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Supplementation with n-3, n-6, n-9 fatty acids in an insulin-resistance animal model: does it improve VLDL quality?

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Insulin-resistance (IR), of increased cardiovascular risk, is characterized by the production of altered VLDL with greater atherogenicity. Dietary fatty acids influence the type of circulating VLDL. But, it is not clear how dietary fatty acids impact VLDL characteristics in IR. Aim: to evaluate the effects of n-3, n-6 and n-9 fatty acid supplementation on preventing atherogenic alterations in VLDL, in a diet-induced IR rat model. Male Wistar rats (180–200 g) were fed: standard diet (control, n = 8) and a sucrose rich diet (30% sucrose in water/12 weeks, SRD; n = 24). Simultaneously, SRD was subdivided into SRD-C (standard diet), and three other groups supplemented (15% w/w) with: fish oil (SRD-n3), sunflower oil (SRD-n6) and high oleic sunflower oil (SRD-n9). Lipid profile, free fatty acids, glucose, and insulin were measured. Isolated VLDL (d < 1.006 g ml^{-1}) was characterized by chemical composition and size (size exclusion-HPLC). In comparison with SRD-C: SRD-n3 showed an improved lipoprotein profile (p < 0.01), with lower levels of insulin and HOMA-IR (p < 0.05). SRD-n6 showed increased levels of HDL-cholesterol and lower insulin levels. SRD-n9 did not exhibit differences in lipid and IR profile, and even favored weight gain and visceral fat. Only SRD-n3 prevented the alterations in VLDL-TG% (54.2 ± 4.4% *vs.* 68.6 ± 8.2, p < 0.05) and showed lower large VLDL-% (22.5[19.7–35.6] *vs.* 49.1[15.5–82.0], p < 0.05), while SRD-n6 and SRD-n9 did not show effects. *Conclusion*: In IR, while n-3 PUFA showed expected favorable effects, supplementation with n-6 PUFA and n-9 MUFA did not prevent atherogenic alterations of VLDL. Thus, the recommendations of supplementation with these fatty acids in general diet should be revised.

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1. Introduction

The prevalence of insulin-resistance (IR) is still increasing around the world. Nowadays IR affects a large number of individuals, being closely related to a higher risk of cardiovascular disease (CVD). Dyslipidemia in the IR state is characterized by elevated fasting triglycerides, altered VLDL and low HDL cholesterol levels.[1] In this context, it has been demonstrated that modifications in VLDL characteristics could increase its atherogenic capacity.[2]

Nutritional and lifestyle modifications are the first steps for the treatment of dyslipidemia, in order to reduce CVD risk. It is generally accepted that the quantity and composition of dietary fat has an impact on plasma lipoprotein concentrations, and surely, on cardiovascular morbidity and mortality.[3]

Current dietary recommendations for general population focus on fatty acids and include the reductions in saturated (SFA) and trans-fatty acids, and emphasize the consumption of mono- and poly-unsaturated fatty acids (MUFA, PUFA).[4] However, there are considerable controversies about the biological responses to dietary fatty acids in IR[5–8] and little is known about their effect on the VLDL characteristics.

Compelling evidence showed that n-3 PUFA decreases plasma triglyceride levels by the reduction of hepatic VLDL synthesis and secretion on the one hand, and also increases the catabolic rate of VLDL by lipoprotein-lipase on the other.[9–11] However, the role of n-9 and n-6 fatty acid series on plasma triglycerides and VLDL is not as clear as for n-3 PUFA.

Regarding the potential beneficial effects of n-9 MUFA on CVD, most of the published studies have been conducted

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using olive oil as the source of fat.[12,13] Even though olive oil is one of the main dietary sources of oleic acid, nowadays high oleic sunflower oil (HOSO; 83% 18: 1 n-9 *cis* and 9% 18: 2 n-6), obtained from hybrid varieties of sunflower, is used to replace saturated and trans fats in the processing of packaged and fried food.[14]

Given the similar composition of both oils, similar beneficial effect on CVD is expected. However, it should be noted that olive oil and HOSO differ in the quantity and quality of sterols and other non-nutritional components, which might determine different biological effects.[14]

Moreover, most studies having addressed the effect of n-6 PUFA supplementation, applied n-6/n-3 together. Only a few studies have assessed the specific influence of dietary n-6 PUFA on IR so far.[5,15]

In previous reports, we observed in IR rats, fed a sucrose rich diet, an increased secretion of larger and triglyceride over-enriched VLDL particles, despite concomitant triglyceride deposits in the liver.[16,17] In addition, we confirmed the predominance of larger VLDL sub-fractions in patients with metabolic syndrome, by implementing size exclusion HPLC.[18] Notably, these kinds of VLDL particles exert endothelial dysfunction.[19,20]

Thus, to our knowledge no studies have addressed the impact of dietary n-3, n-6 and n-9 fatty acids on VLDL composition and size in the same IR model. In this study, we aimed to evaluate the effects of supplementation with EPA and DHA-rich deepwater fish oil (n-3 series), linoleic acid-rich sunflower oil (n-6 series) and oleic acid-rich high oleic sunflower oil (n-9 series) on preventing atherogenic alterations in VLDL, in a diet-induced insulin-resistance rat model.

