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FULL ARTICLE

Assessment of trans-scleral iontophoresis delivery of lutein to the human retina

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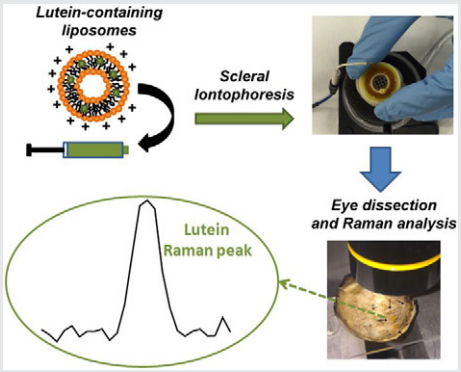
The efficacy of novel scleral iontophoresis device for in situ delivery of lutein to the human retina was assessed by Resonance Raman spectroscopy (RRS) technique. Eight human donor eye globes were used for experiments, 6 of which underwent trans-scleral iontophoresis delivery of lutein and the other 2 were used as controls. The scleral iontophoresis applicator was filled with liposome-enriched 0.1% lutein solution and the generator's current was set at 2.5 mA and delivered for 4 min. A custom RRS setup was used for detecting lutein in the inner sclera, choroid, retinal periphery and macula of treated samples and controls. Forty minutes after iontophoresis, the inner sclera, choroid and retinal periphery were greatly enriched with lutein ($P < .05$); no lutein was found in the same ocular regions of non-treated samples. In the same period, the average concentration of lutein in the macula (4.8 ± 1.7 ng/mm^2) of treated samples was 1.3 times greater than controls (3.7 ± 1.0 ng/mm^2; $P = .4$). Scleral iontophoresis was shown to be effective in delivering lutein to the human retina. Future studies will aim at assessing if this therapeutic strategy is valuable to enrich the macular pigment in human subjects.



KEYWORDS

iontophoresis, lutein, macula, Resonance Raman spectroscopy

1 | INTRODUCTION

Lutein is a dietary carotenoid from the xanthophyll family; together with its isomer zeaxanthin, it is the main component of the human retina's macular pigment.

The main physiological functions ascribed to macular xanthophylls are the protection of the retinal photoreceptors from light damage and their shielding effect against short-wavelength light radiation [1–9]. Lutein and zeaxanthin show a 3-peak absorption curve in the 390 to 540 nm range with peaks at 446 and 462 nm, respectively, rendering these xanthophylls efficient physical quenchers of harmful blue-light. The protective function of xanthophyll pigments to macular photoreceptors is considered to reside in their ability to prevent deleterious oxidative effects of singlet oxygen and related reactive oxygen species through the numerous unconjugated double bonds present in the molecules through physical interaction with the lipid bilayer of cell membranes [3–9].

Age-related macular degeneration (AMD) is the primary cause of blindness among people older than 55 y [10]. Nevertheless, an ever-increasing number of people will be at risk of visual impairment for AMD as populations grow and age [11–15]. AMD development and progression have not yet been fully understood. The most indorsed pathophysiologic pathway of AMD includes a relationship with age and lesions to the photoreceptor/Retinal Pigment Epithelium (RPE)/Bruch's membrane/choriocapillar is complex, which have been associated with light-induced oxidative damage (*blue light hazard*) [6, 7, 16–21].

Epidemiologic and intervention studies have highlighted that the increased dietary intake and/or supplementation of xanthophylls is associated with a decreased risk of AMD incidence or progression [22–27]. In Age-Related Eye Disease Study 2, the risk of progression to advanced AMD was reduced by 10% in patients supplemented with lutein and zeaxanthin in comparison with patients not supplemented [27]. In several other clinical studies, the protective benefit of lutein in adult subjects has been associated with oral supplement intake between 6 and 20 mg per day [23–26].

Although increasing evidences are showing that prolonged oral supplementation of lutein is safe and may be associated with decreased risk of progression from early to advanced AMD stages, compliance of patients is still limited. This is mainly due to the need of daily oral administration for prolonged time. Another constraint of oral supplementation is the limited and variable absorption of lutein and zeaxanthin through the digestive route. New strategies are envisaged to deliver xanthophyll pigments to the macula of patients at risk of AMD progression.

