JOURNAL OF FUNCTIONAL FOODS 14 (2015) 641–649

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Dietary intervention with Salvia hispanica (Chia) oil improves vascular function in rabbits under hypercholesterolaemic conditions

Liliana Sierra [a], *Julieta Roco* [b], *Gabriela Alarcon* [b], *Mirta Medina* [a], *Carina Van Nieuwenhove* [a,c], *María Peral de Bruno* [b,d], *Susana Jerez* [a,b,*]

[a] *Facultad de Ciencias Naturales, Universidad Nacional de Tucumán (UNT), Miguel Lillo 298, San Miguel de Tucumán, Tucumán, Argentina*

[b] *Instituto Superior de Investigaciones Biológicas (INSIBIO, UNT-CONICET), Av Independencia 1800, San Miguel de Tucumán, Tucumán, Argentina*

[c] *Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, 4000 San Miguel de Tucumán, Tucumán, Argentina*

[d] *Facultad de Medicina. Universidad Nacional de Tucumán (UNT), Av. Presidente Kirchner 1900, San Miguel de Tucumán, Tucumán, Argentina*

ARTICLE INFO

Article history:
Received 19 November 2014
Received in revised form 23 February 2015
Accepted 25 February 2015
Available online

Keywords:
Chia oil
Hypercholesterolaemia
Vascular dysfunction
Angiotensin II
Noradrenaline

ABSTRACT

The effects of dietary supplementation with Salvia hispanica (chia) oil on vascular function in hypercholesterolaemic rabbit were examined. Rabbits were fed either regular diet (CD) or 10% chia oil in regular diet or 1% cholesterol diet (HD) or diet containing 1% cholesterol and 10% chia oil (HD-Ch) during 5–6 weeks. HD increased total cholesterol, LDL and triacylglycerol levels. HD-Ch significantly attenuated the triacylglycerol rise and increased alpha linolenic acid (ALA) levels. Aorta from hypercholesterolaemic rabbits exhibited an impaired relaxation response to acetylcholine (Ach), reduced NO-release and increased intima/media ratio. Including chia oil in the HD partially normalized the response to Ach and the intima/media ratio, and totally restored the NO-release. In addition, dietary supplementation with chia oil blunted the contractile response to angiotensin II and noradrenaline. These findings suggest that increased ALA levels induced by dietary chia oil could improve vascular function under hypercholesterolaemic conditions and therefore could serve as a true functional food.

1. Introduction

Elevated plasma levels of total cholesterol and low density lipoproteins (LDLs) play a significant role in atherosclerosis development and subsequent cardiovascular mortality and morbidity (Berenson et al., 1998). Lipid accumulation in blood vessel walls during hypercholesterolaemia produces impairment in endothelium-dependent vasodilation in rabbits, even before morphological changes occur (Jayakody, Senaratne, Thomson, & Kappagoda, 1987; Jerez, Sierra, Peral de Bruno, & Coviello, 2008; Sorensen et al., 1994). The development of interventions to inhibit cholesterol-induced atherosclerosis and the associated vascular dysfunction has received much attention because of this strong association. At present, there is an increasing interest in nutritional interventions that may prevent the development of atherosclerosis and protect against the vascular function abnormalities induced by cholesterol consumption.

* *Corresponding author.* Av. Independencia 1800, Tucumán, Argentina. Tel.: +54 0381 4364120; fax: +54 0381 4364120.
E-mail address: sjerez@herrera.unt.edu.ar (S. Jerez).

http://dx.doi.org/10.1016/j.jff.2015.02.042
1756-4646/

Among dietary interventions that might protect against atheroma and its complications are diets rich in omega-3 fatty acids supplements. The protective effects of diets rich in fish oil are quite strongly supported by experimental, epidemiological and clinical trial data (Goodfellow, Bellamy, Ramsey, Jones, & Lewis, 2000; Kris-Etherton, Harris, & Appel, 2002). Beneficial effects of fish oil supplementation on endothelial function in resistance arteries *in vivo* (Chin & Dart, 1994) and *in vitro* (Goode, Garcia, & Heagerty, 1997) have been reported. Marine-derived long chain n-3 polyunsaturated fatty acids (n-3 PUFAs) [eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] have been shown to improve markers of endothelial function in humans (Din et al., 2013).

Plant-derived n-3 PUFAs [alpha linolenic acid (ALA, 18:3n-3)] can be elongated and desaturated to its long-chain derivatives EPA and DHA. However, some moderate net rise in the level of EPA and no net rise in the levels of DHA were found in subjects fed a diet enriched with flaxseed oil (Burdge & Calder, 2005; Hussein et al., 2005). Since marine EPA and DHA are not as widely available as plant-derived ALA owing to the cost and supply constraints of seafood compared with plant sources, the effect of ALA on endothelial function is therefore of considerable importance, particularly for populations with low consumption or availability of fatty fish. Studies investigating the effects of ALA in hypercholesterolaemic patients demonstrated cardiovascular protective effects including anti-inflammation effects (Zhao et al., 2004, 2007) and beneficial effects on platelet function and arterial compliance (Ros et al., 2004). Clinical observations have demonstrated that the endothelial function index is positively related to the proportion of ALA in young men (Steer, Vessby, & Lind, 2003). Dupasquier et al. (2006) demonstrated that dietary ALA intake in the form of flaxseed limits cholesterol induced atherogenesis as well as abnormalities in endothelial-dependent vasorelaxation. Evidence from clinical trials has also demonstrated that ALA could enhance flow-mediated dilation of the brachial artery 4 h after meals in patients with type 2 diabetes mellitus (Hilpert et al., 2007). All these studies indicate that ALA is an endothelial protective factor. However, little evidence exists about the preventive effects of ALA intake on endothelial dysfunction and underlying mechanisms during the development of hypercholesterolaemia.

Salvia hispanica (chia) is a summer annual herbaceous plant categorized under the mint family (Labiatae), superdivision of Spermatophyta, and kingdom of Plantae. The seed contains from 25 to 40% oil with 60% of it comprising (omega) ω-3 ALA and 20% of (omega) ω-6 linoleic acid. Most of the studies about the beneficial effects of chia were conducted with seeds. In this regard, results from a randomized trial demonstrated that supplementation of conventional therapy with chia seed improves the cardiovascular risk factors in patients with type 2 diabetes mellitus (Vuksan et al., 2007). Chicco, D'Alessandro, Hein, Oliva, and Lombardo (2009) reported that dietary chia seed improves adiposity and normalizes hypertriacylglycerolaemia and insulin resistance in dyslipaemic rats. However, less evidence about the beneficial effects of plant-derived omega-3 rich oils has been found. Ayerza and Coates (2005) demonstrated a decrease of triacylglycerol (TG) and increase of HDL-cholesterol levels in plasma from rats fed chia oil. The n-3/n-6 fatty acid ratio of chia oil ranged from 3.18 to 4.18 (Ayerza & Coates, 2004; Ixtaina et al., 2011), these values being markedly higher than that of most vegetable oils, e.g. canola oil (0.45), olive oil (0.13), soybean oil (0.15), and walnut oil (0.20) (Belitz & Grosch, 1999). In addition, chia oil contained other minor components: about 238–427 mg/kg of tocopherols, mainly gamma tocopherol (85%) and delta-tocopherol, variable concentrations of alpha-tocopherol (0.4–9.9 mg/kg) and undetectable levels of beta-tocopherol. The total polyphenolic content in chia oils ranged from 6.10^{-6} to $2.1.10^{-5}$ mol/kg. The major phenolic compounds were chlorogenic and caffeic acids, followed by myricetin, quercetin and kaempferol (Ixtaina et al., 2011).