2. Methods

2.1. Animals and diets

Male Wistar rats, weighing 180–200 g, were used in the present study. Animals were obtained from the animal laboratory of the Department of Biochemistry, Facultad de Odontología, Universidad de Buenos Aires (Buenos Aires, Argentina), and maintained at 23–25 °C with a 12 h light/12 h dark cycle. Rats received *ad libitum* standard chow diet and water during the experimental period. The present study was carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals,[21] and was approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

Diets were supplemented with different fat sources: with deepwater fish oil (EPA and DHA-rich oil, omega-3 series), sunflower oil (linoleic acid-rich oil, omega-6 series) and with high oleic sunflower oil (HOSO, oleic acid-rich oil, omega-9 series). For diet preparation, standard rodent chow pellets were powdered and mixed with the oils and re-pelleted to contain 15% w/w of each respective oil. The fat amounts were selected considering that 15% of fat supplementation represents about 35% of total kcal in the final diet, which is consistent with the Western diets.[22] Macronutrient composition of the standard diet and the fatty acid composition of fish oil (Parafarm, Saporiti SACIFIA, Buenos Aires, Argentina), sunflower oil (Molinos Río de la Plata, Buenos Aires, Argentina) and HOSO (Ecoop, Cooperativa Obrera, Bahia Blanca, Argentina) are described in Table 1. Feeds were prepared weekly and packed in individual plastic sealed bags in quantities sufficient for one-day feed and kept at −20 °C. Diets were supplied daily and food containers were cleaned before

Table 1 Compositions of standard and oil supplemented diets

Ingredients (g per 100 g)	C	SRD-C	SRD-n3	SRD-n6	SRD-n9
Carbohydrates	44	44	38	38	38
Protein	23	23	14	14	14
Fat	5	5	3	3	3
Oil supplements					
Fish oil			12		
Sunflower oil				12	
High oleic sunflower oil (HOSO)					12
Total fat	5	5	15	15	15
Fat composition					
Total saturated fatty acids	2.1	2.1	4.3	2.3	2.1
Polyunsaturated fatty acids, n-3[a]	—	—	**5.8**	—	—
Polyunsaturated fatty acids, n-6	1.4	1.4	—	**8.2**	1.4
Total monounsaturated fatty acids, n-9	1.5	1.5	4.9	4.5	**11.5**
Fiber	6	6	4	4	4
Ash	9	9	5	5	5
Vitamin mixture	1.0	1.0	0.6	0.6	0.6
Water	12	12	24	24	24
Total kJ	1310	1310	1435	1435	1435

C: control, standard diet consisted of commercially available pellets (Purina chow). Oil-supplemented diets were prepared by adding each respective oil in the control diet. SRD: sucrose rich diet; n-3: deepwater fish oil; n-6: sunflower oil; n-9: high oleic sunflower oil. [a] EPA: 3.50 g%, DHA: 2.34 g%.

being refilled. Food cups were refilled once a day, and food consumption was measured with a Mettler scale PC 4000 (accuracy61 mg). Daily food intake was recorded as kcal per 100 g of body weight per day (kcal per 100 g W per day). Body weight was monitored weekly throughout the experimental period. Eventual dehydration in animals was checked by plasma sodium measurement at the end of the experiment.

2.2. Experimental design

At the beginning of the experiment, animals were divided into two groups: control (C; $n = 8$), receiving standard diet and the other group under a sucrose rich diet (SRD; $n = 32$), receiving 30% sucrose in drinking water for 12 weeks. During the time of the experiment, SRD animals developed an early insulin resistant state.[16] Since the start of the experimental period, the SRD group was subdivided into four groups supplemented with different fat sources, designated as follows: SRD-C (standard diet), SRD-n3 (standard diet + 15% w/w fish oil), SRD-n6 (standard diet + 15% w/w sunflower oil) and SRD-n9 (standard diet + 15% w/w HOSO oil). The oil-supplemented diets were administered throughout the 12 weeks, in parallel to the SRD.

