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Basic nutritional investigation

Arachidonic acid–rich ARASCO oil has anti-inflammatory and antidiabetic actions against streptozotocin + high fat diet induced diabetes mellitus in Wistar rats

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ABSTRACT

Objectives: The aim of this study was to investigate the effects of arachidonic acid (AA)-rich ARASCO oil on high-fat diet (HFD) + streptozotocin (STZ)–induced diabetes mellitus in male Wistar rats and its possible mechanisms of action.
Methods: Male Wistar rats with HFD + STZ–induced diabetes were employed in the present study. ARASCO oil was administered orally for the first 7 d consecutively, followed by once weekly throughout the study (14 wk). At various time points, blood glucose and body weight and oral glucose tolerance tests were measured. At the end of the study, animals were sacrificed to collect plasma and various organs and stored at −80°C. Plasma insulin, tumor necrosis factor-α, interleukin-6, and lipoxin A4 were measured. Expression of the following genes was determined: nuclear factor-κB (*NF-κB*), cyclooxygenase-2 (*COX-2*), 12-lipoxygenase (*12-LOX*) in pancreas and lipocalin 2 (*LPCLN2*) in adipose tissue. Various antioxidants were measured in the plasma and other tissues. Area under the curve and insulin sensitivity index were assessed by computing homeostatic model of assessment for insulin resistance, quantitative insulin check index, Matsuda, and Belfiore indices.
Results: ARASCO oil treatment decreased hyperglycemia, restored insulin sensitivity, suppressed inflammation, enhanced plasma lipoxin A4 levels, and reversed altered antioxidant status to near normal in animals with HFD + STZ–induced diabetes.
Conclusion: These results suggest that ARASCO, a rich source of AA, can prevent HFD + STZ–induced diabetes in Wistar rats owing to its anti-inflammatory action. It remains to be seen whether ARASCO oil is useful in preventing or postponing the development of type 2 diabetes mellitus in humans.

Introduction

Despite the increasing incidence of type 2 diabetes mellitus (T2DM) worldwide, its exact pathobiology remains unclear. The insulin resistance (IR) and glucose intolerance seen in obesity, sedentary lifestyles, and T2DM is associated with low-grade systemic inflammation, as evidenced by high levels of plasma interleukin (IL)-6, tumor necrosis factor (TNF)-α, and high-sensitivity C-reactive protein [1–6]. High calorie intake and hyperglycemic conditions are known to induce production of reactive oxygen species and nitric oxide (NO) free radicals [7]. Clinical studies have shown that oxidative stress is strongly associated with occurrence of T2DM, and the levels of oxidants and oxidative stress–induced tissue damage biomarkers such as hydroperoxides, 8-hydroxy-deoxyguanine, and 8-epi-prostaglandin F2 α were reported to be elevated in pancreas biopsies, plasma, and serum of patients with T2DM [8–11]. High calorie intake and consequent hyperglycemia can cause significant oxidative stress in pancreatic β cells and can cause DNA and protein damage leading to insufficient production of insulin [12]. This suggests that methods designed to inhibit oxidative stress and inflammation may be of benefit in IR.

Our previous studies showed that oils rich in polyunsaturated fatty acids (PUFAs) such as fish oil (rich in eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]), borage oil (rich in γ-linolenic acid [GLA]), and ARASCO (rich in arachidonic acid [AA]) can prevent alloxan-induced T1DM in experimental animals [13]. In this study, it was observed that alloxan induced a significant decrease in the plasma and liver and muscle content of GLA, AA, and EPA. Subsequent studies revealed that of all the fatty acids tested, AA is the most

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effective in preventing alloxan-induced toxicity to rat insulinoma cells in vitro and alloxan-induced T1DM in vivo [14–16]. In an extension of this study, we reported that not only alloxan-induced, but also streptozotocin (STZ)-induced T1DM and T2DM can be prevented by AA [17–19]. These results with AA came as a surprise because AA is believed to possess proinflammatory actions as it forms the precursor to proinflammatory prostaglandin (PG)E2 leukotrienes (LTs) of four series and thromboxane A2 (TXA2). The observation that administration of AA decreased plasma IL-6 and TNF-α levels and suppressed the expression of nuclear factor (NF)-κB in pancreatic tissue of alloxan- and STZ-treated animals led to the suggestion that AA in fact possesses anti-inflammatory actions. This is further supported by the finding that AA treatment led to an increase in the formation of lipoxin A4 (LXA4), a potent anti-inflammatory metabolite of AA [17–19]. These studies suggest that AA may function as an antidiabetic molecule.

To exploit AA as a potential antidiabetic agent, it needs to be produced in large quantities. AA is an unstable compound owing to the presence of four double bonds in its structure and is easily peroxidizable. Hence, other sources of AA, such as oils that contain significant amounts of it, need to be explored.

ARASCO oil is a rich source (~40%) of AA obtained from the culture of soil fungus *Mortierella alpine*. Several preclinical and toxicology studies showed that consumption of ARASCO oil is safe and has been designated as Generally Regarded As Safe by the FDA. A communication from the FDA to this effect is is provided in the Supplementary data. ARASCO oil is also added to infant feed formula [20–23].

In view of its safety profile, we evaluated possible antidiabetic actions of ARASCO oil in HFD- and STZ-induced diabetes in Wistar rats.

Materials and methods

Chemicals

All reagents and chemicals were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). ARASCO oil was obtained from DSM Nutritional products, Inc. (Columbia, MD USA). Complete composition of ARASCO oil as communicated by the manufacturer is provided in Table 1. We reanalyzed the fatty acid composition of ARASCO oil and the composition as detected by us (only major fatty acids) is given Table 2. Polymerase chain reaction (PCR) reagents were obtained from Sigma. PCR primers were purchased from Bioserve (Hyderabad, India).

Experimental animals

Four-wk-old male Wistar rats procured from National Institute of Nutrition (Hyderabad, India) were used in this study. The animals were housed at 25°C room temperature with 12-h dark and 12-h light cycle. Animals weighing ~190 g were segregated into four groups: control, HFD + STZ–induced diabetes, ARASCO, and ARASCO + HFD + STZ–induced diabetes. The control group received phosphate-buffered saline and citrate buffer. The diabetic group received low-dose STZ + HFD. The ARASCO group received 0.1 mL ARASCO oil per animal orally for 7 consecutive d and once a week until the end of the experiment. The combination group received ARASCO + HFD + STZ. The control and ARASCO groups were fed a standard animal chow; the HFD + STZ–induced diabetes and combination groups were fed an HFD (100 g of HFD contained 45 g animal and saturated fat, 10 g sucrose as carbohydrate source, 20 g casein protein, 5 g vitamin/mineral mixture, 20 g wheat bran) daily. Complete details of the protocol and the number of animals included in each group, fatty acid composition of the chow diet, HFD composition, and its fatty acid content are given in Tables 3 to 6. There were six animals per group and the total number of animals used in the study was 120. All experiments were approved by Institutional Animal Ethical Committee.

Induction of HFD + STZ-induced diabetes

HFD + STZ–induced diabetes was employed as per the protocol described in Figure 1A. Freshly prepared STZ in 50 mM citrated buffer pH 4.5 was injected 20 mg/kg body weight intraperitoneally on day 1, and this group also received HFD throughout the study (Fig. 1A). Each animal in the ARASCO and combination groups received 0.1 mL ARASCO oil (composition of ARASCO oil is given in Table 1) orally consecutively for 7 d followed by once a week until the end of the study [17–19]. Our previous study showed that 0.1 mL of PUFA-rich oils is optimum to study their effect in alloxan-induced diabetes, and hence in the present study we used 0.1 mL of ARASCO oil [13]. The protocol of the study and the composition of HFD and the fatty acid content of chow and HFD used are given in Tables 3 to 6. Total number of animals used in the present study were 120. Blood samples were collected from the study groups at various time points: weeks 1, 4, 8, 12, and 14 from the day of STZ injection for various biochemical studies. At the end of each time point (weeks 1, 4, 8, 12, and 14), all the scheduled animals were sacrificed and their blood samples and tissues—pancreas, adipose, and liver—were collected for various studies. Fasting blood glucose was measured by using Accu-Check blood glucose meter (Roche, Branchburg, NJ, USA) and oral glucose tolerance test (OGTT) was performed. The animals were confirmed to have developed diabetes when the fasting blood sugar levels were ≥150 mg/dL. Body weight was measured along with the blood glucose estimation. Plasma, red blood cell, and tissues that were collected were stored at −80°C until further analysis.

Table 1
Chemical composition of ARASCO oil as mentioned by the supplier

Fatty acids	% total
Myristic acid (14:0)	0–2
Palmitic acid (16:0)	3–15
Palmitoleic acid (16:1)	0–2
Stearic acid (18:0)	5–20
Oleic acid (18:1)	5–38
Linoleic acid (18:2)	4–15
Linolenic acid (18:3)	1–5
Arachidic acid (20:0)	0–1
Eicosatrienoic acid (20:3)	1–5
Arachidonic acid (20:4)	38–44
Behenic acid (22:0)	0–3
Docosapentaenoic acid (22:5)	0–3
Lignoceric acid (24:0)	0–3
Chemical analysis	
DHA	<0.1%
EPA	<0.1%–0.16%
Free fatty acid	0.10%–0.27%
Peroxide value	0.12–1.51 meq/kg
Volatiles	<0.01%–0.03%
Non-saponifiables	1.18%–1.73%
Insolubles	<0.01%
Trans fats	<1%
Elemental analysis	ppm
Arsenic	<0.5
Cadmium	<0.1
Chromium	<0.1
Copper	<0.02
Iron	<0.02
Lead	<0.1
Manganese	<0.01
Mercury	<0.04
Molybdenum	<0.05
Nickel	<0.1
Phosphorous	<1
Silicon	280–350
Sulphur	3–6

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Table 2
Percentage of fatty acid composition of ARASCO oil as measured using gas chromatography

Fatty acid	% fatty acid
18:1 ω-9	29.3
18:2 ω-6	6.7
20:4 ω-6	48.2
20:5 ω-3	ND
22:6 ω-3	ND

ND, not detected.

OGTT and insulin sensitivity indices measurement

At the end of each time point, an OGTT was performed on animals with overnight fasting. After the collection of the basal blood sample (considered time point 0), the animals were challenged with an oral glucose load (2 g/kg) administered using a stomach tube. Further blood samples were collected at the end of 30, 60, 90, and 120 min from the retro-orbital plexus. Area under the curve was calculated

Table 3
Protocol of the ARASCO study with composition of HFD diet: ARASCO vs HFD experiment protocol*

No of months	Animals, n	Dosage of ARASCO per group†,‡ (0.1 mL/animal) (A) (mL)	Dosage period of ARASCO (B)	Injections/mo l	Total amount of ARASCO/mo (AxC) in mL	STZ injection
1	60	6	7 continuous daily 0.1 mL	07	42.0	20 mg/kg body weight (only once)
1	48	4.8	Once weekly	04	19.2	
2	36	3.6	Once weekly	04	14.4	
3	24	2.4	Once weekly	04	9.6	
4	12	1.2	Once weekly	04	4.8	
Total volume of ARASCO needed in mL					90	

HFD, high-fat diet; STZ, streptozotocin.
Six animals per group were sacrificed on the morning of the eighth day and end of every month (4 wk) until the end of the study.
(4 mo). Although brain, spleen, and muscle tissues were collected, they were not analyzed in the present study.
*Study was for 4 mo (i.e., 16 wk).
†Groups: control, HFD + STZ, HFD + ARASCO + STZ, ARASCO only.
‡Six animals per group.