In this work, we aimed to assess a novel strategy to deliver lutein to the human retina in situ. The approach consisted of using a scleral iontophoresis device and a positively charged lutein ophthalmic formulation. The device was tested on human donor eye globes and the concentration of lutein in ocular tissues was investigated by Resonance Raman spectroscopy (RRS). It is a highly specific vibrational spectroscopy that has long been used to identify and quantify xanthophyll pigments in the retina [28–34]. Carotenoid molecules are especially suitable for Raman measurements because they can be excited with light overlapping their visible absorption bands. Under this excitation condition, they exhibit a very strong resonance Raman scattering response, with an enhancement factor of about 5 orders of magnitude relative to non-resonant Raman spectroscopy. This enables to detect the characteristic vibrational energy levels of carotenoids through their corresponding spectral fingerprint signature and any off-resonance Raman response from other molecules present in the sampling volume would be strongly suppressed under these conditions [33, 34].

2 | MATERIALS AND METHODS

2.1 | Donor eye bank tissues

Donor human eye globes, which were not suitable for transplantation, were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy). Written informed consent from the next of kin was obtained for the use of samples in research. All human tissues were used in compliance with the guidelines of the Declaration of Helsinki for research involving the use of human tissue, and the experimental protocol was approved by the National Research Council research ethics and bioethics advisory committee. Inclusion criteria included an endothelial cell density (ECD) $\geq$1800 cells/mm^2; exclusion criteria included any corneal or ocular surgery.

Eight eye globes, from different donors, were explanted between 3.5 and 12 h after death (mean cadaver time: 7.2 $\pm$ 2.5 h) and immediately preserved at 4°C in storage medium (Minimum Essential Medium (MEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) modified solution enriched with nutrients and antibiotics/antimicotic in purified water) enriched with 6% dextran. The mean donor age was 68 $\pm$ 3 y. The mean ECD, which was measured using an inverted optical microscopy (Axiovert 25, Carl Zeiss Microscopy, Jena, Germany), was 2200 $\pm$ 200 cells/mm^2. The eye globes, submerged in 6% dextran-enriched solution, were shipped to the laboratory and used within 5 d. Six eye globes (study group; mean age: 69 $\pm$ 3 y) underwent trans-scleral iontophoresis delivery of lutein. The other two eye globes (control group; mean age: 66 $\pm$ 1 y) were used as controls.

2.2 | Lutein ophthalmic solution

The 0.1% lutein ophthalmic formulation was composed of FloraGLO crystalline lutein (KeminFood L.C., Des Moines, Iowa) encapsulated in positively charged liposomes using phospholipon 90H (Lipoid GmbH, Ludwigshafen, Germany), octadecylamine (Sigma-Aldrich, St. Louis, Missouri) and distilled water; encapsulation in liposomes was used to improve lutein solubility in water and to facilitate its flow through ocular tissues.

2.3 | Trans-scleral iontophoresis delivery of lutein to the retina

At the beginning of experiments, each eye globe was gently mounted into a specially designed holder, facing upward. The eye was connected, through tubing, to a column manometer, filled with 0.9% sodium chloride solution, to maintain the pressure inside the eye at 15 mm Hg during experiment. Each eye was pre-conditioned for 20 min before iontophoresis, according to standardized study protocol, which allowed us to achieve a unique pre-stressing reference state of the ocular tissues before testing [35].

The iontophoresis device included a generator, an applicator with an active electrode and a return, passive, electrode. The passive electrode was applied to the optic nerve of the donor eye and the active electrode (cathode), consisting in a plastic bath tube, was applied to the anterior scleral surface. The scleral applicator was gently placed onto the eye and then filled with the positively charged lutein