2.3. Samples

After 12 weeks of diet intervention, food and water were removed at the end of the dark period (7:00 AM). After 4 h of fasting, animals were anesthetized with an intraperitoneal injection of pentobarbital (60 mg per kg body weight), blood samples were obtained by cardiac puncture and rapidly centrifuged at 1500*g* for 15 min. Serum samples were stored at 4 °C for all assessments within the next 48 h.

Liver was removed immediately, weighed and preserved for further analysis. One piece of liver was kept at −70 °C for the assessment of liver lipid content, and another liver specimen was placed in 10% formalin for histological evaluation. Epididymal, perirenal and intestinal fat tissues were also removed and weighed in order to evaluate visceral adiposity.

2.4. Biochemical determinations

Glucose, total cholesterol, and triglycerides were measured by commercial enzymatic kits and HDL-cholesterol was assessed by standardized homogeneous method (Roche Diagnostics GmbH, Mannheim, Germany), in a Cobas c501 autoanalyzer. In order to evaluate atherogenic lipoproteins, non-HDL cholesterol was calculated. Free fatty acids (FFA) were determined by an enzymatic colorimetric method (Randox, UK), and insulin was measured by a sandwich enzyme-linked immunosorbent assay kit using a monoclonal antibody against rat insulin and a polyclonal antibody linked to the enzyme (Rat/Mouse ELISA kit, Linco Research, USA). With the purpose of estimating insulin-resistance, the homeostasis model assessment for insulin resistance index (HOMA-IR) was calculated.[23] All measurements were under good quality control.

2.5. Isolation and composition of VLDL

VLDL was isolated by preparative ultracentrifugation at density $d < 1.006$ g mL^{-1} in a Beckman XL-90 using a fixed-angle rotor type 90 Ti. Each run was performed at 105 000*g*, for 18 h, at 14 °C.[24] Purity of lipoprotein was tested by agarose gel electrophoresis. Isolated VLDL composition was characterized by the following parameters: cholesterol and triglyceride levels, using the methods previously described and phospholipid[25] and protein levels by the Lowry method. Data was expressed as the percentage of each component.

Afterwards, an aliquot of isolated VLDL was subjected to size exclusion chromatography by HPLC, as was previously reported.[18] Briefly, samples were injected into a column TSK-Gel Lipopropack XL, 7.8 mm ID × 30 cm (Tosoh, Japan). Tris acetate/acetate buffer (pH 8) was used as a mobile phase. The flow rate was 0.5 mL min^{-1} and the eluate was monitored at 280 nm. For the conversion of elution time in particle diameter, a standard curve was implemented with size standard latex particles [100 nm (Fluka, Sigma-Aldrich), 39 and 27 nm in diameter (Magsphere INC)].

From chromatograms we could recognize a peak at 9.95 ± 0.10 min with a diameter of 90 ± 3.0 nm, compatible with very large VLDL and chylomicron remnants, another peak at 12.46 ± 0.48 min with an average diameter of 60.0 ± 3.6 nm, corresponding to large VLDL, a majority peak at 22.35 ± 0.05 min and a diameter of 37.3 ± 0.08 nm identified as typical VLDL and finally smaller peaks were detected at longer retention times (from 24 to 32 min) and sizes about 35 to 30 nm, as VLDL remnants. Results are expressed as the percentage of each peak area with respect to total area, using the ChromQuest 4.1 integration program.

2.6. Measurement of liver fat content

Hepatic lipid content was determined by a Folch extraction and evaporation to dryness followed by gravimetric measurement.[26] Pieces of liver were weighed and homogenized with 30 vol of chloroform : methanol (2 : 1). After standing overnight at room temperature, the homogenate was filtrated and partition was performed in a separatory funnel by adding 0.2 vol of 0.05 N aqueous NaCl solution. When the two phases were completely separated, the lower fraction containing the lipids dissolved in it was collected. Anhydrite $CaCl_2$ was added in order to remove water vestiges and once again filtrated to eliminate the salt. The filtrated organic phase was taken to dryness in a rotavapor at 45 °C. The residue was weighed and the lipid content was expressed as weight/weight.

2.7. Histological evaluation

Liver histological examination by light microscopy was performed in a blinded manner. Hepatic tissue samples were fixed overnight, at room temperature in formalin buffer 10%, pH 7 with 0.1 M phosphate-buffered saline. Samples were dehydrated in ethanol, embedded in paraffin wax, and cut with a microtome. The resulting 5 micro-sections were stained with hematoxylin and eosin reagent for evaluation of steatosis. The steatosis pattern was expressed with the presence of macro- and micro-vesicular fat deposits.[27]

2.8. Statistics

All data were expressed as mean ± SD or median and range, depending on data distribution. Statistical analysis was performed with SPSS software version 21.0. The data were subjected to one-way ANOVA, followed by Dunnet's multiple comparisons test or, when appropriate, the statistical significance of differences was determined by a Student's t test. Histological information was analyzed by Fisher's exact test. Correlations between variables were assessed using a Pearson test. P values below 0.05 were considered statistically significant.

