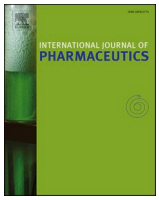
International Journal of Pharmaceutics 649 (2024) 123602

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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



Ophthalmic wild olive (ACEBUCHE) oil nanoemulsions exert oculoprotective effects against oxidative stress induced by arterial hypertension

Á. Santana-Garrido [a,c,1], M. Durán-Lobato [b,1], A. Mate [a,c,*], L. Martín-Banderas [b,*], C. M. Vázquez [a,c]

[a] Departamento de Fisiología, Facultad de Farmacia, Universidad de Sevilla. CL Profesor García González 2, 41012 Sevilla, Spain
[b] Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Sevilla. CL Profesor García González 2, 41012 Sevilla, Spain
[c] Epidemiología Clínica y Riesgo Cardiovascular, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío – Consejo Superior de Investigaciones Científicas – Universidad de Sevilla. Avda. Manuel Siurot s/n, 41013 Sevilla, Spain

ARTICLE INFO

Keywords:
Acebuche oil
Cornea
L-NAME hypertension
Nanoemulsion
Oxidative stress
Retina

ABSTRACT

Oxidative stress plays a key role in several systemic and ocular diseases, including hypertensive eye diseases. In this context, we previously showed that oral administration of wild olive (acebuche, ACE) oil from *Olea europaea* var. *sylvestris* can counteract ocular damage secondary to arterial hypertension by modulating excess reactive oxygen species (ROS) produced by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Therefore, this work describes the development of an ACE oil-based formulation for ocular administration as a local therapy to counteract hypertension-related oxidative damage. Specifically, ACE oil nanoemulsions (NEs) were successfully produced and characterized, exhibiting appropriate features for ophthalmic administration, including a nanometer size (<200 nm), moderate negative ZP, adequate osmolality and pH, and colloidal stability in biorelevant fluids. Likewise, the NEs presented a shear thinning behavior, especially convenient for ocular instillation. *In vivo* evaluation was performed through either intravitreal injection or topical ophthalmic administration in mice with hypertension induced via administration of Nω-nitro-L-arginine-methyl-ester (L-NAME). Both routes of administration reduced hypertensive morphological alterations and demonstrated a noticeable antioxidant effect thanks to the reduction of the activity/expression of NADPH oxidase in cornea and retina. Thus, an ACE oil ophthalmic formulation represent a promising therapy for ocular pathologies associated with arterial hypertension.

1. Introduction

Arterial hypertension (AH), defined as a sustained elevation of systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) values above 140/90 mmHg, is a systemic and multifactorial disease that is considered one of the major risk factors for the development of cardiovascular, renal, and neurological diseases (van Oort et al., 2020). AH is also a risk factor for several vision-threatening ocular conditions due to the fact that the eye is highly vascularized, a situation especially aggravated in the retina and choroid (Fraser-Bell et al., 2017). Even though HA harmful effects on the eye have been established, the precise mechanisms responsible for ocular dysfunction have not yet been fully elucidated.

Despite the multifaceted nature of ocular damage related to AH, the implication of oxidative and inflammatory events is well documented. Oxidative stress has been attributed a major role in the pathogenesis of various systemic and ocular diseases, including age-related macular degeneration, keratoconus, or diabetic retinopathy (Nebbioso et al., 2022; Oduntan and Mashige, 2011).In this regard, recent findings suggest that the enzyme nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX), the major enzymatic source of reactive oxygen species (ROS) in the hypertensive context, plays an important role in hypertension-associated damage not only in the retina and choroid but also in the cornea (Santana-Garrido et al., 2022; Santana-

* Corresponding authors.
E-mail addresses: mate@us.es (A. Mate), luciamartin@us.es (L. Martín-Banderas).
[1] AS-G and MD-L contributed equally to the work presented here and should therefore be regarded as equivalent authors.

https://doi.org/10.1016/j.ijpharm.2023.123602
Received 29 June 2023; Received in revised form 7 November 2023; Accepted 11 November 2023
Available online 13 November 2023

Garrido et al. (2021a)). In addition to the role of NADPH oxidase, there is strong evidence linking NOX-generated ROS with inflammation through the stimulation of cytokines and transcription factors (Morawietz et al., 2023). The multidimensional activity of NADPH oxidase contributes to the pathological mechanisms of eye diseases in which AH is considered a risk factor, such as age-related macular degeneration, or diabetic retinopathy (Fraser-Bell et al., 2017). These findings suggest that modulation of NADPH oxidase at the ocular level might be a useful strategy to mitigate hypertensive damage to ocular tissues, which might eventually contribute to preventing or controlling some of the most prevalent ocular conditions.

The use of natural agents for the prevention and treatment of eye diseases is becoming increasingly common (Choo et al., 2022). Extra virgin wild olive oil (commonly known as "acebuche" (ACE) oil in Andalusia in Southern Spain) is obtained from the wild olive tree (*Olea europaea* var. *sylvestris*) (Green, 2002) and mostly consumed as a 'gourmet' oil. Unlike extra virgin olive oil (EVOO) obtained from the usual cultivated olive tree (*Olea europaea* var. *Europaea),* which has been the focus of extensive literature, only a few studies have dealt with the bioactivity of ACE oil so far. Among the most significant reports, regular dietary consumption of ACE oil has been demonstrated to counteract hypertensive damage by blocking the activity of NADPH oxidase, displaying antioxidant and anti-inflammatory properties at the retinal level in hypertensive mice (Santana-Garrido et al., 2020b; Santana-Garrido et al., 2021b). ACE oil properties can be attributed to its richer composition in tocopherols, triterpene acids and polyphenols (specially secoiridoids) compared to EVOO (Santana-Garrido et al., 2020b). All these components are involved in a variety of biological activities, and they have been postulated as powerful tools for the prevention and management of cardiovascular and neurological diseases (Angeloni et al., 2017; Gorzynik-Debicka et al., 2018; Reboredo-Rodríguez et al., 2018). The outstanding properties of ACE oil motivate research on the development of adequate pharmaceutical forms for local delivery in ocular structures, which might maximize its beneficial effect on the eyes and, consequently, reduce the onset of ocular pathologies associated to AH.

Lipophilic drugs are traditionally attributed low and variable bioavailability (Beig et al., 2013). In addition, the maximum amount of active drug that can reach the vitreous through systemic delivery, to treat posterior segment disorders, is often well below optimum, due to the restrictive character of ocular barriers (Singh et al., 2020). These facts, together with the retinoprotective effects attributed to ACE oil-enriched diets (Santana-Garrido et al., 2020b), led us to hypothesize that direct ocular administration of ACE oil could represent a novel and promising approach to treat AH-related corneal and retinal pathologies.

The treatment of posterior segment ocular diseases represents a challenge, due to the complex anatomy and physiology of the eye. Indeed, the eyeball contains several blood-ocular barriers that restrict drug delivery to the target site of action (Varela-Fernández et al., 2020). Briefly, for topical administration, the drug of interest must overcome precorneal limitations such as tear drainage, the low dwelling volume of the eye, and the presence of mucin and lipids in lacrimal fluid (Singh et al., 2020; Varela-Fernández et al., 2020); the restrictive character of the cornea and conjunctiva towards drug transport; and the blood-aqueous barrier (BAB) (Varela-Fernández et al., 2020). For these reasons, intravitreal (IV) drug administration is the most frequent treatment option in posterior segment ocular therapies (e.g., uveitis and retinopathies), while the number of topical formulations for this purpose is very limited (Kim and Woo, 2021).

Advanced formulations based on nanocarrier drug delivery systems represent a promising strategy in this area (Souto et al., 2019). They have been reported to overcome the limitations of conventional formulations restricting drug bioavailability by improving the interaction with ocular biological barriers (Le Bourlais et al., 1998; Saati et al., 2010) while maintaining a liquid pharmaceutical form, thus allowing for topical instillation and a potentially better patience compliance. Specifically, lipid-based nanocarriers are especially interesting due to the fact that their components, i.e. lipids and emulsifiers, can be selected from Generally Recognized as Safe (GRAS) excipients by the FDA (Garcia et al., 2022; Santalices et al., 2017). Among these nanocarriers, nanoemulsions (NEs) stand out owing to several features, such as avoiding burst release and drug leakage, presenting improved tolerance upon administration, and offering ease of production and scalability with low associated production costs (Garcia et al., 2022), among others. Finally, the possibility of using ACE oil as the main constituent of the oily droplets of a nanoemulsion (NE), instead of dissolving it within a hydrophobic matrix, would allow maximizing the amount of pharmacologically active ingredient loaded in the formulation.

With this background, the current work presents a proof-of-principle that an ophthalmic formulation for local administration of ACE oil, intended for topical instillation, may be a feasible and promising approach for the development of therapies against AH-related ocular pathologies. Specifically, a NE of ACE oil is proposed based on the distinct advantages of NEs as ocular drug delivery nanocarriers for lipophilic compounds, along with the possibility of maximized ACE oil loading, as mentioned above. ACE oil NEs were produced and characterized in terms of their physicochemical properties, rheology, and stability in biorelevant fluids. Finally, *in vivo* evaluation of the NEs was carried out in a hypertensive rodent model upon both instillation and ~~intravitreal~~ IV administration, paying especial attention to morphological changes and antioxidant properties in corneal and retinal layers.

2. Materials and methods

2.1. ACE oil information and general materials

ACE oil was obtained from wild olive trees located at Sierra de las Nieves (Málaga, Spain). ACE fruits came from an early harvest (November) and the oil was obtained at room temperature by centrifugation in a two-phase system (Madureira et al., 2022), following standard protocols to comply with the extra virgin olive definition (Regulation (EU), 2013). The oil was kept in a sufficient quantity to guarantee the homogeneity of the products used for the study and was stored in an insulated cellar with controlled temperature, protected from light and away from any source of potential undesirable flavors. The chemical composition of this ACE oil has been described previously (Santana-Garrido et al., 2020b). Acetone (HPLC grade) was acquired from Panreac Química (Barcelona, Spain). Tween 80 (polyethylene oxide sorbitan mono-oleate, polysorbate 80) (Tw80) was purchased from Acofarma (Barcelona, Spain). Sodium chloride, NaCl, sodium bicarbonate, calcium chloride dehydrate, potassium chloride (all ACS reagent grade, ≥99.0 %) hydrochloric acid (ACS reagent, 37 %), sodium hydroxide solution (1.0 N, Bioreagent) and PBS tablets were provided by Merck Life Science S.L.U. (Madrid, Spain). Deionized and filtered water was used in all the experiments (Milli-Q Academic, Millipore, Molsheim, France).

