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Exploring the Potential of Wild Olive (Acebuche) Oil as a Pharm-Food to Prevent Ocular Hypertension and Fibrotic Events in the Retina of Hypertensive Mice

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Scope: Our laboratory has previously described the antioxidant and anti-inflammatory potential of a wild olive (acebuche, ACE) oil against hypertension-associated vascular retinopathies. The current study aims to analyze the antifibrotic effect of ACE oil on the retina of hypertensive mice. Methods and results: Mice are rendered hypertensive by administration of NG-nitro-L-arginine-methyl-ester (L-NAME) and simultaneously subjected to dietary supplementation with ACE oil or a reference extra virgin olive oil (EVOO). Intraocular pressure (IOP) is measured by rebound tonometry, and retinal vasculature/layers are analyzed by fundus fluorescein angiography and optical coherence tomography. Different fibrosis-related parameters are analyzed in the retina and choroid of normotensive and hypertensive mice with or without oil supplementation. Besides preventing the alterations found in hypertensive animals, including increased IOP, reduced fluorescein signal, and altered retinal layer thickness, the ACE oil-enriched diet improves collagen metabolism by regulating the expression of major fibrotic process modulators (matrix metalloproteinases, tissue inhibitors of metalloproteinases, connective tissue growth factor, and transforming growth factor beta family). Conclusion: Regular consumption of EVOO and ACE oil (with better outcomes in the latter) might help reduce abnormally high IOP values in the context of hypertension-related retinal damage, with significant reduction in the surrounding fibrotic process.

1. Introduction

Due to its remarkable bioactive characteristics, extra virgin olive oil (EVOO) from *Olea europaea* L. has been recognized by numerous authors as one of the healthiest components of the Mediterranean diet.[1,2] Among the benefits of regular EVOO consumption is the reduction of the risk of cardiovascular and neurodegenerative diseases, cancer, and mortality[3–5] due to its antioxidant, anti-inflammatory, antifibrotic, and antitumoral properties.[6]

The benefits of EVOO are attributed to its rich composition in bioactive compounds. Its lipid profile is characterized by a high content in monounsaturated fatty acids,[7] especially oleic acid. Polyunsaturated fatty acids such as linoleic (C18:2) and α-linolenic (C18:3) acids, pigments, and phosphatides are also found in EVOO.[8] Noteworthy is the large number of minor compounds (e.g., sterols, tocopherols, triterpenes, polyphenols) capable of modulating different pathways related to redox state, homeostasis, and inflammation.[9–12]

Very few studies have reported so far on healthy products obtained from the wild olive tree (*Olea europaea var. sylvestris*), of which the primitive lineage that abounds in areas such as Andalusia in Southern Spain is commonly known as "acebuche" (ACE). Compared to cultivated olive trees, ACE trees grow in hard-to-reach places and on unsettled land, and the fruits are smaller.

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In terms of organoleptic properties, ACE oil is spicier, more bitter, and fruitier than standard EVOOs. ACE oil also differs from EVOO in the proportion of minor components, showing a greater amount of triterpene acids and tocopherols, and a higher ratio secoiridoids/ortodiphenols.[13,14] Exploring these differences may be worthwhile to confirm if they can be extrapolated to the bioactivity of the oils.

Unlike the extended use of EVOO as an effective pharm-food tool against cardiovascular diseases, its beneficial effect in the context of ocular diseases is rather limited. In vivo and in vitro approaches have evidenced the neuroprotective effect of minor components such as hydroxytyrosol[15] and oleuropein[16] in diabetic retinopathy.[17,18] Interestingly, we have recently described the first evidence of the retinoprotective effects of ACE oil- and EVOO-enriched diets, with better outcomes in favor of the former, in a context of arterial hypertension. Thus, dietary intake of these oils delayed the onset and progression of hypertension-related retinal damage by reducing superoxide anion ($O_2^{\cdot-}$) production through modulation of the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase.[14] We have also reported the anti-inflammatory properties of ACE oil in the retina of hypertensive mice through reduction of pro-inflammatory cytokines and regulation of anti-inflammatory agents that abolished retinal dysfunction.[19] Inflammation alters retinal microcirculation and favors retinal neurodegeneration through different effectors such as cytokines, which eventually produce retinal damage, edema, or neovascularization.[20] In this sense, the implementation of dietary interventions based on ACE oil and EVOO supplements could help reduce ocular damage in the setting of retinal pathologies.

Oxidative stress and inflammation are important triggers of tissue regeneration and fibrosis. Sustained elevation of blood pressure contributes to the progression of fibrotic processes leading to target organ damage by arterial fibrosis and extracellular matrix (ECM) remodeling.[21] Thus, hypertension favors the cross-linkage of collagen (the main ECM protein) making it insoluble and, therefore, increasing its deposition in the tissues to produce vascular remodeling and subsequent pathological events.[22,23] Focusing on retinopathies, fibrosis is a common feature in neovascular retinopathies such as proliferative retinopathy, diabetic retinopathy, and macular degeneration; it is also the most common finding, along with neovascularization, behind retinal vision loss.[24] In this context, collagen seems to participate in fibrotic ocular pathological mechanisms, including in the retina.[25] Nevertheless, it is still unknown how increased blood pressure favors fibrosis in the development/progression of retinopathies. Hence, the aim of this study was to describe the possible antifibrotic effect of ACE oil in the eyes of mice with hypertension induced by chronic treatment with NG-nitro-L-arginine-methyl-ester (L-NAME). To this end, retinal imaging analyses and intraocular pressure readings were performed along with different fibrosis-related parameters that were measured in the retina and choroid of normotensive and hypertensive mice with or without dietary oil supplementation.

2. Experimental Section

2.1. Materials

ACE oil was supplied by Alcazarín Reunidos FP, S.L. (Málaga, Spain). Dietary pellet composition (ROD14IRR) was purchased in Sodispan Research (Altromin, Germany). Chemical reagents used in the study include absolute ethanol (Merck. Art. 1.00983.1000), ECL Prime Western Blotting Detection reagent (Cytiva. Art. RPN2232), Blotting-Grade Blocker (Bio-Ra Laboratories. Art. 170–6405), bovine serum albumin (Sigma-Aldrich. Art. A6793), Diva decloaker (Biocare Medical. Art. DV2004), DAPI Fluoromount-G® (DAPI, Southern Biotech. Art. 0100–20), High-Capacity cDNA Archive kit (Applied Biosystems. Art. 4322171), L-NAME (Sigma-Aldrich. Art. N5751), iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories. Art. 17255121), ketamine (Ketamidor, Richter Pharma AG), paraformaldehyde (Panreac Applichem. Art. 131328.1211), phosphate buffered saline (PBS, Sigma-Aldrich. Art. P4417-50TAB), phosphatase inhibitor (Sigma-Aldrich. Art. P004), protease inhibitor (Roche. Art. 04693116001), RIPA lysis buffer (Merck Milipore. Art. 20–188), TRIzol (Thermo Fisher Scientific. Art. 15596018). Other specific chemical compounds were mentioned in the sections below.

2.2. Experimental Design

The experiments followed the European Union (EU) Directive 2010/63/EU and the National (RD 53/2013) guidelines for the care and use of Laboratory animals, and were reviewed and approved by the competent Institutional Animal Care and Use Committee (approval reference #13/03/2019/031, issued by Junta de Andalucía, Dirección General de la Producción Agrícola y Ganadera). 10–12 week-old male C57B/6J mice (average body weight = 24 – 25 g), obtained from the Center for Animal Production and Experimentation at the University of Seville (Spain), were distributed into six groups of 15 animals each: 1) Control group, normotensive mice fed a standard commercial diet; 2) ACE group, normotensive mice fed a commercial diet supplemented with 12% w/w of acebuche oil; 3) EVOO group, normotensive mice supplemented with 12% of extra virgin olive oil; 4) L-NAME group, hypertensive mice administered 45 mg L-NAME/kg body weight/day and fed a commercial diet; 5) LN+ACE group, L-NAME-treated hypertensive mice supplemented with 12% of ACE oil; and 6) LN+EVOO group, L-NAME-treated hypertensive mice supplemented with 12% of EVOO. The animals were kept in these conditions for 6 weeks with continuous control of food and water intake and housed in a regulated environment under standard conditions (23 ± 1 °C, 12 h/12 h light/dark cycles). Upon harvesting, animal samples were collected as described below and assigned to different experiments as specified in the figure legends.