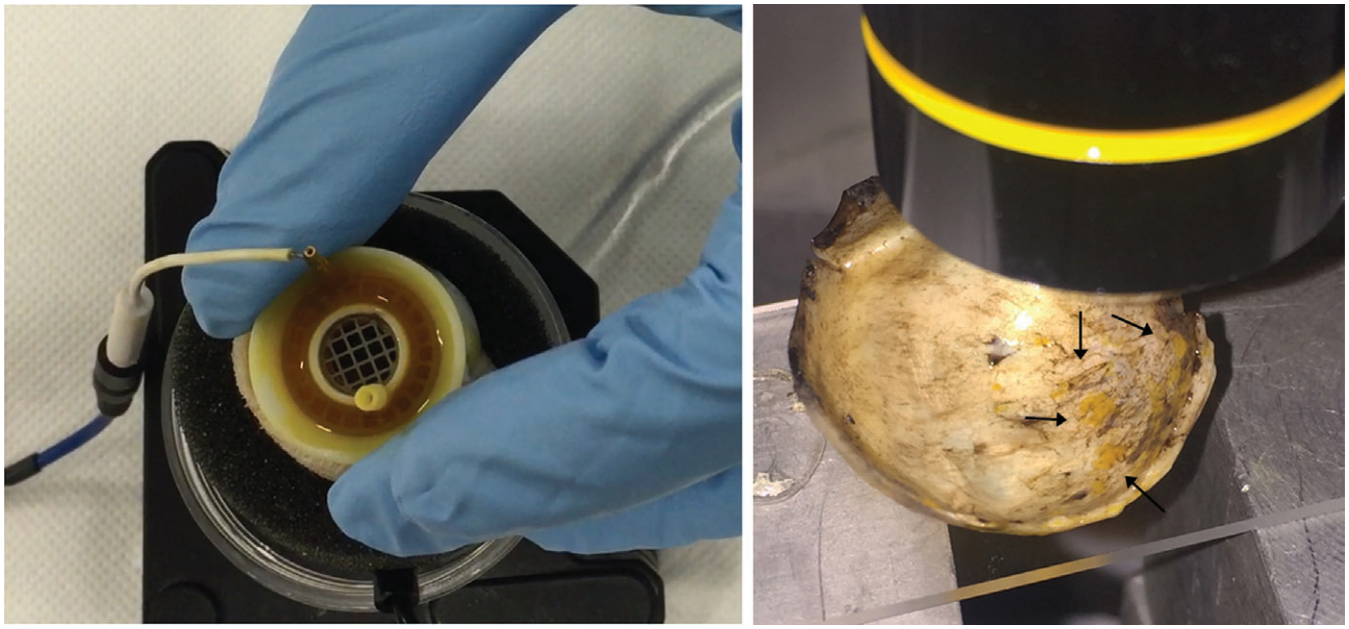


FIGURE 1 Scleral iontophoresis on human donor eye globes. (A) Scleral iontophoresis was performed on donor human eye bank tissues. Each eye globe was mounted into a purpose-designed holder to mimic as much as possible the in vivo condition. The active electrode, consisting of a plastic tube placed onto the sclera, was filled with liposome-enriched 0.1% lutein solution in the scleral reservoir and connected to the generator. The passive electrode was clamped to the optic nerve. (B) Forty minutes after scleral iontophoresis, the eye globe was excised using standardized technique. The sclera, choroid, peripheral retina and macula were analyzed by RRS to assess the concentration of lutein. In this panel, the inner sclera shows several bright yellow spots providing macroscopically evidence of trans-scleral diffusion of lutein (black arrows)

ophthalmic solution. The generator's current was set at 2.5 mA and was delivered for total 4 min. The resulting current density was 1.9 mA/cm^2 (surface of scleral electrode was 1.34 cm^2).

After iontophoresis, each eye globe was maintained, facing upward, in the eye holder with the pressure inside the eye at 15 mm Hg for 40 min. Thereafter, eyecups were prepared by using a standardized procedure, which consisted in removing the iris, lens and vitreous body to perform RRS measurements on the inner sclera, choroid and retina (Figure 1 and Figure S1, Supporting Information).

2.4 | Resonance Raman spectroscopy

A custom RRS setup was mounted on an optical bench and used for detecting lutein in eye tissues of treated samples and controls (Figure 2). A single mode optical fiber with collimating lens was used to focus the 473 nm laser beam through a dichroic mirror and a microscope objective (×10, $N_A = 0.25$), onto the sample, which was placed on a x,y,z micro-positioning stage. The power of the laser beam was set at 0.75 mW at the focal plane. The beam divergence after the collimating lens and the beam size illuminating the objective's pupil was adjusted to obtain a 1-mm spot