Table 4
High fat diet composition (100 g)

Sl. No	Ingredient	Quantity (g)	Calories
1	Ghee	35	315
2	Vanaspathi	10	90
3	Sucrose	10	40
4	Casein protein	20	80
5	Vitamin and mineral mix	5	–
6	Wheat bran	20	60
Total		100	585

Table 5
Fatty acid composition of ghee and Vanaspati

Fatty acid (%FA)	Ghee (clarified butter)	Vanaspati
C4:0 (butyric)	0.22 ± 0.08	
C6:0 (caproic)	0.30 ± 0.13	
C8:0 (caprylic)	0.47 ± 0.20	
C10:0 (capric)	1.87 ± 0.34	
C12:0 (lauric)	2.81 ± 0.38	0.36 ± 0.18
C14:0 (myristic)	11.81 ± 0.57	1.18 ± 0.11
C16:0 (palmitic)	39.13 ± 1.15	53.33 ± 4.75
C18:0 (stearic)	13.89 ± 1.17	5.97 ± 1.04
C20:0 (arachidic)	0.52 ± 0.12	0.82 ± 0.24
C14:1 (myristoleic)	0.95 ± 0.17	
C16:1 (palmitoleic)	1.86 ± 0.16	
C18:1 ω9 c (oleic)	23.19 ± 1.46	29.10 ± 4.78
C18:2 ω6 c (linoleic)	2.00 ± 0.62	4.73 ± 1.81
C18:3 ω3 (linolenic)	0.55 ± 0.11	
C18:1 t	0.44 ± 0.09	4.52 ± 2.40
TSFA	71.02 ± 1.86	61.63 ± 4.86
TMUFA	26.44 ± 1.63	33.62 ± 3.91
TPUFA	2.34 ± 0.64	4.73 ± 1.81
TPUFA/TSFA	0.04	0.08

TMUFA, total monounsaturated fatty acids; TPUFA, total polyunsaturated fatty acids; TSFA, total saturated fatty acids.
Values are mean ± SD.

for the glucose and insulin estimated at various time points 0, 30, 60, 90, and 120 min of OGTT. Insulin sensitivity index (ISI) was assessed by computing homeostatic model of assessment for IR (HOMA-IR), quantitative insulin check index (QUICKI), Matsuda, and Belfiore indices. HOMA was computed to determine the IR and ISIs as described previously [18].

Estimation of NO, LP, and antioxidant enzymes from in vivo study samples

NO, lipid peroxides (LP), and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx), and glutathione-S-transferase (GST) were measured in the plasma and various tissue homogenates of all animals in the study. The methods followed for these estimations have been described previously [17–19,24,25].

Table 6
Composition (including fatty acid content) of laboratory chow diet

Crude protein	%	18.9
Sugar	%	4.7
Crude fat	%	3.5
Fatty acid species		
Palmitic acid (C16:0)	mg/g^{-1}	4.8
Stearic acid (C18:0)	mg/g^{-1}	0.97
Palmitoleic acid (C16:1)	mg/g^{-1}	0.03
Oleic acid (C18:1)	mg/g^{-1}	5.3
Linoleic acid (C18:2)	mg/g^{-1}	17.1
Arachidonic acid (C20:4)	mg/g^{-1}	<0.1
SFA	mg/g^{-1}	6.2
MUFA	mg/g^{-1}	5.7
PUFA	mg/g^{-1}	19.4
Total energy	kj/g^{-1}	17.7

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;SFA, saturated fatty acids.

Estimation of plasma LXA4

LXA4 was measured in the plasma samples of all study animals using enzyme-linked immunosorbent assay (ELISA; Oxford Biomedical Research Company, Rochester Hills, MI, USA) per the manufacturer's instructions.

Estimation of plasma insulin

Plasma insulin levels were estimated by ultrasensitive rat insulin ELISA kit (Crystal Chemical Inc, Belvidere, IL, USA) per the manufacturer's instructions.

Estimation of plasma TNF-α

TNF-α was measured in the plasma samples at various time periods of the study (days 10, 20, and 30 after the first STZ injection) using Quantikine TNF-α Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA).

Estimation of plasma IL-6

IL-6 was measured in the plasma samples at various time periods of the study (days 10, 20, and 30 after the first STZ injection) using Rat IL-6 ELISA Kit (Abcam, Cambridge, MA, USA).

Gene expression studies in pancreatic and adipose tissues

Isolation of RNA and cDNA synthesis

Trizol reagent method was used to isolate the RNA from homogenized pancreas and adipose tissues; cDNAs were then synthesized by reverse transcription from 1 μg of total RNA using SuperScript First Strand Synthesis for real-time quantitative (qRT)-PCR (Invitrogen, Carlsbad, CA, USA). Both RNA isolation and RT-PCR were done according to the manufacturer's instructions.

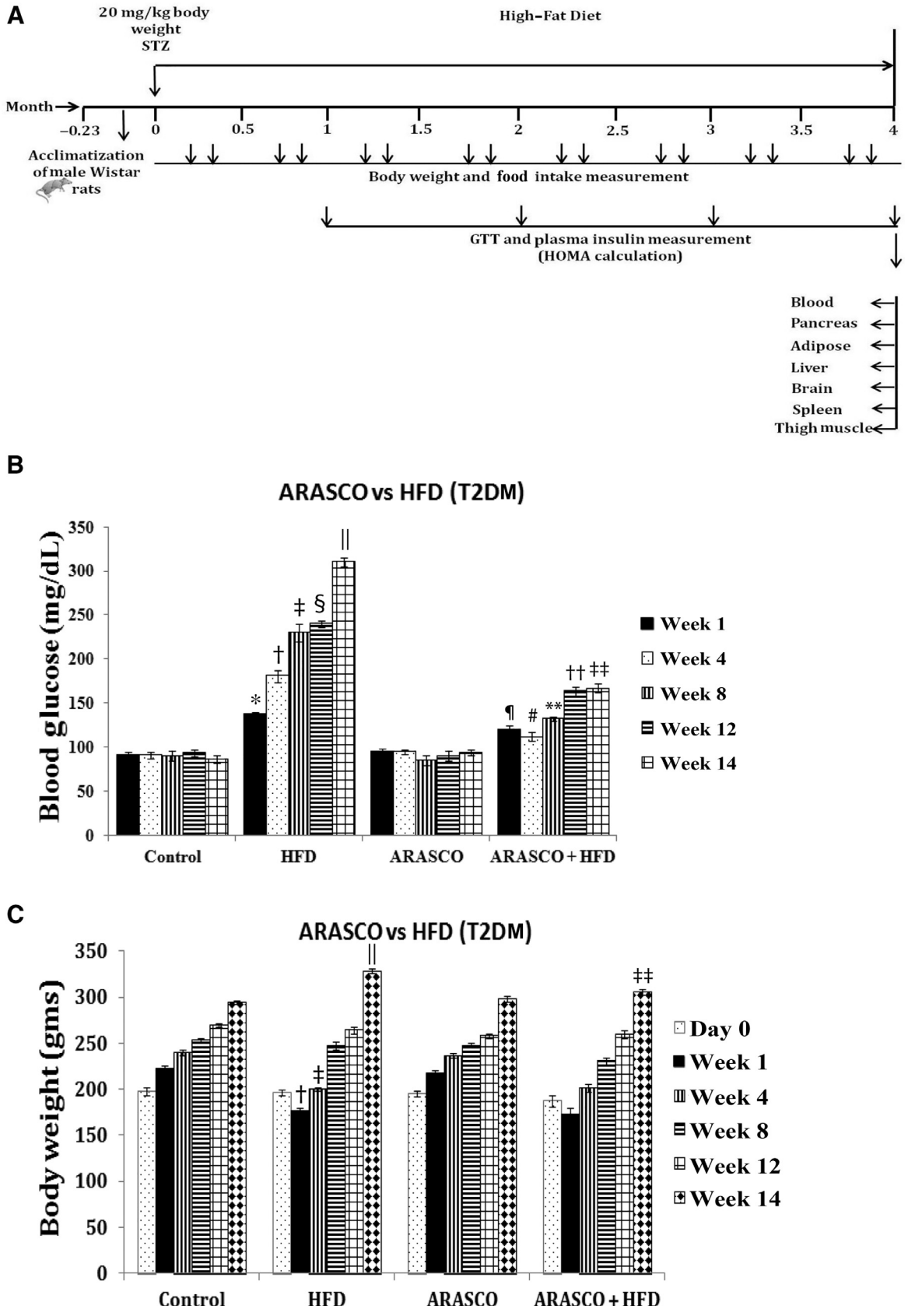


Fig. 1. (**A**) STZ + HFD–induced diabetes protocol used in the study. Experiment Protocol: After 7 d of acclimatization, diabetes mellitus was induced by 20 mg/kg body weight STZ and HFD. All animals, except placebo and diabetic control, received 0.1 mL ARASCO oil orally consecutively for 1 wk during the first week and later once in every week until the end of the study. Although brain, spleen, and muscle tissues were collected, they were not analyzed in the present study. (**B**) Blood glucose of animals in various

Table 7
Sequence of gene primers used in semi quantitative PCR studies

S. No	Gene	Forward	Reverse	Product size, bp
1	*p65 Nf-κB*	5′-CCTAGCTTTCTCTGAACTGCAAA-3′	5′- GGGTCAGAGGCCAATAGAGA-3′	71
2	*COX-2*	5′-TGCATGTGGCTGTGGATGTCATCAA-3′	5′-CACTAAGACAGACCCGTCATCTCCA-3′	583
3	*12-LOX*	5′-GGGCCACTGCAGTTCGTGA-3′	5′-CGGCCTCTGCGCTCATC-3′	120
4	*LPCLN2*	5′-AGTAGGATCCCAGGACTCAACTCAGAACTTG-3′	5′-AGTACTCGAGTCAGTTGTCAATGCATTGGTC-3′	557
5	*β-actin*	5′-TGAGCGCAAGTACTCTGTGTGGAT-3′	5′-TAGAAGCATTTGCGGTGCACGATG-3′	617

PCR, polymerase chain reaction.