2.3. Dietary Interventions (Oil Supplementation) and Treatments

A regular dietary pellet composition (ROD14IRR, Sodispan Research, Altromin, Germany) was supplemented, where

applicable, with 12% w/w of ACE oil or EVOO (1–1.5 mL of oil kg^{-1} body weight per day in humans), as specified in Section 2.1. The study had previously reported the chemical composition of these oils and the steps to prepare the final mixes.[14] Particular care was taken to ensure the freshness of powder–oil mixtures, which were prepared regularly in small batches.

Olives from both cultivated and wild olive trees were harvested at Sierra de las Nieves (Málaga, Spain), and ACE oil/EVOO were produced following similar extraction methods according with the standards protocols to comply with extra virgin olive oil definition. In hypertensive mice (L-NAME, LN+ACE, and LN+EVOO groups), the concentration of the blood pressure-increasing drug in the drinking water was adjusted throughout the experimental period according to the animals' body weight and water intake, and considering previous experiments conducted in the laboratory using this animal model.

2.4. In Vivo Retinal Imaging

Retinal fundus, blood flow, and thickness were explored using the Phoenix MicronIV image-guided Optical Coherence Tomography (OCT; Pleasanton, CA, USA) imaging system in anesthetized mice (3% isoflurane). Protocols for the fundus fluorescein angiography (FFA) were previously established in the laboratories.[26] Briefly, 10% fluorescein sterile solution (30 mg kg^{-1}; Alcon Nordic, Copenhagen, Denmark) was subcutaneously administered to mice immediately before starting a 5-min FFA imaging recording with fixed camera exposure settings. The fluorescein intensity from FFA images was evaluated in the different experimental groups by using ImageJ (imagej.nih.gov/ij). This image processing and analysis software were also used to evaluate the thickness of retinal layers on OCT images of central retinas at the end of the dietary intervention/treatment period. OCT images were taken with vertical and horizontal scan beam to ensure analysis of the same retinal point from both perspectives. The eyes were always aligned with respect to the measurement beam to ensure that the optic nerve head was at the center. Each animal's measurement was the average of at least four segments near the central area; in turn, the values plotted correspond to average values of at least seven animals from each experimental group.

2.5. Intraocular Pressure (IOP)

Intraocular pressure (IOP) was measured in awaked and unrestrained mice using a rebound tonometer (Tonoblab, Icare) and following protocols described elsewhere.[27] Readings were taken at the same time in the morning to minimize circadian IOP oscillations. IOP values (each one representing the average from five consecutive measurements) were recorded at the beginning, middle, and end of the 6-week treatment period. The mice were habituated to being manipulated for this technique from the week before the start of the treatments.

2.6. Tissue Harvesting and Homogenization

Mice were deeply anesthetized with a mix of ketamine (75 mg kg^{-1} i.p.) and diazepam (10 mg kg^{-1} i.p.) and euthanized by cervical dislocation. Following previous protocols,[14] the retinas were rapidly dissected under a binocular stereo microscope. Retinal homogenates from five mice (each one with the two pooled retinas from the same animal) were prepared for each experimental group in 50 mM phosphate buffer saline (PBS, pH 7.4) containing protease inhibitors (Sigma Aldrich-Roche, Madrid, Spain) and using a Potter–Elvehjem tissue grinder. Homogenates were centrifuged for 10 min at 10 000 × *g* and the supernatants were recovered to determine the protein concentration by the Bradford method.[28]

2.7. Histology Slide Preparation and Immunohistofluorescence

Paraffin sections of 5 μm thickness were obtained from both eyes (five mice per group) following administration of 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) by intravitreal injection; then, eyes were post-fixed in 4% PFA for 24 h. These sections were used for localization and expression of COL4α1 and TGFβ receptor isoforms by immunohistofluorescence staining on retina and choroid in deparaffinized eye sections. Antigen retrieval compound Diva Decloaker (Biocare Medical, LLC, Pacheco, CA, USA) and primary antibodies listed in **Table 1** were used for immunostaining. Goat anti-rabbit Alexa Fluor 488 (Cat. No. CSA3211) and Goat anti-mouse Alexa Fluor 647 (Cat. No. CSA3808) were used as fluorescent secondary antibodies, where appropriate, and sections were mounted with DAPI Fluoromount-G. Immunofluorescence staining intensity was measured in all retinal layers with the same acquisition settings in parallel images from the different experimental groups using ImageJ software (imagej.nih.gov/ij).

2.8. Real-Time PCR

Following the TRIzol RNA isolation method (Thermo Fisher Scientific, Madrid, Spain) in freshly dissected retinas (five mice per group and the two retinas pooled for each animal), reverse transcription reactions were run as previously described[29] and using the specific primers listed in **Table 2**. Gene products were amplified in a CFX96 real-time PCR system (Bio-Rad, Madrid, Spain). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to quantify the relative changes in mRNA expression following the $2^{-\Delta\Delta Ct}$ method.

2.9. Western Blotting

Western blotting analyses were performed in retinal homogenates containing equal amounts of proteins (40–50 μg), as previously described.[14] Specific antibodies used were listed in **Table 3**. Quantitative analysis was performed by optical densitometry (GE Healthcare Life Sciences, USA) using β-actin as a loading control in the same membranes.

2.10. Statistical Analyses

All results were presented as means ± SEM. One-way ANOVA followed by post-hoc Tukey multiple comparison test was performed with GraphPad InStat Software (San Diego, CA, USA, v.

Table 1. Antibodies used for immunofluorescence studies.

Primary antibody	Origin	Dilution	Reference
Anti- COL4α1	Mouse monoclonal	1:200	Santa Cruz Biotechnology
Anti- TGFβ-RI	Mouse monoclonal	1:100	Santa Cruz Biotechnology
Anti- TGFβ-RII	Mouse monoclonal	1:100	Santa Cruz Biotechnology
Anti-CD31	Rabbit monoclonal	1:200	Rockland Immunochemicals (Limerick, PA)

Table 2. Primers used for real-time PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
TGFβ1	TGGAGCAACATGTGGAACTC	GTCAGCAGCCGGTTACCA
TGFβ2	AGGAGGTTTATAAAATCGACATGC	TAGAAAGTGGGCGGGATG
TGFβ3	AGTGGCTGTTGAGGAGAGAGT	GTGTGGGTTGTGGTGATCCTT
TGFβ-RI	GCAGCTCCTCATCGTGTTG	AGAGGTGGCAGAAACACTGTAAT
TGFβ-RII	CCATGGCTCTGGTACTCTGG	ATGGGGGCTCGTAATCCTT
MMP2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
MMP3	GTCTTTGAAGCATTTGGGTTTCTC	GGTGTCATCCATAGCTCCTG
MMP9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
TIMP1	CCAGAGCAGATACCATGATGG	CCACAGAGGCTTTCCATGAC
TIMP2	GAATCCTCTTGATGGGGTTG	CGTTTTGCAATGCAGACGTA
COL1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
COL1α2	GGTGAGCCTGGTCAAACGG	ACTGTGTCCTTTCACGCCTTT
COL3α1	CCTGGCTCAAATGGCTCAC	GACCTCGTGTTCCGGGTAT
COL4α1	CTAACGGTTGGTCCTCACTG	CGTGGGCTTCTTGAACATCTC
COL4α2	GGCTTCATCAAAGGAGTCAAGG	CCCAATGTCACCAAAGTCCC
GAPDH	GCCAAAAGGGTCATCATCTCCGC	GGATGACCTTGCCCACAGCCTTG

3.10), and differences were considered statistically different at $p < 0.05$. Based on the stability of the values of the variables considered in this study, each one of the samples was sufficiently representative of the population of the group to which it belongs. Accordingly, the application of the Central Limit Theorem guarantees the non-violability of the hypotheses prior to the application of the ANOVA and post-hoc tests for the comparison of means.

3. Results

3.1. ACE Oil Corrects Blood Flow, Retinal Thickness, and IOP Abnormalities Found in L-NAME Animals

Retinal fundus examination revealed no remarkable findings in the central retina at the end of the treatment (**Figure 1**A, top row). On the other hand, FFA images (Figure 1A, bottom) demonstrated a reduction in fluorescein intensity in the L-NAME group in comparison with all other groups. Quantification of fluorescein signals confirmed a significant decay (≈60%; $p < 0.05$) at both 1 and 5 min in hypertensive mice compared with the control group (Figure 1C). Interestingly, the LN+ACE group displayed a fluorescence intensity similar to that of untreated mice, while a remaining signal reduction of ≈35% was measured in the LN+EVOO group.