In a previous work we demonstrated that feeding a high cholesterol diet (HD) for a short term induces vascular dysfunction characterized by: (a) reduced Ach-relaxation and nitric oxide (NO)-release; (b) endothelium-dependent increase of the angiotensin II (Ang II) response (Jerez et al., 2008); (c) lipid metabolism-alteration and moderate changes of vascular morphology (Medina et al., 2014). Therefore the objective of the present study was to determine the effects of dietary supplementation with Salvia hispanica (chia) oil on vascular function and morphological changes development during early hypercholesterolaemic conditions. We hypothesize that dietary chia oil would demonstrate a protective effect against cholesterol-induced endothelial dysfunction and vascular contractile abnormalities.

2. Materials and methods

2.1. *Animals and diets*

Experiments were reviewed and approved by our Institutional Animal Care and Use Committee (Bioethics Committee of the School of Medicine of the National University of Tucuman, Argentina). Thirty two male Flanders hybrid rabbits (Cabaña Los Prietos, Las Talitas, Tucumán, Argentina), weighing 800–900 g on arrival, were individually housed in metal cages in a room with controlled temperature, humidity, and a 12-h light cycle. Animals were randomly assigned to four groups of eight animals. Animals were fed for 5–6 weeks. The four diets included a control diet (CD) of regular rabbit chow (Ganave, Pilar, Argentina), a 10% chia oil-supplemented chow (CD-Ch), a 1% cholesterol-supplemented chow (HD), and a diet supplemented with 1% cholesterol and 10% chia oil (HD-Ch). All diets were dry mixed and repelleted to incorporate the added components. Rabbits were fed 100 g of the appropriate dietary treatment per day. The total intake of ALA was about 4.8 g per day according to the recommendation for humans of the American Heart Association (Kris-Etherton, Harris, & Appel, 2003). Water was given ad libitum. The chia oil was prepared by extracting seed oil craft under cold pressed systems without the use of chemical additives to give a natural, high quality product. Oil fatty acid composition (%) was: myristic 0.04, palmitic 6.6, stearic 2.3, palmitoleic 0.1, oleic 6.7, linoleic 20.4, and linolenic 63.0. The animals were weighed before dietary manipulation and every day throughout the period of the experiment.

2.2. *Blood sampling and analysis*

Blood samples were taken at baseline (0 week) and after 5–6 weeks of dietary intervention from the marginal ear vein before

daily feeding. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), LDL cholesterol (LDL-C) and TG were measured using colorimetric reactions with commercial kits (Wiener, Rosario, Argentina). Total lipids were extracted from the plasma samples according to the method of Folch, Lees, and Sloane Stanley (1957). They were derivated with HCl/methanol solution according to Van Nieuwenhove, Cano, Chaia, and Gonzalez (2007). One µl of fatty acid methyl ester (FAME), dissolved in hexane, was injected into a gas chromatograph (GC, Model 6890N, Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector (Agilent Technologies, Wilmington, DE) and an automatic injector (Model 7683, Agilent Technologies Shanghai, China) into an HP-88 capillary column (100 m × 0.25 mm × 0.20 µm, Agilent Technologies, Wilmington, DE). GC conditions involved an injector with a temperature of 255 °C. The initial oven temperature of 75 °C was increased to 165 °C at 8 °C/min and was held there for 35 min. It was then increased to 210 °C at 5.5 °C/min and maintained for 2 min, and afterwards to 240 °C at 15 °C/min and held for 3 min. The detector temperature was 280 °C. Nitrogen was used as a carrier gas at a flow rate of 18 ml/min at 38 psi. C17:0 was used as the internal standard. FAME was identified and quantified by comparison with the retention times and peak areas of standards (Sigma, St Louis, MO, USA). Results were expressed as g/100 g of FAME.

2.3. *Preparation of tissues*

After 5–6 weeks of dietary treatment, rabbits were anaesthetized with ketamine (75 mg/kg). The aortas were excised and immediately placed in cold Krebs (128 mM NaCl, 14.4 mM NaHCO3, 1.2 mM NaH_2PO_4, 4.7 mM KCl, 0.1 mM disodium salt of ethylenediaminetetraacetic acid, 2.5 mM $CaCl_2$, 11.1 mM glucose, pH 7.2). The aortas were carefully dissected from the distal end of the aortic arch to the base of the diaphragm. The aortas were cleaned of adventitial tissue and prepared for vascular function testing.

2.4. *Experimental protocol for assessing vascular response*

Aortic tissue, dissected into 5-mm-width rings, was fastened in an organ bath with surgical wire, perfused with the Krebs solution, aerated with 95% O_2 and 5% CO_2, and equilibrated at 37 °C and pH 7.4. Isometric contractions were measured using force–displacement transducers and recorded under an initial tension of 2 g, which was found to be the optimal tension for KCl-induced contraction (96 mM). All the preparations were allowed to equilibrate for 120 min and washed with Krebs solution at 15-min intervals. Aortic rings from all diet groups were stimulated with 96 mM KCl, which causes membrane depolarization and stimulates Ca^{2+} entry from the extracellular space (Karaki et al., 1997). Once the KCl contraction reached a plateau, the tissue was rinsed with Krebs solution and allowed to return to basal tension. Then, to check endothelial function, aortic rings were contracted with phenylephrine (Phe) 5.10^{-6} M and exposed to the endothelium-dependent vasorelaxant acetylcholine (Ach, 10^{-8} M to 5.10^{-6} M). Thus, a concentration–response curve (CRC) was constructed. To check contractile response to Ang II and noradrenaline (NA), aortic rings were exposed to increasing doses either of Ang II (10^{-10} to 10^{-6} M) or NA (10^{-8} to 10^{-4} M) to construct CRCs.