3. Results

There were no differences in calorie intake between C and SRD-C (Table 2). In the fatty acids supplemented groups, food intake was increased in all groups in comparison with SRD-C ($p < 0.01$). However, water intake was decreased in SRD-n6, increased in SRD-n9, and unchanged in SRD-n3. Transforming food and water consumption into calories, energy intake was increased in SRD-n3 and SRD-n9 in comparison with SRD-C, but not in SRD-n6.

Control, SRD-C, SRD-n3, SRD-n6 groups exhibited similar weight gain throughout the treatment period; however, SRD-n9 presented an increased body weight gain compared to other groups ($p < 0.01$), Table 2.

Serum metabolic status of all studied groups can be appreciated in Table 3.

When compared to C, as expected, rats on SRD-C showed an abnormal lipid–lipoprotein profile compatible with IR. Furthermore, SRD-C presented higher FFA, insulin levels and HOMA-IR ($p < 0.05$), without differences in glucose concentrations. Although there were no differences in body weight gain, SRD-C showed an increase of visceral adipose tissue ($p < 0.05$), and also an increase in liver weight ($p < 0.05$). Besides, liver fat content was significantly increased in SRD-C rats ($p < 0.01$) and the histological study revealed moderate micro- and macrovesicular steatosis, $p = 0.0037$ (Chi^2 test), compatible with hepatic steatosis (Fig. 1A and B).

When evaluating the influence of fatty acid supplementation on the IR status, in comparison with those IR rats without supplementation (SRD-C), SRD-n3 showed decreased levels of triglycerides, non-HDL cholesterol and FFA ($p < 0.01$) whereas HDL cholesterol was increased ($p < 0.05$). In addition, insulin levels ($p < 0.05$) and HOMA-IR ($p < 0.01$) were lower, Table 3. SRD-n6 did not show changes in triglyceride and non-HDL cholesterol levels however, showed increased HDL-cholesterol levels. In this group insulin levels were lower in comparison with SRD-C, although HOMA-IR and FFA were unchanged. SRD-n9 did not exhibit any difference in the lipid profile, FFA, insulin and HOMA-IR when compared to SRD-C.

Table 2 Dietary intakes and body weight in groups throughout the experimental period

Variable	C ($n = 8$)	SRD-C ($n = 8$)	SRD-n3 ($n = 8$)	SRD-n6 ($n = 8$)	SRD-n9 ($n = 8$)
Food intake, g per day per 100 g b.w.	6.0 ± 2.6	2.9 ± 1.4[a]	4.9 ± 2.0[b]	4.7 ± 2.2[b]	6.0 ± 2.3[b]
Water intake, ml per day per 100 g b.w.	9.6 ± 1.7	11.7 ± 2.4[a]	10.1 ± 1.8	8.9 ± 2.3[c]	13.6 ± 5.9[c]
Total calorie intake, kJ per day per 100 g b.w.	85.7 ± 10.2	96.4 ± 6.9	126.9 ± 10.9[c]	117.6 ± 11.7	159.7 ± 25.3[b]
Body weight gain	238 ± 27	255 ± 30	236 ± 15	262 ± 41	307 ± 35[b]

Data is expressed as mean ± SD. b.w. = body weight. C: control; SRD: sucrose rich diet; n-3: deepwater fish oil; n-6: sunflower oil; n-9: high oleic sunflower oil. [a] $p < 0.0001$ *vs*. C. [b] $p < 0.01$. [c] $p < 0.05$ *vs*. SRD-C.

Table 3 Effect of 12 weeks of sucrose rich diet and fatty acid supplementation on visceral adipose tissue mass, liver and serum parameters