Retinal layer thickness measurements from representative OCT images (Figure 1A, middle row) revealed a significant ($p < 0.05$) reduction of ganglion cell layer (GCL), outer nuclear layer (ONL), outer segments (OS), and retinal pigment epithelium/choroid (RPE/CH) in the L-NAME group, accompanied by thicker inner plexiform layer (IPL; Figure 1B). Simultaneous administration of diets supplemented with ACE oil or EVOO partially reversed these modifications in hypertensive animals. No significant differences were found in the inner nuclear layer (INL) and outer plexiform layer (OPL) among the six experimental groups.

Tonometry measurements showed a profound ($p < 0.05$) rise in IOP values in L-NAME-treated mice both after 3 weeks of treatment (≈15.5 vs ≈13.5 mm Hg in L-NAME and Control groups, respectively) and at the end of the treatment (≈17.5 vs ≈13.5, respectively; Figure 1D). Both ACE oil- and EVOO-enriched diets prevented this alteration, with a more notable effect in favor of the former (≈13/13.5 in LN+ACE vs ≈14.5/15.5 mmHg in LN+EVOO after 3 weeks of treatment/at the end of the treatment, respectively). Neither ACE oil nor EVOO administration altered IOP values in normotensive mice.

3.2. ACE Oil-Supplemented Diet Can Reduce Fibrosis by Modulating Collagen Expression

As shown in **Figure 2**A,B, immunofluorescence studies indicated a significant ($p < 0.05$) elevation of COL4α1 expression in GCL, IPL, OPL, OS, and RPE/CH (87.7-, 42.2-, 160-, 3.01- and 3.92-fold increase, respectively) in L-NAME mice in comparison with the Control group. Simultaneous dietary supplementation with the oils revealed a relevant ($p < 0.05$) downregulation of COL4α1 expression in hypertensive animals, especially in the LN+ACE group. No remarkable expression of COL4α1 was detected in GCL, IPL, and OPL in Control, ACE, EVOO, and LN+ACE group, whereas a clearly visible signal was detected at these layers in the LN+EVOO group.

Different collagen isoforms (COL1α1, COL1α2, COL3α1, COL4α1, and COL4α2) were also upregulated in total retina homogenates obtained from L-NAME-treated mice group (≈2–3 fold in comparison with the Control group; Figure 2C–G). Again, ACE oil here demonstrated its ability to reduce the gene expression of these collagen isoforms, both in normotensive conditions (≈50% average reduction in ACE vs Control group) and under induced hypertensive phenotype (≈41% reduction in LN+ACE vs Control group; $p < 0.05$). Although the EVOO-rich diet also managed to lower collagen expression in hypertensive animals, it was not as effective as ACE oil.

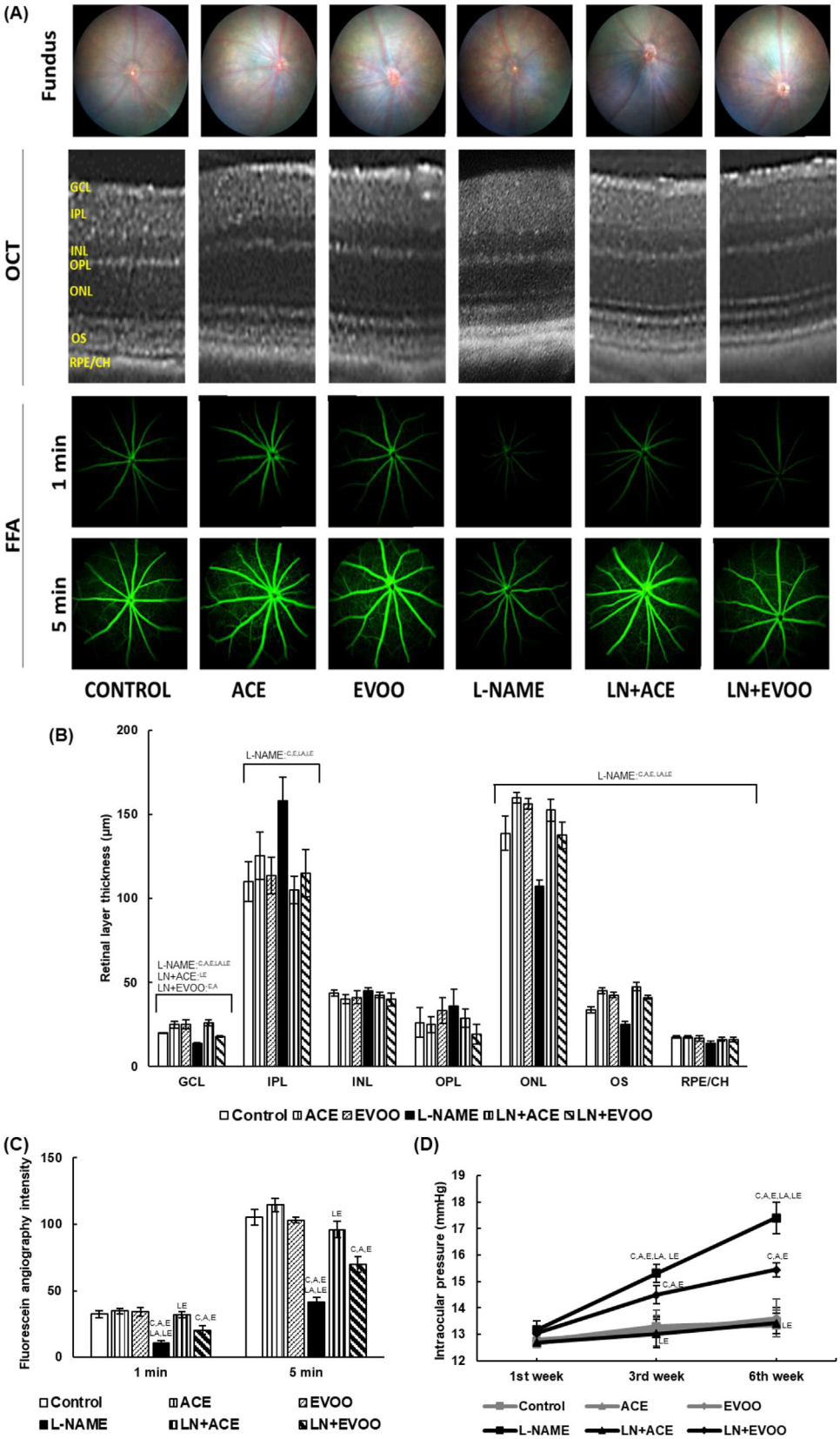


Figure 1. A) Retinal imaging analysis. Representative images of retinal fundus (top), optical coherence tomography (OCT) of retinal layers (middle), and fundus fluorescein angiography (FFA) acquired at two separate times (bottom). Retinal layer thickness in OCT images (B) and fluorescence intensity from

Table 3. Antibodies used for Western blotting analysis.

Primary antibody	Origin	Dilution	Secondary antibody	Dilution	Reference
Anti-CTGF	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	Santa Cruz Biotechnology (Santa Cruz, CA)
Anti-TGFβ1	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TGFβ2	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TGFβ3	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TGFβ-RI	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TGFβ-RII	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- MMP2	Mouse monoclonal	1:2000	Goat anti-mouse	1:3000	
Anti- MMP3	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- MMP9	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TIMP1	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti- TIMP2	Mouse monoclonal	1:1000	Goat anti-mouse	1:2000	
Anti-β-Actin	Mouse monoclonal	1:20 000	Goat anti-mouse	1:30 000	

3.3. Modulation of Collagen Metabolism by ACE Oil-Rich Diets

To assess collagen metabolism, we analyzed the expression of the main enzymes responsible for collagen remodeling, i.e., matrix metalloproteinase enzymes (MMPs; **Figure 3**), and that of their inhibitors (tissue inhibitors of metalloproteinases, TIMPs; **Figure 4**A–D). While the protein expression of MMP2 and MMP9 isoforms did not significantly differ ($p > 0.05$) between the Control and L-NAME groups, MMP3 appeared to be overexpressed in the latter at this level. On the contrary, these MMP isoforms were downregulated (≈30% reduction on average) at the mRNA level in hypertensive mice. Interestingly, ACE oil-enriched diets induced marked overexpression of MMPs in both normotensive and hypertensive animals. MMP isoforms also underwent mRNA upregulation in mice supplemented with EVOO-rich diets but to a lesser extent compared with the effect of ACE oil. In fact, MMP levels were unaffected by EVOO administration in hypertensive retinas.