2.5. *Nitrite measurement*

Nitrite was measured with the use of Griess reagent as previously described (Jerez, Peral de Bruno, & Coviello, 2001). Aortic rings cut longitudinally (3 mm wide) from rabbits fed the CD or rabbits fed the HD treated or untreated with chia oil were placed in a 1 ml organ bath containing Krebs solution at 37 °C and aerated with 95% O_2 and 5% CO_2. ACh 10^{-6} M was added to the bath and samples (500 µL) were collected from the incubation medium 20 min after the Ach-stimulation. Nitrite was analysed with Griess reagent [N-(50 µL 1-naphthylethylenediamine 0.2% and 450 µL sulphanilamide 0.1%)]. The tubes were kept at room temperature for 10 to 15 min until a full pink colour developed. Absorbance was measured at 540 nm using a spectrophotometer (Metrolab 1000, Metrolab, Argentina). Amount of nitrite was estimated from a standard curve of sodium nitrite by using regression analysis ($y = a + bx$). Only curves with a correlation coefficient >0.95 were used. Nitrite was expressed as picomol/mg tissue as wet weight/ml.

2.6. *Vascular morphology*

Histological analysis of segments of the thoracic aorta (adjacent to the aortic arch) from the four experimental groups was performed. The aorta was rinsed with normal saline solution and preserved in 10% formaldehyde buffered solution (pH 7.4) for the next step. From each sample, serial sections were made (3–5 sections/aorta). The 7 µm sections were stained by haematoxylin-eosin method. Media and intima thickness were measured by image analysis with the software Media Cybernetics® Image-Pro Plus TM. The ratio between the tunica intima and the tunica media was calculated. A code number was assigned to each section observed.

2.7. *Statistical analysis*

All data are expressed as mean ± SE. The pD_2 (negative log of molar concentration of agonist inducing 50% of the maximal contraction) and the maximal contractile response (E_{max}) were calculated using a curve-fitting analysis program. The significance of the differences between and within the groups was examined with an analysis of variance (ANOVA) for repeated measures followed by a Duncan's test. The differences in the mean values between the four diet groups were tested by one way ANOVA followed by a Duncan's test. Pearson's correlation coefficients were used to assess relationships between normally distributed variables. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Diet composition and animal weights*

Fatty acid composition of experimental diets is described in Table 1. Animal body weights did not differ significantly among

Table 1 – Fatty acid composition of the experimental diets.

Fatty acid	CD	CD-Ch	HD	HD-Ch
Myristic 14:0	0.27 ± 0.1	0.21 ± 0.02	0.20 ± 0.01	0.22 ± 0.02
Palmitic C16:0	18.9 ± 0.1	15.31 ± 0.16	16.7 ± 0.47	15.9 ± 0.49
Stearic C18:0	1.62 ± 0.3	1.38 ± 0.04	2.32 ± 0.13	2.02 ± 0.05
Total SFA	21.4 ± 0.1	16.9 ± 0.2	19.2 ± 0.5	18.1 ± 0.5
Palmitoleic C16:1	0.13 ± 0.1	0.10 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
Oleic C18:1 n9	17.2 ± 0.1	19.04 ± 0.32	14.3 ± 0.37	15.1 ± 0.3
Total MUFA	17.6 ± 0.1	20.0 ± 0.3	15.6 ± 0.4	15.2 ± 0.3
Linoleic C18:2 n6	50.0 ± 0.5	55.86 ± 0.37	60.2 ± 0.42	56.9 ± 0.88
Linolenic C18:3 n3	6.3 ± 0.04	8.15 ± 0.06	6.35 ± 0.21	9.82 ± 0.04
Total PUFA	56.7 ± 0.5	64.0 ± 0.4	66.6 ± 0.4	66.7 ± 0.9

CD: control diet; HD: high cholesterol diet; CD-Ch: control diet supplemented with 10% chia oil; HD-Ch: high cholesterol diet supplemented with 10% chia oil. Values are expressed as % (mean ± SE) from total fat (3%) of chow diet.

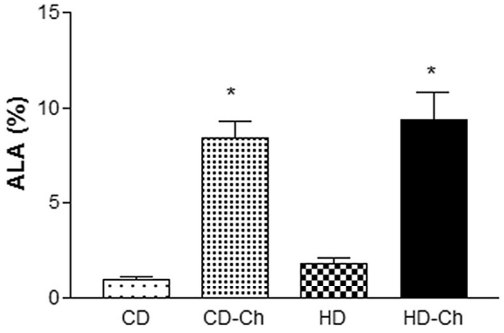

Fig. 1 – % of alpha linolenic acid (ALA) in the plasma of rabbits fed a control diet (CD); rabbits fed a control diet supplemented with 10% of chia oil (CD-Ch); rabbits fed a diet rich in 1% cholesterol (HD); rabbits fed a diet rich in cholesterol 1% supplemented with 10% chia oil (HD-Ch). Each data point shows the mean of 8 animals and vertical lines indicate SE. *P < 0.05 indicates differences statistically significant between the ALA levels of rabbits fed diets supplemented with chia oil (CD-Ch or HD-Ch) and their respective controls (CD or HD).

the four groups before feeding (0 wk) or at the end of the feeding trials (5–6 weeks) (Table 2), which suggests that the energy content of the experimental diets did not differ significantly.

3.2. *Effects on lipid concentrations*

After 5–6 weeks of dietary treatment, animals fed a high cholesterol diet (HD and HD-Ch groups) had increase in plasma TC levels compared with the CD and CD-Ch groups (Table 2). The addition of 10% chia oil to the CD or to the HD did not modify plasma levels of TC. Plasma TG levels were also elevated in the HD group. The addition of chia-oil to the HD significantly attenuated this rise (Table 2).

Plasma ALA levels increase by 8 and 5 fold in CD-Ch and HD-Ch groups compared with the CD and the HD groups respectively (Fig. 1). Plasma EPA levels were 0.24 ± 0.02% in rabbits fed a CD, 0.30 ± 0.05% in rabbits fed a HD and undetectable in rabbits fed diets enriched with chia-oil. Plasma DHA levels were 1.97 ± 0.07% in rabbits fed a CD, 2.10 ± 0.05% in rabbits fed a HD and undetectable in rabbits fed diets enriched with chia-oil.

3.3. *Effects of dietary chia-oil and cholesterol on vascular contractile response*

3.3.1. *Response to acetylcholine*

Aortic relaxation responses were monitored after precontraction with 5.10^{-6} M Phe as a function of the dietary interventions. Ach (10^{-8} to 5.10^{-6} M) caused endothelium-dependent relaxation in a concentration–response manner in all diet groups. Aortic rings from the HD group exhibited significantly less endothelium-dependent relaxation in response to Ach than the CD group after 5–6 weeks of dietary interventions (Fig. 2). Chia-oil added to the diet partially prevented these cholesterol-induced defects. Furthermore, chia oil addition to CD improves significantly the affinity to Ach.

