comparing Db/db and CD1 mice by a two-way ANOVA analysis, we found significant differences in the antioxidant capacity in adipose tissue (F=0.808, p=0.493, Bonferroni: LY vs CO= 0.002); antioxidant capacity in plasma (F= 0.642, p=0.590, Bonferroni: CL vs CO= 0.000, MD vs LY= 0.038, MD vs CO= 0.002, CO vs LY= 0.000); lipid peroxidation in adipose tissue (F= 1.044, p=0.377, Bonferroni=NS), and lipid peroxidation in plasma (F= 0.735, p= 0.534, Bonferroni=NS).

4. DISCUSSION

The results of this study show that the consumption of *n-3* fatty acids from microalgae administered in the form of a supplement has beneficial effects on the total antioxidant capacity in adipose tissue of diabetic mice and in the plasma of healthy mice, as these values did not decrease significantly in comparison with the group consuming saturated fat.

Our results on BMI are different from those reported in C57B1/6 mice, where fish-oil supplementation caused no changes [36]. With respect to blood glucose, Kalupahana *et al*., found that EPA supplementation protects glucose

Table 2. Effect of consumption of EPA and DHA fatty acids extracted from microalgae on the Antioxidant capacity and lipoperoxidation.

-	-	RC	MD	LY	CO	F (p)	Bonferroni (p<0.05)
	-	n=6	n=6	n=6	n=6	-	-
Db/db mice							
Antioxidant capacity *µM Trolox equivalents*	Adipose Tissue+	405.02	473.59	615.67	332.84	--- (**0.001**+)	RC vs LY, CO vs LY
	Plasma++	706.90± 22.14	639.20± 18.80	730.35± 41.00	379.27± 35.60	9.341 (**0.000**)	RC vs CO, MD vs CO, LY vs CO
Lipid peroxidation *µM MDA equivalents*	Adipose Tissue++	0.43± 0.07	0.44± 0.03	0.45± 0.08	0.60± 0.04	2.624 (0.062)	NS
	Plasma	0.08± 0.01	0.08± 0.01	0.08± 0.01	0.10± 0.01	1.437 (0.245)	NS
CD1 mice							
Antioxidant capacity, *µM Trolox equivalents*	Adipose Tissue++	487.31± 64.43	424.32± 65.67	545.48± 81.35	378.81± 53.84	0.883 (0.457)	NS
	Plasma++	1103.49± 79.78	930.64± 32.23	1132.97± 85.75	804.12± 26.29	8.679 (**0.000**)	RC vs CO, LY vs CO
Lipid peroxidation, *µM MDA Equivalents*	Adipose Tissue++	0.07± 0.01	0.08± 0.01	0.06± 0.01	0.07± 0.01	0.171 (0.915)	NS
	Plasma++	0.42± 0.11	0.46± 0.07	0.44± 0.02	0.46± 0.11	1.046 (0-382)	NS

+ Data are presented as median (p50); Kruskal-Wallis H Test as data were not normally distributed.
++ Data are presented as means ± SD; One-way ANOVA for comparison of differences between all groups.
NS: Non-significant.

metabolism in C57B1/6 mice [37]. The lower intake of food in our Db/db mice may be explained by the fact that *n*-3 supplementation may induce a modification in adiponectin production thus regulating food intake [38]. Additionally, Shklyaev *et al*., [39] showed that an increase in adiponectin in Sprague-Dawley rats promoted a reduction in food intake and body weight, however, the effect on our db/db mouse model may have been different particularly with respect to weight gain due to the metabolic characteristics of these animals. Another explanation for the differences in food intake may be that the MD diet may have had a more satiating effect than the rodent chow alone or with the addition of LY or CO by oral gavage.

Several studies have previously shown the close relationship between obesity, hyperglycaemia states and the generation of reactive oxygen species [40-43] as well as the association with low levels of antioxidants [44, 45] and increased lipid peroxidation [46, 47].

The determination of total antioxidant capacity in tissue and plasma is of great importance, since it gives a more complete measure of the antioxidant mechanisms present in the organism [45]. TBARS assay for lipid peroxidation applied on different types of samples has also been very useful [48-52]. However, to date, there are few studies that focused on the analysis of the antioxidant effect of the consumption of a specific nutrient [53].