2.2. Production of NEs

NEs were prepared following a solvent displacement technique reported elsewhere (Crecente-Campo et al., 2019; Garzoli et al., 2020; Jafari et al., 2020) with some modification. Briefly, 30 mg of ACE oil were dissolved with 2.5 mL of acetone. In addition, a 100 mg/mL Tw80 solution in acetone was prepared. Next, 200 μL of Tw80 solution were added over the ACE solution, and the resulting mix was briefly homogenized by vortexing. Subsequently, the resulting ACE/Tw80 solution was added over 10 mL of PBS under stirring (600 rpm, RT15 power IKA® Werke, Staufen, Germany) in a fume hood. The organic solvent was then evaporated by maintained stirring inside the fume hood at room temperature for 2 h, yielding a final volume of 10 mL of formulation (0.3 % w/v of ACE oil). Subsequently, NEs were concentrated down to 0.5 mL by ultrafiltration (6 % w/v of ACE oil) (Ultracel 100 KDa

Amicon) at 15 °C and 4.000 x g (Eppendorf 504R centrifuge, Eppendorf AG, Germany). Whenever needed, the formulations were stored at 4 °C until further assessment, and their main physicochemical properties re-evaluated immediately before use. In the case of NEs batches intended for *in vivo* studies, the formulation was prepared in a biological safety cabinet (Telstar BIO II A, Telstar, Terrasa (Barcelona, Spain)) using previously autoclaved material and filtered through sterile, low-binding PVDF syringe filters with 0.22 μm pore size (Merck, Barcelona, Spain) for sterilization (Ismail et al., 2020; Jurišić Dukovski et al., 2020; Rebibo et al., 2022). Size, count rate and ZP were monitored prior to administration.

2.3. Nes characterization methods

2.3.1. Particle size and distribution and zeta potential

The mean diameter and size distribution of the NEs were measured by Dynamic Laser Light Scattering (DLS), and their Zeta Potential (ZP) by Laser Doppler Anemometry, both in a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern UK). Measurements were carried out in triplicate by diluting an aliquot of NEs with purified water at 25 ± 0.5 °C.

2.3.2. Nes morphology analysis

The morphological characterization was performed by image analysis obtained by Transmission Electron Microscopy (TEM) in a ZEISS LIBRA 120, 120Kv (Carl Zeiss Iberia, Madrid, Spain) using a previously described negative-staining method (Durán-Lobato et al., 2015) with slight modifications. Beforehand, a previously prepared 2 % w/v uranyl acetate (Klang et al., 2012) solution was filtered through a 0.2 μm PVDF filter to eliminate potential precipitates from storage. Then, 20 μL of NE were added onto 300-mesh copper grids and the liquid excess was removed with filter paper after 5 min. Following, 20 μL of the uranyl solution were added to the grid, and the liquid excess was removed with filter paper after 5 min. Finally, the grids were allowed to dry overnight at room temperature and then observed by TEM.

2.3.3. Osmolality measurements

The osmolality of NEs was measured by determination of the freezing point in an osmometer (Löser™ Osmometer, Fisher Scientific SL, Madrid, Spain). The osmolality was initially presumed to be near isotonicity due to the composition of the aqueous media (i. e. PBS) and amenable to be regulated by adjusting buffer concentration. Measurements were performed in triplicate.

2.3.4. Rheology analysis

The rheology of the NEs was analyzed with a hybrid interfacial shear rheometer (REO-KSV Nima, Biolin Scientific UK, Manchester, UK). A shear deformation was applied onto the interface with a magnetized probe. The probe is oscillated using a mobile magnetic trap. The movement of the probe is recorded with a high-resolution camera, and the rheological properties are subsequently calculated. The testing temperature was fixed at 25 °C, and measurements taken for the shear rate range of 0–200 s^{-1}. In order to attain a sample volume feasible for analysis, several batches of the formulation were prepared and collected as a single pool, which was subsequently analyzed to ensure that the physicochemical properties of the NEs were maintained.

2.3.5. Colloidal stability in biorelevant medium

To assess the colloidal stability of the NEs in a biological medium resembling *in vivo* conditions, the formulations were incubated in Simulated Lacrimal Fluid (SLF) (Shastri et al., 2010; Taranbir et al., 2010) (1:5 v/v) at 37 °C and 300 rpm up to 2 h (Reimondez-Troitiño et al., 2016) (Titramax 1000, Comecta, Abrera, Barcelona, Spain). At predetermined intervals, samples were withdrawn and their size, size distribution, and Derived Count Rate (DCR) monitored by DLS measurement to assess the maintenance of both size properties and droplet concentration, as previously described (Niu et al., 2018; Thwala et al., 2016).

2.4. In vivo experiments with ACE oil NEs

2.4.1. Study design and pharmacological treatments

The experimental design was conducted in accordance with the European Union (EU) Directive 2010/63/EU and the national (RD 53/2013) guidelines for the care and use of laboratory animals and was 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, supplied by the Center for Animal Production and Experimentation (University of Seville, Spain), were randomly distributed into two groups housed during 6 weeks with free access to food and water under standard conditions (23 ± 1 °C, 12 h/12 h light–dark cycles): (1) Normotensive mice (Control group) and (2) hypertensive mice (via administration of 45 mg Nω-nitro-L-arginine methyl ester (L-NAME)/kg body weight/day) (L-NAME group). After the 6 weeks of treatment, L-NAME animals were subsequently distributed into different groups depending on whether they received ~~intravitreal~~ IV injections or topical instillation of ACE oil NEs: (3) L-NAME mice treated with a single IV injection of 1 μL of ACE oil NEs (LN + 1IVACE group); (4) L-NAME mice treated with two IV injections of 1 μL of ACE oil NEs (LN + 2IVACE group); and (5) L-NAME mice treated with one drop of ACE oil NEs instilled on the cornea every 8 h for a total period of 5 days (LN + DACE group). Concerning IV treatments, the first IV injection of ACE oil NEs was done on day 1 (d1) immediately after the end of the 6-week treatment with L-NAME, both in LN + 1IVACE and LN + 2IVACE groups. Two days after this first IV injection (d3), LN + 1IVACE animals were sacrificed, whereas LN + 2IVACE mice were treated with the second IV injection of ACE oil NEs, the latter being euthanized two days later (d5). In turn, ACE oil drop instillation for 5 days was also initiated upon completion of the 6-week treatment with L-NAME in the LN + DACE group. In all groups, L-NAME administration was continued until sacrifice. All ophthalmological treatments were carried out in anesthetized animals under controlled isoflurane inhalation vapor (3 %).

2.4.2. Animal monitoring

Systolic (SBP) and diastolic (DBP) blood pressure was measured weekly by the indirect method of tail-cuff occlusion in conscious animals using a NIPREM 645 pressure recorder (CIBERTEC, Barcelona, Spain). Blood pressure values were calculated as the average of three to four consecutive records.

2.4.3. Ocular tissue harvesting and homogenization

Mice were euthanized by cervical dislocation. Following previous protocols (Santana-Garrido et al., 2022; Santana-Garrido et al., 2020b), the corneas and retinas were rapidly dissected under a binocular stereo microscope. Cornea and retina homogenates were prepared for each experimental group in 50 mM phosphate buffer saline (PBS, pH 7.4) containing protease and phosphatase inhibitors (Sigma Aldrich-Roche, Madrid, Spain) and using a Potter-Elvehjem tissue grinder. Homogenates were centrifuged for 10 min at 10000 x g and the supernatants were recovered to determine the concentration of proteins by the Bradford method (Bradford, 1976).

2.4.4. NADPH oxidase activity measurements

NADPH oxidase activity was measured both in cornea and retina homogenates by lucigenin-enhanced chemiluminescence, following routine protocols in our laboratory (Santana-Garrido et al., 2022; Santana-Garrido et al. (2020a)). To confirm the source(s) of superoxide anion ($O_2^{\cdot-}$), homogenates samples were preincubated for 5 min at 37 °C with the following inhibitors at 0.1 mmol/L: diphenyleneiodonium, DPI (inhibitor of flavoproteins; Sigma-Aldrich, Madrid, Spain); oxypurinol (inhibitor of xanthine oxidase; Sigma-Aldrich, Madrid, Spain); rotenone (mitochondrial chain inhibitor of electro transport; Sigma-Aldrich,

Madrid, Spain).

2.4.5. Real-Time PCR

RNA from corneal and retinal tissue was isolated using a well-established TRIzol® RNA isolation method (Thermo Fisher Scientific, Madrid, Spain) (Santana-Garrido et al., 2022; Santana-Garrido et al., 2021b). A retrotranscription reaction was performed as previously described (Chomczynski and Sacchi, 2006). Table 1 lists the specific primers used for the amplification of gene products 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 $2^{-2\Delta\Delta Ct}$ method.

2.4.6. Corneal and retinal histomorphometry studies

~~Intravitreal~~ IV injections of 4 % paraformaldehyde (PFA) in PBS (1X: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) were administered in the eyeballs. Then, the eyeballs were post-fixed in 4 % PFA for 24 h, and subsequently processed for paraffin embedding. The thickness of corneal and retinal layers was measured in deparaffinized hematoxylin/eosin-stained sections (H/E) as previously reported (Santana-Garrido et al., 2022; Santana-Garrido et al., 2020b) and using ImageJ-NIH freeware (v. 2.0.0) (https://imagej.nih.gov/).

2.4.7. ROS measurements in the cornea and retina

Paraffin-embedded eye sections were used to estimate *in situ* ROS production by dihydroethidium (DHE) staining (MedChemExpress, Madrid, Spain, Cat. No. HY-D0079) in the cornea and retina, following a protocol previously used in our laboratory (Santana-Garrido et al., 2022; Santana-Garrido et al., 2020b). The specificity of DHE staining in deparaffinized sections was evaluated by preincubation with 100 U/mL polyethylene glycol-conjugated superoxide dismutase (PEG-SOD; Sigma Aldrich, S9549) for 30 min at 37 °C. An Olympus DP73 fluorescence microscope (Tokyo, Japan) and Image J-NIH freeware (v. 2.0.0) were used to measure staining intensities. All images were taken using the same exposure time and magnification. Results were expressed as relative to the Control group.

2.4.8. Retinal relative bioavailability comparison

Preliminary approaches of relative bioavailability were done based on both the reduction on DHE and NADPH oxidase units (pharmacological availability, PA%) in the retina for each formulation. PA% was calculated using the following formula estimated from previous authors (Niu et al., 2012):

$$PA\% = \frac{UR_{formulation} \text{ x } AO_{1IVACE}}{UR_{1IVACE} \text{ x } AO_{formulation}} \text{ x } 100\%$$

- $UR_{formulation}$, the reduction of DHE signal or NADPH oxidase activity done by ACE oil administered using different formulations (i.e., oral, topical instillation or 2 IV ACE oil NEs).
- $AO_{formulation}$, dose of ACE oil necessary to reduce 1 unit of DHE signal or NADPH activity using different formulations (i.e., oral, topical instillation or 2 IV ACE oil NEs).
- UR_{1IVACE}, the reduction of DHE signal or NADPH oxidase activity done by one IV injection of ACE oil NEs (1IVACE).
- AO_{1IVACE}, dose of ACE oil necessary to reduce 1 unit of DHE signal or NADPH activity using one IV injection of ACE oil NEs (1IVACE).

The UR is compared in each formulation with the values of one IV injection of ACE oil NEs (1IVACE), as it is the closest administration to the retina.

2.5. Statistics

Regarding *in vitro* results, statistical analysis (Prism 8, GraphPad Software, LLC) was performed using Student's *t*-test and one-way analysis of variance (ANOVA) to define the significance between two groups and between more than two groups, respectively. All experiments were performed in triplicate. Results are presented as mean value ± standard deviation (SD). Data with $p < 0.05$ was considered significant. As per *in vivo* results, they are presented as mean ± SEM. One-way ANOVA followed by a post-hoc Tukey's multiple comparison test was performed with GraphPad InStat Software (San Diego, CA, USA, v. 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 is 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 and discussion

3.1. Production and characterization of ACE oil NEs

The dietary intake of an ACE-enriched diet has postulated as a novel strategy to prevent hypertensive damage in the eye (Santana-Garrido et al., 2021b; Santana-Garrido et al., 2020b). This fact has been attributed to its rich chemical composition in pharmacologically active components such as polyphenols, triterpene acids, tocopherols, and others (Lee et al., 2020; Marcelino et al., 2019; Saibandith et al., 2017). However, to date its effect has only been tested through oral administration, which might not be offering the maximum efficiency owing to potentially reduced bioavailability of several components (Naeem et al., 2022). Among the large number of potential strategies for improving the oral bioavailability of phytocompounds (Tan et al., 2021), ocular local administration, such as IV injections and especially topical instillation, would offer a higher chance to overcome ocular barriers and maximize drug bioavailability in the eye (Singh et al., 2020).