3.3.2. *Response to vasoconstrictors agonists*

The response of aortic rings from animals fed the different dietary regimens was investigated as a function of the contractile agonists. No differences in KCl-induced vasoconstriction were observed in any groups following 5–6 weeks of dietary

Table 2 – Plasma levels of lipids, glucose and weight values from rabbits fed a control diet (CD), a CD supplemented with 10% chia oil (DC-Ch), a high cholesterol diet (HD), a HD supplemented with 10% chia oil (HD-Ch).

	CD	CD-Ch	HD	HD-Ch
Total cholesterol (mg/dl)	59 ± 6	53.4 ± 16.2	872 ± 114[a]	783.57 ± 278.7[a]
HDL-cholesterol (mg/dl)	26.7 ± 4.1	25.3 ± 4.5	164 ± 45[a]	128.57 ± 46.9[a]
LDL-cholesterol (mg/dl)	23.8 ± 3.1	24.6 ± 4.0	666 ± 99[a]	704.99 ± 290.6[a]
Triacylglycerol (mg/dl)	91.7 ± 14.1	120 ± 29	222 ± 33	102 ± 2[b]
Blood glucose (mg/dl)	109 ± 5	125 ± 6	122.3 ± 3.6	122 ± 2
Weight (g)	2072 ± 178	2043 ± 63	2088 ± 44	1852 ± 110

Data are expressed as mean ± SE of 8 rabbits.

[a] $P < 0.05$ indicates statistically significant differences between rabbits fed a HD or a HD-Ch and rabbits fed a CD or a CD-Ch.

[b] $P < 0.05$ indicates statistically significant differences between rabbits fed a HD-Ch and rabbits fed a HD. (One way ANOVA and Duncan´s post test.)

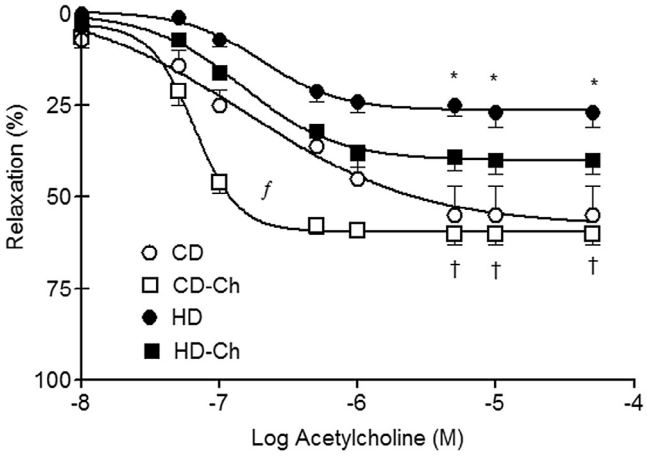

Fig. 2 – Endothelium-dependent relaxation in response to acetylcholine (Ach) in aortic rings following pre-contraction with a submaximal dose of phenylephrine (5.10^{-6} M). CD: arteries from rabbits fed a control diet; CD-Ch: arteries from rabbits fed a control diet supplemented with 10% of chia oil; HD: arteries from rabbits fed a diet rich in 1% cholesterol; HD-Ch: arteries from rabbits fed a diet rich in cholesterol 1% supplemented with 10% chia oil. Each data point shows the mean of 8 experiments and vertical lines indicate SE. *P < 0.05 indicates differences statistically significant between HD and all the other diet groups. †P < 0.05 indicates differences statistically significant between CD-Ch and HD-Ch. (Two way ANOVA with repeated measures and Duncan's post test.) *f*P < 0.05 indicates shift to the right of the concentration response curve to Ach from CD-Ch with respect to all the diet groups. (One way ANOVA and Duncan´s post test.)

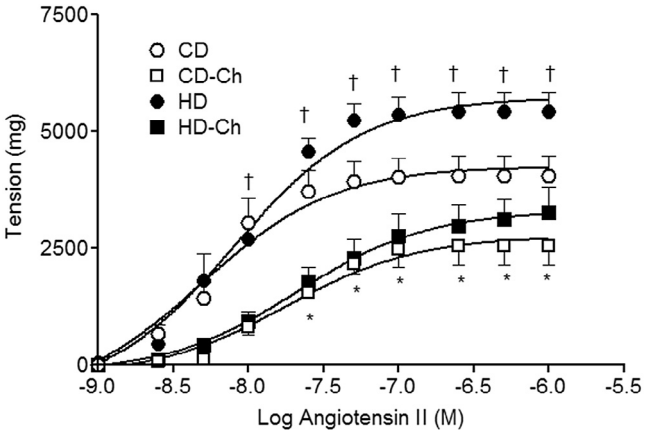

Fig. 4 – Concentration response curves to angiotensin II. CD: arteries from rabbits fed a control diet; CD-Ch: arteries from rabbits fed a control diet supplemented with 10% of chia oil; HD: arteries from rabbits fed a diet rich in 1% cholesterol; HD-Ch: arteries from rabbits fed a diet rich in cholesterol 1% supplemented with 10% chia oil. Each data point shows the mean of 8 experiments and vertical lines indicate SE. *P < 0.05 indicates differences statistically significant between rabbits fed a CD-Ch and rabbits fed a CD. †P < 0.05 indicates differences statistically significant between rabbits fed a HD-Ch and rabbits fed a HD. (Two way ANOVA with repeated measures and Duncan's post test.)

treatment (Fig. 3). Aortic preparations from cholesterol-supplemented group contracted significantly more in response to Ang II than did the CD (Fig. 4). The addition of 10% chia oil to the CD significantly reduced the E_{max} and affinity to Ang II and NA (Figs. 4 and 5). Furthermore, chia oil not only block the increase of E_{max} and affinity to Ang II that was observed in rabbits fed a HD but even blunted the E_{max} with respect to the CD. Desensitization to NA was observed in rabbits fed a HD-Ch (Table 3).

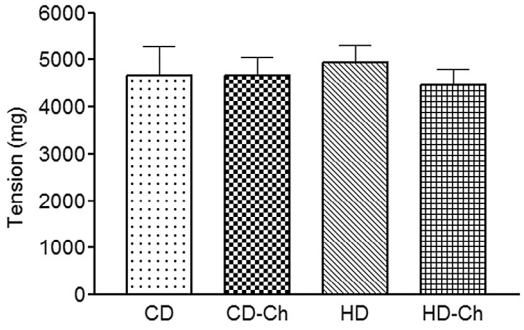

Fig. 3 – Contractile response to 96 mM KCl. CD: arteries from rabbits fed a control diet; CD-Ch: arteries from rabbits fed a control diet supplemented with 10% of chia oil; HD: arteries from rabbits fed a diet rich in 1% cholesterol; HD-Ch: arteries from rabbits fed a diet rich in cholesterol 1% supplemented with 10% chia oil. Each data point shows the mean of 8 experiments and vertical lines indicate SE.