Unlike MMPs, TIMP1, and TIMP2 isoforms were overexpressed in the L-NAME group compared to control normotensive mice (≈2.6 fold for protein expression and 7.3/3.1-fold, respectively, for gene expression; $p < 0.05$). In this case, both oils managed to reverse these alterations to a similar extent and reached values close to those observed in the Control group (Figure 4A–D). In turn, the calculation of MMP9/TIMP1 (Figure 4E) and MMP2/TIMP2 (Figure 4F) ratios returned a reduction of both ratios in hypertensive animals (≈49% and ≈54% relative to Control, respectively; $p < 0.05$). These tendencies were reversed by both oil diets in normotensive and hypertensive conditions, especially in mice fed ACE oil. In fact, the values exceeded those observed in control animals.

3.4. Role of the TFGβ Family in Retinal Inflammation and Fibrosis in L-NAME-Induced Hypertension

Figure 5A shows a significant ($p < 0.05$) increase in the protein expression of profibrotic growth factor CTGF (connective tissue growth factor) in the L-NAME group compared to Control (2.62-fold increase) and to all other animal groups (which behave the same in this regard). Similar findings were found regarding the expression of transforming grow factor beta (TGFβ) isoforms (TGFβ-1, TGFβ-2, and TGFβ-3; Figure 5B–G). Thus, the hypertensive phenotype presented with upregulation ($p < 0.05$) of all these TGFβ ligands, both at protein (≈2-fold) and gene (≈3-fold) levels. All changes detected in Western blotting experiments were reversed in the LN+ACE and LN+EVOO groups. On the other hand, mRNA expression analyses yielded significant differences between mice fed with either ACE oil or EVOO; thus, compared to the Control group values, TGFβ-1 and TGFβ-2 were significantly reduced in both the ACE and the LN+ACE groups, while the values in the EVOO and LN+EVOO groups tended to remain in the range of controls (Figure 5C,E). Interestingly, the reducing effect of both oils on the mRNA expression of TGFβ-3 was more pronounced in hypertensive groups (LN+ACE and LN+EVOO) than in normotensive groups (ACE and EVOO; Figure 5G).

Figure 6A shows the immunolocalization of TGFβ-RI and TGFβ-RII isoforms in the retina and their association with retinal endothelial cells. Immunofluorescence signals were generally weak in all groups except in non-supplemented hypertensive mice (L-NAME group). In addition, merged colocalization signals between TGFβ-R isoforms and the endothelial marker CD31 demonstrated the presence of TGFβ receptors manly in retinal endothelial cells. These results correlate with the protein and gene expression of these receptors (Figure 6B–E)

FFA images (C) were quantified by ImageJ software. D) Intraocular pressure (IOP) measurements along the experimental period. Values are expressed as mean ± SEM of at least seven animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; RPE/CH, retinal pigment epithelium/choroid.

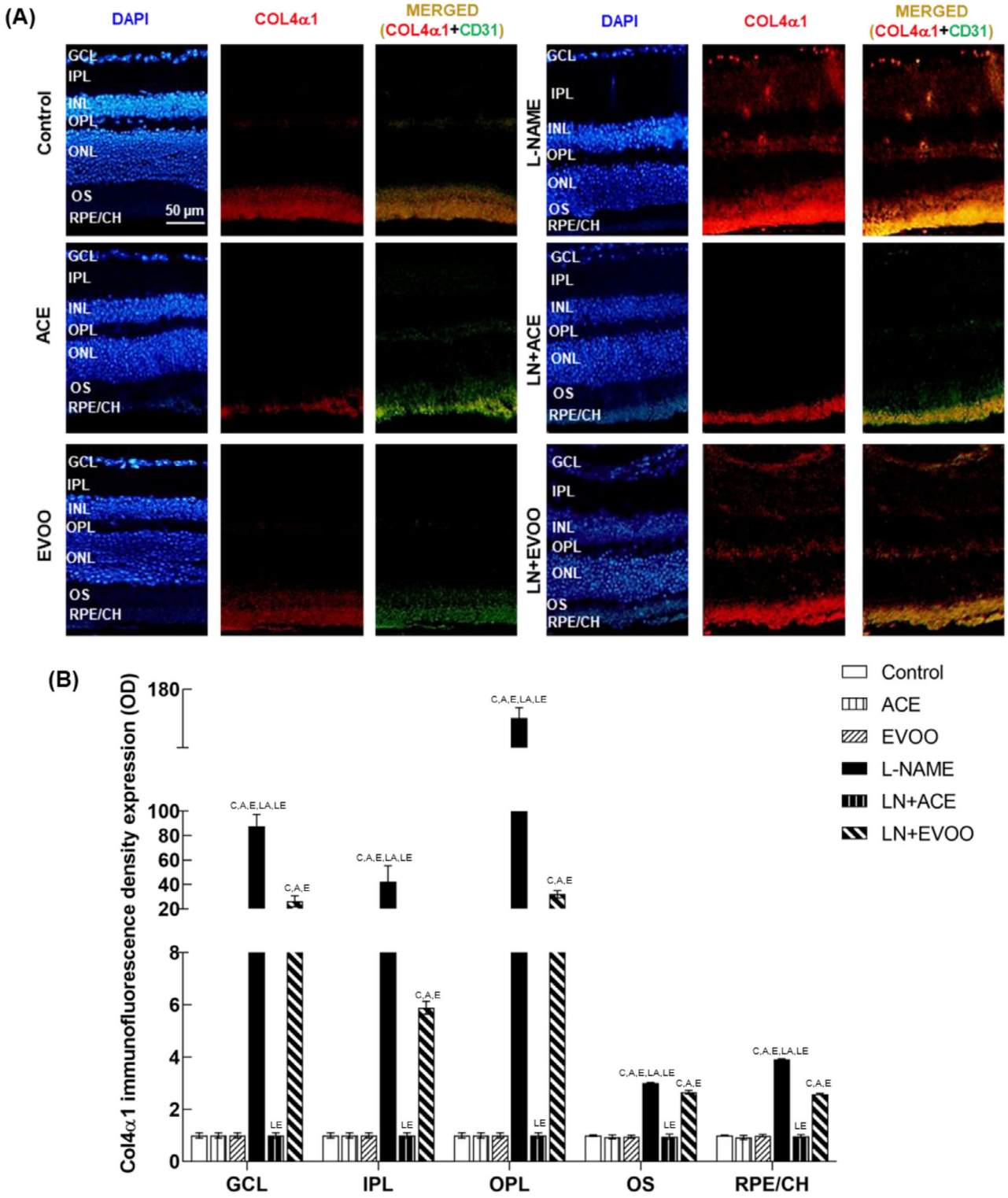


Figure 2. A) Col4α1 expression (red color) and double staining with endothelial marker (anti-CD31, green color) in retinal layers from each experimental group, where the merge is represented in yellow color. Nuclei staining with DAPI (blue color) was used to delimit the different retinal layers. Magnification: 10×. B) Quantitative analyses of fluorescence signal of Col4α1 relative to Control group. C–G) Gene expression of Col1α1 (C), Col1α2 (D), Col3α1 (E), Col4α1 (F), and Col4α2 (G) in retinal homogenates. The quantitative fold changes in gene expression were determined relative to the corresponding value for GAPDH housekeeping gene. Values are expressed as mean ± SEM of five animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments; RPE/CH, retinal pigment epithelium/choroid.