A primary aim of this work was to design and produce an ACE oil pharmaceutical form amenable for ophthalmic administration. In this regard, several aspects were considered for the selection of the most convenient dosage form. Firstly, a liquid form enabling topical instillation was desirable, since this administration modality is overall considered the most convenient and easiest mode of drug application to the eye, leading to better patient compliance and representing around 90 % of the ophthalmic topical preparations currently employed (Le Bourlais et al., 1998; Saati et al., 2010). Secondly, a nanocarrier formulation was expected to overcome limitations associated to conventional formulations leading to low bioavailability, including precornea loss, *trans*-conjunctival systemic absorption, and lacrimal drainage (Le Bourlais et al., 1998; Saati et al., 2010). For instance, less than 5 % of the drug administered with such formulations is expected to reach the aqueous humor, with the remaining of the drug being drained out without absorption (Araújo et al., 2009; Taskar et al., 2017). Among

Table 1
Primers used for real-time PCR.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
NOX1	GGTTGGGGCTGAACATTTTTC	TCGACACACAGGAATCAGGAT
NOX2	CCCTTTGGTACAGCCAGTGAAGAT	CAATCCCACGTCCCACTAACATCA
NOX4	CCAAATGTTGGGCGATTGTGT	CAGGACTGTCCGGCACATAG
GAPDH	GCCAAAAGGGTCATCATCTCCGC	GGATGACCTTGCCCACAGCCTTG

the nanotechnology-based approaches described so far in the literature, lipid-based nanocarriers stand out mostly because most of the ingredients needed for their production (mainly solid lipids, oils, and emulsifiers) are in the category of Generally Recognized As Safe (GRAS) excipients by the FDA (Garcia et al., 2022; Santalices et al., 2017). Specifically, NEs offer several advantages over other lipid-based nanocarriers, including ease of production and scalability, and low associated production costs (Garcia et al., 2022). Furthermore, NEs were shown to overcome several drawbacks associated to other nanocarriers, such as burst release, drug leakage, poor stability and vision blurring effect, among others (Garcia et al., 2022), and have also shown improved tolerance and reduced irritation (Ousler et al., 2007). In addition, several mechanisms for improved drug absorption and bioavailability have been attributed to these nanocarriers, including increased wettability of the tear film due to the interaction of the emulsifiers with the mucin and lipid layers (Alany et al., 2006) and to the enhancement of the spreading coefficient (Zhang et al., 2020), overall acting as drug-depots; opening of tight junctions, leading to improved drug penetration (El-Ridy et al., 2015; Jiao (2008)); and inhibition of the P-glycoprotein (P-gp) efflux pump activity (Yadav et al., 2018). Finally, the composition of a nanoemulsion was expected to allow vehiculizing much higher concentrations of ACE oil in a liquid formulation compared to any other type of nanocarrier. Specifically, a nanoemulsion is mainly composed of nanosized droplets of an oil excipient as material core, stabilized by emulsifiers, where a lipophilic drug is dissolved in concentrations typically below 0.5 % w/w (Garcia et al., 2022). By employing ACE oil as both active ingredient and oil material core, its concentration in the final form could be maximized.

For the production of ACE oil NEs, Tween 80 (Tw80, a widely employed emulsifier suitable for ophthalmic dosage forms) was selected on account of its high HLB value (Jafari et al., 2020), aiming at obtaining an oil-in-water (O/W) nanoemulsion (Liu et al., 2019). The emulsifier is expected to occupy the o/w interface of the oil droplets, preventing globule aggregation (Villalobos-Castillejos et al., 2018) and hence instability processes of coalescence, creaming, phase inversion and Ostwald ripening (McClements, 2012). In addition, surfactants with high HLB values usually promote corneal permeation (Calvo et al., 1996; Tayel et al., 2013), and specifically Tw80 has been associated with improved wettability and topical retention times upon ophthalmic administration (Singh et al., 2020; Zhang et al., 2020). PBS, a buffer with optimum capacity around physiological pH and with adequate tonicity, was employed as aqueous phase. A solvent displacement methodology was followed (Fig. 1), which allows obtaining NEs with low energy input and mild conditions (Crecente-Campo et al., 2019; Singh et al., 2017). NEs of homogenous macroscopic aspect, size below 200 nm and moderate negative ZP were obtained (Table 2), adequate for ophthalmic administration. In fact, nanocarriers of size greater than 300 nm have been associated to increased aggregation and disturbance to the vision (Gorantla et al., 2020). In addition, the engineered nanometer size and subsequent large interfacial area of NEs has been attributed with enhanced corneal penetration (Shafiq et al. (2007)). Regarding ZP, and considering the non-ionic character of Tw80, the moderate negative surface charge was attributed to either potential dissociation of fatty acids from ACE oil and/or adsorption of negative ions from the aqueous media on non-polar residues at the interface, as traditionally observed in oil droplets within ion-containing water (Hou et al., 2021). An ultrafiltration process allowed obtaining a concentrate of the NEs under mild conditions and eliminate potential molecules of emulsifier not attached to the surface of the nanostructures, while maintaining the size and ZP of the nanocarriers (Table 2). In addition, NEs stored up to two weeks at 4 °C maintained their main physicochemical properties (Table 2). While further optimization of the composition and production conditions of the NEs was feasible, the results obtained validated the selection of the emulsifier and its employed concentration to produce ACE oil NEs of adequate properties and stability for its subsequent evaluation as an ophthalmic formulation.

Table 2
Physicochemical parameters of NEs evaluated before and after concentration by ultrafiltration, and after storage (4 °C) for up to two weeks. Results are presented as average value ± standard deviation (SD) (n = 3).

	Size (nm)	PDI	ZP (mV)	Osmolality (mOsm/Kg)	pH
Before ultrafiltration	175.6 ± 7.4	0.080	−18.0 ± 1.6	N/A	6–7
After ultrafiltration	162.3 ± 6.5	0.154	−15.6 ± 0.5	326 ± 7.2	6–7
After 2-week storage (4 °C)	170.1 ± 8.6	0.087	−17.8 ± 1.4	N/A	6–7

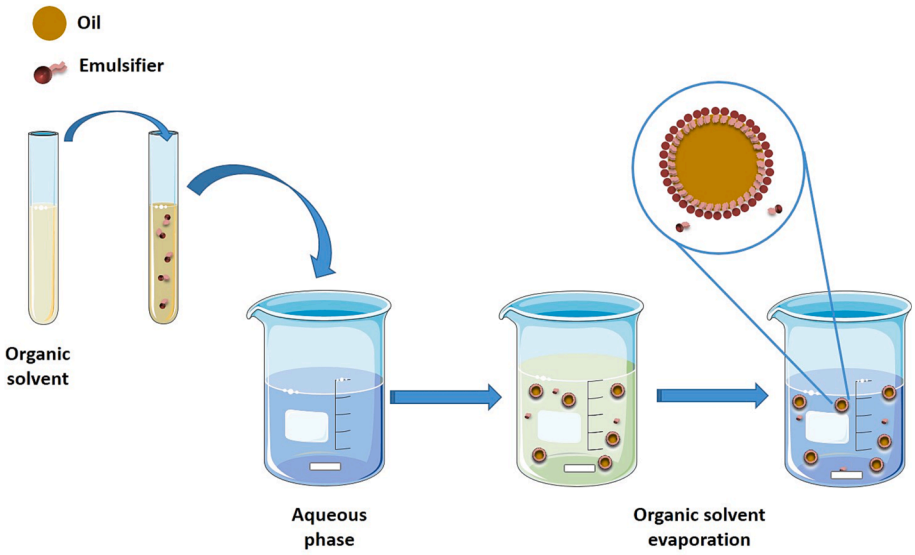


Fig. 1. Schematic representation of the production methodology of the ACE oil NEs, following a solvent displacement procedure. An organic solution containing the ACE oil and the emulsifier is poured onto the aqueous phase under stirring, leading to the instant formulation of droplets stabilized by the emulsifier at the interface. The organic solvent is then allowed to evaporate.

3.2. Technological properties of ACE oil NEs: morphology, pH, osmolality, and rheology

TEM imaging (Fig. 2) revealed a population of spherical droplets of nanometer size in accordance with the results obtained by DLS analysis (Table 2). The pH of the NEs was found to be 6–7 (Table 2), which was attributed to the buffer capacity of the PBS employed to that purpose as outer aqueous phase in the formulation. This value was considered adequate for ophthalmic administration, since the appropriate pH range for topical formulations was reported to be from 6.6 to 7.8 (López-Alemany et al., 1999) and even NEs with pH in the range 5.5 to 5.9 were found suitable for ocular instillation (Ismail et al., 2020).

Osmolality represents another key aspect determining the compatibility of a formulation for ocular administration. The osmolality of the tear film in the normal and non-dry eye was reported to be of about 302 mmol/kg (Tomlinson et al., 2006), while additional studies revealed that the osmolality of the open eyes varied between 231 and 446 mOsm/Kg due to fluid evaporation (Terry and Hill, 1978). Subsequently, and based on drop volume, overall solutions with osmolality values lower than 100 mOsm/Kg or higher than 640 mOsm/Kg were considered as eye-irritant (Van Ooteghem, 2019). The NEs described here displayed an osmolality value of 326 ± 7.2 mOsm/Kg, well within the indicated range and close to the optimum value, which was again attributed to the use of PBS as the external aqueous phase.

Finally, the evaluation of the rheology of the NEs showed a shear thinning or pseudoplastic behavior (non-Newtonian) (Al-Zahrani and Al-Fariss, 1998; Kumar and Mandal, 2018), where the apparent viscosity decreased in response to an increase of shear rate (Fig. 3). This decrease in viscosity is attributed to the alignment of nanoemulsion droplets along the flow streamline and the extrication of the droplets with the rise in shear stress (Al-Zahrani and Al-Fariss, 1998; Samanta et al., 2010). A decreased viscosity in response to shear rate improves the flowability of the nanoemulsion (Kumar and Mandal, 2018) and hence favors the instillation, while an increased viscosity in absence of shear rate favors an increased residence time at the ocular surface. Of note, the ocular shear rate is considered to be large ranging from 0.03 s^{-1} during interblinking periods to 4.250 – 28.550 s^{-1} (Shastri et al., 2010), supporting the convenience of a higher viscosity under conditions of low shear rate (Lin and Sung, 2000). Thus, the pseudoplastic behavior of the NEs constitute an added valuable feature for its use as ophthalmic topical formulation.