Semiquantitative PCR

The expression of genes *P65 NF-κB, 12-LOX, COX-2,* and β-*actin* studied by using semi-quantitative PCR was as follows: 95°C for 2 min initial denaturation; 95°C for 30 s denaturation; 64°C, 54°C, 60°C, and 52.5°C for 30 s annealing for *p65 NF-κB, 12-LOX, COX-2,* and β-*actin,* respectively; followed by 72°C for 30 s extension and 72°C for 5-min final extension; and overall 35 cycles were performed for pancreatic tissue.

In adipose tissue, PCR was performed to study the expression of *p65 NF-κB, 12-LOX, COX-2,* and β-*actin* as follows: 95°C for 2 min initial denaturation; 95°C for 30s denaturation; 64°C, 63°C, 60°C, and 52.5°C for 30s annealing for *p65 NF-κB, LPCLN2,* and β-*actin*, respectively; followed by 72°C for 30s extension and 72°C for 5-min final extension; and overall 35 cycles were performed. PCR products were observed and analyzed by electrophoresis on 1.5% (w/v) agarose gel in 1 × TAE buffer at 100 V. Quantification was performed by taking the ratio of gene of interest and β-actin and calculated as percentage comparing with respective control. The details of primer genes *NF-κB, 12-LOX, COX-2, LPCLN2,* and β-*actin* are presented in Table 7. PCR was performed in Eppendorf 5331 Master cycler. Quantification of genes was done by Major Science image analysis software.

Statistical analysis

Experiments were performed by taking six animals in each group. All results are expressed as mean ± SEM. The obtained values were analyzed by using two-way analysis of variance or Tukey's range test.

Results

ARASCO treatment improves the blood glucose and body weight in animals with STZ + HFD–induced diabetes

Fasting blood glucose was observed to be consistently elevated in animals with HFD + STZ-induced diabetes throughout the study, indicating development of diabetes. At the end of week 14 of the study, fasting blood glucose levels hovered around 320 mg/dL in animals with diabetes. As shown in the protocol (Fig. 1A), 0.1 mL of ARASCO was administered orally for the first 7 consecutive d followed by once a week until the end of the study. The experiment was performed for 14 wk and blood samples and tissues were collected at the end of weeks 1, 4, 8, 12, and 14 for various studies. ARASCO treatment restored ($P < 0.05$) fasting blood glucose levels to near normal in animals with HFD + STZ–induced diabetes at all time points studied (Fig. 1B). ARASCO treatment alone did not have any adverse effect on plasma glucose levels and no side effects were observed in the rats. The animals with HFD + STZ–induced diabetes showed an increase in body weight (Fig. 1C) owing to accumulation of subcutaneous fat (data not shown). ARASCO treatment ($P < 0.05$) prevented the fat deposition in HFD + STZ–treated rats.

ARASCO treatment improved hyperinsulinemia and glucose intolerance

In HFD + STZ–treated animals, hyperinsulinemia was seen possibly, secondary to hyperglycemia and IT. It is known that chronic systemic inflammation induced by HFD + STZ can lead to IR and hyperglycemia. The results shown in Figures 2 and 3 revealed that ARASCO treatment significantly reduced ($P < 0.05$) glucose intolerance and IR in animals with HFD + STZ–induced diabetes. The OGTT studies showed that ARASCO treatment can decrease plasma blood glucose levels to a significant degree in animals with HFD + STZ–induced diabetes. The area under curve studies of insulin (Fig. 3) showed that HFD + STZ–induced hyperinsulinemia in animals with diabetes is reduced to a significant degree by ARASCO treatment ($P < 0.05$). In contrast, no changes in plasma insulin and glucose levels were seen in ARASCO control animals.

ARASCO treatment restored plasma TNF-α and IL-6

HFD + STZ treatment is known to induce low-grade systemic inflammation, as evidenced by an increase in plasma IL-6 and TNF-α levels (Fig. 4C and D). Throughout the study, both IL-6 and TNF-α levels in the plasma were elevated in animals with HFD + STZ–induced diabetes compared with the control group ($P < 0.05$). ARASCO treatment restored ($P < 0.05$) plasma IL-6 and TNF-α levels to near ARASCO-alone–treated control values, except in week 8 of the study. It is interesting to note that ARASCO-treated control animals also showed significant increase in plasma levels of IL-6 and TNF-α compared with the untreated control group (Fig. 4C and D). It can be seen that the plasma insulin levels for weeks 1, 4, 8, and 12 (given in Fig. 4A) in the ARASCO-treated animals with HFD + STZ–induced diabetes were restored to near normal (compared with untreated controls) and ARASCO-alone–treated animals and are significantly lower than in the animals with HFD + STZ–induced diabetes. These results suggest that ARASCO treatment suppressed hyperinsulinemia seen in animals with HFD + STZ–induced diabetes.

Previously, we showed that LXA4, an anti-inflammatory metabolite of AA, had potent antidiabetic actions [17,19]. Hence, in the present study, we measured plasma LXA4 levels to know whether ARASCO treatment can influence LXA4 formation. It is seen from the results given in Figure 4B that there is a significant increase in the plasma levels of LXA4 in ARASCO-supplemented animals with HFD + STZ–induced diabetes. On the other hand, animals with

groups of the study. HFD group refers to STZ + HFD–induced diabetes group; whereas ARASCO + HFD refers to STZ + HFD + ARASCO group. STZ + HFD–induced diabetes group is considered to have developed T2DM because they have significant amounts of plasma insulin levels. Blood glucose estimation was performed in all animals at each time point (1, 4, 8, 12, and 14 weeks) until the end of the study. All values are expressed as mean ± SEM. $^{*}P \leq 0.05$ compared with week 1 control values. $^{\dagger}P \leq 0.05$ compared with week 4 control values. $^{\ddagger}P \leq 0.05$ compared with week 8 control values. $^{\S}P \leq 0.05$ *compared* with week 12 control values. $^{\|}P \leq 0.05$ compared with week 14 control values. $^{\P}P \leq 0.05$ compared with week 1 of HFD + STZ–induced diabetes. $^{\#}P \leq 0.05$ compared with week 4 of HFD + STZ–induced diabetes. $^{**}P \leq 0.05$ compared with week 8 of HFD + STZ–induced diabetes. $^{\dagger\dagger}P \leq 0.05$ compared with week 12 of HFD + STZ–induced diabetes. $^{\ddagger\ddagger}P \leq 0.05$ compared with week 14 of HFD + STZ–induced diabetes. (**C**) Body weight of animals in various groups of the study. Body weight of rats treated with AA ± HFD + STZ–induced diabetes: The measurements were taken once in 10 d until the end of the study. All values are expressed as mean ± SEM. $^{*}P \leq 0.05$ compared with the untreated control, $^{\dagger}P < 0.05$ compared to other weeks in the same group. AA, arachidonic acid; GTT, glucose tolerance test; HFD, high-fat diet; HOMA, homeostatic model of assessment; T2DM, type 2 diabetes mellitus; STZ, streptozotocin.

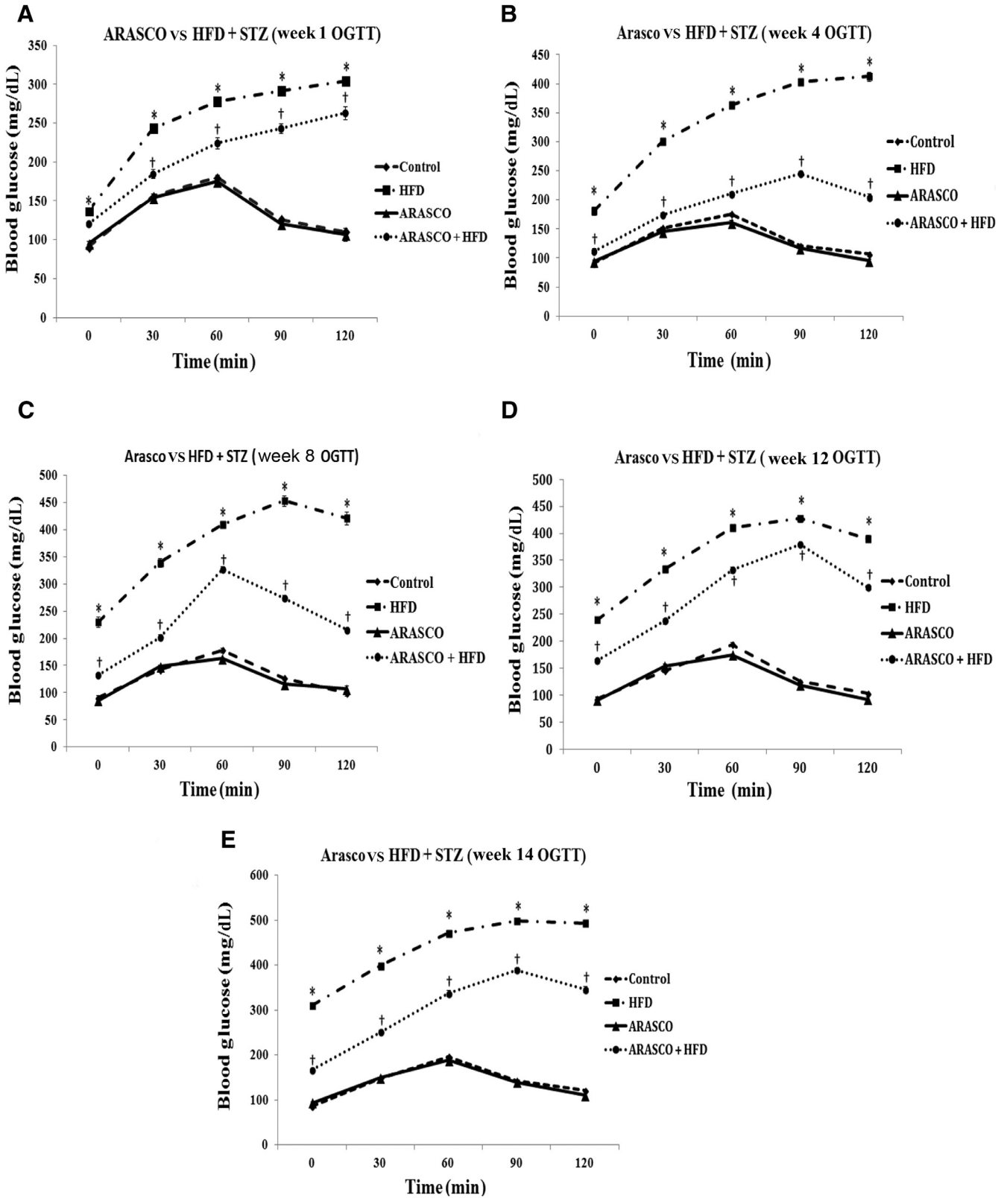


Fig. 2. Effect of ARASCO on HFD + STZ–induced diabetes: OGTT values. Estimation of plasma glucose was performed by OGTT at 0, 30, 6 0, 90, and 120 min in all groups of animals. All values are expressed as mean ± SEM. $^{*}P \leq 0.05$ compared with untreated control. $^{\dagger}P \leq 0.05$ compared with HFD + STZ–induced diabetes. HFD, high-fat diet; OGTT, oral glucose tolerance test; STZ, streptozotocin.