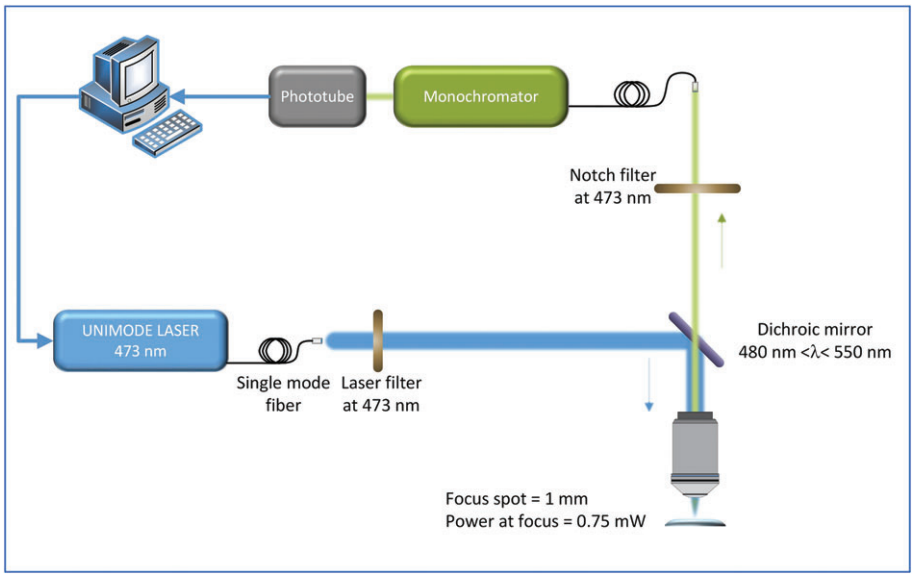


FIGURE 2 Schematic setup of the RRS used in the present study. The full list of components is described in the main text. The ocular tissues were excited by laser light at 473 nm wavelength; the backscattered light was imaged by a Raman spectrometer

diameter at the focal plane. This allowed us to improve instrument sensitivity and to decrease the power density for avoiding tissue damage. The laser source wavelength was chosen to obtain the resonance response and to decouple more efficiently the fluorescence background contribution. No other biomolecule with significant concentrations in the human retina exibits similar resonant enhancement at 473 nm excitation wavelength; therefore, carotenoid RRS spectra are remarkably free of confounding Raman responses.

A camera was used to observe the area to illuminate. After passing through the dichroic mirror and a notch filter, the backscattered light was collected by a multimode fiber with collimation lens. Before entering the slit of the spectrometer, the collected light passed through a fiber adapter to match the numerical aperture of the spectrometer ($N_A = 0.05$).

The spectrometer (BM-100, B&M Spektronik GmbH, Munich, Germany) has 1 m focal length and uses an holographic grating with 1200/mm. The spectrometer entrance and exit slits were both set at 500 μm and the spectral resolution was 10 cm^{-1}. The detector at the exit slit of the spectrometer was a cooled photomultiplier (R943–02, Hamamatsu Photonics, Hamamatsu, Japan). The spectrometer and data acquisition were computer controlled as well as the shutter (placed along the excitation path), which was open only during data acquisition to avoid unnecessary laser exposure to the sample.

For each eye, the RRS measurements were performed on four regions in various ocular tissues, such as the inner sclera at the site of iontophoresis delivery (i.e., the perilimbal sclera facing toward the ciliary body), the choroid and the retinal mid-periphery, which included the region of the retina surrounding the vascular arcades and the optic nerve head, and the macula. Measurements were performed in three areas across each region to collect enough data to correctly estimate data in the treated and control eyes.

The quantitative analysis of lutein was performed using a calibration procedure and a mathematical method. Calibration of the device was done by measuring the height of the Raman characteristic peak of lutein at 1530 cm^{-1} for a series of lutein solutions with known concentration values (i.e., from 0.001% to 0.002%). This procedure permitted to determine, from the resonant Raman characteristic peak, the amount of lutein in the areas of interest of excised donor eye tissues. The background originating from the fluorescence of the retinal compounds was subtracted through an automated standard minimization method. After the background subtraction, the surface concentration of lutein in the ocular tissues was obtained from the height of the Raman peak at 1530 cm^{-1}. The Raman signal emitted from the outer retinal layers, which may be absorbed by lutein located in the inner layers, was estimated negligible. In this study, 1000 Raman counts at 1530 cm^{-1} corresponds to 1 ng/mm^2 of xanthophyll pigment.