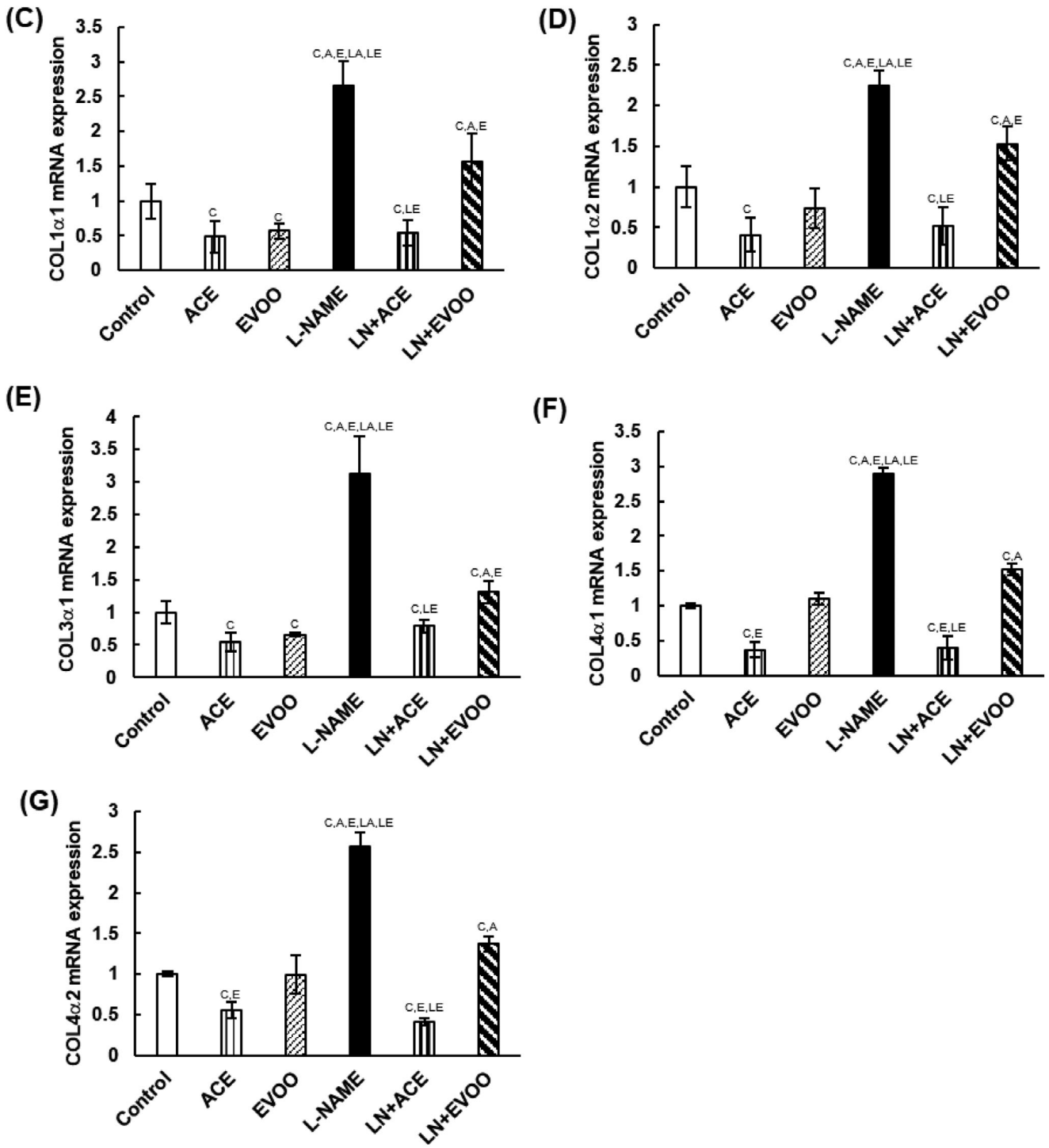


Figure 2. *Continued*

and confirm the ability of both ACE oil and EVOO to modulate the expression of TFGβ family members.

4. Discussion

The present study was conducted using a model of arterial hypertension based on L-NAME-mediated depletion of nitric oxide (NO). In agreement with previous studies in our laboratory,[14,19] neither L-NAME treatment nor oil supplementation altered feeding behavior or weight gain among experimental groups. On the other hand, blood pressure values were elevated at the end of the treatment in the L-NAME group, as expected (Figure S1, Supporting Information). We have recently reported various alterations in the oxidative status and inflammation-related processes in the

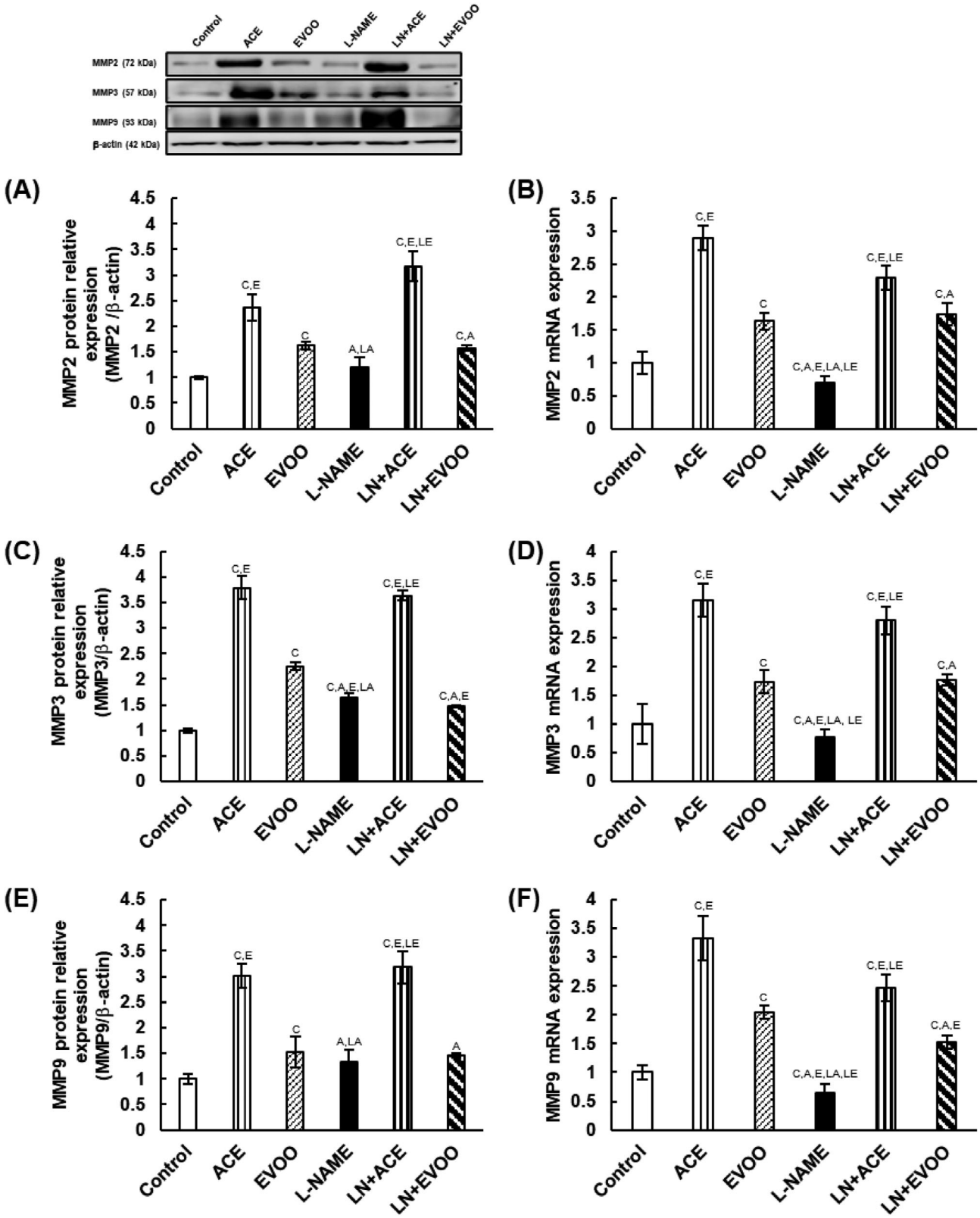


Figure 3. Collagen metabolism analyzed by protein and gene expression of MMP2 (A, B), MMP3 (C, D), and MMP9 (E, F) in the retina of all experimental groups. The quantitative fold changes in gene expression were determined relative to the corresponding value for GAPDH housekeeping gene. Values are expressed as mean ± SEM of five animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO.