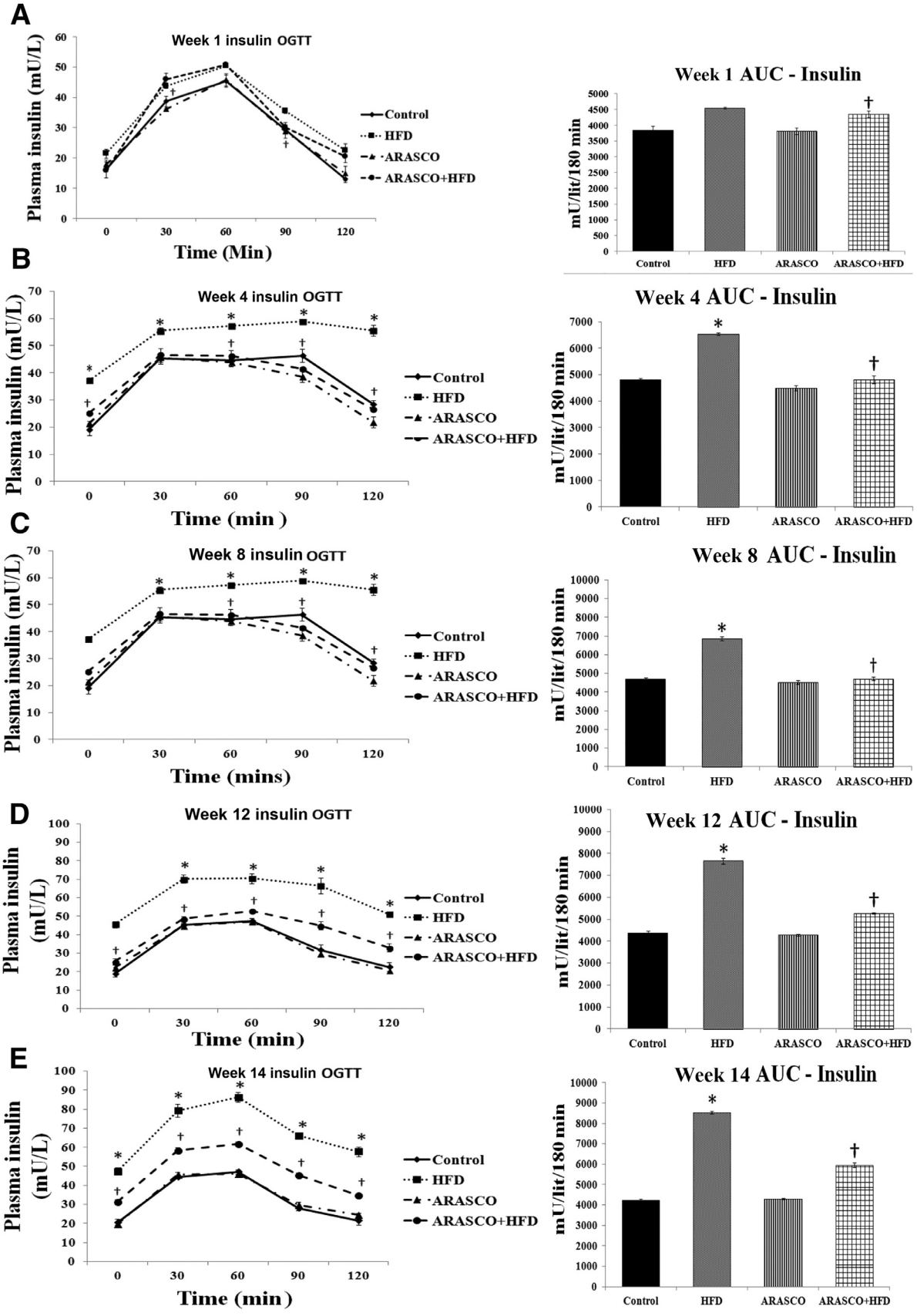


Fig. 3. Effect of ARASCO on HFD + STZ-induced diabetes: AUC values of insulin. Estimation of plasma insulin was done during OGTT and AUC concentrations were estimated for plasma insulin in all groups of animals by using Tapezoidal rule. All values are expressed as mean ± SEM. *$P \leq 0.05$ compared with untreated control. †$P \leq 0.05$ compared with HFD + STZ–induced diabetes. AUC, area under curve; HFD, high-fat diet; OGTT, oral glucose tolerance test; STZ, streptozotocin.

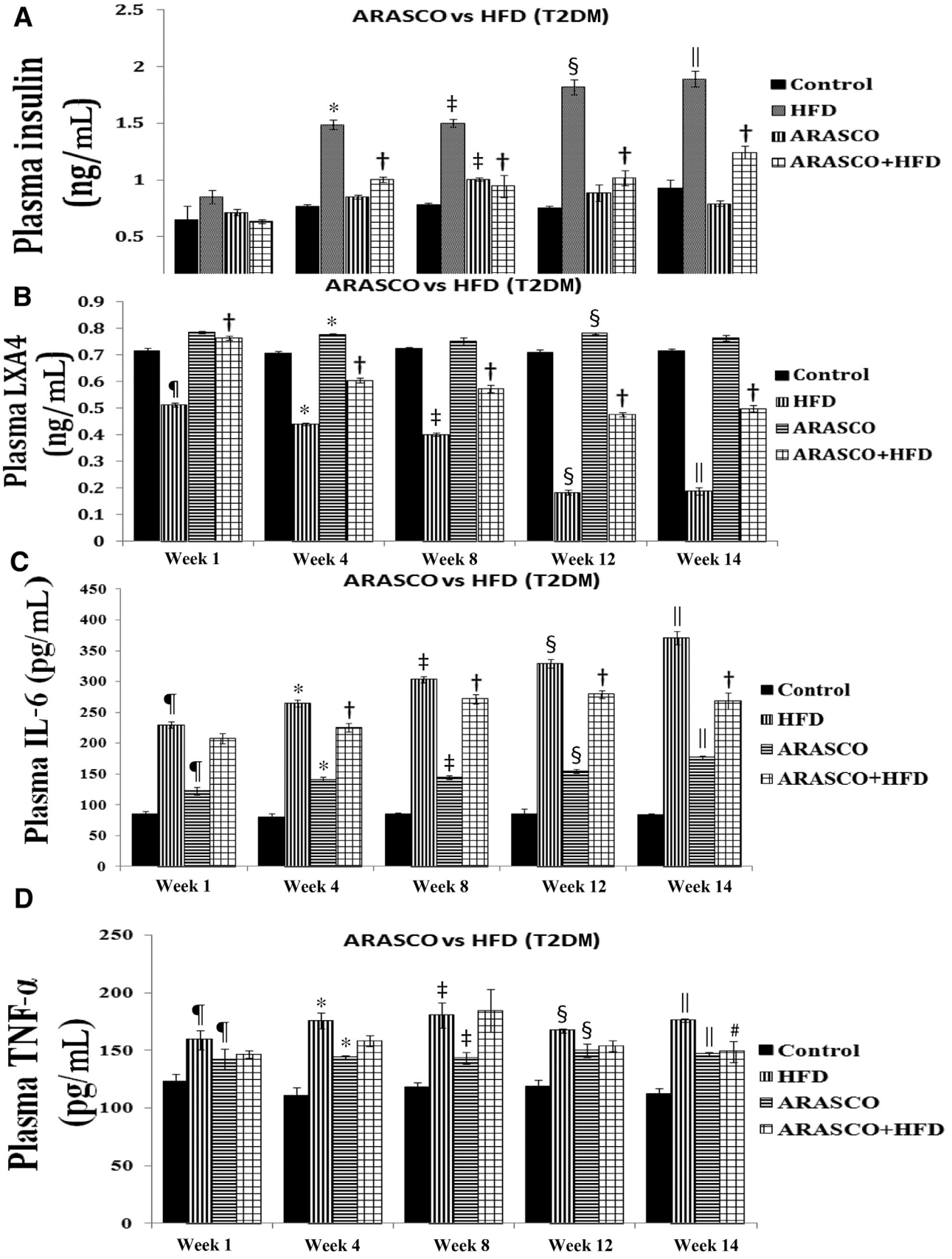


Fig. 4. Effect of ARASCO on HFD + STZ-induced diabetes: plasma insulin, LXA4, TNF-α, and IL-6 estimation. (**A**) Plasma insulin levels of all animals at the end of the study. Insulin estimation was done by using ultrasensitive rat insulin ELISA kit (Crystal Chemical Inc, Belvidere, IL, USA). (**B**) Measurement of LXA4 levels in plasma of all combinations of ARASCO ± HFD + STZ-induced diabetes animals at the end of each time point of study. (**C**) Plasma IL-6 was measured in all animals. IL-6 estimation was done by using Rat

Table 8
Insulin indices

Group	HOMA-IR	QUICKI	Matsuda	Belfiore
Control	0.76 ± 0.01	0.31 ± 0.001	3.39 ± 0.19	1 ± 0
STZ + HFD	5.91 ± 0.16*	0.24 ± 0.004*	0.46 ± 0.01*	0.23 ± 0.001*
ARASCO control	0.74 ± 0.02	0.31 ± 0.001	3. 08 ± 0.07	0.95 ± 0.02
ARASCO + STZ + HFD	2.10 ± 0.15†	0.27 ± 0.002†	1.13 ± 0.05†	0.54 ± 0.023†

HFD, high-fat diet; HOMA-IR, homeostatic model assessment; QUICKI, quantitative insulin sensitivity check index; STZ, streptozotocin.
*$P < 0.05$ compared with control.
†$P < 0.05$ compared with STZ + HFD–induced diabetes group.

HFD + STZ–induced diabetes showed significantly reduced plasma LXA4 levels compared with control animals. In fact, it can be seen that the animals with STZ + HFD–induced diabetes had a gradual and persistent decrease in plasma LXA4 levels from week 1 to week 8 and the lowest levels were seen in weeks 12 and 14 (Fig. 4B). This suggests that continued administration of HFD produces a persistent and gradual fall in LXA4 formation in STZ-treated animals.