Variable	C ($n = 8$)	SRD-C ($n = 8$)	SRD-n3 ($n = 8$)	SRD-n6 ($n = 8$)	SRD-n9 ($n = 8$)
Visceral adipose tissue (g)	13.0 ± 3.4	18.1 ± 4.5[a]	17.4 ± 1.9	22.2 ± 5.4	23.9 ± 6.0[c]
Epididymal adipose tissue (g)	5.2 ± 1.9	6.9 ± 2.4[a]	5.1 ± 0.7	7.8 ± 2.6	8.6 ± 3.1
Perirenal adipose tissue (g)	4.1 ± 1.1	6.9 ± 2.1[b]	6.2 ± 1.3	8.4 ± 1.9	9.4 ± 2.0[c]
Intestinal adipose tissue (g)	3.7 ± 1.6	4.3 ± 1.4	6.1 ± 0.8	6.0 ± 1.5	6.0 ± 2.1
Liver weight (g)	14.2 ± 1.5	16.1 ± 1.8[a]	15.3 ± 1.5	16.3 ± 2.3	16.4 ± 3.8
Liver fat (g)	90 ± 44	148 ± 33[a]	120 ± 45	165 ± 48	144 ± 31
TG (mmol L^{-1})	0.6 ± 0.2	1.8 ± 0.9[b]	0.7 ± 0.2[d]	1.2 ± 0.6	1.2 ± 0.5
Total-chol (mmol L^{-1})	1.43 ± 0.18	1.30 ± 0.21	1.12 ± 0.21	1.43 ± 0.19	1.27 ± 0.13
HDL-chol (mmol L^{-1})	1.01 ± 0.16	0.62 ± 0.10[b]	0.83 ± 0.16[d]	0.88 ± 0.13[d]	0.75 ± 0.08
Non-HDL-chol (mmol L^{-1})	0.47 ± 0.13	0.73 ± 0.23[a]	0.29 ± 0.11[d]	0.55 ± 0.08	0.52 ± 0.13
Glucose (mmol L^{-1})	7.2 ± 1.0	8.7 ± 1.5	8.8 ± 1.1	8.4 ± 0.8	7.8 ± 1.3
FFA (mmol L^{-1})	0.43 ± 0.11	0.69 ± 0.13[b]	0.29 ± 0.08[d]	0.84 ± 0.16	0.54 ± 0.14
Insulin (pmol L^{-1})	110 (60–213)	401 (219–1220)[b]	120 (64–247)[c]	130 (114–175)[d]	343 (215–751)
HOMA-IR	5.9 (3.0–7.6)	15.6 (5.5–48.5)[a]	6.7 (2.1–18.0)[c]	7.2 (5.6–9.2)	16.3 (7.5–39.9)

Data is expressed as means ± SD or median (range) for skewed distributed data. TG, triglyceride; chol, cholesterol; FFA, free fatty acids; HOMA-IR, homeostasis model assessment insulin-resistance index. C: control; SRD: sucrose rich diet; n-3: deepwater fish oil; n-6: sunflower oil; n-9: high oleic sunflower oil. [a] $p < 0.05$. [b] $p < 0.001$ *vs*. C. [c] $p < 0.05$. [d] $p < 0.01$, *vs*. SRD-C.