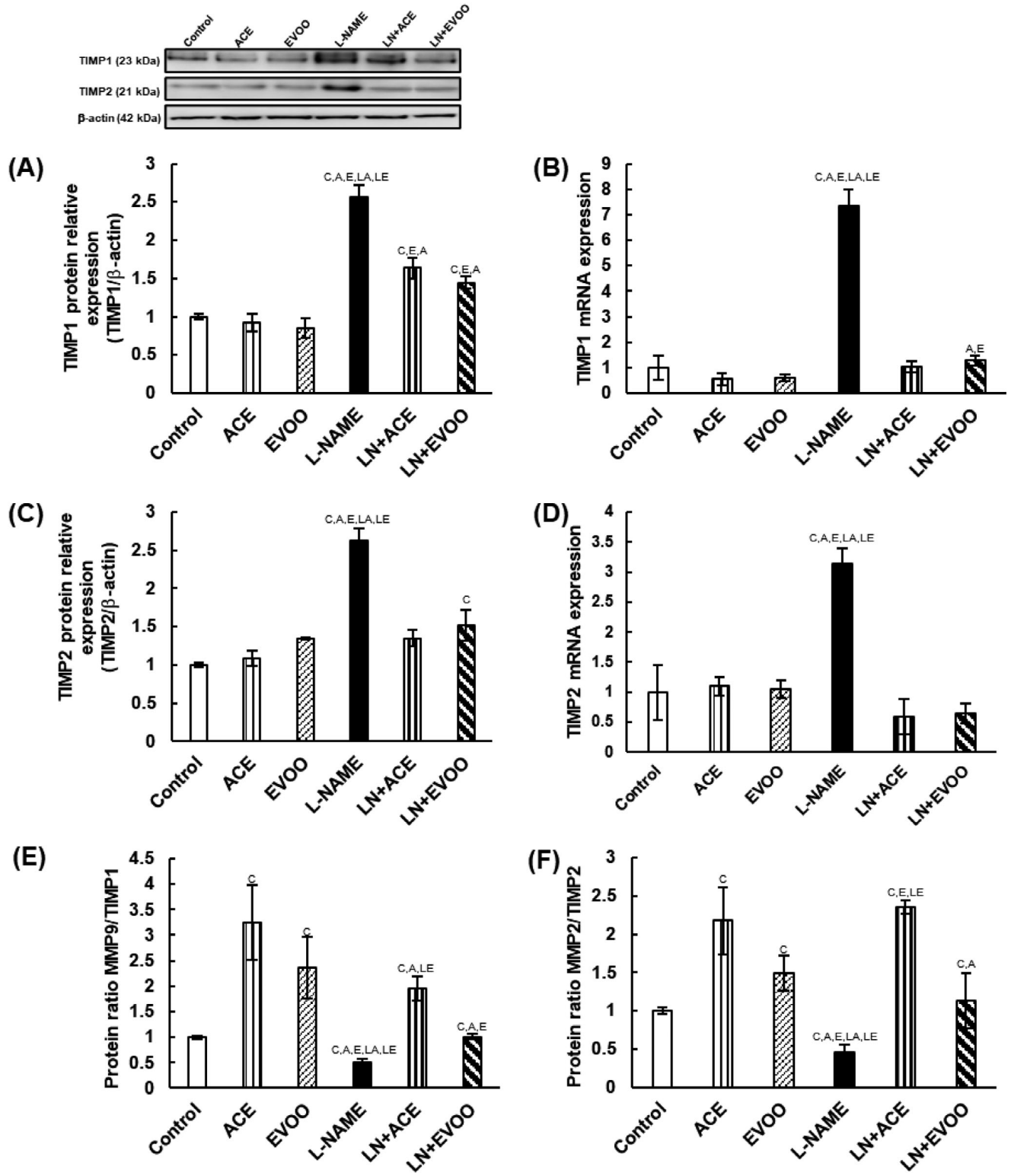
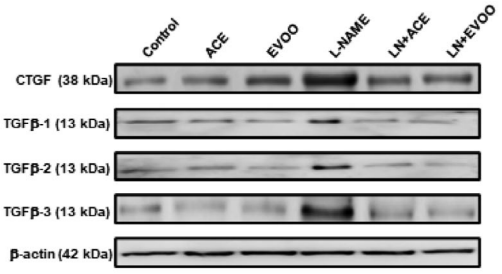
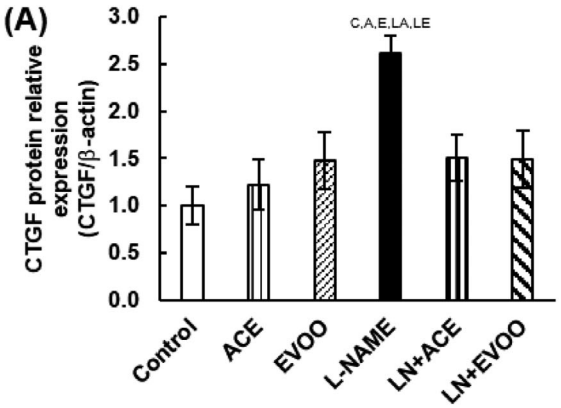
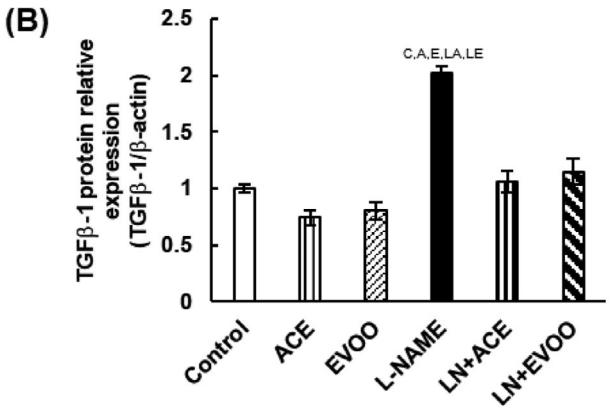
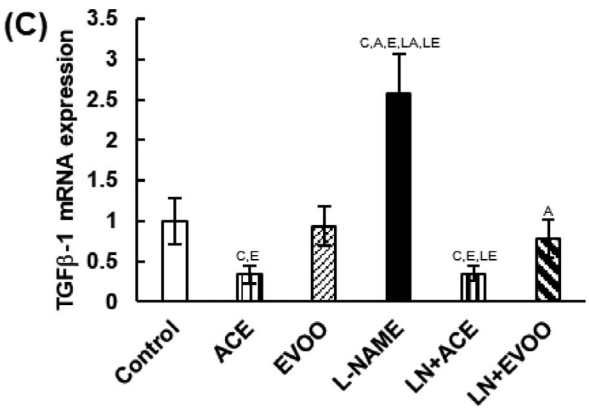


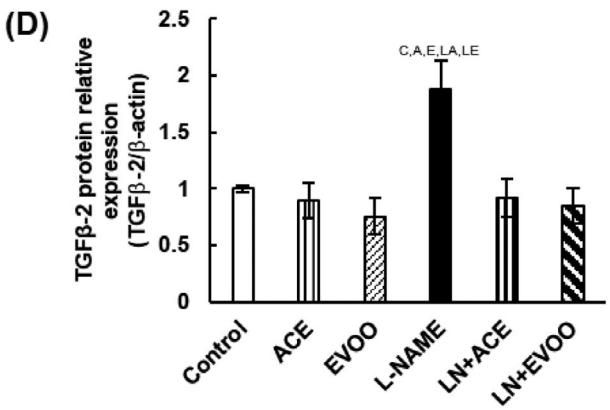
Figure 4. Activation status of MMP proteins analyzed by protein and gene expression of its inhibitors TIMP1 (A, B) and TIMP2 (C, D), and ratios MMP9/TIMP1 (E) and MMP2/TIMP2 (F) in the retina of all experimental groups. The quantitative fold changes in gene expression were determined relative to the corresponding value for GAPDH housekeeping gene. Values are expressed as mean ± SEM of five animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