3.4. *Effects of dietary chia-oil and cholesterol on Ach-stimulated nitrite release*

The release of NO from all diet group rabbit aortas was determined as nitrites by using the Griess Method. The release of NO from endothelial cells of each aorta stimulated by ACh is shown in Fig. 6. Ach-stimulated NO release was lower in rabbits fed a HD than rabbits fed a CD. Addition of chia oil to the diet normalized the Ach stimulated-NO release in rabbits fed a HD.

3.5. *Histological changes*

Representative photographs of the morphological changes in the aorta from the four groups stained with haematoxylin-eosin method are shown in Fig. 7. Histological examination showed moderate thickening of the tunica intima in arteries from rabbits fed the HD. The addition of chia-oil to the HD significantly reduced the intima-media ratio (CD: 0.045 ± 0.002 vs HD: 0.78 ± 0.05 vs HD-Ch: 0.29 ± 0.02; $P < 0.01$, one way ANOVA and Duncan's post test).

A significant correlation was found between intima-media ratio and E_{max} to Ang II ($r = 0.90$, $P < 0.01$) and pD_2 to NA ($r = 0.86$, $P < 0.01$) in rabbits fed a HD supplemented with chia oil.

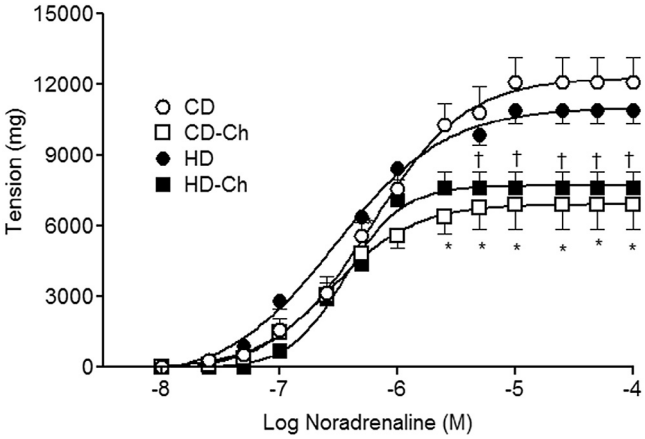

Fig. 5 – Concentration response curves to noradrenaline. CD: arteries from rabbits fed a control diet; CD-Ch: arteries from rabbits fed a control diet supplemented with 10% of chia oil; HD: arteries from rabbits fed a diet rich in 1% cholesterol; HD-Ch: arteries from rabbits fed a diet rich in cholesterol 1% supplemented with 10% chia oil. Each data point shows the mean of 8 experiments and vertical lines indicate SE. *P < 0.05 indicates differences statistically significant between rabbits fed a CD-Ch and rabbits fed a CD. †P < 0.05 indicates differences statistically significant between rabbits fed a HD-Ch and rabbits fed a HD. (Two way ANOVA with repeated measures and Duncan's post test.)

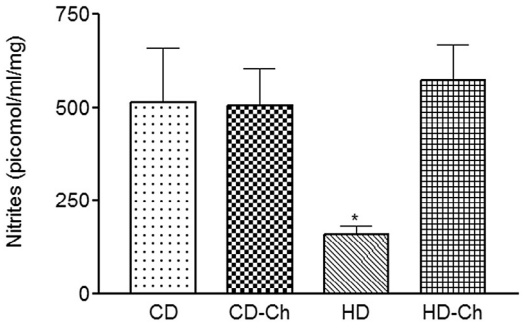

Fig. 6 – Release of nitrites by endothelium intact aortas from rabbits fed a control diet (CD), control diet supplemented with 10% chia oil (CD-Ch), high cholesterol diet (HD) and high cholesterol diet supplemented with 10% chia oil (HD-Ch). Values are shown as mean ± SE of 12 experiments. *P < 0.05 indicates statistical significance between rabbits fed a HD and rabbits fed a HD-Ch, a CD and a CD-Ch. (One way ANOVA and Duncan´s post test.)

4. Discussion

We have demonstrated for the first time that dietary intervention with chia oil can improve the vascular dysfunction under hypercholesterolaemic conditions. Chia oil addition increased the Ach-relaxation, normalized NO-release and reduced the intima/media ratio in rabbits fed a HD. Furthermore chia oil reduced contractile response to Ang II and NA in both diet groups. These findings support its protective effects on the vascular function. Dietary intervention with chia oil to the atherogenic diet also mitigated the cholesterol-induced rise in plasma TG levels. These results are consistent with previous reports using chia seed (Chicco et al., 2009) but are in conflict with other reports that found chia seed supplementation does not modify blood lipids in type 2 diabetes (Vuksan et al., 2007) or in overweight adults (Nieman et al., 2009). Because dietary chia oil supplementation did not alter circulating cholesterol levels, either in the presence or in the absence of additional cholesterol in the diet, it is clear that its beneficial effects were not achieved through a cholesterol-lowering action. Considering that fibre is responsible for the cholesterol-lowering action of grains, it is expected that chia oil has no effect on cholesterol levels.

According to previous work from our laboratory (Jerez et al., 2008; Jerez, Sierra, Scacchi, & Peral de Bruno, 2010; Medina et al., 2014), the present study demonstrated a deleterious effect of HD on the vascular function. Impairment of vascular relaxation as well as increase of the contractile response and affinity to Ang II was found. Chia oil improved the potency of Ach but did not modify its efficacy in arteries from rabbits fed a CD. Moreover NO release from arteries of rabbits fed a CD supplemented with chia oil was similar to NO release from arteries of rabbits fed a regular diet. These results imply that the chia oil-enriched diet has partially beneficial effects on endothelial function on its own compared with a regular diet. In addition, we observe no morphological changes of the aorta

Table 3 – Maximal contractile response (E_{max}) and pD_2 to noradrenaline and angiotensin II in rabbit aortic ring.

	Noradrenaline		Angiotensin II	
	E_{max} (mg)	pD_2	E_{max} (mg)	pD_2
CD	11,237 ± 1,234	6.43 ± 0.14	4117 ± 414	7.82 ± 0.08
CD-Ch	7042 ± 1,008[a]	6.56 ± 0.21	2755 ± 521[a,c]	7.73 ± 0.08
HD	11,210 ± 642	6.55 ± 0.04	5556 ± 400[b]	8.04 ± 0.04[b]
HD-Ch	8507 ± 608[a]	6.03 ± 0.11[a]	3473 ± 539[a]	7.88 ± 0.33[a]

Values are expressed as means ± SE of 8 experiments. CD: control diet; HD: high cholesterol diet; CD-Ch: control diet supplemented with 10% chia oil. HD-Ch: high cholesterol diet supplemented with 10% chia oil.

[a] $P < 0.01$ indicates statistically significant differences between rabbits fed a CD-Ch or a HD-Ch and rabbits fed a CD or a HD.