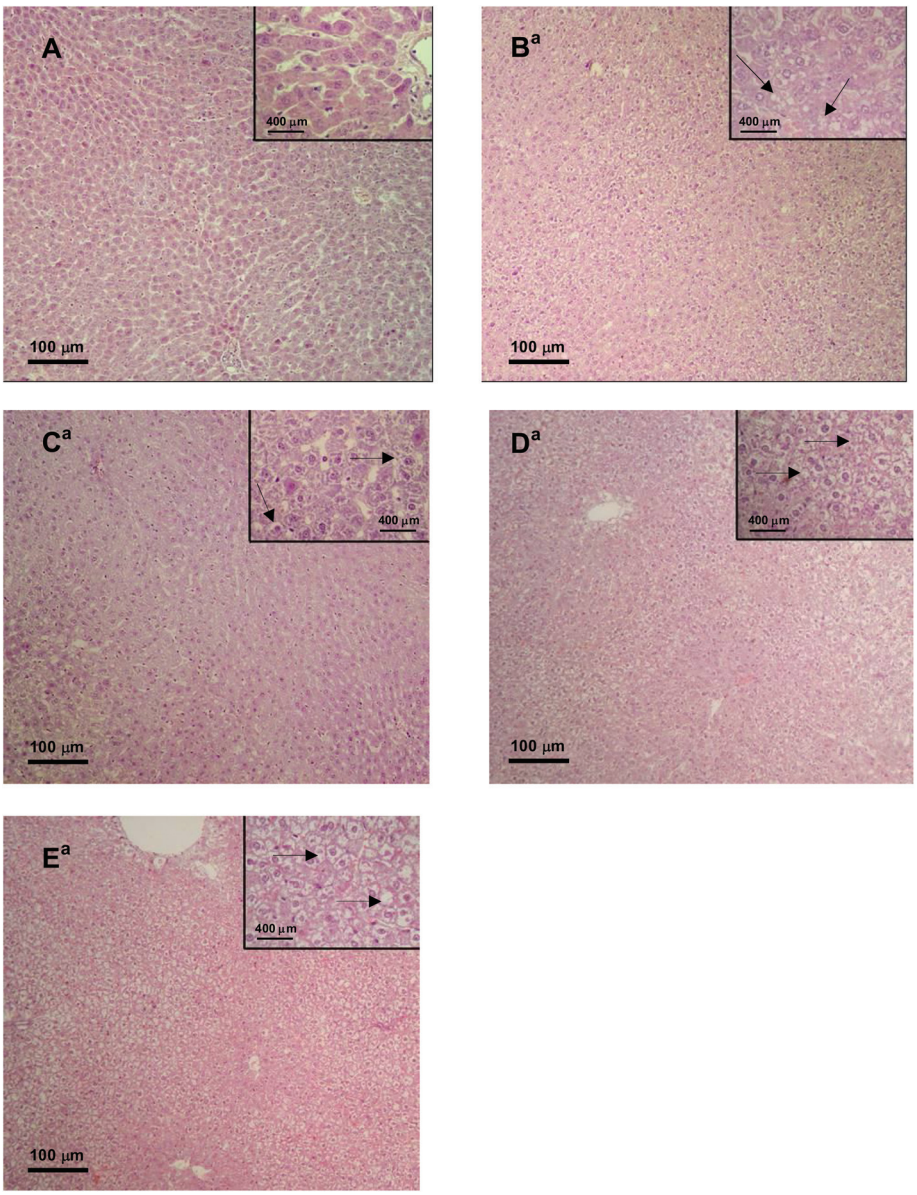

Fig. 1 Hematoxylin and eosin stain in liver of control (A), SRD-C (B), SRD-n3 (C), SRD-n6 (D) and SRD-n9 (E). Arrows indicate the macro and micro-vesicular lipid deposits present in all groups of rats fed SRD. Original magnification at ×100 and ×400. [a] $p = 0.0037$ *vs.* C. SRD: sucrose rich diet; n-3: deepwater fish oil; n-6: sunflower oil; n-9: high oleic sunflower oil.

Visceral and hepatic fat content were also evaluated as part of the IR state. Only SRD-n9 presented an increase in visceral fat content, that positively correlated with the increase in body weight gain ($r = 0.88$, $p = 0.004$). No differences were observed between SRD groups in liver weight and hepatic fat content, Table 3. In accordance, the histological study has not revealed differences in hepatic fat deposits between fatty acid supplemented groups and SRD-C (Fig. 1). Therefore, the fatty acid supplementation did not prevent the development of liver fat deposit in SRD.

Table 4 shows the isolated VLDL percentage chemical composition on the one side, and sub-fraction proportions on the other. As expected, in comparison with C, VLDL from SRD-C has triglyceride over-enrichment ($p < 0.05$) with a greater proportion of large VLDL sub-fraction, detected by size exclusion HPLC ($p = 0.041$). Of note, the increase in the proportion of large VLDL positively correlated with VLDL triglyceride content (%) ($r = 0.816$, $p = 0.002$) and showed a tendency to correlate with the raise in FFA levels ($r = 0.644$; $p = 0.066$).

When evaluating the fatty acid supplementation on VLDL, in comparison with SRD-C, only SRD-n3 prevented the alterations in triglyceride content (54.2 ± 4.4% *vs.* 68.6 ± 8.2, $p < 0.05$), that could be explained by the decreased fatty acid levels ($r = 0.689$, $p = 0.043$). In relation to VLDL sub-fractions, SRD-n3 showed a lower proportion of large VLDL (22.5 [19.7–35.6] *vs.* 49.1 [15.5–82.0], $p < 0.05$), with a subsequent increase in the proportion of typical VLDL in comparison with SRD-C

Table 4 Percent chemical composition and sub-fractions of VLDL isolated from control, sucrose rich diet and fatty acid supplementation groups

Chemical composition (w/w)					
	C	SRD-C	SRD-n3	SRD-n6	SRD-n9
Triglycerides (%)	51 ± 10	68.6 ± 8.2[a]	54.2 ± 8.9[b]	62 ± 10	65 ± 6
Cholesterol (%)	5.9 ± 1.9	5.5 ± 1.5	5.4 ± 1.2	5.0 ± 1.3	6.2 ± 0.9
Phospholipids (%)	24.0 ± 5.7	14.2 ± 7.8[a]	8.2 ± 4.6	22.7 ± 7.2	13.7 ± 4.5
Proteins (%)	19.9 ± 7.1	12.8 ± 3.3[a]	22.2 ± 7.2[b]	9.9 ± 4.7	15.6 ± 7.2
Proportion of VLDL sub-fractions					
Very large	9.0 (2.3–23.5)	7.9 (5.9–24.8)	9.1 (5.5–9.9)	6.1 (4.3–7.5)	23.8[b] (2.3–53.5)
Large	20.9 (5.1–41.3)	49.1[a] (15.5–82.0)	22.5[b] (19.7–35.6)	65.7 (61.4–70.4)	63.5 (56.6–71.4)
Typical	59.8 (34.1–71.3)	24.2[a] (10.2–70.8)	56.1[b] (49.3–61.9)	20.2 (17.7–23.4)	9.5[b] (5.3–23.3)
VLDL remnants	4.5 (2.6–20.3)	3.4 (0.0–9.4)	8.2 (3.6–12.5)	7.7[b] (7.6–7.9)	1.7 (0.0–2.5)