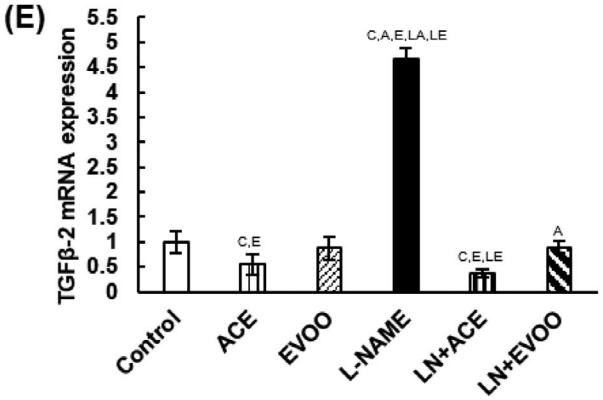


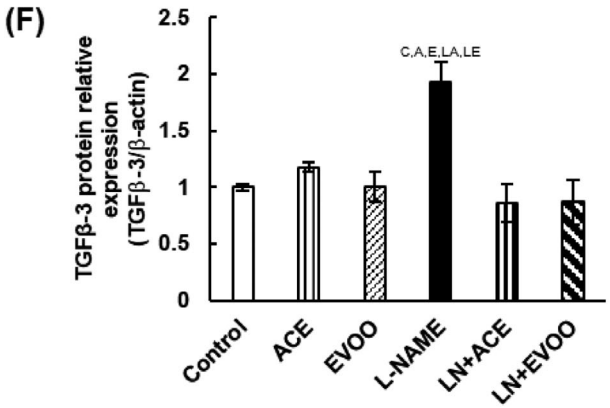


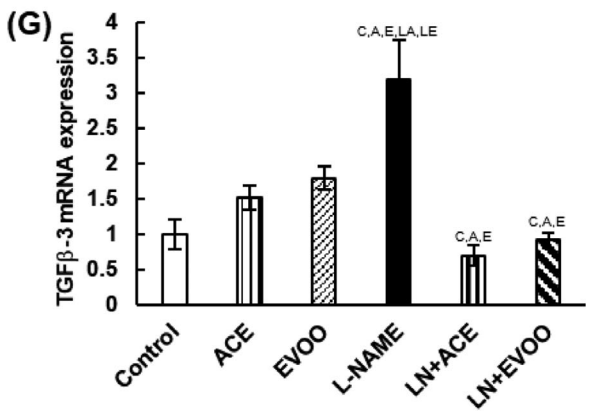












choroid/retina of rodents with arterial hypertension induced by L-NAME administration.[14,30] Chronic treatment with L-NAME yields a well-established animal model presenting with severe hypertension according to current guidelines.[31] These hypertensive animals have been shown to develop structural changes in the retinal layers[14,30] and exhibited retinal dysfunction on electroretinography recordings,[19] as typically observed in hypertensive retinopathy.[32] Such changes were prevented by dietary interventions based on 12% w/w ACE oil or EVOO supplementation; these supplements are in line with previous studies in rodents by our group and others, and could be extrapolated to regular consumption of around 1-1.5 mL of oil/Kg body weight per day in humans.

In the current study, we observed that L-NAME hampers fluoresceine progression in retinal vessels, which might suggest a reduction of retinal blood flow in hypertensive mice. The slow progression of fluorescein in retinal vessels was prevented by ACE oil- and EVOO-enriched diets, indicating an improvement in retinal blood flow produced by some of their components. Indeed, previous studies in our group revealed the ability of ACE oil to prevent vascular remodeling and restore systemic vascular function in L-NAME-treated rats.[33] The better outcomes observed in this regard in favor of ACE oil could be partly explained by differences in minor compounds with vasoactive properties, such as triterpene acids (e.g., oleanolic and maslinic acid).[34,35] Additional studies in hypertensive patients have highlighted the presence of arterial sclerosis and vascular contraction due to enhanced intravascular pressure in the choroid[36] and reduced retinal blood flow.[37]

Regarding IOP values (whose elevation constitutes another harmful risk factor of retinopathy), L-NAME-treated animals presented an elevation of this parameter in the middle and at the end of the 6-week follow-up period; an alteration that was completely or partially abolished when hypertensive mice received dietary supplementation with ACE oil or EVOO, respectively. Several reports have indicated an association between nutritional supplements and the development of glaucoma.[38] In this sense, the deficiency in ω-3 polyunsaturated fatty acids has been found to produce retinal ganglion cell dysfunction that could increase IOP.[39] Although the fatty acid composition is very similar between both oils, we have found significant differences in minor components (e.g., sterols, tocopherols, triterpenes, and secoiridoids/ortodiphenols)[14] that might also be responsible for selective modulation of this parameter.

Pathological fibrosis is a common feature in many retinal diseases,[40] and oxidative and inflammatory events play a crucial role in its development. In our experimental design, the increased expression of COL4α1 in retinal layers (GCL, plexiform layers, OS, and RPE/CH) from hypertensive mice was accompanied by upregulation of TGFβ receptor isoforms detected by immunofluorescence staining. Interestingly, chronic L-NAME treatment induced morphological changes in the same retinal layers, especially in GCL, where we have also reported increased production of superoxide anion and a higher expression of NADPH oxidase isoforms (NOXs) and glial fibrillary acidic protein (GFAP), as a marker of Müller cell activation.[14,30] These changes were accompanied by retinal dysfunction in electroretinography recordings in inner retinal cell layers of hypertensive animals[14,19] and may be indicative of retinal ganglion cell loss leading to increased IOP. All these alterations were reversed when hypertensive mice received dietary supplementation with ACE oil or EVOO.

It is well known that a reduction in NO increases the volume of trabecular meshwork cells and outflow resistance, which also causes an elevation in IOP.[41] Evidence suggests that stimulation of endothelial nitric oxide synthase (eNOS) activity results in improved outflow function and reduced IOP, while inhibition of eNOS produces the opposite effect.[41] In a previous study, we found a reduction in NO levels and inactivation of eNOS in the retina of hypertensive mice that could be abolished by dietary supplementation with ACE oil/EVOO.[14] Together, our results suggest that the IOP elevation observed in L-NAME hypertensive mice might be prevented through a beneficial effect on NO metabolism after regular consumption of the oils.

The retinal layer thickness assessed by OCT showed a reduction in GCL, ONL, OS, and RPE/CH, together with an increase in IPL, in L-NAME-treated animals. Our OCT measurements are slightly different from previous results obtained in hematoxylin/eosin-stained retinal sections of hypertensive mice, were no changes in ONL were reported.[14] Although several authors have demonstrated a qualitative correlation between OCT and retinal histology measurements,[42,43] this controversy might be due to tissue changes during the paraffin embedding process. It must also be noted that other authors have reported a significant thinning of IPL in ocular hypertension.[44] This finding is not in agreement with our observation that L-NAME-induced hypertensive animals present increased IOP and a thicker IPL. Amongst other possible mechanisms, the latter might be due in part to axonal swelling, fibrotic effects, or activation of Müller glial cells by neuronal injury, as previously observed in *retinitis pigmentosa* or Leber congenital amaurosis.[45,46] Furthermore, since IOP augmented in a less abrupt and intense manner in L-NAME-treated animals than is normally found in traditional animal models of glaucoma, different physiological responses may exist between these models.

Collagen I and IV are considered major candidates able to promote ocular fibrosis. As such, their detection is of special relevance in retinal pathologies such as retinal injuries,[47] diabetic retinopathy,[48,49] or age-related macular degeneration.[50] Furthermore, hypertension can modify the turnover and expression of collagen I and III, favoring the fibrotic process in different organs[51]; and collagen IV has been implicated in the development of microvascular complications.[52] Therefore, the regulation of collagen isoforms, especially type IV collagen, by nutraceutical agents might be a useful target to reduce the risk of hypertension-driven retinal vascular damage. In the current

Figure 5. Protein expression of profibrotic growth factor CTGF (A) and protein/gene expression of TGFβ family ligands TGFβ-1 (B, C), TGFβ-2 (D, E), and TGFβ-3 (F, G) in the retina of all experimental groups. The quantitative fold changes in gene expression were determined relative to GAPDH in each corresponding group. Values are expressed as mean ± SEM of five animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO.