ARASCO treatment enhanced insulin sensitivity in HFD + STZ–induced diabetes

HOMA, QUICKI, Matsuda, and Belfiore indices were used to compute insulin sensitivity and resistance in week 14 plasma samples of the study. HOMA computation model was used to assess the IR in animals with HFD + STZ–induced diabetes. The HOMA value (Table 8) was raised ($P < 0.05$) to 5.91 ± 0.16 in the animals with HFD + STZ–induced diabetes, which is approximately a five-fold increase compared with controls (0.76 ± 0.01). No significant change was observed in HOMA value in the ARASCO-alone–treated animals. In contrast, ARASCO treatment reduced ($P < 0.05$) IR (2.10 ± 0.15) in animals with HFD + STZ–induced diabetes. QUICKI, Matsuda, and Belfiore indices were used to assess insulin sensitivity in animals with HFD + STZ–induced diabetes. In animals with HFD + STZ–induced diabetes, regression ($P < 0.05$) of QUICKI value to 0.24 ± 0.004 was observed compared with values for the control group (0.31 ± 0.001; Table 8). On the other hand, ARASCO ($P < 0.05$) restored the QUICKI index to 0.27 ± 0.002 in animals with HFD + STZ–induced diabetes. The Matsuda and Belfiore indices in animals with HFD + STZ–induced diabetes were reduced ($P < 0.05$) by almost threefold (i.e., 0.46 ± 0.01 and 0.23 ± 0.001) compared with control values (3.39 ± 0.19 and 1 ± 0, respectively; Table 8). ARASCO treatment significantly ($P < 0.05$) restored Matsuda and Belfiore indices to 1.13 ± 0.05 and 0.54 ± 0.023, respectively. These values are closer to near normal in animals with HFD + STZ–induced diabetes (Table 8). There was no change in the values of these indices in animals treated with ARASCO alone compared with the control rats.

ARASCO treatment suppresses the expression of genes p65 Nf-κB *and* LPCLN2 *in HFD + STZ–induced diabetes mesenteric adipose tissue*

High calorie intake is known to upregulate the inflammatory genes such as *p65, NF-κB,* and *LPCLN2* and thus lead to the onset of low-grade systemic inflammation seen in obesity and T2DM and HFD + STZ–induced diabetes. Results shown in Figure 5A and B clearly demonstrate that animals with HFD + STZ–induced diabetes have significantly ($P < 0.05$) elevated expression of both *p65 NF-κB* in pancreas and adipose tissue and *LPCLN2* in adipose tissue, which were significantly downregulated by ARASCO treatment ($P < 0.05$). On the other hand, administration of ARASCO alone did not affect their expression. The increased expression of COX-2 enzymes was also decreased by ARASCO treatment in animals with HFD + STZ–induced diabetes especially in the pancreatic tissue as shown in Figure 5A.

ARASCO restored antioxidants to near normal in animals with HFD + STZ–induced diabetes

Because HFD + STZ–induced diabetes is likely to be associated with enhanced prooxidant status, we measured possible changes in the activity of various antioxidant enzymes owing to administration of ARASCO in various groups of the present study. The results shown in Table 9 demonstrated that ARASCO can restore SOD, CAT, GST, and GPx, NO, and lipid peroxides (LPO) levels in the plasma, pancreas, adipose, and liver tissues to near normal in animals with HFD + STZ–induced diabetes. These results suggest that ARASCO treatment significantly ($P < 0.05$) counters the oxidative stress induced by HFD + STZ–induced diabetes in Wistar rats.

ARASCO versus AA on plasma glucose and insulin levels and insulin resistance in HFD + STZ–induced DM

ARASCO oil is not only rich in AA (~40%) but also contains significant amounts of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), linoleic acid (LA), α-linoleic acid (ALA), and eicosatrienoic acids (see Tables 1 and 2 for the fatty acid content and composition of ARASCO oil). Hence, it is not clear whether the presence of AA in ARASCO oil is as effective as that of pure AA in preventing HFD + STZ–induced DM and IR. To verify this possibility, we studied the effect of pure AA against HFD + STZ-induced diabetes in Wistar rats. In this study, we used 0.1 mL of ARASCO oil, which results in ~40 mg of AA (as ARASCO oil contains ~40% AA and so 0.1 mL = 100 mg will deliver ~40 mg of AA), whereas pure AA was given at the rate of only 10 μg/animal orally. The results of this comparative study are shown in Figures 6 to 8. It is evident from these results that the pure AA-supplemented group showed lower plasma glucose, higher plasma insulin, less IR, and an increase in insulin sensitivity compared with the ARASCO group (see Figs. 6–8).

IL-6 ELISA Kit (Abcam, Cambridge, MA, USA). IL-6 measurement was done in plasma all combinations of ARASCO ± HFD + STZ-induced diabetes animals at the end of each time point of study. (**D**) Plasma TNF-α was measured in all animals. Quantikine TNF-α Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA). TNF-α measurement was done in plasma collected all combinations of ARASCO ± HFD + STZ–induced diabetes treated animals at the end of each time point of study. All the values are expressed as mean ± SEM. All values expressed as mean ± SEM. *$P \leq 0.05$ compared with week 4 control values. †$P \leq 0.05$ compared with HFD + STZ-induced diabetes. ‡$P \leq 0.05$ compared with week 8 control values. §$P \leq 0.05$ compared with week 12 control values. ||$P \leq 0.05$ compared with week 14 control values. ¶$P \leq 0.05$ compared with week 1 control values. #$P < 0.05$ compared to corresponding control. ELISA, enzyme-linked immunosorbent assay; HFD, high-fat diet; IL, interleukin; LXA, lipoxin A; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor; STZ, streptozotocin.

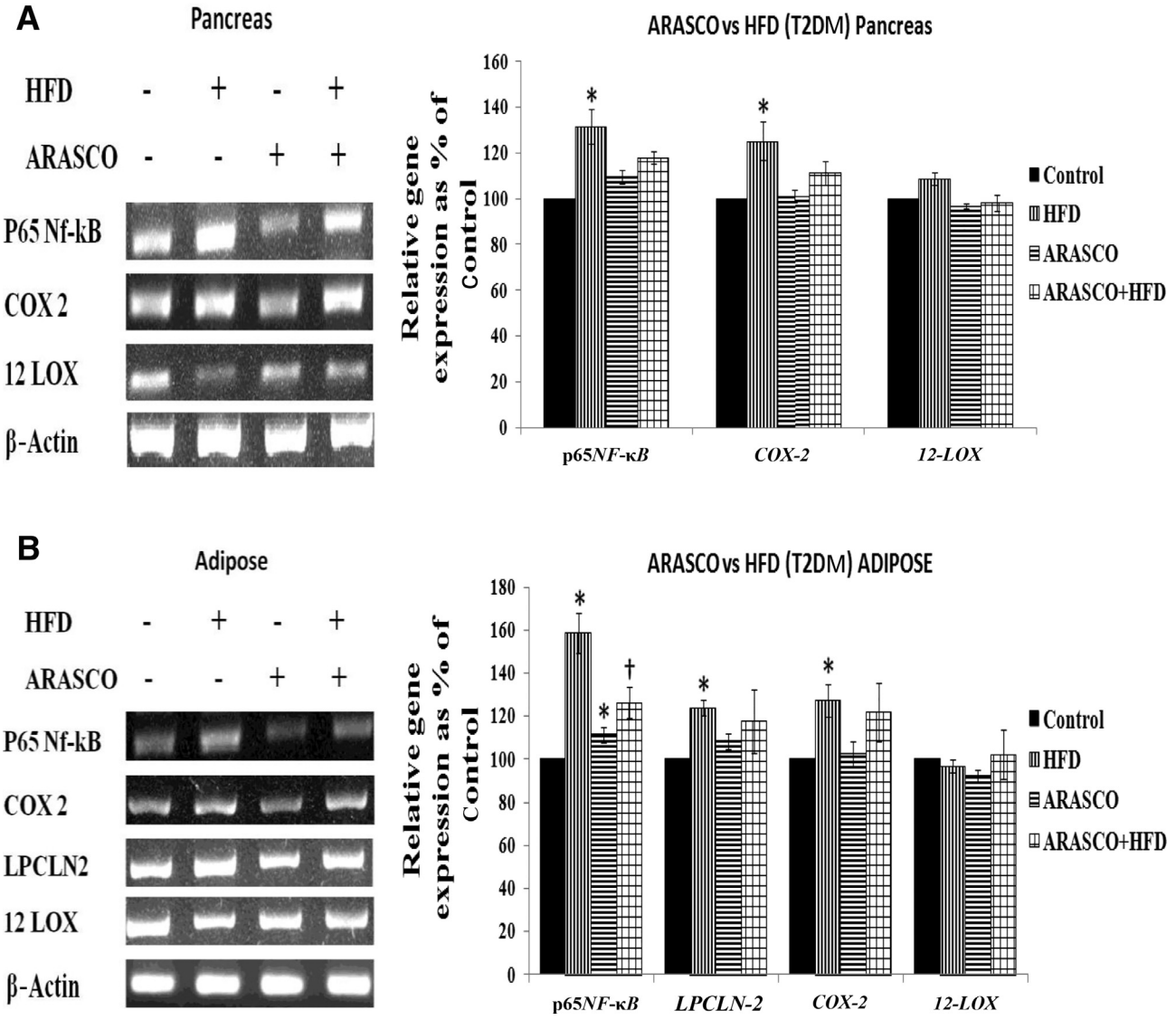


Fig. 5. Gene expression studies in pancreas and adipose tissue. Gene expression studies were performed in pancreas and adipose tissue collected at end of the experiment. In pancreas, the percentage of change in the expression of genes: *12-LOX, COX-2,* and *NF-κB* were studied in ARASCO ± HFD + STZ–induced diabetes animals by using semi-quantitative PCR method. The equality of sample loading was confirmed by β-*actin* gene expression. Quantification of genes was done by Major Science image analysis software. In adipose tissue, the percentage of change in the expression of genes *12-LOX, COX-2, LPCLN2,* and *NF-κB* were studied in ARASCO ± HFD + STZ–induced diabetes animals by using semi-quantitative PCR method. The equality of sample loading was confirmed by β-*actin* gene expression. Quantification of genes was done by Major Science image analysis software. All values are expressed as mean ± SEM. $^{*}P \leq 0.05$ compared with untreated control. $^{\dagger}P \leq 0.05$ compared with HFD + STZ–induced diabetes. It may be noted here that in the figures STZ refers to HFD + STZ–induced diabetes group and STZ + ARASCO refers to HFD + STZ + ARASCO–treated group. ARASCO group refers to ARASCO-only–treated control group. HFD, high-fat diet; PCR, polymerase chain reaction; STZ, streptozotocin.