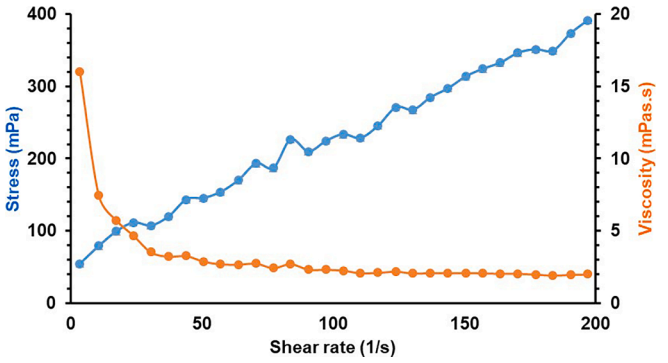


Fig. 3. Rheological evaluation of the NEs, showing a pseudoplastic behavior (non-Newtonian) where the apparent viscosity decreases in response to an increase in shear stress. Results are presented as average value ± standard deviation (SD) (n = 3).

3.3. Colloidal stability in biorelevant medium

Considering that the attributes of NEs for drug improved bioavailability are intrinsically related to its nanometer size (Gorantla et al., 2020; Shafiq et al. (2007)), as mentioned above, the capacity of the NEs to maintain their size upon *in vivo* administration is critical for the performance of the formulation. Hence, to assess the colloidal stability (Fig. 4) of the formulations in a biologically relevant medium simulating *in vivo* conditions, the NEs were incubated in SLF for up to 2 h, a far longer time than theoretically required for the interaction with the corneal epithelium (Reimondez-Troitiño et al., 2016). Size and DCR monitoring, the latter indicative of the concentration of nanostructures remaining in suspension (Niu et al., 2018; Thwala et al., 2016), showed that the NEs successfully preserved their size and concentration during the time frame assayed, indicating their suitability for administration.

3.4. AH induced by L-NAME administration

The importance of AH as a risk factor for many pathologies, including retinopathies and other ocular diseases (Dziedziak et al., 2022), makes it necessary to identify novel therapeutic approaches to reduce its harmful effect in hypertensive target organs such as the eye. L-NAME-treated animals constitute a traditional model of arterial hypertension secondary to nitric oxide depletion, which results in general

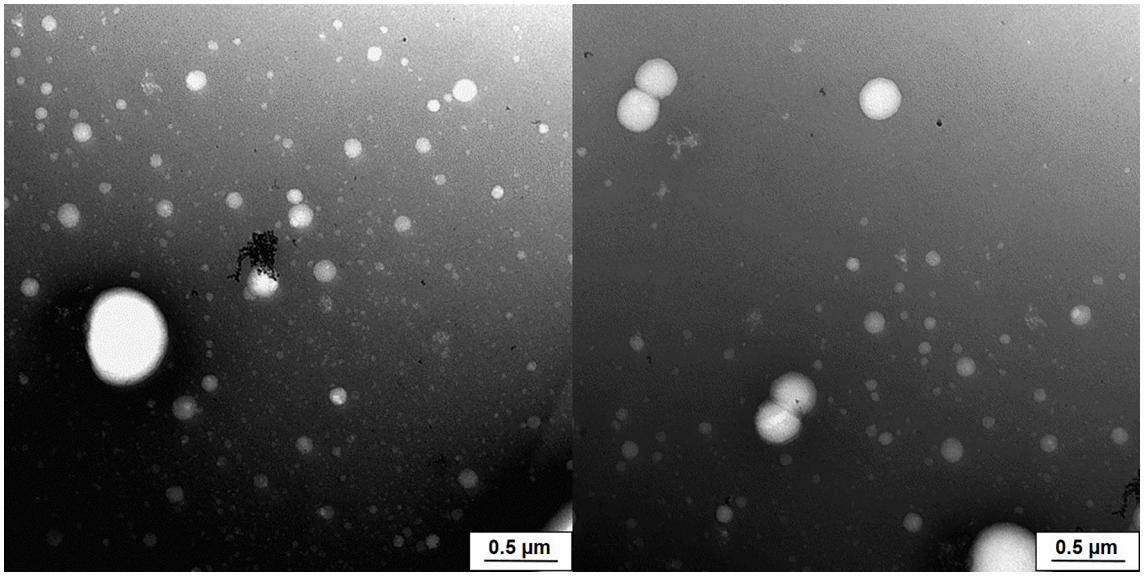


Fig. 2. TEM images of the ACE oil NEs.

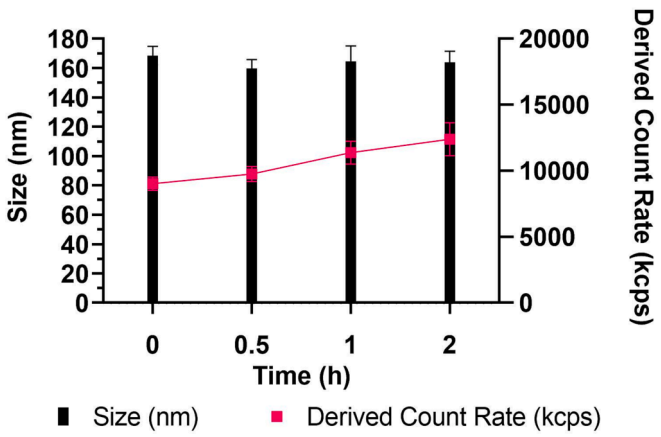


Fig. 4. Colloidal stability of the NEs evaluated in incubation in SLF (37 °C, 300 rpm, 2 h) as a function of size and derived Count Rate (DCR) monitoring by DLS. Results are presented as average value ± standard deviation (SD) (n = 3).

vasoconstriction and consequently in a significant rise in both SBP and DBP (Leong et al., 2015). As expected, sustained elevations of SBP (194 mmHg) and DBP (101 mmHg) values were found in all the L-NAME groups, without any modification in the animals' weight gain or feeding behavior (Figure S1). Besides, this hypertensive model mimics much of functional, molecular, and pathological mechanisms in the setting of AH (Zambrano et al., 2013; Dornas and Silva, 2011) Previous reports from our group demonstrated that the L-NAME model reproduces some ocular findings found in hypertensive patients, such as retinal dysfunction, local oxidative stress and inflammation, and other ocular abnormalities (Santana-Garrido et al., 2021b; Santana-Garrido et al. (2021a); Santana-Garrido et al., 2020b). Moreover, the most recent studies from our group revealed the first evidence that the L-NAME model also presents increased intraocular pressure (IOP) and altered corneal morphology (Santana-Garrido et al., 2022). All these data support the L-NAME model as a valuable source to optimize new pharmacological treatments against ocular hypertensive damage.

3.5. *ACE oil NEs prevent histomorphometry changes in hypertensive corneas and retinas*

Representative images of H/E-stained corneas and retinas are shown in Fig. 5A and 5C. As previously described by our group (Santana-Garrido et al., 2022), L-NAME hypertensive corneas displayed a noticeable reduction in corneal epithelium (CEP) (~28 %), stroma (ST) (~52 %) and corneal endothelium (CEN) (~28 %) in comparison with the Control group (Fig. 5B). The corneal morphology changes observed in the L-NAME group might be explained by the progressive increase of IOP in this animal model (Santana-Garrido et al., 2022). Corneal thinning seems to be associated with disease progression and poorer outcomes, for instance in glaucoma patients (Herndon, 2004), which is often associated with vascular damage with lower response to IOP fluctuations (Lesk et al., 2006).

Next, the effect of the topical administration of ACE oil NEs was evaluated. To enable a quantitative assessment of the pharmacological availability of this approach, IV injections of NEs were administered as a positive control of maximum bioavailability at the targeted location. Histopathological analyses of corneal and retinal sections from control mice subjected to topical or intravitreal administration of ACE oil NEs or its vehicle (Tween 80) showed no signs of toxicological events associated with the treatments, thereby suggesting the safety of the formulation (Figure S2). Interestingly, both the ~~intravitreal~~ IV and topical administration of ACE oil NEs were able to prevent corneal thinning in L-NAME animals, reaching values close to those of the Control group in LN + 2IVACE (~60, ~174, ~11 μm of thickness in CEP, ST, and CEN, respectively) and in LN + DACE groups (~69, ~171, ~11 μm of thickness in CEP, ST, and CEN, respectively). Although the administration of 1 IV injection of ACE oil NEs showed efficacy to prevent morphological reduction (~55, ~160, ~9 μm of thickness in CEP, ST and CEN respectively), its effect was lower in comparison to LN + 2IVACE and LN + DACE. As expected, topical administration of ACE oil NEs displayed better findings than IV administration in the CEP layer. On the other hand, similar effects were found after topical instillation and two IV injections of ACE oil NEs in ST and CEN. This might be due to the amphiphilic characteristics of corneal layers which may be affecting the effect of topical administration of ACE oil in deeper corneal layers, since CEP is a hydrophobic epithelium whereas the ST is a hydrophilic tissue (Cholkar et al., 2013). This fact indicates that topical ACE oil NEs administration might have a preference by CEP and be more limited to penetrate the ST to reach the CEN, despite the fact that nanoemulsions have been described as an effective formulation to improve corneal absorption (Bottos et al., 2013). Regarding the effect of IV injections of ACE oil NEs on corneal tissue, some authors have demonstrated the effect of IV treatments on the cornea structure, which could be due to the fact that CEN favors the transport of nutrients from the aqueous humor to the rest corneal tissues physiologically (Bayat et al., 2020) (Klyce, 2020). Thus, while one single IV ACE oil NEs injection seems to be far from the topical effects, repeated injections may be increasing the dose of ACE oil NEs and allow similar effects to those obtained with the use of topical administration in the ST and CEN.

As expected from previous observations (Santana-Garrido et al., 2020b), the morphometric analysis of retinal layer thickness in L-NAME animals revealed significant reductions of ~ 19 %, ~13 %, and ~ 15 % in the ganglion cell layer (GCL), outer segments (OS), and retinal pigmentary epithelium/choroid (RPE/CH), respectively, in comparison to the Control group (Fig. 5D). IV treatments with ACE oil NEs showed greater effects to counteract L-NAME-induced morphometric changes at this level, with retinal thickness in the LN + 2IVACE group being similar to the Control group (Fig. 5C-D). While the administration of one single IV injection of ACE oil NEs (LN + 1IVACE group) and drop instillation (LN + DACE group) also resulted in positive outcomes, their effect was less pronounced in this case. On the other hand, the inner plexiform layer (IPL) was ~ 10 % thicker in the L-NAME group in comparison to the Control group. Although LN + DACE reduced IPL thickness, IV-treated groups reached values closer to the Control group. Furthermore, both ~~intravitreal~~ IV and topical approaches resulted in thinning/widening of the outer plexiform layer (OPL) and the outer nuclear layer (ONL), respectively, even though there were no differences between Control and L-NAME group in this regard. No modifications were found in the thickness of the inner nuclear layer (INL) among any of the groups.

Similar retinal morphology modifications have been reported in hypertensive patients with arterial sclerosis and vascular contraction, showing thinning of GCL, OS, and RPE/CH layers that was explained by high intravascular pressure in the choroid (Akay et al., 2015) and decreased retinal blood flow (Lim et al., 2019). Thus, partial recovery of retinal morphology suggests a positive modulation of these parameters (i.e., IOP and retinal blood flow) after administration of ACE oil NEs. In terms of formulation, the IV administration of our ACE oil-based NEs might be more effective than the topical route to counteract L-NAME-dependent morphometric changes, which might be in part due to greater penetration of bioactive compounds in the retina. However, IV injection is an invasive technique with the risk of adverse events that can be overcome with the topical route (Tawfik et al., 2022). Therefore, topical treatment may be a worthwhile administration, although longer treatments should be evaluated to confirm increased efficacy similar to IV treatments.