2.5 | Statistical analysis

Sample size calculation was based on the assumption that virtually no lutein enriches the inner sclera and choroid of control eyes as well as on the RRS resolution. The statistical power of the test was calculated (G*Power, available at http://www.gpower.hhu.de/) to reach 85% (β) to find average lutein concentration differences of 1.5 ng/mm^2 (allocation ratio 3:1; SD: 20%; $\alpha = 0.05$) between ocular tissues in treated and control eyes. The Wilcoxon test for unpaired data was used to compare data between groups using a commercial software program (KyPlot, KyensLab Inc., Tokyo, Japan).

3 | RESULTS

Scleral iontophoresis was successfully performed in all eye globes. After dissection, each eye was inspected macroscopically; the presence of lutein was revealed by yellow spots

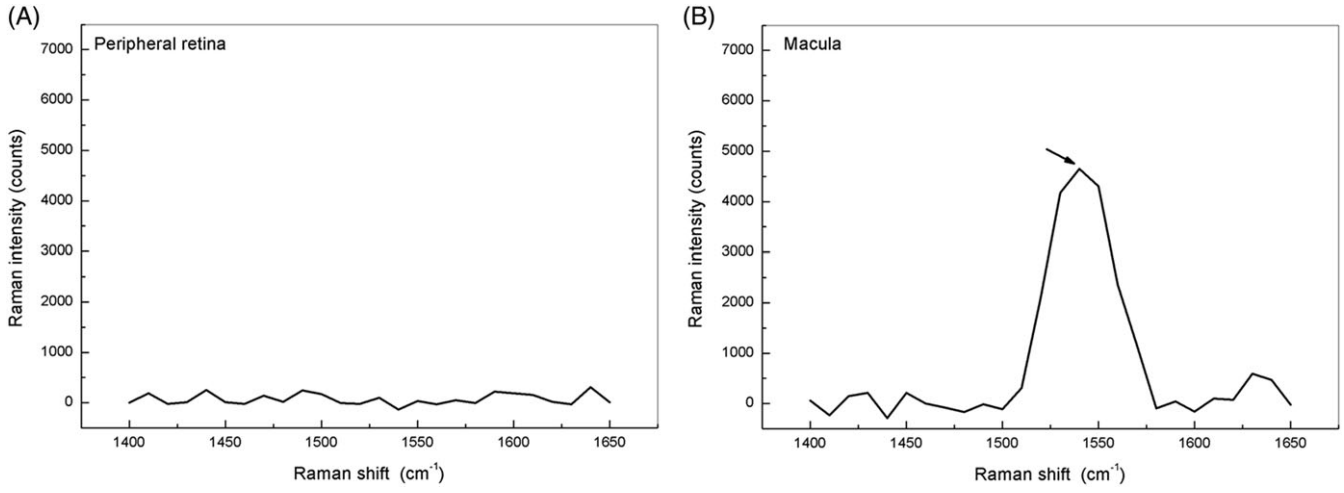


FIGURE 3 (A) Resonance Raman spectra obtained from the peripheral retina of a control eye. There is no detectable Raman peak corresponding to xanthophyll molecules. (B) Resonance Raman spectra obtained from the macula of a control eye. The macula contains the highest concentration of xanthophyll pigments in the retina of human eyes (arrow). Two-thirds of the total xanthophyll concentration is in the Henle fiber layer of the macula; the remaining one-third is in the photoreceptors' outer segment