Results are expressed as means ± SD or median (range) for skewed distributed data. [a] $p < 0.05$ *vs.* C. [b] $p < 0.05$ *vs.* SRD-C. C: control; SRD: sucrose rich diet; n-3: deepwater fish oil; n-6: sunflower oil; n-9: high oleic sunflower oil.

Table 5 Statistical power of studied variables

	Statistical power
Weight gain	0.8909
Visceral adipose tissue	0.8916
Liver weight	0.3265
Hepatic fat content	0.5038
Triglycerides	0.8748
HDL-cholesterol	0.9461
Non-HDL-cholesterol	0.9233
Insulin	0.8901
HOMA-IR	0.8201
VLDL-triglyceride (%)	0.5421
Large VLDL	0.9934
Very large VLDL	0.6143
Typical VLDL	0.9942
FFA	0.9965

Power analyses was performed with G*Power 3.1.9.2 (Universität Düsseldorf, Germany). HOMA-IR: homeostasis model assessment for insulin resistance index, FFA: free fatty acids.

(p = 0.008), suggesting that fish oil supplementation would improve VLDL quality in IR. SRD-n9 maintained a similar proportion of large VLDL than SRD-C, together with an increase in very large sub-fractions, Table 4. VLDL sub-fractions profile in SRD-n6, were also similar to SRD-C, without changes in typical or large VLDL. Therefore, the supplementation with sunflower oil n-6 and HOSO n-9 would not prevent VLDL alterations in IR.

Finally, *post hoc* power analysis was conducted using the software G*Power 3.1.9.2 (Universität Düsseldorf, Germany). As shown in Table 5, the power of most statistically significant variables was within the acceptable range, between 0.5038 and 1.000. For liver weight, that showed the lowest statistical power, we used our data as preliminary results to calculate the sample size, and found that 3 times more animals in each group would have been needed to achieve a power of at least 0.80.

4. Discussion

In the present study we evaluated the possible preventive effect of dietary oil supplementation with unsaturated fatty acids (n-3, n-6 and n-9 series) on plasma metabolic parameters, visceral adipose tissue, liver fat content and VLDL composition and size, in an animal model of early IR induced by a sucrose rich diet. We found that only SRD-n3 improved VLDL quality beyond the ameliorated metabolic parameters, while SRD-n6 and SRD-n9 did not prevent the atherogenic alterations in VLDL. Although SRD-n6 showed some improvements in the lipid and IR profile, SRD-n9 favored visceral fat deposition and weight gain. To our knowledge this is the first time that VLDL characteristics were evaluated in this context.

The recent AHA/ACC Guidelines on Lifestyle Management to Reduce Cardiovascular Risk recommend a dietary pattern that achieves 5–6% of calories from SFA,[4] being the unsaturated fat preferred as replacement for SFA. Moreover, it is well known that the rising prevalence of the IR state contributes to increasing CVD risk. In the recent years, the consumption of unsaturated fatty acids has rapidly grown. However, the manner in which dietary fatty acid composition might impact IR is to date controversial.[28,29]

Rats fed a sucrose rich diet constitute a well-known experimental model of IR, and as previously demonstrated, 12 weeks of treatment is an appropriate period to achieve an early IR state.[16,30] In fact, the results we have observed in SRD-C were those expected for the development of an IR state and are in agreement with previous results.

Rats under SRD and supplemented with n-3, n-6- and n-9 fatty acids, showed some variances in response to diet differences. Even though SRD-n3 and SRD-n9 increased caloric intake throughout the studied period, only SRD-n9 resulted in weight gain. This may be explained since n-3 PUFA induces an up-regulation in the expression of genes involved in beta-oxidation and a down-regulation of genes related to lipogenesis, preventing the increase in body weight.[31] In addition, SRD-n3 showed less water intake, however it must be taken into account that the preparation of supplemented foods contained water with sucrose 30%, in order to counteract possible differences in water consumption and to ensure the achievement of the IR state.