The effect of supplementation of *n-3* fatty acids from fish oil on oxidative stress has shown inconclusive results. A study on patients with Alzheimer's disease, who were supplemented with n-3 fish oil for 6 months, showed that it had no effect on the modulation of the formation of free radicals [54]. However, a study on patients with a high risk of psychosis showed that the antioxidant effect of *n-3* fatty acids only occurred significantly when they were supplemented with vitamin E together with *n-3* fatty acids [55]. Also, a study conducted by Di Nunzio *et al*., showed that supplementation with polyunsaturated fatty acids except for DHA, increased cellular susceptibility to oxidative stress [56]. On the other hand, a study carried out on Wistar rats, showed that the consumption of EPA and DHA in different proportions had a positive effect on inflammatory markers and oxidative stress [57].

Regarding the use of microalgae as sources of *n-3* fatty acids and their antioxidant effect, currently, the evidence is limited, and most studies have focused on the first analysis of the content and their possible antioxidant capacity [58, 59]. A study conducted by Maadane *et al.,* demonstrated the antioxidant capacity of the microalgae *Nannochloropsis gaditana* and *Chlorella sp*. using the DPPH radical scavenger assay [60]. It is important to mention that these species belong to the same family of microalgae used in this study.

As for studies conducted on animal models for determining the antioxidant effect of *n-3* fatty acids from microalgae; Haimeur *et al.,* demonstrated that the supplementation with fatty acids of microalgae improved oxidative state by decreasing the production of MDA, increasing the concentrations of glutathione peroxidase in the liver and platelets [61].

Our study did not show a significant effect in the group fed with modified diet (MD); however, a study conducted in broilers fed with corn and soybean enriched with microalgae biomass (*Nannochloropsis oceanica*, belonging to the *Eustigamatophiceae* family) at several doses, showed that the liver, plasma, breast and thighs of chickens were high in EPA and DHA, suggesting that microalgae can be used to produce chicken meat enriched with *n-3* fatty acids [62].

In our study, lipid peroxidation was not affected by supplementation or consumption of *n-3* from microalgae, however, the group supplemented with coconut oil (CO) showed significantly higher concentrations of MDA in diabetic mice. The above findings do not agree with the studies by Narayanankutty *et al.,* and Alves *et al.,* in which they show that supplementation with coconut oil decreases the serum MDA concentrations in rats fed with a high-fructose diet and hypertensive Wistar rats [63, 64]. We think that this difference in results is explained by the time of exposure and the dose administered to the experimental animals; while we administered an established dose of pure coconut oil orally for 8 weeks, the other studies used coconut oil added into modified diets, which may lead to variable intake of coconut oil and for a period of 4 weeks.

CONCLUSION

The present study concludes that the use of *n-3* fatty acids extracted from microalgae could be a useful strategy to combat oxidative stress in chronic diseases such as diabetes mellitus by increasing the total antioxidant capacity, however, more studies are necessary.

LIST OF ABBREVIATIONS

AGES	=	Advanced Glycation End Products
BMI	=	Body Mass Index
DHA	=	Docosahexaenoic Acid
ENSANUT 2016 M	=	Encuesta Nacional de Salud y Nutrición Medio Camino 2016 (Mexican National Survey of Health and Nutrition Mid-way 2016)
EPA	=	Eicosapentaenoic Acid
MDA	=	Malondialdehyde
OD	=	Optical Density
PUFAs	=	Polyunsaturated Fatty Acids
RNS	=	Reactive Nitrogen Species
ROS	=	Reactive Oxygen Species
TAC	=	Total Antioxidant Capacity Determination
TBARS	=	Thiobarbituric Acid Reactive Substances Fluorometric Assay
UAEMex	=	Universidad Autónoma del Estado de México (Autonomous University of the State of Mexico)

AUTHORS' CONTRIBUTIONS

Gutierrez-Pliego LE performed the supplementation protocol, collection of samples, and the statistical analysis and also wrote the paper.

Martínez-Carrillo BE designed the research, collected data, performed the statistical analysis and wrote the paper.

Resendiz-Albor AA analysed data and wrote the paper.

Valdés-Ramos R designed the research, performed the statistical analysis and wrote the paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Research Committee of the School of Medicine of the Universidad Autónoma del Estado de México in Toluca, Mexico (Protocol Number: UAEM 4507/2018/CI).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. Animal care and experimental procedures were carried out in accordance with the guidelines of the Mexican Ministry of Health for the Production and Care of Laboratory Animals (NOM-062-ZOO-1999 Ministry of Agriculture, Mexico City, Mexico) and the Internal Regulations for the Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author, [RVR], on reasonable request.

CONFLICT OF INTERESTS

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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