Discussion

Both obesity and T2DM are assuming epidemic proportions in several countries. This increase in the incidence of obesity and T2DM has been attributed to lack of exercise and consumption of high-fat or calorie-dense foods. Despite this, it is not clear how exactly HFD and calorie-dense food increase obesity and T2DM. To understand the mechanisms involved in the obesity and the T2DM epidemic, several models of human T2DM have been tried and developed to investigate the pathophysiology of the disease and to evaluate various therapeutic strategies of the same. Patients developing T2DM often have obesity, although it is well known that patients of South East Asian descent are often thin, may have abdominal obesity, and contain more percentage of abdominal fat than Europeans with T2DM [26,27]. Hence, it is important to develop and employ animal models that show reduced insulin sensitivity along with compensatory excess basal insulin secretion and hyperproinsulinemia that are clinically relevant to human T2DM. Such models of T2DM are important and required to achieve the aim of testing new and better therapeutics. Both genetic spontaneous diabetes models and experimentally induced non-spontaneous diabetes models exist for this purpose. An example of an experimentally induced animal model of T2DM is the HFD + STZ rat model. In this model, a combination of HFD to induce hyperinsulinemia, IR, or glucose intolerance followed by treatment with the β-cell toxin STZ that produces a significant reduction in functional β-cell mass (28–34) is employed. It was found that these two stressors mimic the pathology of human T2DM diabetes, although on a shorter time scale than found in humans. Several previous studies revealed that the HFD + STZ diabetes model is far superior

Table 9
Various antioxidant enzymes measured in different organs in the study

		NO	LPO	CAT	SOD	GPx	GST
Plasma	Control	0.83 ± 0.04	0.31 ± 0.03	3038 ± 175	48.50 ± 5.2	3674 ± 410	58 ± 1.9
	HFD	1.35 ± 0.05*	0.83 ± 0.04*	3802 ± 206*	77.7 ± 8.8*	4426 ± 194*	81 ± 2.00*
	ARASCO	0.9 ± 0.04	0.33 ± 0.02	2852 ± 138	57.23 ± 9.32	4031 ± 416	61 ± 1.40
	ARASCO+HFD	1.01 ± 0.03	0.38 ± 0.02†	3194 ± 109	61.33 ± 3.89†	4491 ± 618†	61 ± 2†
Pancreas	Control	0.78 ± 0.05	0.47 ± 0.03	3049 ± 202	161 ± 13.75	3606 ± 502	43.3 ± 1.5
	HFD	1.38 ± 0.03*	0.78 ± 0.03*	5125 ± 360	221 ± 9.8*	6587 ± 442*	68.7 ± 3.4*
	ARASCO	0.79 ± 0.04	0.51 ± 0.01	3216 ± 139	124.75 ± 12	4276 ± 469	49.67 ± .67
	ARASCO+HFD	1.24 ± 0.06†	0.49 ± 0.02†	4013 ± 279†	146.06 ± 8.8†	4401 ± 546†	59.18 ± 1.0†
Liver	Control	3.3 ± 0.08	0.23 ± 0.02	2315 ± 194	78.44 ± 4.4	2952 ± 350	40.40 ± 1.83
	HFD	1.3 ± 0.07*	0.98 ± 0.06*	2761 ± 126	56.5 ± 3.4*	3052 ± 335	39 ± 1
	ARASCO	0.72 ± 0.03*	0.36 ± 0.01	2662 ± 173	93.04 ± 6.01	2993 ± 269	44 ± 2.03
	ARASCO+HFD	0.92 ± 0.06†	0.55 ± 0.02†	2794 ± 98†	94.84 ± 3.3†	2928 ± 364†	43 ± 1
Adipose	Control	1.15 ± 0.12	0.34 ± 0.03	2424 ± 166	57.4 ± 3.6	3766 ± 651	34 ± 1
	HFD	2.6 ± 0.16*	1.77 ± 0.06*	5029 ± 800*	67 ± 6.10*	3719 ± 228	36 ± 4
	ARASCO	0.8 ± 0.06*	0.6 ± 0.05	2512 ± 125	61 ± 8	3526 ± 418	34.4 ± 1.72
	ARASCO+HFD	1.64 ± 0.09†	1.61 ± 0.06†	4273 ± 617	60 ± 8†	5905 ± 105	33.38 ± 2.48

CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; HFD, high-fat diet; LPO, lipid peroxide; NO, nitric oxide; SOD, superoxide dismutase, STZ, streptozotocin.
HFD refers to STZ + HFD–induced diabetes group; ARASCO + HFD group refers to ARSCO-treated STZ + HFD–induced diabetes group.
*$P < 0.05$ compared with control.
†$P < 0.05$ compared with STZ + HFD control.

to HFD or STZ alone in terms of IR, compensatory hyperinsulinemia, and glucose intolerance [28–34], thus implying that controls of HFD or STZ are not needed when this HFD + STZ model is employed in a given study. In view of this, in the present study, we used only four groups of animals: control, HFD + STZ, ARASCO, and HFD + STZ + ARASCO.

In view of the current epidemic of obesity and T2DM that is taking a huge toll on individual's health and consequently society at large, their associated morbidity and mortality calls for identifying safe, reliable, and robust endogenous or synthetic molecules that are of significant benefit. The results of the present study suggest that ARASCO oil could be of such benefit. The major component of ARASCO, AA, is an endogenous bioactive lipid molecule that has significant role in various physiologic and pathologic processes. AA forms a precursor of not only proinflammatory prostaglandins, thromboxanes, and leukotrienes but also of potent anti-inflammatory LXA4. In a previous study, we showed that supplementation of ARASCO orally can prevent alloxan-induced T1DM in Wistar rats [13]. Alloxan-treated animals showed decreased plasma, liver, and muscle content of GLA, di-homo-γ-linolenic acid (DGLA), and AA in their phospholipid fraction. ARASCO supplementation restored AA content to normal levels in the plasma, liver, and muscle tissues [13]. In an extension of this study, we observed that of all the fatty acids tested, AA is the most potent in preventing both alloxan and STZ-induced type 1 and T1DM and T2DM, respectively [14–19]. Because AA is expensive and unstable, we sought to find an alternative source. The results of the present study clearly demonstrated that ARASCO oil that is rich in AA (~ 40%) can prevent HFD + STZ–induced diabetes in Wistar rats.

HFD + STZ–induced diabetes produced oxidative stress (in the form increase in the formation of LPOs and NO and alteration in the activities of SOD, CAT, and GSH), increased the plasma levels of IL-6 and TNF-α, and enhanced expression of *NF-κB* and *LPCL2* events that are known to induce low-grade systemic inflammation (see Figs. 4 and 5 and Table 9) in addition to hyperglycemia and hyperinsulinemia. All these abnormalities reverted to near normal in ARASCO-supplemented HFD + STZ–induced diabetes animals. It is noteworthy that ARASCO-treated animals showed an increase in plasma LXA4 levels that suggests that AA has significant anti-inflammatory actions that may underlie its beneficial action in HFD + STZ–induced diabetes. This is supported by our previous study [17,18] where we observed that AA treatment of STZ-induced T2DM animals have increased plasma levels of LXA4 compared with Wistar rats treated with STZ alone. Furthermore, LXA4 administration attenuated both STZ and alloxan-induced type 1 and T2DM and T1DM, respectively [17–19], suggesting that the beneficial actions of AA are mediated by increased formation of LXA4, a potent anti-inflammatory molecule. The increase in plasma IL-6 and TNF-α levels (TNF-α > IL-6) and LXA4 (especially in weeks 4 and 12 of the present study) in ARASCO-supplemented control group is interesting. The increase in plasma IL-6 and TNF-α levels, despite an increase in plasma LXA4 levels in the ARASCO-treated control animals that is known to suppress the production of these proinflammatory cytokines, is rather intriguing.

Despite the belief that high plasma IL-6 and TNF-α cause IR and glucose intolerance, some studies have shown that mildly elevated plasma levels of these cytokines could increase insulin sensitivity and glucose tolerance [35–37]. These studies revealed that low levels of TNF-α infusion reduces basal insulin levels but does not affect the insulin response to glucose. On the other hand, some studies have shown that infusion of low-levels of IL-6 enhances insulin sensitivity and glucose uptake. Incubation of 3 T3-L1 cells with low levels of (~800 u/mL) IL-6 increased glucose transportation into the cells by increasing the expression of GLUT1 transporters on the cells [36].

It is noteworthy that IL-6 stimulates secretion of the pro-survival incretin hormone glucagon-like peptide 1 by α cells and acts directly on β cells to stimulate insulin secretion in vitro. IL-6 was found to activate signal transducer and activator of transcription 3 (STAT3), a transcription factor involved in autophagy. It was reported that stimulation of autophagy by IL-6 is regulated via inhibition of mammalian target of rapamycin complex 1 (mTORC1) and activation of Akt, ultimately leading to increases in autophagy enzyme production. Thus, pretreatment with IL-6 rendered β cells resistant to apoptosis induced by proinflammatory cytokines. Because autophagy is critical for β-cell homeostasis, especially under conditions of stress, direct stimulation of autophagy by IL-6 may result in protection of β cells from stress-induced apoptosis [38]. Thus, the increase in IL-6 levels in ARASCO-treated animals seen in the present study may result in increased survival of pancreatic β cells and hence, prevention of HFD + STZ–induced diabetes noted. The changes observed in plasma IL-6, TNF-α, and LXA4 levels seen in ARASCO-supplemented STZ + HFD–treated animals may explain the anti-inflammatory and antidiabetic actions of AA-rich ARASCO oil.