3.6. *Corneal and retinal ROS depletion by ACE oil NEs*

Images of DHE-stained corneas can be observed in Fig. 6A. L-NAME

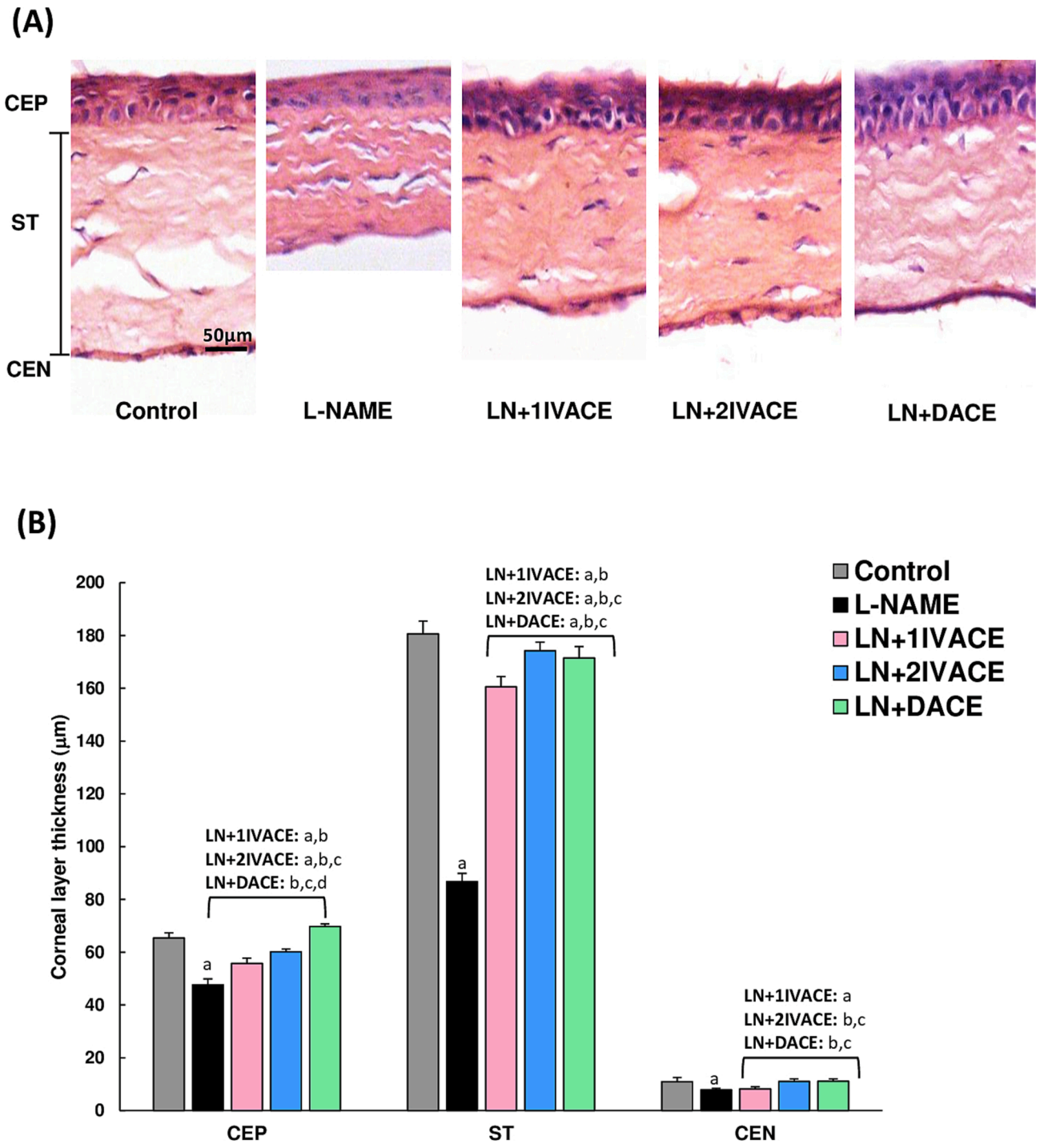
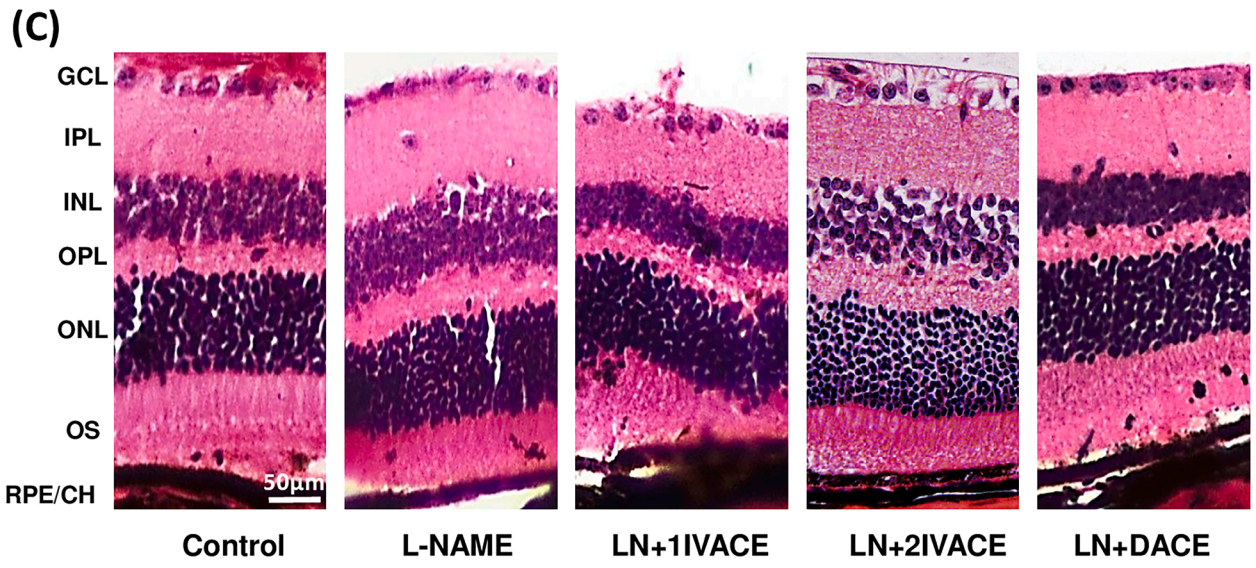


Fig. 5. (**A**) Representative corneal images of hematoxylin/eosin staining and (**B**) corneal layer thickness in all the experimental groups. (**C**) Representative retinal images of hematoxylin/eosin staining and (**D**) retinal layer thickness in all the experimental groups. Magnification: 10x. Values are expressed as mean ± SEM of seven animals per group: $^{a}p < 0.05$ vs. Control; $^{b}p < 0.05$ vs. L-NAME; $^{c}p < 0.05$ vs. LN + 1IVACE; $^{d}p < 0.05$ vs. LN + 2IVACE. **Animal groups**: Control: untreated animals; L-NAME: hypertensive mice (via administration of 45 mg Nω-nitro-L-arginine methyl ester (L-NAME)/kg body weight/day); LN + 1IVACE: L-NAME mice treated with 1 IV injection of ACE oil NEs; LN + 2IVACE: L-NAME mice treated with 2 IV injection of ACE oil NEs; LN + DACE: L-NAME mice treated with 1 instilled drop of ACE oil NEs in the cornea each 8 h. **Abbreviations**: CEN: corneal endothelium; CEP: corneal epithelium; GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer; OS: outer segment; RFU: relative fluorescence units; RPE/CH: retinal pigment epithelium/choroid; ST: stroma.

corneas displayed an intensified signal in CEP (approximate ~ 2.5-fold increase) and sharp ~ 12- and ~ 100-fold increases in ST and CEN, respectively, in comparison to the Control group (Fig. 6B-D). Regarding IV treatments, diminished DHE staining was obtained in corneas from LN + 1IVACE and LN + 2IVACE groups, with a higher the reduction in the latter. Interestingly, the instillation of ACE oil NEs drops on the cornea displayed a higher reduction of corneal ROS, displaying ~ 49 % DHE signal reduction in CEP compared with the Control group; in turn,



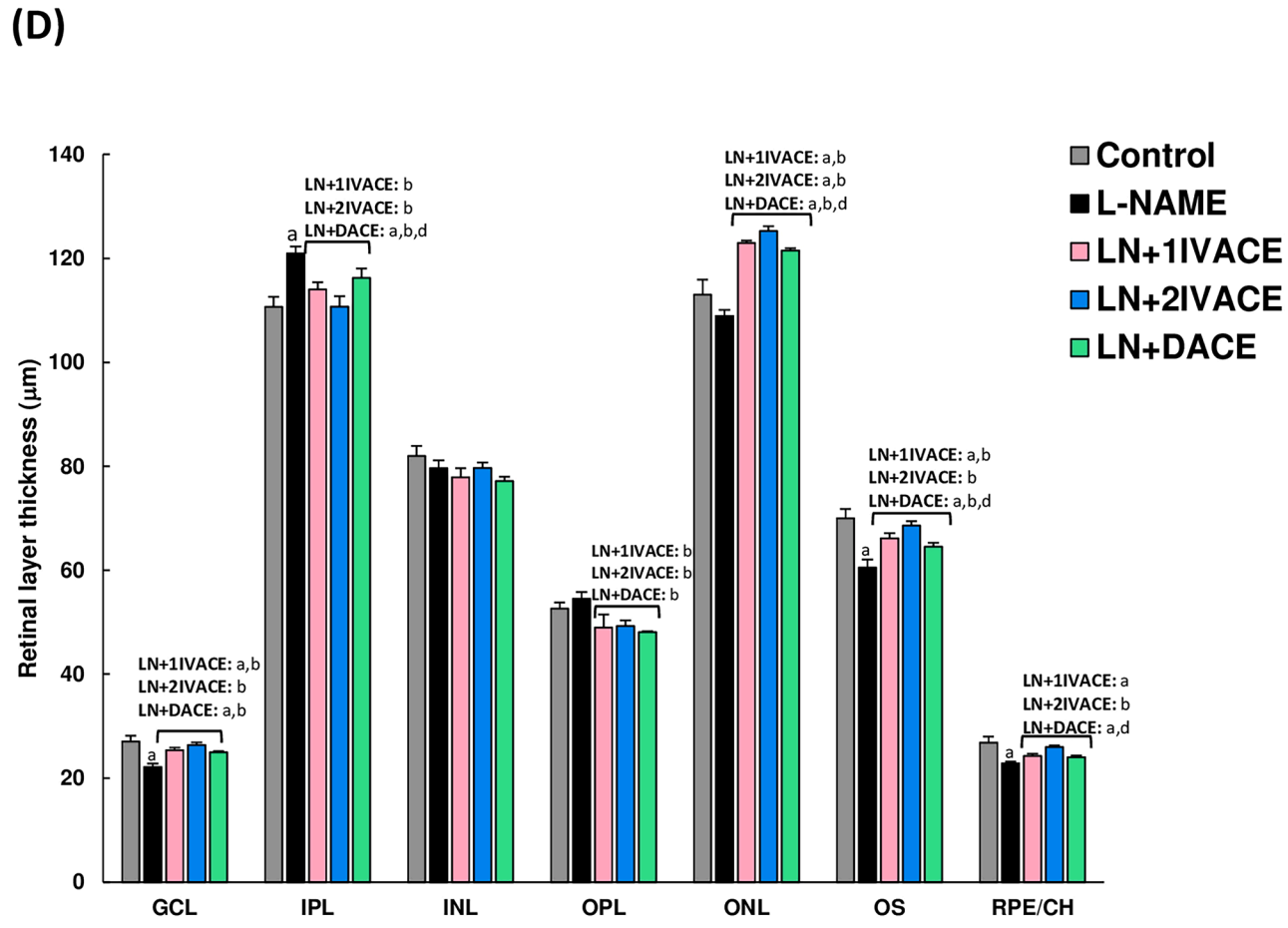


Fig. 5. (*continued*).