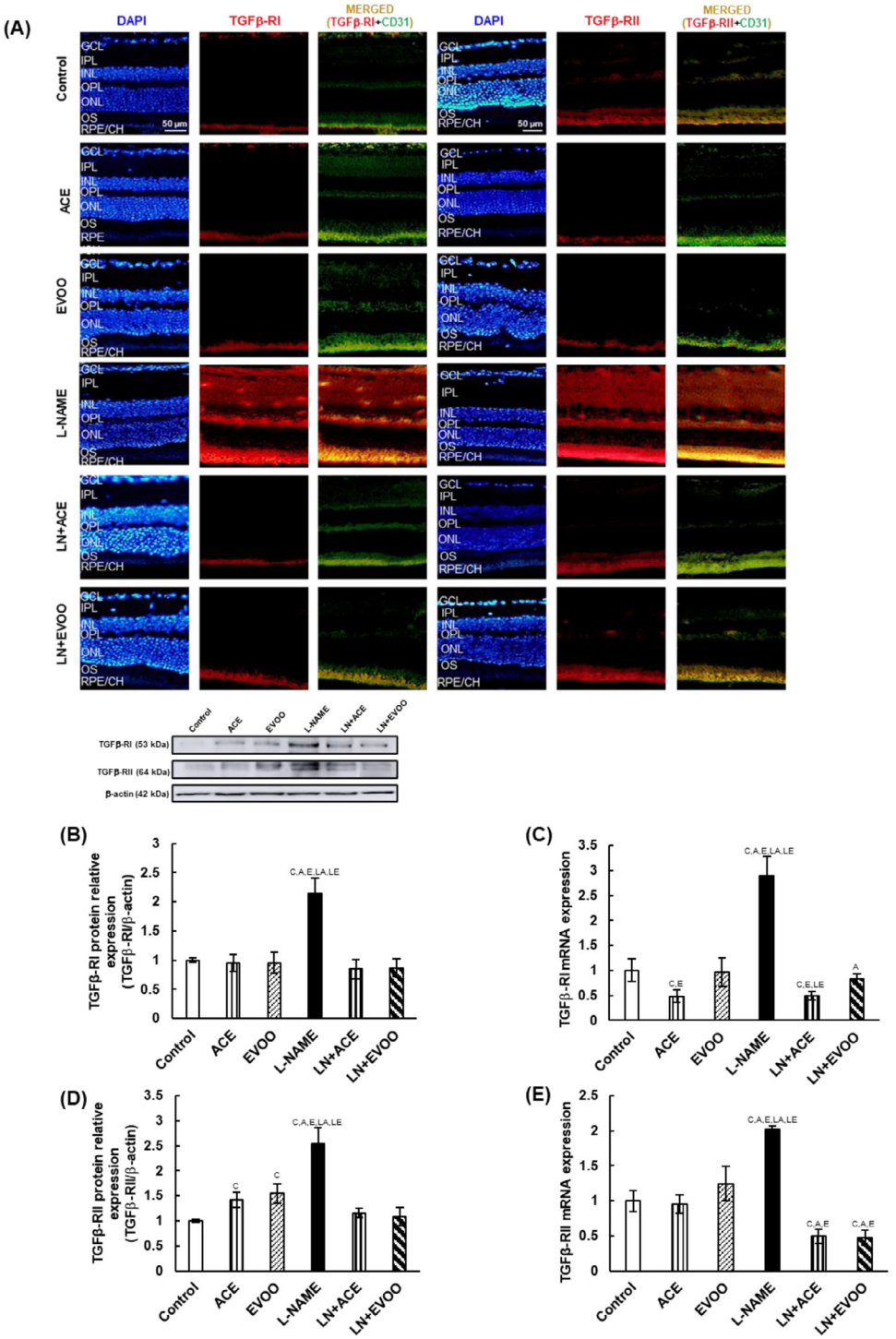


Figure 6. A) Localization and expression of TGFβ family receptors TGFβ-RI (left) and TGFβ-RII (right) explored by immunolocalization (red color) and double staining with CD-31 (green color) in retinal layers, where the merge is represented in yellow color. Nuclei staining with DAPI (blue color) was used to delimit the different retinal layers. Magnification: 10×. B–E) Protein/gene expression of TGFβ family receptor TGFβ-RI (B, C) and TGFβ-RII (D, E) in the retina of all experimental groups. The quantitative fold changes in gene expression were determined relative to GAPDH in each corresponding group. Values are expressed as mean ± SEM of five animals per group: $^{C}p < 0.05$ versus Control; $^{A}p < 0.05$ versus ACE; $^{E}p < 0.05$ versus EVOO; $^{LA}p < 0.05$ versus LN+ACE; $^{LE}p < 0.05$ versus LN+EVOO. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; RPE/CH, retinal pigment epithelium/choroid.

study, the greater colocalization of collagen IV with the specific endothelial cell maker (CD31) in the retina and choroid of L-NAME-treated mice could indicate that the fibrotic process in these animals occurs mainly at these vascular beds. Interestingly, the major reduction of collagen expression induced by ACE oil over EVOO is a notable indication of its competence to regulate fibrotic processes and to protect the retinal vasculature.

In view of the low MMP/TIMP ratio found in L-NAME-treated mice, we hypothesized that arterial hypertension tends to induce collagen accumulation in the retina, and that ACE oil- and EVOO-enriched diets were able to reverse this alteration as a possible defense mechanism to avoid excessive collagen deposition in the eye. These results are not in agreement with previous studies showing that Mediterranean diet and some EVOO components, such as polyphenols[53] and oleocanthal.[54] decreased the expression and activity of MMPs, although these findings occurred in normotensive conditions. In addition, it has been suggested that the upregulation of MMP2 and MMP9 is due to a response of the organism to modulate ECM in glaucoma.[55] In any case, it should be noted that the regulation of MMP expression/activity is complex and can be influenced in different pathological conditions by molecules such as cytokines, growth factors, or hormones.[23] In fact, the expression of MMP3 appeared to be different at protein and mRNA levels in our hypertensive animal model. For this reason, it would be of interest to further investigate the regulatory mechanisms of MMP isoforms to elucidate how they are altered by AH at the retinal level.

To explore additional mechanisms involved in the antifibrotic properties of ACE oil in hypertensive retinas, we also quantified the relative gene/protein expression of CTGF and TGFβ family members. The TGFβ family plays a crucial role in the induction of fibrosis and participates in the transcription of numerous genes highly associated with profibrotic effects and inflammation. Its activity can be either attenuated by nuclear receptors factors such as peroxisome proliferator activated receptor gamma (PPARγ), or aggravated by cytokines such as IL-1β or TNF-α.[56] For this reason, TGFβ inhibition has been explored as a novel therapy in different pathologies that present with inflammatory/fibrotic retinopathy[57–59]; indeed, TGFβ-1 has been postulated as a potential biomarker of diabetic retinopathy.[60] The efficacy of ACE oil-supplemented diets to prevent inflammatory and fibrotic events might be due to a reduction of CTGF and TGFβ expression in the retina. This effect is likely related to recent observations showing that ACE oil can induce the expression of PPARγ, PPARα, and anti-inflammatory cytokine IL-10, while downregulating pro-inflammatory cytokines, in the retina and choroid of L-NAME-induced hypertensive mice.[19]

Other authors have described the efficacy of some minor components contained in ACE oil and EVOO to inhibit TGFβ. For instance, oleanolic acid attenuated fibrosis through depletion of TGFβ isoforms and receptors in a rodent model of ureteral obstruction,[61] and hydroxytyrosol has been proven effective in reducing inflammatory events by inhibiting TGFβ signaling.[62] Ursolic acid, which has also been described as a TGFβ antagonist,[63,64] reduced fibrosis and inflammation in a model of myocardial infarction by blocking massive cytokine generation and collagen deposition.[65] Recently, maslinic acid demonstrated its ability to inhibit p38 MAPK phosphorylation and reduce TGFβ-1 signaling in a mouse model of drug-induced myocardial fibrosis.[66] Another recent study describes oleocanthal as a promising compound to modulate TGFβ-mediated fibrosis by modulating oxidative and inflammatory biomarkers such as NADPH oxidases, interleukins, and MMPs.[67] Therefore, the greater effects of ACE oil compared to EVOO in terms of blocking profibrotic CTGF and TGFβ signals in hypertensive retinas could be attributed to its higher content in these and/or other minor components.

In summary, our results support a novel application of ACE oil- and EVOO-supplemented diets as a nutraceutical tool to avoid alterations in retinal blood flow, regulate IOP values and prevent fibrotic events and retinal dysfunction in a hypertensive context. It is unclear, however, whether the retinal fibrotic processes observed in our experimental conditions are directly caused by the elevation of intraocular pressure or if they are due rather to a systemic effect of L-NAME treatment. In fact, L-NAME can sustain NO depletion and excessive ROS production, which can cause oxidative, inflammatory, and fibrotic events that may be targeted by minor compounds present in the oils. Overall, the better outcomes of ACE oil, compared to an equivalent EVOO of similar origin and preparation, makes it a suitable candidate to be considered as an effective pharm-food against eye pathologies aggravated by arterial hypertension.

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Conflict of Interest

Part of the results reported here were used by the authors for patent applications 1) Use of acebuche oil in the retinal damage associated to arterial hypertension (application number P202030625, 23 June 2020); and 2) Acebuche oil to for use as an ocular hypotensive agent (application number P202230081, 3 February 2022), at the Oficina Española de Patentes y Marcas (OEPM), Ministerio de Industria, Comercio y Turismo.

Author Contributions

Á.S.-G.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. C.R.-G.: Investigation, Writing – review & editing. H.A.: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. C.M.V.: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Super-

vision, Project administration, Funding acquisition. A.M.: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Keywords

acebuche, arterial hypertension, fibrosis, olive oil, retina

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