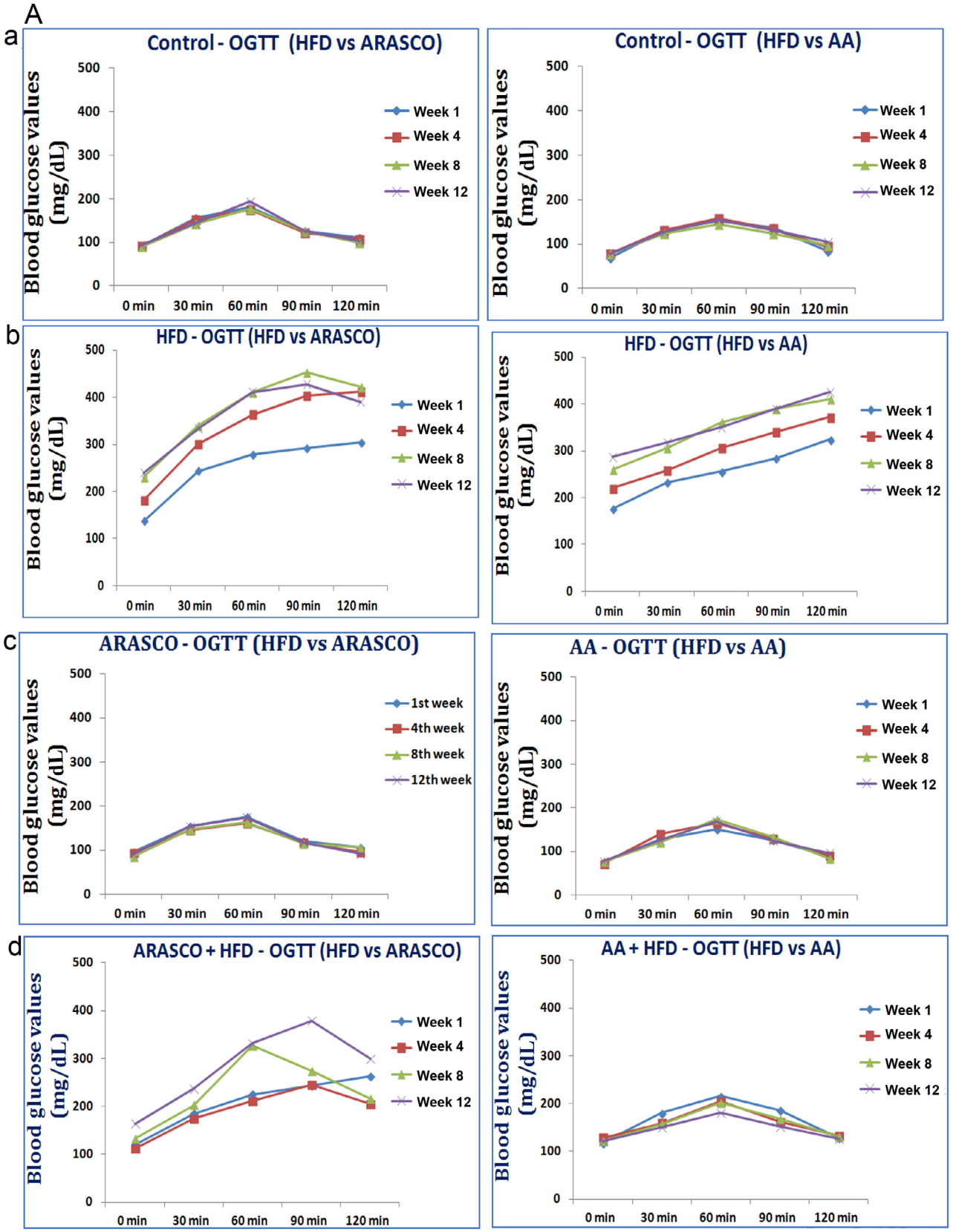
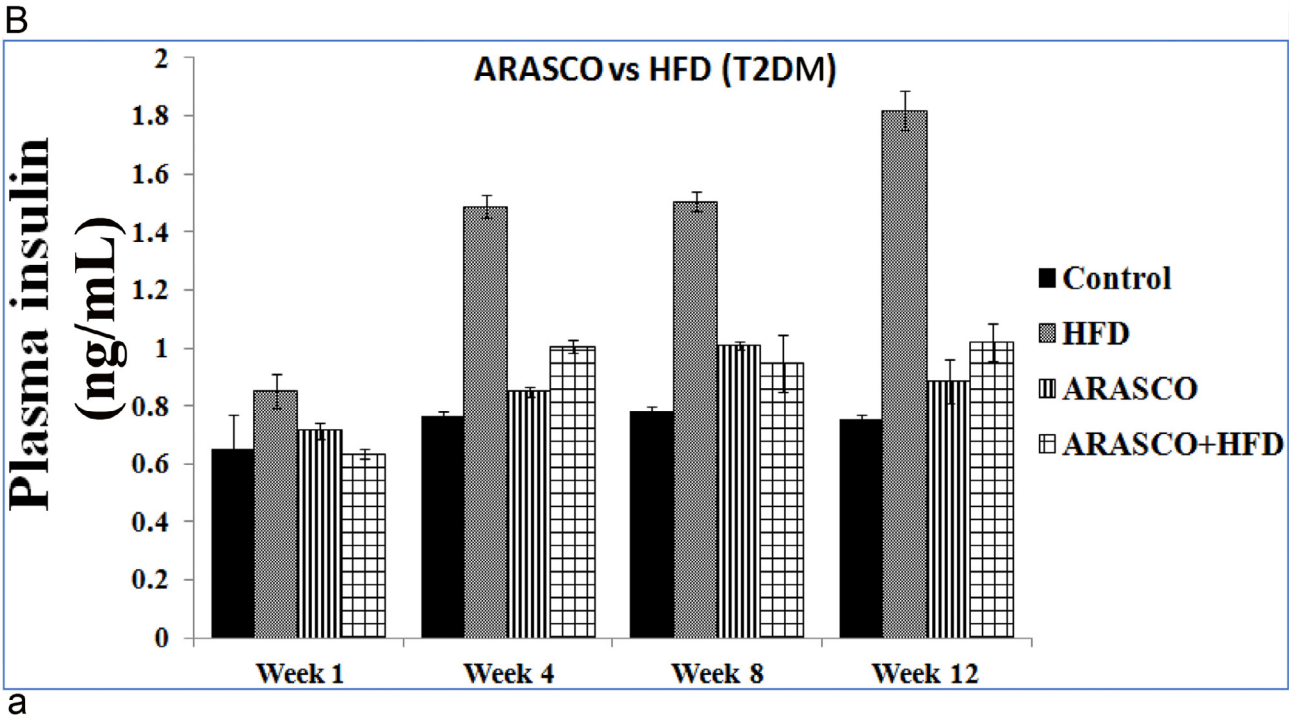


Fig. 6. (**A**) Comparison of OGTT between ARASCO and AA-treated Wistar rats that were induced to develop diabetes using STZ + HFD treatment performed at the end of the study. (a) OGTT in the control group of ARASCO study (HFD vs ARASCO) and the control group of the AA study (HFD vs AA). (b) OGTT in animals in the ARASCO group that had STZ + HFD–induced diabetes (HFD vs ARASCO) and those in the AA group that had STZ + HFD–induced diabetes (HFD vs AA). (c) OGTT in ARASCO control animals (HFD vs ARASCO) and AA control animals (HFD vs AA). (d) OGTT in STZ + HFD + ARASCO–treated animals (HFD + ARASCO) and STZ + HFD + AA (HFD + AA). **(B)** Plasma insulin levels at different time periods of the study (at weeks 1, 4, 8, and 12 of the study) in ARASCO and AA treated STZ + HFD–induced diabetes groups. (a) Plasma insulin levels in ARSCO (0.1 mL orally for the first 7 d and subsequently 0.1 mL/wk) treated STZ + HFD–induced diabetes animals. (b) Plasma insulin levels in AA (10 μg/animal given orally for the first week and later once weekly until the end of the study) treated STZ + HFD–induced diabetes animals. AA, arachidonic acid; HFD, high-fat diet; OGTT, oral glucose tolerance test; STZ, streptozotocin.



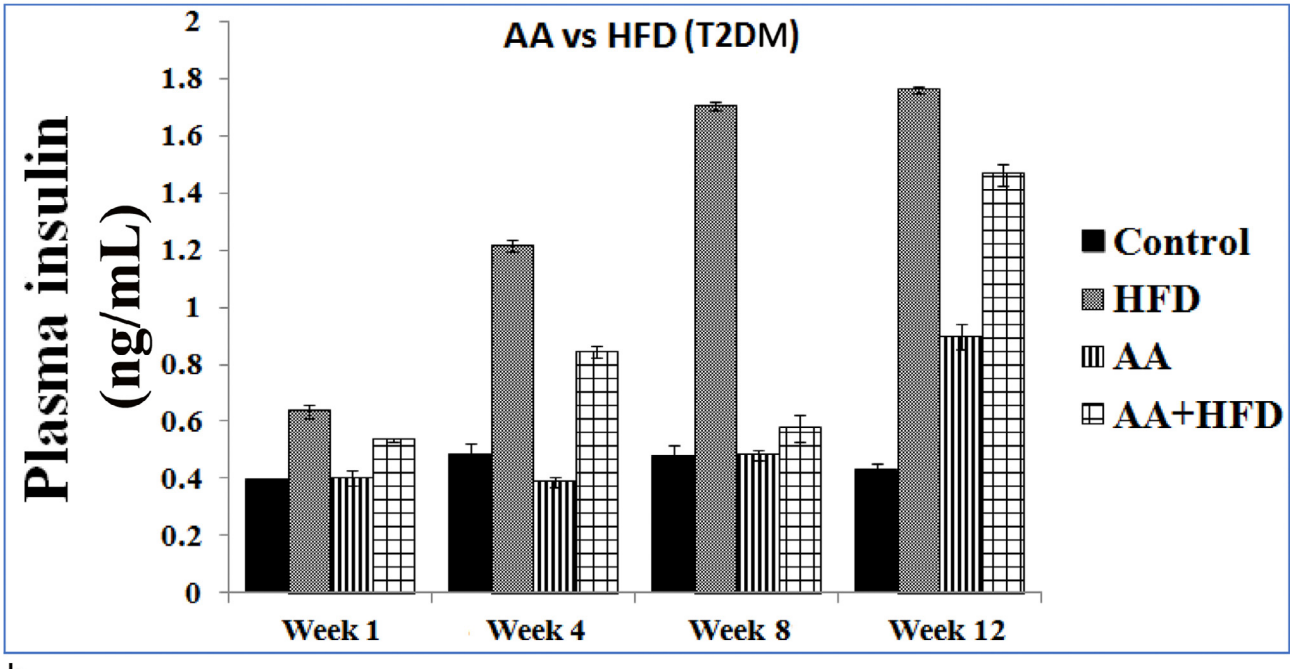


Fig. 6 Continued.

It was reported that in a nonobese diabetic (NOD) transgenic mice expressing TNF-α under the control of the rat insulin II promoter, TNF-α expression in the islets led to insulitis, composed of CD4+ T cells, CD8+ T cells, and B cells, yet they were protected from the development of T1DM. It was found that expression of TNF-α in islets downregulated splenic cell responses to autoantigens, suggesting that local expression of TNF-α protects NOD mice from autoimmune diabetes by preventing the development of autoreactive islet-specific T cells [39]. It is not clear whether such a phenomenon is responsible for the prevention of HFD + STZ–induced diabetes in the present study.

From the above observations, it may be suggested that the rise in plasma IL-6 and TNF-α levels seen in ARASCO-treated animals signifies its (AA) regulatory role in IR and glucose intolerance and prevention of HFD + STZ–induced diabetes observed in the present study.