DHE signals in ST and CEN reached values similar to those in the Control group.

As shown in Fig. 7A, DHE signals were clearly more intense in retinal layers from the L-NAME group. Unlike in corneal layers, IV treatments showed a greater therapeutical effect than topical ACE oil NEs administration. Specifically, DHE signal reductions of ~ 25 %, ~20 %, ~44 %, ~18 %, and ~ 26 % in the GCL, INL, ONL, OS, and RPE/CH were found in LN + 1IVACE animals, and the effect was even higher in the LN + 2IVACE group (~35 %, ~45 %, ~52 %, ~32 %, and ~ 36 % reductions in GCL, INL, ONL, OS and RPE/CH, respectively; Fig. 7B). In

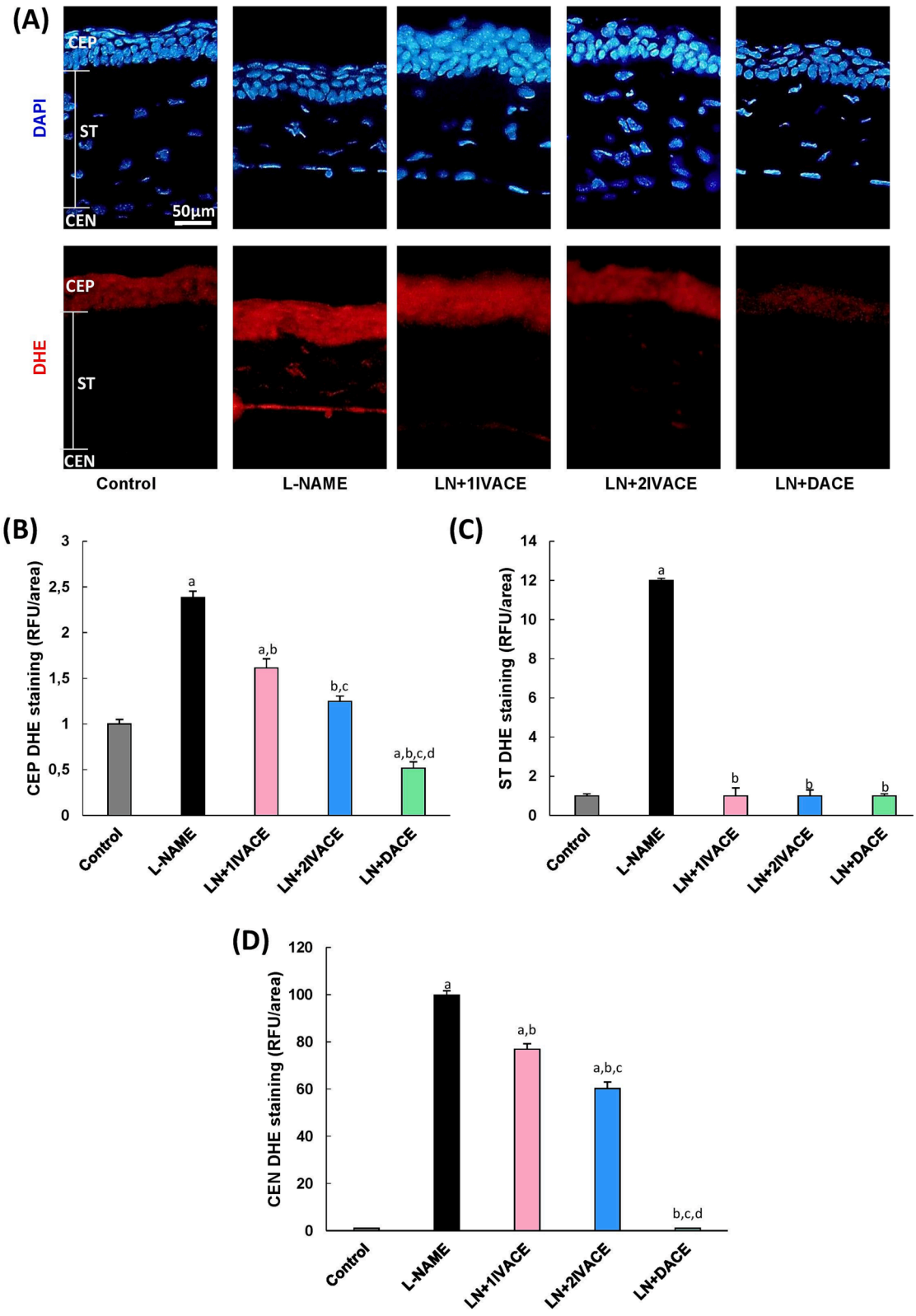


Fig. 6. (**A**) Reactive oxygen species (ROS) production in the cornea visualized by DHE labeling (red color) together with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining (blue color). (**B-D**) Relative fluorescence intensity in **A** relative to that of the Control group and quantified using the Image J software. Magnification: 10x. Values are expressed as mean ± SEM of seven animals per group: $^{a}p < 0.05$ vs. Control; $^{b}p < 0.05$ vs. L-NAME; $^{c}p < 0.05$ vs. LN + 1IVACE; $^{d}p < 0.05$ vs. LN + 2IVACE. **Animal groups**: Control: untreated animals; L-NAME: hypertensive mice (via administration of 45 mg Nω-nitro-L-arginine methyl ester (L-NAME)/kg body weight/day); LN + 1IVACE: L-NAME mice treated with 1 IV injection of ACE oil NEs; LN + 2IVACE: L-NAME mice treated with 2 IV injection of ACE oil NEs; LN + DACE: L-NAME mice treated with 1 instilled drop of ACE oil NEs in the cornea each 8 h. **Abbreviations**: CEN: corneal endothelium; CEP: corneal epithelium; RFU: relative fluorescence units; ST: stroma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

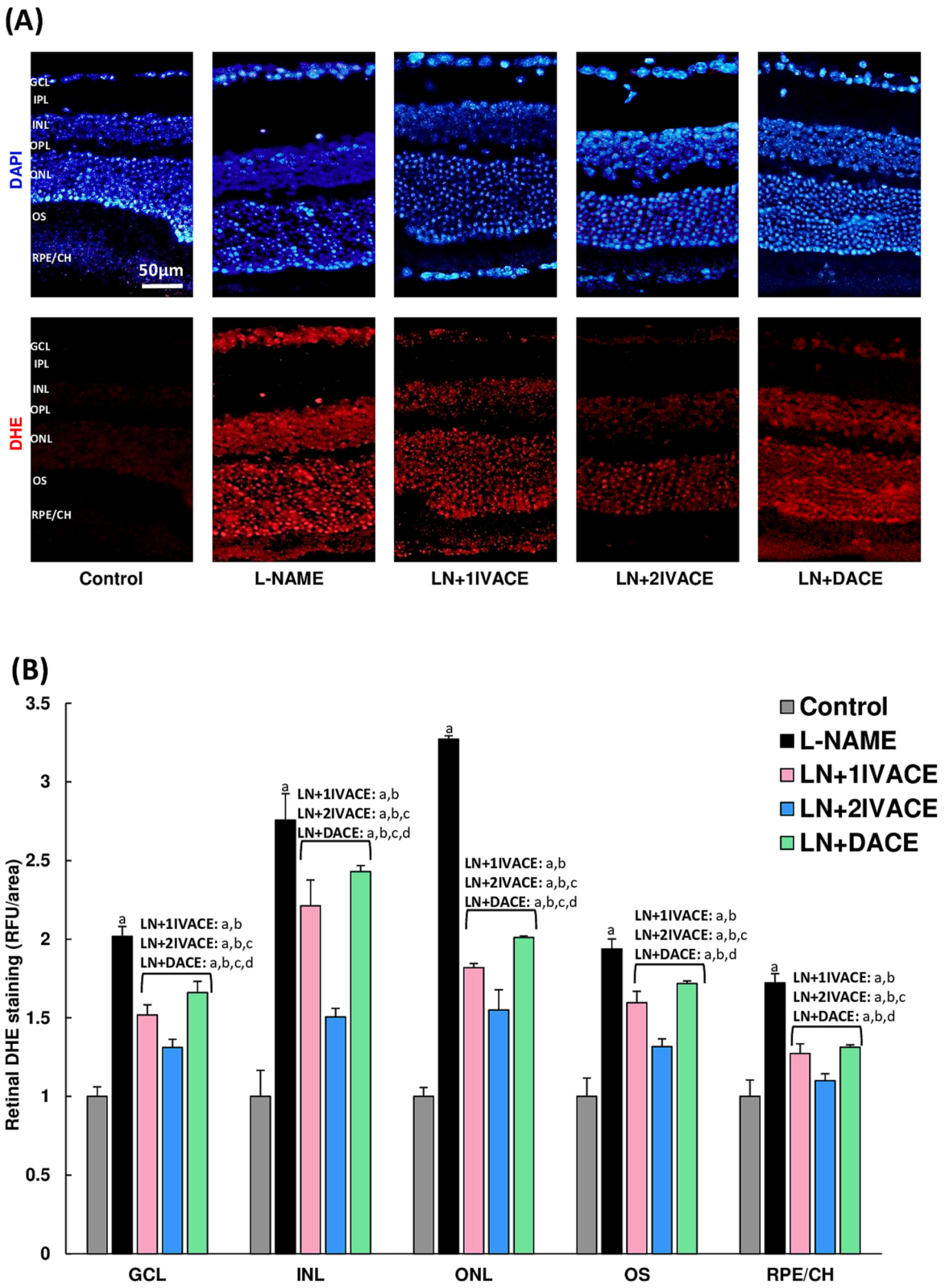


Fig. 7. (**A**) Reactive oxygen species (ROS) production in the retina visualized by DHE labeling (red color) together with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining (blue color). (**B**) Relative fluorescence intensity in **A** relative to that of the Control group and quantified using the Image J software. Magnification: 10x. Values are expressed as mean ± SEM of seven animals per group: $^{a}p < 0.05$ vs. Control; $^{b}p < 0.05$ vs. L-NAME; $^{c}p < 0.05$ vs. LN + 1IVACE; $^{d}p < 0.05$ vs. LN + 2IVACE. **Animal groups**: Control: untreated animals; L-NAME: hypertensive mice (via administration of 45 mg Nω-nitro-L-arginine methyl ester (L-NAME)/kg body weight/day); LN + 1IVACE: L-NAME mice treated with 1 IV injection of ACE oil NEs; LN + 2IVACE: L-NAME mice treated with 2 IV injection of ACE oil NEs; LN + DACE: L-NAME mice treated with 1 instilled drop of ACE oil NEs in the cornea each 8 h. **Abbreviations**: GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer; OS: outer segment; RFU: relative fluorescence units; RPE/CH: retinal pigmentary epithelium/choroid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