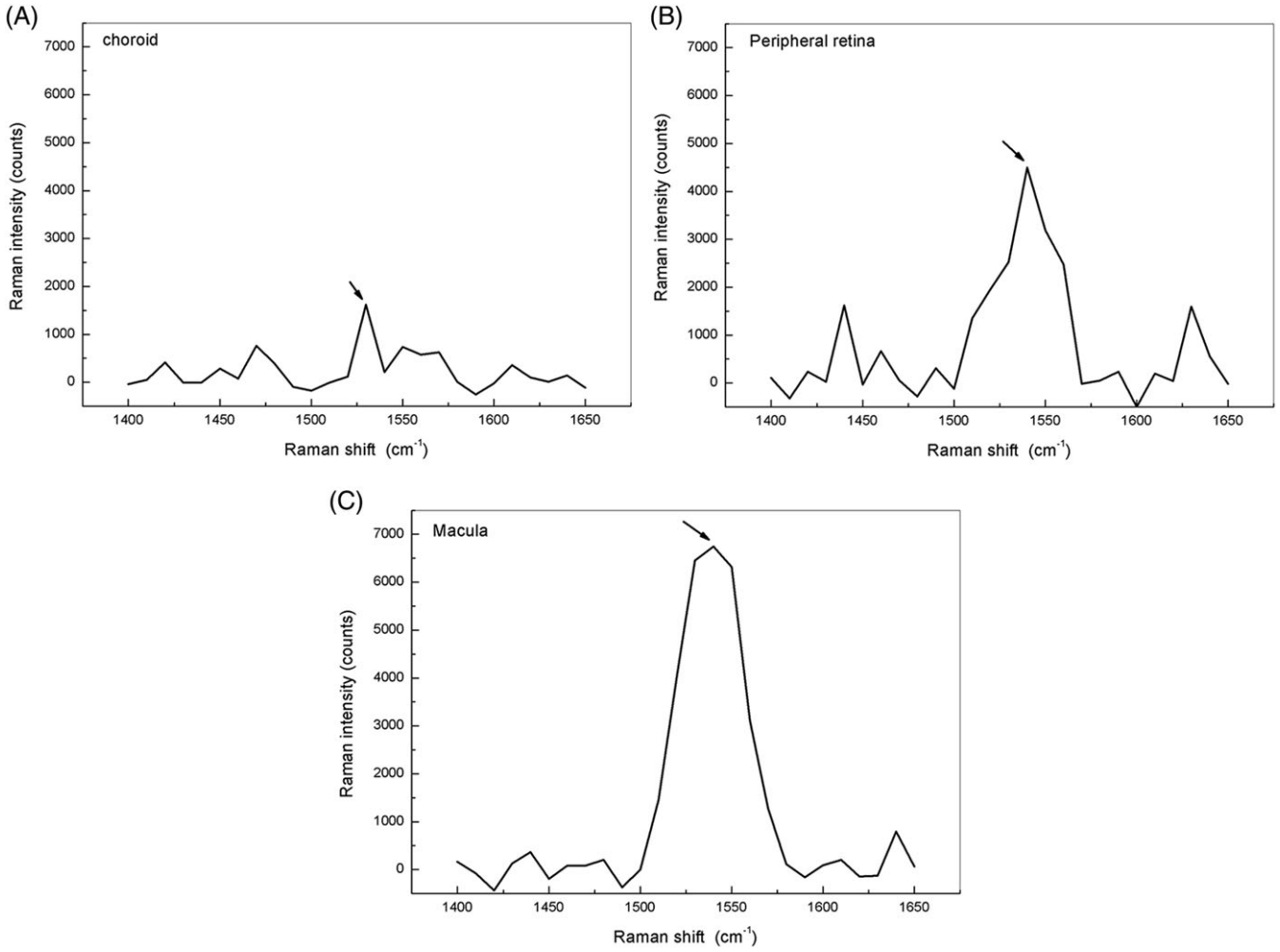


FIGURE 4 Resonance Raman spectra obtained for the (A) choroid, (B) peripheral retina and (C) macula after scleral iontophoresis delivery of lutein, respectively. The peripheral retina was greatly enriched with lutein (panel B, arrow). Scleral iontophoresis was effective to enrich the macula of a donor eye with lutein 40 min after treatment (panel C, arrow)

on the inner sclera of all treated eyes (Figure 1). The spots were spread across the inner sclera at the site of iontophoresis delivery. Control eyes did not show any yellow spot on the inner sclera.