Regarding the plasma biochemical parameters, the supplementation with n-3 PUFA improves the IR state as well as

the dyslipidemia observed in SRD-C. Our results are in line with several epidemiological, human and animal studies that have demonstrated that n-3 PUFA have positive physiological effects on IR and lipid metabolism.[32,33] Conversely, SRD-n6 had a poor effect in improving dyslipidemia, only evidenced by an increase in HDL cholesterol levels without affecting other parameters. Some animal studies describe a greater hypolipidemic effect on triglyceride, LDL and total cholesterol levels.[5,34] However, important clinical studies in humans showed that when n-6 PUFA is consumed, a decrease in HDL cholesterol levels and an increase in LDL susceptibility to oxidation are observed as a consequence.[35,36] Therefore, the effects of n-6 PUFA on lipid profile still remain controversial.

As regards to n-9 MUFA, the source we have used herein was high oleic sunflower oil which is widely distributed in industrialized products, and would represent a closer approach to an actual situation than olive oil supplementation does. In general population, studies carried out with olive oil, report that n-9 MUFA can lower blood pressure, fasting glucose and improve lipid and IR parameters, while others observed neutral responses.[7,37] In the present study, SRD-n9 did not attenuate any lipid or IR parameters, including visceral and liver fat content. According to Gillingham *et al*., the influence of MUFA-rich diets in preventing the risk factors of IR are inconsistent.[37]

The most important contribution of this study is the evaluation of VLDL characteristics. In previous reports, we observed modifications in circulating VLDL, in both human and animal models of IR.[16,18] Moreover, we demonstrated that this type of VLDL can alter the endothelial function, which favors the development of atherogenesis.[19]

When evaluating the effect of fatty acid supplementation on VLDL characteristics, only SRD-n3 is able to normalize VLDL triglyceride content, contributing to a greater predominance of typical VLDL sub-fractions. In contrast, neither SRD-n6 nor SRD-n9 neutralized VLDL alterations; this result was expected since no favorable effects were observed on lipid and IR profile. These results suggest once again that VLDL is a consequence of the IR state.

Among the few studies reporting the effect of n-3 PUFA on VLDL, by means of kinetic assays, Barrett *et al*. showed that its supplementation in obese men decreases plasma VLDL-triglyceride concentration by decreasing hepatic secretion rate.[38,39] Other authors assessed the influence of n-6 PUFA intervention on VLDL sub-fractions, separated by HPLC, in overweight-obese men, finding a decrease in VLDL cholesterol and triglyceride; however the comparison was made against medium-chain fatty acid supplementation.[40]

Perona *et al*. evaluated VLDL fatty acid composition after olive oil supplementation, observing modifications in lipid composition of VLDL, linked to a reduction in serum triglyceride concentrations. These changes in VLDL lipid composition would be influenced, in part, by olive oil phenolic compounds.[41,42] Surely, future studies addressing the evaluation of fatty acid composition in VLDL, isolated from an IR animal model under the PUFA/MUFA diets -as implemented in the present study-, would complete the knowledge of this question.

In order to compare results and to optimally test a proposed hypothesis, it must be taken into account that there are several controversial outcomes among the published studies, regarding the effects of unsaturated fatty acids on body weight, insulin-resistance and lipid metabolism. These differences could be due to the use of diverse animal models and different type and formulation of PUFAs/MUFA, dose and duration of interventions, as well as if fatty acid sources are used as substitution or as supplementation.

Herein we compared the effect of dietary supplementation with three different oils, widely spread in human consumption, on VLDL composition in IR. Sunflower oil and HOSO are generally used as raw oil in human consumption and fish oil is commonly sold in pharmacies as supplement, thus we considered interesting to evaluate the effect of the whole oils on VLDL, instead of the action of individual components.

Other authors have evaluated the effect of the cod liver oil supplementation on IR.[43] Nevertheless, cod liver oil contains high amounts of vitamin A and D as well as cholesterol which could impact lipoprotein metabolism. In this sense, in the present study, the fish oil used as a n-3 source was obtained by the pressing of whole fish, without significant concentrations of vitamin A and D or cholesterol. However, it is important to bear in mind that EPA and DHA were present in variable concentration. Importantly, these fatty acids have different metabolic effects. Further studies should be conducted to evaluate the individual effect of each fatty acid.

In summary, while n-3 PUFA showed expected favorable effect, supplementation with n-6 PUFA and n-9 MUFA did not prevent the atherogenic alterations of VLDL produced in IR. Thus, the proposal of the supplementation of n-6 PUFA and n-9 MUFA -provided by HOSO- in general diet recommendations, should be revised.

Conflict of interest

There are no conflicts of interest to disclose.

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