[b] $P < 0.01$ indicates statistically significant differences between rabbits fed a HD and rabbits fed a CD, a CD-Ch and a HD-Ch.

[c] $P < 0.01$ indicates statistically significant differences between rabbits fed a CD-Ch and rabbits fed a CD. (Two way ANOVA and Duncan's post test.)

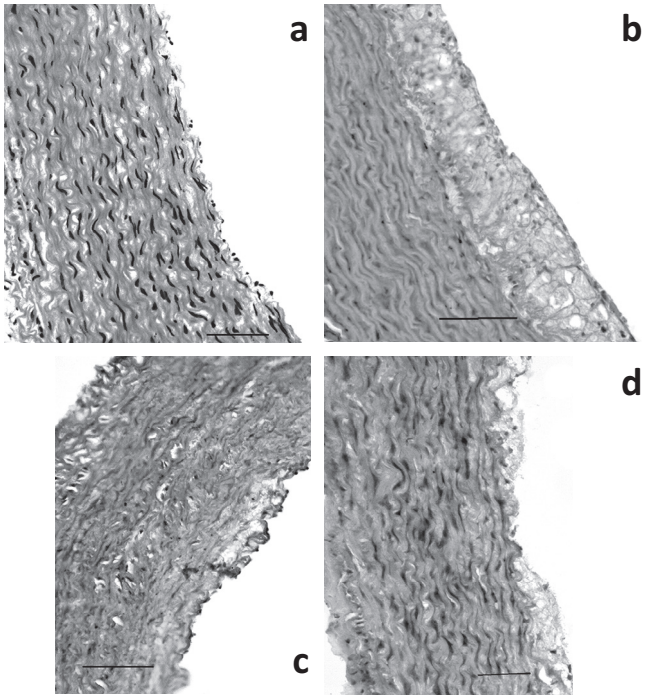

Fig. 7 – Representative microphotographs of aorta sections from rabbits fed a control diet (a), a high cholesterol diet (b), a control diet supplemented with 10% chia oil (c), a high cholesterol diet supplemented with 10% chia oil (d). Sections were stained with haematoxylin/eosin after isolation from rabbits fed the experimental diets during 5–6 weeks, as described in the "Materials and methods" section. Bar indicates 50 μm. Magnification × 40.

from rabbits fed a chia oil-enriched diet compared with a regular diet. These data suggested that chia oil is not altering the intrinsic characteristics of endothelial cells but instead selectively attenuates the detrimental changes induced by cholesterol. As was stated previously, chia-oil did not achieve this effect by altering the circulating cholesterol levels.

In spite of ALA being the main fatty acid present (64.5–69.3%) in chia oils, other bioactive compounds as tocopherols and flavonoids have been found in low concentrations. According to the Zutphen Elderly Study (Hertog, Feskens, Hollman, Martijn, & Kromhout, 1993), subjects consuming the greatest amount of flavonoids (>29 mg/day) have a 68% lower cardiovascular risk. However, no relation was found between dietary intake of tochopherols and cardiovascular mortality (Buijsse, Feskens, Kwape, Kok, & Kromhout, 2008; Saremi & Arora, 2010). In the present study, dietary incorporation of flavonoids in chia oils was only 0.057 μg/day. On the other hand, dietary chia seed increases levels of ALA and EPA in the plasma from humans (Nieman et al., 2009; Vuksan et al., 2007) and rats (Ayerza & Coates, 2007) when compared with the control. Partially in agreement with these authors we found increased levels of ALA and undetectable levels of EPA and DHA in the plasma from rabbits fed a diet supplemented with chia-oil with respect to rabbits fed a CD or a HD. Therefore, it is reasonable to hypothesize that ALA induced the protective changes observed. In support of this contention, ALA levels have been shown to reduce serum markers of vascular inflammation and endothelial activation (Rallidis et al., 2004; Zhao et al., 2004) and reduce platelet aggregation (Vas Dias, Gibney, & Taylor, 1982). Furthermore, circulating ALA levels have been positively associated with endothelium-dependent vasodilation in normocholesterolaemic and hypercholesterolaemic subjects (Ros et al., 2004; Steer et al., 2003). n-3 PUFAs display antiatherogenic effects through direct modulation of NO production and release (Abeywardena & Head, 2001). NO is synthesized from L-arginine by endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). NO regulates vascular relaxation and inhibits key atherosclerotic processes such as platelet aggregation, monocyte adhesion and vascular smooth muscle cell (VSMC) proliferation and migration. The cellular mechanisms by which n-3 PUFAs improve endothelial function remain unclear. However, recent reports (Zhang et al., 2013) demonstrated that ALA prevents diabetes-induced endothelial dysfunction by enhancing eNOS activity.

Addition of chia oil to the diets blunted the E_{max} to Ang II and NA in both diet groups. Furthermore, in hypercholesterolaemic rabbits, chia oil blocked the sensitization to Ang II and induced desensitization of the contractile response to NA. However, we observed no effects in KCl-induced vascular tension generation, which suggests that chia oil did not induce a general defect in smooth muscle function. In disagreement with our results, Dupasquier et al. (2006) found defects in both KCl and NA-contractile response in aorta from rabbits fed a HD supplemented with flaxseed. These authors claim that the protective action of dietary flaxseed on vascular response was selective. Flaxseed protected against the changes in vascular relaxation but did not protect against the contractile dysfunction induced by the elevation in circulating cholesterol. Kenny et al. (1992) report that n-3 PUFAs significantly reduced forearm vascular resistance responses to Ang II in normotensive men. These changes are associated with a reduction in plasma TG. Moreover, the vascular response to NA and phentolamine has been found unchanged. In the present work, the beneficial effects of chia oil addition on vascular reactivity to Ang II and NA were similar in CD and HD in spite of the plasma TG reduction in rabbits fed a HD. Furthermore, there was no relationship between the reduction of plasma TG levels and the Ang II or NA contractile response. As was stated previously, vascular function is related to the proportions of saturated fatty acids and ALA in healthy subjects (Sarabi, Vessby, Millgård, & Lind, 2001; Steer et al., 2003). The mechanisms involved are not known, but may relate to changes in membrane fatty acid composition and fluidity. The membrane fluidity is known to modify receptor activity and other physiological functions of the cell membrane, such as passage of ions and electrical potential across the membrane (Spector & Yorek, 1985). Fatty acids have been reported to directly increase the activity of Ca^{2+}-activated-K^+ channels in rabbit smooth muscle cells (Ahn, Kim, Lee, Kang, & Kang, 1994; Clarke, Petrou, Walsh, & Singer, 2002). Activation of Ca^{2+}-activated-K^+ channels leads to hyperpolarization of the cell membrane. This effect causes reduction of $_i[Ca^{2+}]$ and diminution of the contractile response. Considering that chia oil increases ALA and EPA concentrations in plasma and different tissues, we can hypothesize that chia oil reduces contractile response to Ang II and NA by this pathway.