In the inner sclera, choroid and peripheral retina, the Resonance Raman peak of lutein was observed only in treated eyes. No peak around the 1530 cm^{-1} shift was found in control eyes (this means that lutein concentration was virtually 0 ng/mm^2 in these regions of non-treated eyes). Figure 3 shows the Resonance Raman spectra obtained from the retina of a representative control eye. In control eyes, the average concentration of lutein in the macular area was 3.7 ± 1.0 ng/mm^2; 40 min after iontophoresis, the average concentration of lutein in the macula (4.8 ± 1.7 ng/mm^2; $P = .4$) was 1.3 times greater than controls. Figure 4 shows the resonance Raman spectra obtained from the retina of a representative treated eye. Table 1 shows the average concentration of lutein measured by RRS in the study and control groups.

4 | DISCUSSION

We presented a novel strategy to deliver lutein directly to the retina of human eyes using a scleral iontophoresis device. The concentration of lutein enriching the retina in human donor eyes was assessed by using RRS. RRS was crucial for the aim of this work due to its molecular selectivity [36, 37]. Indeed, other spectroscopic techniques, such as fluorescence, do not allow for discriminating directly the retinal xanthophylls. In addition, for fluorescence

TABLE 1 Concentration of lutein in ocular tissues (ng/mm^2, M ± SD)

	Inner sclera	Choroid	Peripheral retina	Macula
Controls ($n = 2$)	0	0	0	3.7±1.0
Iontophoresis ($n = 6$)	16 ± 9.0[a]	1.2 ± 0.3[a]	2.5 ± 1.3[a]	4.8 ± 1.7

[a] $P < .05$ between study and control groups.

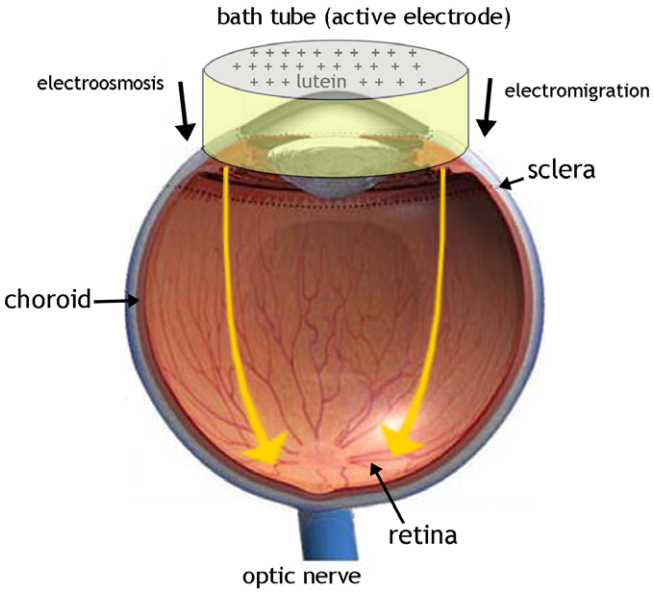


FIGURE 5 Scleral iontophoresis delivers lutein efficiently to the retina. During iontophoresis, lutein, in forms of liposome aggregates, passes through the sclera and the ciliary body and reaches the peripheral retina. Once lutein is delivered to the peripheral retina, it diffuses through the tissue's components toward the macular region (orange arrows). The electric mobility facilitates transport of lutein toward the macula across the retinal tissue's structures

techniques, emission strongly depends on the environment, preventing the calibration procedure [38].

We quantified the amount of lutein that reached the ocular tissues after iontophoresis and compared the results with untreated eyes. Forty minutes after iontophoresis, a great amount of lutein, which was not found in controls, enriched the inner sclera and peripheral retina of human donor eyes; macular lutein concentration in treated eyes was on average 1.3 times greater than controls. These findings indicated that lutein delivered by scleral iontophoresis enters the sclera and flows efficiently and rapidly to the retinal tissue, reaching the macula (Figure 5). The concentration values of lutein in the macula of control eyes was consistent with the results of previous in vivo study in 70-y-old adult subjects [33], thus supporting the accuracy of the storage protocol and methodology used in the current work. As the primary scope of this study was to assess efficacy of in situ delivery of lutein to the human retina by scleral iontophoresis, we did not aim at identifying zeaxanthin and lutein in the macula separately [39] nor at determining the best protocol of lutein delivery to the macula, which would be object of further work.