In this context, one may argue that all the observed changes are unlikely to be due to the AA content in ARASCO since ARASCO also contains several other SFAs, MUFAs, and PUFAs (see Tables 1 and 2 for the fatty acid content and composition of ARASCO oil), although all of them are in the range from 1% to 15% and may also play a role in the various actions observed in the present study. Earlier, we

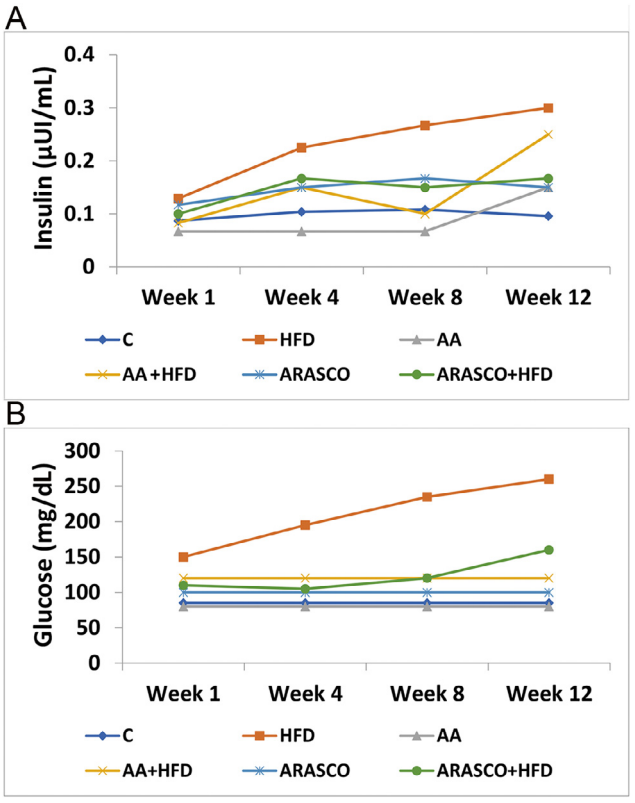


Fig. 7. Fasting plasma insulin (**A**) and glucose (**B**) levels in AA and ARASCO-treated STZ + HFD–induced diabetes Wistar rats at different time periods (weeks 1, 4, 8, and 12 of the study). Same data in Fig. 6B but given in the form of a line figure for better appreciation of the differences in the two (ARSCO and AA) treatments. AA, arachidonic acid; HFD, high-fat diet; STZ, streptozotocin.

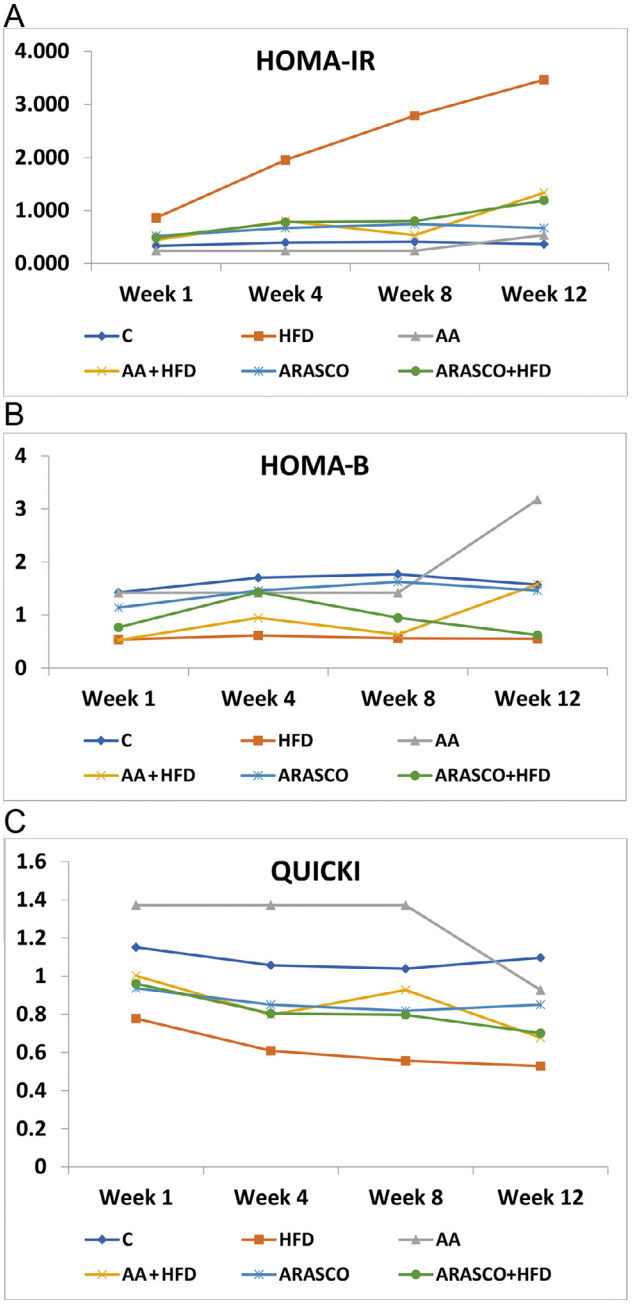


Fig. 8. Estimation of insulin resistance (**A**) (HOMA-IR), β-cell function (**B**) (HOMA B), and QUICKI (**C**) in ARASCO and AA-treated STZ + HFD–induced diabetes animals. Optimal range: 1 (0.5–1.4). <1 means insulin sensitive, which is optimal. >1.9 indicates early insulin resistance. >2.9 indicates significant insulin resistance. High HOMA-IR and low HOMA-B were independently and consistently associated with an increased diabetes risk. AA, arachidonic acid; HFD, high-fat diet; HOMA-IR, homeostatic method of assessment for insulin resistance; QUICKI, quantitative insulin sensitivity check index; STZ, streptozotocin.

performed studies with pure individual fatty acids (including SFA: stearic acid: 18:0; MUFA: oleic acid: 18:1 ω-9; LA: 18:2 n-6; ALA: 18:3 ω-3; GLA: 18:3 ω-6; DGLA: 20:3 ω-6; AA: 20:4 ω-6; EPA: 20:5 ω-3 and DHA: 22: 6 ω-3) and observed that of all the fatty acids tested, AA was the most potent in protecting against alloxan-induced DM [14–16]. In an extension of this study, we also noted that both COX and LOX enzyme inhibitors did not prevent the antidiabetic action of AA against alloxan- and STZ–induced diabetes [14–19], suggesting that AA itself is active. Subsequent studies revealed that enhanced formation of LXA4 occurs and could be responsible for the beneficial action of AA seen in these studies [17–19]. These results were confirmed by gene expression studies wherein it was noted that STZ induced significant decreases in 5-LOX and 12-LOX enzymes and substantial increases in COX-2 expression, whereas AA treatment restored COX-2, 5-LOX, and 12-LOX activities to near normal levels that are essential for formation of LXA4, coinciding with the increased circulating levels of LXA4. Furthermore, LXA4 prevented both alloxan- and STZ-induced diabetes in experimental animals [17–19]. Based on these studies, we believe that AA brings about its antidiabetic and anti-inflammatory actions by enhancing the formation of LXA4. Because other fatty acids such as SFA, OA, LA, GLA, DGLA, ALA, EPA, and DHA are comparatively less potent than AA in preventing chemically induced diabetes and are also not the precursor of LXA4, it is reasonable to assume that AA and its anti-inflammatory metabolite LXA4 are the mediators of the beneficial action of ARASCO observed in the present study.

To verify this possibility further, we studied the effect of pure AA on the development and progression of diabetes in STZ + HFD–treated Wistar rats. These results obtained with AA were compared with those of ARASCO. It may be mentioned here that in the present study, we used 0.1 mL of ARASCO oil, which results in about 40 mg of AA (as ARASCO oil contains ~40% AA and so 0.1 mL = 100 mg will deliver about 40 mg of AA), whereas when we supplemented pure AA orally to the animals, each animal received only 10 μg. Thus, the difference in the amount of AA received by Wistar rats in the ARASCO groups compared with the group that received pure AA is enormous (pure AA-administered animals received 10 μg versus 40 mg of AA in the ARASCO groups, a difference of about 40 × 1000/10 = 4000 times). Results obtained

in this study (AA versus ARASCO) are given in Figures 6 to 8. These results showed that the plasma glucose, insulin, and calculated HOMA and QUICKI indices for IR and sensitivity, respectively, in Wistar rats with STZ + HFD-induced DM are more favorably inclined toward less severe diabetic status as evidenced by lower plasma glucose, higher plasma insulin, less IR, and an increase in insulin sensitivity in the pure AA-supplemented group compared with the ARASCO group (see Figs. 6–8). It is apparent from the results of this study that pure AA is more beneficial than ARASCO at the doses used despite the fact that ARASCO oil contains significantly higher amounts of AA. These results imply that possibly, other fatty acids (especially SFAs) present in ARASCO oil may interfere with the beneficial actions of AA. It is not clear whether this differences in the beneficial actions of AA and ARASCO is due to differences in the conversion of AA to LXA4. This is a distinct possibility because we found that the plasma levels of LXA4 in AA-treated rats was found to be ~0.6 ng/mL, whereas in the ARASCO-treated rats it hovered ~0.5 ng/mL, despite the presence of increased amounts of AA in ARASCO. In a similar fashion, even the plasma levels of IL-6 and TNF-α showed distinct differences between AA- and ARASCO-treated groups. In AA-treated STZ + HFD rats, plasma levels of IL-6 were ~210 pg/mL, whereas in the ARASCO group they were ~250 pg/mL; whereas plasma TNF-α levels were ~250 pg/mL in AA-treated and 150 pg/mL in ARASCO-treated animals (data not shown). On the other hand, the expressions of *NF-κB, COX-2*, and *12-LOX* in AA- versus ARASCO-treated rats were 100 versus 120; 150 versus 110 and 175, versus 100 as percent of control, respectively. These results suggest that there are distinct differences in the plasma levels of glucose, insulin, LXA4, and expression of various genes concerned with eicosanoid metabolism in those treated with ARASCO versus AA, which may explain why pure AA is more beneficial than ARASCO. This proposal needs further confirmation.

Yet another factor that could explain differences in the beneficial actions seen between AA and ARASCO may lie in their absorption and incorporation into the tissues. It is likely that pure AA may be absorbed and incorporated into tissues better than the AA present in ARASCO. Hence, we estimated plasma phospholipid concentrations of AA in Wistar rats that received 0.1 mL of AA and 0.1 mL of ARASCO for 5 d. The results of this preliminary study showed that plasma levels of AA in ARASCO-treated animals increased by 113.26%, whereas after AA supplementation the increase was ~160% compared with control (unpublished data not shown), suggesting that absorption of AA, when pure AA was given, is significantly higher than the ARASCO treatment. This may explain why pure AA-treated animals showed better response than those treated with AA-rich ARASCO oil.

These beneficial results obtained in animal studies with ARASCO in the present study are supported by the observation that hepatic and plasma levels of AA were significantly lower in obese and T2DM rats than in the control animals [40]. Additionally, we reported that patients with T2DM have low plasma levels of LXA4 [41,42]. Thus, the results obtained in the present animal study support the contention that low plasma levels of LXA4 may predispose or even enhance the risk for and progression of diabetes in humans.

Conclusion

Results of the present study and other evidence suggest that AA and LXA4 have potent anti-inflammatory and antidiabetic actions. ARASCO oil, a rich source of AA, can ameliorate oxidative stress, suppress production of proinflammatory cytokines such as IL-6 and TNF-α, inhibit *NF-κB* expression, restore antioxidant defenses to normal—thus ultimately preventing development of HFD + STZ–induced diabetes. ARASCO oil is non-toxic and safe to administer to humans. It remains to be seen whether long-term supplementation can prevent or postpone the development of T2DM.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2019.05.007.

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