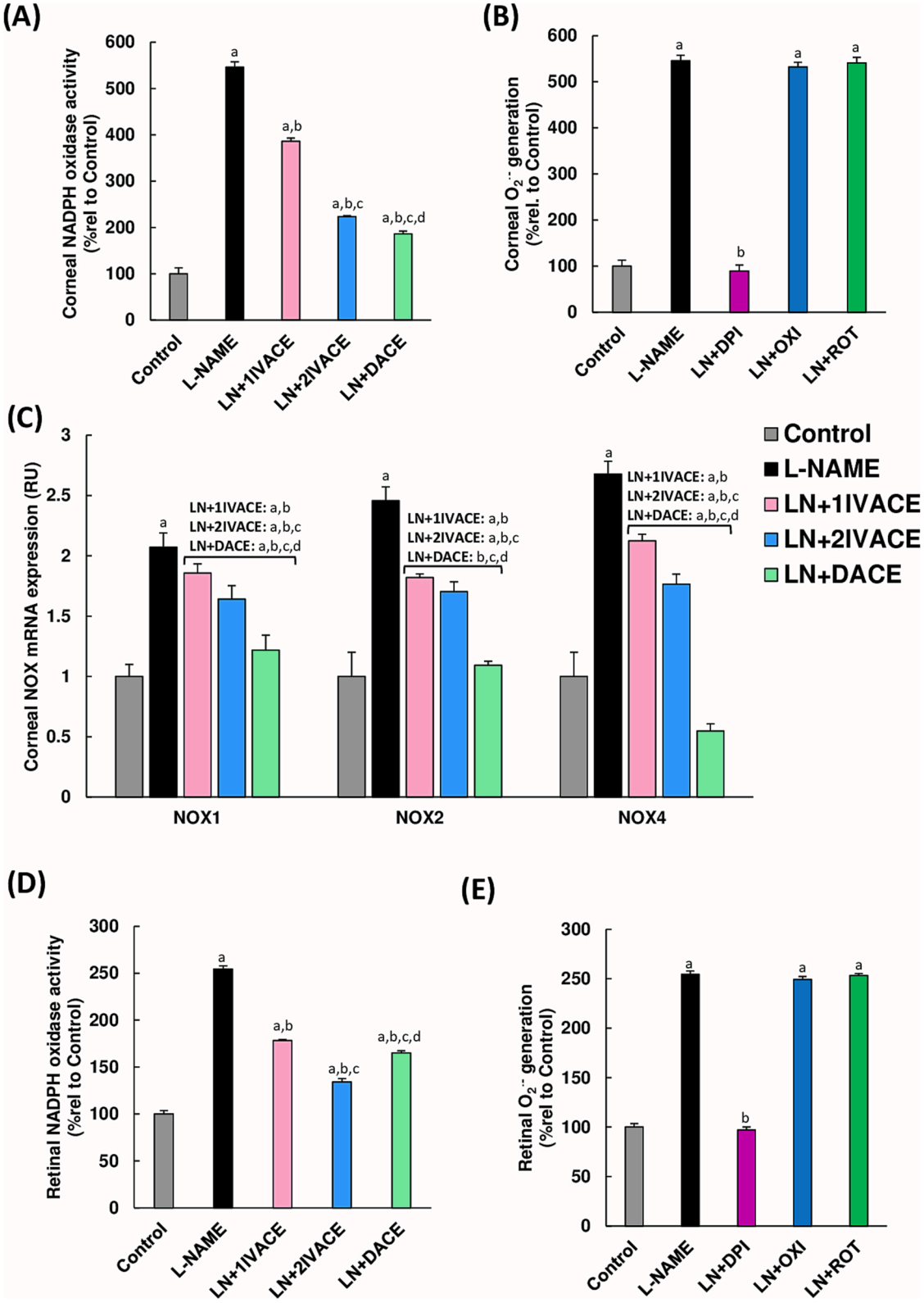


Fig. 8. Relative NADPH oxidase activity in the (A) cornea and (D) retina homogenates. Characterization of the primary source of superoxide anion in the (B) cornea and (E) retina via preincubation with different inhibitors: DPI (diphenyleneiodonium chloride, inhibitor of flavoproteins); OXI (oxypurinol, xanthine oxidase inhibitor); ROT (rotenone, inhibitor of mitochondrial electron transport chain). mRNA expression of NOX isoforms (NOX1, NOX2 and NOX4) in the (C) cornea and (F) retina of the experimental groups. Quantitative fold changes in gene expression were determined relative to the corresponding value for the housekeeping gene GAPDH. Values are expressed as mean ± SEM of seven animals per group: $^{a}p < 0.05$ vs. Control; $^{b}p < 0.05$ vs. L-NAME; $^{c}p < 0.05$ vs. LN + 1IVACE; $^{d}p < 0.05$ vs. LN + 2IVACE. **Animal groups**: Control: untreated animals; L-NAME: hypertensive mice (via administration of 45 mg Nω-nitro-L-arginine methyl ester (L-NAME)/kg body weight/day; LN + 1IVACE: L-NAME mice treated with 1 IV injection of ACE oil NEs; LN + 2IVACE: L-NAME mice treated with 2 IV injection of ACE oil NEs; LN + DACE: L-NAME mice treated with 1 instilled drop of ACE oil NEs in the cornea each 8 h. **Abbreviations**: RU: relative units.

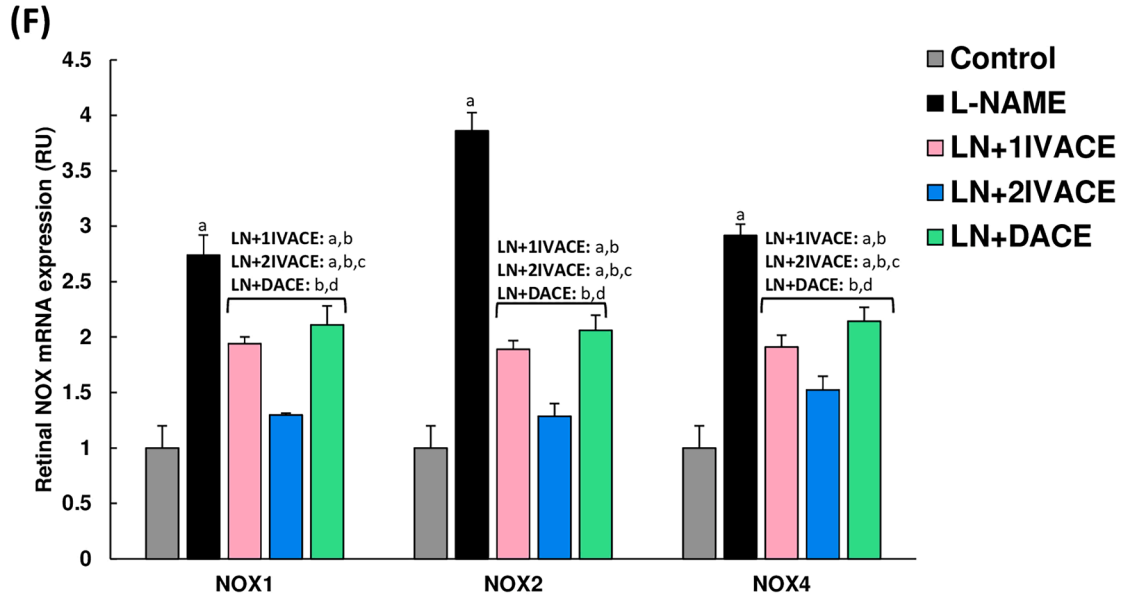


Fig. 8. (*continued*).

turn, the LN + DACE group displayed DHE signal reductions of ~ 18 %, ~12 %, ~39 %, ~11 %, and ~ 24 % in the same respective retinal layers in comparison to the L-NAME group.

3.7. ACE oil NEs modulate NADPH oxidase activity/expression in the cornea and retina

NADPH oxidase, the major enzymatic source of reactive oxygen specifies (ROS) in the hypertensive context (Camargo et al., 2018; Small et al., 2018), has a pivotal role in the local production of $O_2^{\cdot-}$ in hypertensive corneas and retinas (Santana-Garrido et al., 2022, 2021a). In the cornea, a significant (5-fold) increase in the activity of NADPH oxidase was found in the L-NAME group relative to the Control group (Fig. 8A), an alteration that was softened by IV administration (~29 % and ~ 59 % reduction in LN + 1IVACE and LN + 2IVACE, respectively). The effect was more pronounced (~66 % reduction in LN + DACE vs. L-NAME) after topical administration of ACE oil NEs. These results paralleled NOX gene expression at corneal level (Fig. 8C), where NOX1, NOX2 and NOX4 were downregulated after single (~10 %, 26 %, and 21 % reduction) and double (~21 %, 31 %, and 34 % reduction) IV injections, with a greater effect in topically-treated animals (~41 %, 56 %, and 79 % reductions for NOX1, NOX2 and NOX4, respectively).

In the retina, the upregulation of NADPH activity found in the L-NAME group (~2.5-fold relative to Control group) was reduced by ~~intravitreal~~ IV (~30 % and 47 % reductions in LN + 1IVACE and LN + 2IVACE groups, respectively) and topical (~35 % in LN + DACE group) administration of ACE oil NEs (Fig. 8D). In this case, the counteracting effect on NOX expression was more noticeable in IV-treated animals, with reductions of ~ 29 % (NOX1), 51 % (NOX2), and 35 % (NOX4) in LN + 1IVACE animals; and ~ 53 % (NOX1), 67 % (NOX2) and 48 % (NOX4) in the LN + 2IVACE group; respective values were 23 %, 47 %, and 27 % in the LN + DACE group (Fig. 8F). As shown in Fig. 8B and 8E, no changes were observed in corneal and retinal superoxide production in hypertensive mice following preincubation with oxypurinol and rotenone; on the other hand, DPI did restore $O_2^{\cdot-}$ generation back to control values, thus suggesting that relevant free radical production came entirely from the NADPH oxidase system.

Altogether, our findings on ROS production and NADPH oxidase activity suggest that IV administration of ACE oil NEs appears to be more effective at reducing retinal oxidative stress, whereas topical treatment displayed better outcomes at the corneal level in this regard. Furthermore, we can conclude that multiple IV injections of ACE oil NEs reinforce the effects of the single dose, suggesting a cumulative effect or a higher amount of formulation reaching the relevant ocular tissues. Our results might be attributable to the delivery of minor components contained in ACE oil, since many of them have demonstrated antioxidant properties, including polyphenols (Gorzynik-Debicka et al., 2018), triterpene acids (Gudoityte et al., 2021), or tocopherols (Di Vincenzo et al., 2019). Some of the antioxidant properties of polyphenols (Calabriso et al., 2016; Wang et al., 2019) and triterpene acids (Nie et al., 2021; Yang et al., 2015) are associated with downregulation of NADPH oxidase activity and NOX isoforms, which might explain NADPH modulation together with ROS depletion in ACE oil NEs-treated animals. Consequently, ACE oil NEs might be acting by avoiding the activation of other mechanisms such as the uncoupling of the endothelial nitric oxide synthase (eNOS) enzyme (Wu et al., 2021), and/or the activation of pro-oxidant/inflammatory nuclear factor kappa B (NF-κB) (Ávila et al., 2019), as previously postulated after oral administration of ACE oil (Santana-Garrido et al., 2021b; Santana-Garrido et al., 2020b). Considering that ACE oil NEs contain ACE oil droplets, it could be hypothesized that the anti-inflammatory properties associated to ACE oil consumption could be extrapolated to our formulation, although the magnitude of such an inference should be explored further. Similarly, since oral administration of ACE oil managed to prevent hypertensive retinal dysfunction, estimated by electroretinography (Santana-Garrido et al., 2021b), ACE oil NEs may exert an analogous effects in this regard based on its significant antioxidant effect. Therefore, this proof-of-concept study opens the door to evaluating other beneficial properties of ACE oil NEs based on the beneficial modulation of NADPH oxidase.