Although oral supplementation of xanthophyll pigments has been found to be effective to enrich the macular pigment in subjects with AMD, the benefits observed through this route of administration, which is known to present some limitation in efficiency associated to the highly variable absorption of carotenoids, could be potentially improved by supplying lutein directly to the eye. Due to eye mechanical barriers, delivery of molecules to the retina in therapeutically adequate concentrations and with minimal side-effects may be challenging. In situ applications have been used to overcome this problem; however, slow delivery systems, such as implants and intravitreous injections, represent significantly invasive and expensive strategies of delivering xanthophyll pigments toward the macula [40].

Iontophoresis is a well-known drug-delivery strategy and has been used in ophthalmology for decades [41, 42]. It consists in transferring charged molecules to a target tissue through the application of a low-intensity electric field and has been shown to be safe both in ex vivo [35, 43–46] and in vivo studies [47–49]. Current densities up to 4.0 mA/cm^2 for 10 min through the sclera of rabbits have been shown to cause no alterations to eye function or structure [43]. In addition, studies on ocular tolerance to scleral iontophoresis in human subjects have shown that current densities up to 5.0 mA/cm^2 caused no significant alterations in the ophthalmic assessments, nor gave rise to clinical symptomatology [41, 47]. In this study, we did not find any macroscopically damage to the sclera or the intraocular tissues of treated eyes (current density was 1.9 mA/cm^2).

The mechanism of action of iontophoresis is still under investigation. When subjected to an electric field, molecules are exposed to three transport mechanisms: passive diffusion, electromigration and electroosmosis [50–53]. The passive contribution can be negligible in the eye due to its low permeability and is usually very small compared to the other two transport mechanisms; electromigration, which depends on the physicochemical characteristics, charge and concentration of the molecule is the primary electrotransport mechanism for ionized molecules (it can be considered as the ordered movement of ions in the presence of an electric field); electroosmosis produces the motion of the solvent carrying the molecule and is induced by an electric potential applied across an ionized membrane. Since the sclera is negatively charged [54], the electroosmotic flow enhances anodic delivery of a positively charged formulation, supported by the electrical flux of anionic nature.

In the current experiment, we waited for 40 min before investigating the lutein concentration in ocular tissues based on the results of preliminary work [55]. More experiments are needed to fully understand the kinetics of xanthophyll pigments flow from the scleral delivery site to the macula. In previous study, it has been evidenced that current flow was perpendicular to the applied scleral electrode [56]; therefore, electromigration can be primarily influenced by the chemophysical characteristics of the therapeutic substance, because they determine its electric mobility and transport pathway. In vitro studies have been shown to overestimate intraocular drug delivery because of the lack of dynamic elimination pathways, such as choroidal vasculature [57]. Nevertheless, mathematical models may be helpful to predict drug clearance due to intraocular dynamic barriers [58].

Possible clinical applications of macular enrichment with lutein by scleral iontophoresis include treating patients

at more risk of AMD progression. A pilot study will assess the feasibility of this approach in adult subjects soon.

5 | CONCLUSION

We presented scleral iontophoresis as an innovative method for in situ delivery of xanthophyll pigments to the human retina. The methodology was effective to enrich the intraocular tissues with lutein. Further studies are needed to assess whether this could be a valuable approach to enrich the macular pigment and eventually prevent local oxidative damage in patients at risk of AMD progression.

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CONFLICTS OF INTEREST

Kemin Food LC funder provided support in the form of salaries for S.H.S. during the period the study was performed, but did not have any additional role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. G.L., V.V. and N.M. were consultants for Vision Engineering Italy srl during the period the study was performed. M.L is CEO of Vision Engineering Italy srl. P.R. is owner of the patent concerning the iontophoresis device used in this work. This does not alter our adherence to all the journal policies on sharing data and materials, as detailed online in the guide for authors.

AUTHOR CONTRIBUTIONS

M.L., G.L., N.M., V.V. conceived, designed and performed the experiment, analyzed data and wrote and revised the paper. P.R., S.H.S. analyzed data and wrote and revised the paper.

AUTHOR BIOGRAPHIES

Please see Supporting Information online.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Standardized method for in situ excision of tissues from human ocular globe. Forty minutes after scleral iontophoresis of lutein, eyecups were prepared by gently removing the iris, lens and vitreous body. The ocular tissues of interest were exposed and the resonance Raman spectroscopy measurements were performed on the retina, choroid and sclera. The arrow highlights the macula.

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