The intima-media ratio was positively related to the Ang II contractile response and to the NA affinity in rabbits fed a HD-Ch. Reduction in the ratio is an index of improvement of the endothelial function (Poredos, 2011). Thus, these results would imply that improvement of endothelial function in rabbits fed a HD-Ch may account for the reduction in the Ang II response and the NA-desensitization identified in our study. ALA supplementation has been demonstrated to inhibit angiotensin-converting enzyme and mRNA levels in spontaneously hypertensive rats (Ogawa, Suzuki, Aoyama, & Takeuchi, 2009). Considering that the endothelial function is improved by chronic inhibition of the angiotensin-converting enzyme (Varin et al., 2000), this mechanism, together with the increase of NO release and the cell hyperpolarization, may explain the reduced contraction to Ang II and NA in rabbits fed a HD-Ch identified in the present study.

5. Conclusion

Our data demonstrate for the first time that chia oil can protect vascular function against deleterious effects of early hypercholesterolaemia. We have shown that dietary chia oil can improve endothelium-dependent vascular relaxation and can reduce the agonist-induced vascular contractility even in the presence of high cholesterol levels. Its ability to protect the vascular function reinforces the view that chia oil could serve as a functional food and may be a dietary strategy to limit the progression of vascular dysfunction.

Acknowledgments

This work was supported by grants from the Consejo de Investigaciones de la Universidad Nacional de Tucumán (PIUNT I521/1), Consejo de Investigaciones Científicas y Técnicas de la República Argentina (CONICET PIP 11-232, and institutional funds from INSIBIO (Instituto Superior de Investigaciones Biológicas).

REFERENCES

Abeywardena, M. Y., & Head, R. J. (2001). Long chain n-3 polyunsaturated fatty acids and blood vessel function. *Cardiovascular Research*, 52, 361–371.

Ahn, D. S., Kim, Y. B., Lee, Y. H., Kang, B. S., & Kang, D. H. (1994). Fatty acids directly increase the activity of Ca(2+)-activated K + channels in rabbit coronary smooth muscle cells. *Yonsei Medical Journal*, 35, 10–24.

Ayerza, R., & Coates, W. (2004). Composition of chia (Salvia hispanica) grown in six tropical and subtropical ecosystems of South America. *Tropical Science*, 44, 131–135.

Ayerza, R., & Coates, W. (2005). Ground chia seed and chia oil effects on plasma lipids and fatty acids in the rat. *Nutrition Research*, 25, 995–1003.

Ayerza, R., & Coates, W. (2007). Effect of dietary α-linolenic fatty acid derived from Chia when fed as ground seed, whole seed and oil on lipid content and fatty acid composition of rat plasma. *Annals of Nutrition and Metabolism*, 51, 27–34.

Belitz, H. D., & Grosch, W. (1999). *Food chemistry* (2nd ed.). Berlin, Germany: Springer-Verlag.

Berenson, G. S., Srinivasan, S. R., Bao, W., Newman, W. P., Tracy, R. E., & Wattigney, W. A. (1998). Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *The New England Journal of Medicine*, 338(23), 1650–1656.

Buijsse, B., Feskens, E. J. M., Kwape, L., Kok, F. J., & Kromhout, D. (2008). Both α- and β-carotene, but not tocopherols and vitamin C, are inversely related to 15-year cardiovascular mortality in Dutch elderly men. *The Journal of Nutrition*, 138, 344–350.

Burdge, G. C., & Calder, P. C. (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reproduction, Nutrition, Development*, 45, 581–597.

Chicco, A. G., D'Alessandro, M. E., Hein, G. J., Oliva, M. E., & Lombardo, Y. B. (2009). Dietary chia seed (Salvia hispanica L.) rich in alpha-linolenic acid improves adiposity and normalises hypertriacylglycerolaemia and insulin resistance in dyslipemic rats. *The British Journal of Nutrition*, 101, 41–50.

Chin, J. P. F., & Dart, A. M. (1994). Therapeutic restoration of endothelial function in hypercholesterolemic subjects: Effect of fish oils. *Clinical and Experimental Pharmacology & Physiology*, 21, 749–755.

Clarke, A. L., Petrou, S., Walsh, J. V., & Singer, J. J. (2002). Modulation of BKCa channel activity by fatty acids: Structural requirements and mechanism of action. *American Journal of Physiology*, 283, C1441–C1453.

Din, J. N., Sarma, J., Harding, S. A., Lyall, K., Newby, D. E., & Flapan, A. D. (2013). Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in patients with a previous myocardial infarction: A randomised controlled trial. *British Medical Journal Open*, 3, e003054. doi:10.1136/bmjopen-2013-003054.

Dupasquier, C. M., Weber, A. M., Ander, B. P., Rampersad, P. P., Steigerwald, S., Wigle, J. T., Mitchell, R. W., Kroeger, E. A., Gilchrist, J. S., Moghadasian, M. M., Lukas, A., & Pierce, G. N. (2006). Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *American Journal of Physiology, Heart and Circulatory Physiology*, 291, H2987–H2996.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry*, 226, 497–509.

Goode, G. K., Garcia, S., & Heagerty, A. M. (1997). Dietary supplementation with marine fish oil improves in vitro small artery endothelial function in hypercholesterolemic patients. A double-blind placebo-controlled study. *Circulation*, 96, 2802–2807.

Goodfellow, J., Bellamy, M. F., Ramsey, M. W., Jones, C. J., & Lewis, M. J. (2000). Dietary supplementation with marine omega-3 fatty acids improves systemic large artery endothelial function in subjects with hypercholesterolemia. *Journal of the American College of Cardiology*, 35, 265–270.

Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Martijn, B., & Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet*, 342, 1007–1011.

Hilpert, K. F., West, S. G., Kris-Etherton, P. M., Hecker, K. D., Simpson, N. M., & Alaupovic, P. (2007). Postprandial effect of n-3 polyunsaturated fatty acids on apolipoprotein B-containing lipoproteins and vascular reactivity in type 2 diabetes. *The American Journal of Clinical Nutrition*, 85, 369–376.

Hussein, N., Ah-Sing, E., Wilkinson, P., Leach, C., Griffin, B. A., & Millward, D. J. (2005). Long-chain conversion of [13C]linoleic acid and alpha-linolenic acid in response to marked changes in their dietary intake in men. *Journal of Lipid Research*, 46, 269–280.

Ixtaina, V. Y., Martinez, M. L., Spotorno, V., Mateo, C. M., Maestri, D. M., Diehl, B. W., Nolasco, S. M., & Tomás, M. C. (2011). Characterization of chia seed oils obtained by pressing and solvent extraction. *Journal of Food Composition and Analysis*, *24*, 166–174.