Different components isolated from natural products have been previously included in NEs with optimal results in terms of delivery and antioxidant properties by oral route, including polyphenols (LIANG et al., 2021; Nazareth et al., 2021) and triterpene acids (Alvarado et al., 2015). However, with the exception of few nanoemulsion formulations of chemical drugs (e.g. cyclosporine A or difluprednate) (Onugwu et al., 2023), the number of formulations that contain natural drugs intended for ocular use is scarce. In this regard, the efficacy of topical administration of riboflavin together with docosahexaenoic acid has been reported in the cornea of an animal model mimicking dry eye syndrome (Lidich et al., 2019), as has the use of cannabidiol to reduce IOP values (Senapati et al., 2022). Furthermore, Huang et al (Huang et al., 2021) reported the alleviation of retinal inflammation and vascular leakage by

topical (eye drop) nanoemulsion of fenofibrate in rats with diabetes and age-related macular degeneration. Interestingly, the latter described that fenofibric acid (active form of fenofibrate) concentrations were found higher in the cornea than in the retina of these mice after topical administration. All these results suggest the efficacy of NEs for ocular purposes and let us to hypothesize that topical administrations of NEs might display better outcomes in the cornea than in the retina because they are administered directly on this ocular structure. Hence, our topical administration of ACE oil NEs might allow ACE oil's minor components (e.g., polyphenols and tocopherols) to initiate their effects at the corneal level immediately after administration, while reaching the choroid and retina would require a periocular path and *trans*-corneal and/or *trans*-sclera delivery. It also seems plausible that topical administration possibly needs a longer time frame and/or more repetitive administrations to reach similar effects in the retina than in the cornea. Nonetheless, IV administration permit ACE oil's compounds to act directly on the retina due to overcoming many ocular barriers and, consequently, displaying positive outcomes quickly. Besides, some of ACE oil's compounds could be metabolized differently in each ocular layer, which could justify part of the variability effects depending on the route of administration (Argikar et al., 2017). However, it should be considered that IV injections are of delicate nature and can be associated to sight-threatening complications such as endophthalmitis, retinal detachment and/or retinal vasculitis (Patel et al., 2022; Weinmann et al., 2020). In addition, key technological parameters must be carefully addressed and monitored when developing formulations specifically intended for IV administration, including syringeability, density, viscosity, and aeration, among others (Patel et al., 2022; Sadeghi et al., 2022; Weinmann et al., 2020). Again, topical administration, as initially targeted with this formulation, may be overall a more convenient alternative despite needing a higher dose regimen in this context. In any case, ACE oil NEs could represent a promising therapy for prevention and treatment of many ocular pathologies associated with oxidative events related to NADPH oxidase activation, including retinal (Jarrett and Boulton, 2012; Li et al., 2015; Singh et al., 2016) and corneal (Chan et al., 2016; Hakami et al., 2020) conditions.

3.8. ACE oil NEs exerts higher bioactivity than ACE oil-enriched diets in a lower time frame

As already mentioned, the importance of oxidative stress in the pathogenesis of ocular diseases justifies the use of local antioxidants as a useful tool to prevent or control ocular tissue damage. In this way, our results validated the proof-of-concept of the feasibility of an ophthalmic topical formulation based on ACE oil as a promising therapy against AH-related corneal and retinal pathologies. In the retina, the estimation of PA% has been done by comparing previous data obtained in our lab after oral administration of ACE oil (Santana-Garrido et al., 2021b; Santana-Garrido et al., 2020b) with the ACE oil NEs formulations reported in the current study. Considering one IV injection of ACE oil NEs (1IVACE) as the administration with the maximum PA% (100 % of PA%) as it is the closest administration to the retina, the analysis suggest a greater efficacy of IV ACE oil NEs administration when compared with ACE-enriched diet (~0.005 % of PA% compared with 1 IV ACE oil NES injection), which might be due to a higher ACE oil bioavailability reached after IV injection. On the other hand, the PA% of the topical administration of ACE oil NEs was higher (~5% of PA% compared with 1 IV ACE oil NES injection) than oral administration but lower than one IV ACE oil NEs injection. These results might explain why we obtained greater effects in a shorter time with the IV injection. The %PA of two IV injections of ACE oil was lower than 1IVACE (~67 % of PA% compared with 1 IV ACE oil NES injection) because the effect was not proportional. This might indicate that ACE oil is accumulating in the ~~intravitreal~~ IV space and, therefore, further research is necessary to optimize the time frame assayed for two injections (Table 3).

Interesting questions arise regarding the mechanisms through which NEs led to remarkable pharmacological bioavailability. Several pathways of drug penetration to several sections of the eye are considered for topically applied formulations (Singh et al., 2020), including penetration through the tear film and cornea to the aqueous humor and vitreous and finally reaching the retina and systemic circulation; and periocular *trans*-scleral path reaching the choroid and the retina. In addition, diffusion may generally take place through paracellular and transcellular routes. Finally, several mechanisms by which NEs enhance drug interaction with ocular barriers have been identified, generally attributed to the capacity of the hydrophobic components and amphiphilic excipients to interact with epithelial cell membranes and tear components, as well as the high interfacial area available for interaction provided by the small size of the droplets (Shafiq et al., 2007b). These mechanisms include: i) tear film stabilization by interaction of the NEs lipids with the lipid layer of the tear film and/or interaction of surfactants with the mucin layer, hence increasing residence time (Shafiq et al., 2007b; Singh et al., 2020); ii) inhibition of P-gp activity (Katragadda et al., 2006); and iii) tight junction opening (Jiao, 2008b). Specifically, high HLB surfactants, such as Tw80, have been reported to promote corneal permeation (Calvo et al., 1996; Tayel et al., 2013). While *in vitro*, *ex vivo* and *in vivo* biodistribution studies with ACE oil NEs would provide interesting information, the complex composition of ACE oil and the fact that its pharmacological action has not been attributed so far to any specific component poses a challenge for the tracking of the active pharmaceutical ingredient(s). Further studies aiming at discerning the role of each ACE oil component and their tracking upon administration should provide valuable information for optimization of the formulation and suitable ocular therapies. In addition, the proposed

Table 3
Dosing parameters applied in each administration modality and resulting dose needed to achieve a relative unit of pharmacological effect in the retina. *Intravitreal injections were not applied daily. **Abbreviations**: DHE: Dihydroethidium; NADPHox: NADPH oxidase activity; PA%: Pharmacological availability.

Type of administration	Dose	Dosage	Daily dose	Cumulative dose	Amount needed to reduce 1 DHE relative unit	PA% (DHE reduction) relative to 1 IV NES	Amount needed to reduce 1 NADPHox relative unit	PA% (NADPHox reduction) relative to 1 IV NES
Diet	0.12 mL ACE oil/g feed daily	5 g feed daily for 6 weeks	0.6 mL ACE oil	25.2 mL ACE oil	~68.2 μL ACE oil	0.004 %	~246 μL ACE oil	0.005 %
Topical NEs	0.0025 mL ACE oil/ drop ~~daily~~	1 drop/8h per eye for 4 days	0.0075 mL ACE oil	0.0225 mL ACE oil	~0.071 μL ACE oil	4.23 %	~0.25 μL ACE oil	5.2 %
Intravitreal NEs (1 injection)	0.001 mL ACE oil/ injection	1 injection per eye	0.001 mL ACE oil*	0.001 mL ACE oil	~0.003 μL ACE oil	100 %	~0.013 μL ACE oil	100 %
Intravitreal NEs (2 injections)	0.001 mL ACE oil/ injection	2 injections per eye, with a 3-day period in between	0.001 mL ACE oil*	0.002 mL ACE oil	~0.0053 μL ACE oil	56.60 %	~0.017 μL ACE oil	76.50 %

ACE oil NEs offer many possibilities for further optimization, including the incorporation of alternative emulsifiers and targeting moieties, with the possibility of size modulation through the modification of the composition and production parameters. Moreover, the inclusion *in-situ* gelling agents (Singh et al., 2020) to the aqueous phase of the NEs aiming at increasing the residence time at the ocular surface could also be considered.

4. Conclusions

ACE oil NEs amenable for ophthalmic administration were successfully produced. The NEs exhibited a nanometer size below 200 nm, moderate negative ZP, adequate osmolality and pH and colloidal stability in biorelevant fluids. Likewise, rheological characterization revealed a shear thinning behavior, especially convenient for ocular instillation of the formulation. *In vivo* experiments carried out in L-NAME-treated hypertensive animals revealed antioxidant properties of ACE oil NEs by modulation of the enzyme NADPH oxidase, which demonstrated the ability to cross ocular barriers and to counteract hypertensive damage in ocular tissues. Further studies on the pharmacological role of ACE oil components, as well as the mechanisms of the NE that enabled a successful interaction with ocular barriers, are warranted to provide additional information for further potential optimization of the formulation, as well as for the development of alternative related ocular therapies. Overall, our results highlight the potential use of ACE oil as a pharmaceutical and, particularly, point to ACE oil-based ophthalmic formulations as feasible and promising therapies against vascular ocular pathologies associated with AH.

Funding.

The present study was supported in part by Grant 18.06.07.3004 PID2019-109002RB-I00 funded by MCIN/AEI/https:10.13039/501100011033; Consejería de Economía, Conocimiento, Empresas y Universidad, Junta de Andalucía (2020/275 and 2021/188; CTS-584); and VI PPIT Universidad de Sevilla (I.3A1 Ayudas para la movilidad internacional del personal dedicado a la investigación). ASG holds an FPU predoctoral fellowship from Ministerio de Ciencia, Innovación y Universidades (FPU17/03465) .

CRediT authorship contribution statement

Á. Santana-Garrido: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **M. Durán-Lobato:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **A. Mate:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **L. Martín-Banderas:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **C.M. Vázquez:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

M. D-L is especially grateful to the VI Plan Propio de Investigación from the Vicerrectorado de Investigación (Universidad de Sevilla) for the postdoctoral fellowship Contrato de Acceso al Sistema Español de Ciencia, Tecnología e Innovación (grant number USE-19533-Y). Authors are also grateful to Claudia Reyes-Goya from the Biology and Microscopy Services of CITIUS for her technical assistance with osmolality evaluation; to Juan Luis Ribas from the Microscopy Services of CITIUS for his technical help with TEM imaging; and to M. Dolores Domínguez from the Functional Characterization Services of CITIUS for her assistance with rheology characterization. The authors are also grateful to Alcazarín Reunidos FP, S.L. (Monda, Málaga, Spain) for providing the extra virgin wild olive oil used in this study.

Funding.

M D-L VI Plan Propio de Investigación from the Vicerrectorado de Investigación (Universidad de Sevilla) for the postdoctoral fellowship Contrato de Acceso al Sistema Español de Ciencia, Tecnología e Innovación (grant number USE-19533-Y).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.123602.

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