Jayakody, L., Senaratne, M., Thomson, A., & Kappagoda, T. (1987). Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circulation Research*, *60*, 251–264.

Jerez, S., Peral de Bruno, M., & Coviello, A. (2001). Efecto de antagonistas de los receptores de angiotensina II y a2 adrenergicos sobre la liberacion de NO inducida por angiotensina II. *Medicina*, *61*, 431–436.

Jerez, S., Sierra, L., Peral de Bruno, M., & Coviello, A. (2008). Endothelial dysfunction and improvement of the angiotensin II reactivity in hypercholesterolemic rabbits: Role of cyclooxygenase metabolites. *European Journal of Pharmacology*, *580*, 182–189.

Jerez, S., Sierra, L., Scacchi, F., & Peral de Bruno, M. (2010). Hypercholesterolemia modifies angiotensin II desensitization and cross talk between alpha 1-adrenoceptor and angiotensin AT1 receptor in rabbit aorta. *European Journal of Pharmacology*, *635*, 149–155.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K., Harada, K., Miyamoto, S., Nakazawa, H., Won, K. J., & Sato, K. (1997). Calcium movements, distribution, and functions in smooth muscle. *Pharmacological Reviews*, *49*, 157–230.

Kenny, D., Warltier, D. C., Pleuss, J. A., Hoffmann, R. G., Goodfriend, T. L., & Egan, B. M. (1992). Effect of omega-3 fatty acids on the vascular response to angiotensin in normotensive men. *The American Journal of Cardiology*, *70*, 1347–1355.

Kris-Etherton, P. M., Harris, W. S., & Appel, L. J., for the AHA Nutrition Committee. (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, *106*, 2747–2757.

Kris-Etherton, P. M., Harris, W. S., & Appel, L. J., for the AHA Nutrition Committee. (2003). Omega-3 fatty acids and cardiovascular disease: New recommendations from the American heart association. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *23*, 151–152.

Medina, M., Alberto, M. R., Sierra, L., Saad, S., Isla, M. I., & Jerez, S. (2014). Hypercholesterolemia increases plasma saturated and n-6 fatty acids altering prostaglandin homeostasis and promotes endothelial dysfunction in rabbits. *Lipids*, *49*, 685–693.

Nieman, D. C., Cayea, E. J., Austin, M. D., Henson, D. A., McAnulty, S. R., & Jin, F. (2009). Chia seed does not promote weight loss or alter disease risk factors in overweight adults. *Nutrition Research*, *29*, 414–418.

Ogawa, A., Suzuki, Y., Aoyama, T., & Takeuchi, H. (2009). Dietary alpha-linolenic acid inhibits angiotensin-converting enzyme activity and mRNA expression levels in the aorta of spontaneously hypertensive rats. *Journal of Oleo Science*, *58*, 355–360.

Poredos, P. (2011). Markers of preclinical atherosclerosis and their clinical relevance. *The Open Atherosclerosis & Thrombosis Journal*, *4*, 1–10.

Rallidis, L. S., Paschos, G., Papaioannou, M. L., Liakos, G. K., Panagiotakos, D. B., Anastasiadis, G., & Zampelas, A. (2004). The effect of diet enriched with alpha-linolenic acid on soluble cellular adhesion molecules in dyslipidaemic patients. *Atherosclerosis*, *174*, 127–132.

Ros, E., Nunez, I., Perez-Heras, A., Serra, M., Gilabert, R., Casals, E., & Deulofeu, R. A. (2004). Walnut diet improves endothelial function in hypercholesterolemic subjects: A randomized crossover trial. *Circulation*, *109*, 1609–1614.

Sarabi, M., Vessby, B., Millgård, J., & Lind, L. (2001). Endothelium-dependent vasodilation is related to the fatty acid composition of serum lipids in healthy subjects. *Atherosclerosis*, *156*, 349–355.

Saremi, A., & Arora, R. (2010). Vitamin E and cardiovascular disease. *American Journal of Therapeutics*, *17*, 56–65.

Sorensen, K. S., Celermajer, D. S., Georgakopoulos, D., Hatcher, G., Betteridge, D. J., & Deanfield, J. E. (1994). Impairment of endothelium-dependent dilation is an early event in children with familiar hypercholesterolemia and is related to the lipoprotein levels. *The Journal of Clinical Investigation*, *93*, 50–55.

Spector, A. A., & Yorek, M. A. (1985). Membrane lipid composition and cellular function. *Journal of Lipid Research*, *26*, 1015–1035.

Steer, P., Vessby, B., & Lind, L. (2003). Endothelial vasodilatory function is related to the proportions of saturated fatty acids and alpha-linolenic acid in young men, but not in women. *European Journal of Clinical Investigation*, *33*, 390–396.

Van Nieuwenhove, C., Cano, P. G., Chaia, A. P., & Gonzalez, S. (2007). Chemical composition and fatty acids content of buffalo cheese from northwest Argentina: Effect on lipid composition of mice tissues. *Journal of Food Lipids*, *14*, 232–242.

Varin, R., Mulder, P., Tamion, F., Richard, V., Henry, J. P., Lallemand, F., Lerebours, G., & Thuillez, C. (2000). Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in heart failure. Role of nitric oxide, prostanoids, oxidant stress, and bradykinin. *Circulation*, *102*, 351–356.

Vas Dias, F. W., Gibney, M. J., & Taylor, T. G. (1982). The effect of polyunsaturated fatty acids on the n-3 and n-6 series on platelet aggregation and platelet and aortic fatty acid composition in rabbits. *Atherosclerosis*, *43*, 245–257.

Vuksan, V., Danawhitham, J. L., Sievenpiper, A. L., Jenkins, A., Rogovik, R. P., Bazinet, E. V., & Amir, H. (2007). Supplementation of conventional therapy with the novel grain Salba (Salvia hispanica L.) improves major and emerging cardiovascular risk factors in type 2 diabetes. *Diabetes Care*, *30*, 2804–2810.

Zhang, W., Fu, F., Tie, R., Liang, X., Tian, F., Xing, W., Li, J., Ji, L., Xing, J., Sun, X., & Zhang, H. (2013). Alpha-linolenic acid intake prevents endothelial dysfunction in high-fat diet-fed streptozotocin rats and underlying mechanisms. *VASA. Zeitschrift für Gefässkrankheiten*, *42*, 421–428.

Zhao, G., Etherton, T. D., Martin, K. R., Gillies, P. J., West, S. G., & Kris-Etherton, P. M. (2007). Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. *The American Journal of Clinical Nutrition*, *85*, 385–391.

Zhao, G., Etherton, T. D., Martin, K. R., West, S. G., Gillies, P. J., & Kris-Etherton, P. M. (2004). Dietary alpha-linolenic acid reduces inflammatory and lipid cardiovascular risk factors in hypercholesterolemic men and women. *The Journal of Nutrition*, *134*, 2991–2997.