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RETINAL DISORDERS

Macular peeling-induced retinal damage: clinical and histopathological evaluation after using different dyes

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Abstract

Purpose To describe functional and histopathological findings after macular peeling using different dyes.

Methods Prospective, randomized, comparative, interventional, and immunohistochemical study. Forty-five eyes from 45 patients with idiopathic epiretinal membrane (ERM) underwent pars plana chromovitrectomy with ERM and inner limiting membrane (ILM) using trypan blue 0.15% + brilliant blue 0.05% + lutein 2% in group 1 (15 eyes), trypan blue 0.15% + brilliant blue 0.025% + polyethylene glycol 3350 4% in group 2 (15 eyes), and indocyanine green 0.05% in group 3 (15 eyes). We evaluated visual acuity (VA) and macular sensitivity (MS) preoperatively, 1, 3, and 6 months after surgery. The expression of glial fibrillary acidic protein (GFAP) and neurofilament protein (NF) was assessed immunohistochemically on the ILMs peeled as markers of glial and neuronal cells.

Results In group 1, both mean VA and MS were significantly better at 1 and 3 months after surgery ($P < 0.05$), whereas no significant difference was found after 6 months. GFAP and NF expression was significantly lower in group 1 ($P < 0.05$).

Conclusions The ERM/ILM peeling is thought to rip off the intraretinal tissue, based on the amounts of GFAP and NF in the specimens. The use of lutein dyes reduces iatrogenic stress to the retinal tissue and allows a faster functional recovery in the first 3 months after surgery, suggesting a less iatrogenic adhesion to the retinal tissue.

Keywords Dye · Epiretinal membrane · Glial fibrillary acidic protein · Macular peeling · Müller cells · Neurofilament protein · Pars plana vitrectomy

Introduction

Epiretinal membranes (ERMs) are a common eye disease, in which cellular proliferation and metaplasia lead to the formation of avascular fibrocellular tissue at the vitreoretinal interface. [1, 2] ERMs can be associated with other ocular diseases, such as intraocular inflammation, diabetic retinopathy, retinal surgery, retinal vascular diseases, and trauma; they are classified as secondary or idiopathic (iERMs). [2, 3] An ERM is composed mainly of extracellular matrix proteins and retinal and extraretinal cells, such as glial cells, macrophages, hyalocytes, myofibroblasts, fibroblasts, and retinal pigment epithelial (RPE) cells. [4–6] The histological classification divides ERMs in two types: simple and complex. [7] The simple ERM comprises a single layer of glial cells, laminocytes, that are positive to intermediate filament glial fibrillary acidic protein (GFAP) antibodies and grow directly on the internal limiting membrane (ILM). [8] The complex type is a multilayer of cells that migrate through microscopic defects in the ILM on the surface of the retina after a posterior vitreous detachment (PVD), with remnants of native vitreous between the ERM and the ILM. [4, 9] However, ERM formation is not only an epiretinal but also an intraretinal phenomenon that induces outer and inner retinal neuron damage,

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changes in macroglia and microglia, and increased production of GFAP within Müller cells. [10, 11] Morphologically, the ILM is formed by the footplates of Müller cells. GFAP interacts with surface receptors, cytoskeleton, and the glial extracellular matrix, playing a role in cellular adhesion. [12] Indeed, the epiretinal traction induces Müller cells' activation, characterized by reactive gliosis, cellular hypertrophy, and upregulation of GFAP, as non-specific response of Müller cells to retinal injury and diseases. [2, 13, 14] It has been shown that the GFAP content in ERM correlates with tractional forces. [6] The increased GFAP is required for Müller cell gliosis and acts as a bridge between the ILM and Müller cells, [15, 16] making stronger the adhesion between the Müller cells and both ILM and the epiretinal tissue, with consequent increased risk of morphological retinal damage after ERM/ILM peeling. [17] The Müller cells support the foveola structurally, binding the photoreceptor cells; ILM peeling results in foot Müller cells damage, inducing their intraretinal collapse, ultrastructural damage to the inner retinal surface and vertical gliosis. [17, 18] GFAP and neurofilament protein (NF) can be used as indirect immunohistochemical markers for retinal damage; indeed, their expression can be evaluated to quantify the iatrogenic mechanical damage to retinal tissue after macular peeling. [12, 19]

Chromovitrectomy has gained popularity because it minimizes trauma to the underlying neuroretinal tissue due to enhanced visualization of ERM, ILM, and vitreous; nevertheless, the dyes can themselves cause intraretinal damage. [20]

The aim of this pilot study was to describe the presence of GFAP and NF on the ILM after the iERM is peeled and to evaluate the functional outcomes using different dyes.

Material and methods

Study design

A prospective, randomized, comparative, interventional, and immunohistochemical study was designed. Institutional Review Board (IRB) approval was obtained (Protocol Number 287/15) and the study was conducted according to the Declaration of Helsinki. The patients were enrolled form March to June 2016, after receiving the IRB approval. Informed consent was obtained from all individual participants included in the study. The surgeries were performed by three experienced surgeons: MRR (Ophthalmic Clinic of University of Naples "Federico II"), BP (S. Anna Hospital of Brescia), and CM (Polytechnic University of Marche).

Inclusion criteria

For this study, we enrolled patients older than 18 years old who were affected by iERM.

Exclusion criteria

Pregnant women and patients affected by diabetic ERM, severe systemic disease, or any untreated/uncontrolled ocular disease were excluded.

Participants

Forty-five eyes from 45 consecutive patients underwent pars plana vitrectomy (PPV) and ERM/ILM peeling in June and July 2016. Data on age, gender, and past ocular history were collected. The ophthalmic examination included best-corrected visual acuity (BCVA) evaluation, slit-lamp biomicroscopy, dilated fundus exam, and macular sensitivity (MS). The exam was performed by one of two blinded physicians (MF and GlC) preoperatively (baseline) and then at 1, 3, and 6 months after surgery.

Patients were randomly grouped according to the dye used during macular peeling using the software package "randomizeR" version 1.3:

- group 1 (trypan blue (TB) 0.15% + brilliant blue (BBG) 0.05% + lutein 2% (Doubledyne, Kemin Pharma, Oeiras, Portugal), $n = 15$);
- group 2 (TB 0.15% + BBG 0.025% + polyethylene glycol (PEG) 3350 4% (Membraneblue-Dual®, DORC International, Zuidland, The Netherlands), $n = 15$);
- group 3 (indocyanine green (ICG) 0.05% (Diagnostic Green, Aschheim-Dornach, Germany), $n = 15$).

We chose the dyes among those commercially available. Every surgeon performed the same number of surgeries (five patients per group).

Surgical technique description

All patients underwent local anesthesia with a retrobulbar block. Phacoemulsification was performed before vitrectomy in phakic patients. All patients underwent trans-conjunctival, sutureless PPV using Constellation 25 G+ Total Plus Vitrectomy Pak (Alcon Laboratories, Inc., Fort Worth, TX) and Xenon (Alcon Laboratories, Inc) light sources. The conjunctiva was displaced and the valved trocars were introduced with a one-step technique. A core and peripheral vitrectomy was performed and the induction of a complete PVD was carried out by aspiration. The dye was injected through a cannula, left on the retina surface for 30 s of open infusion and then washed out in the fluid-filled eye (wet method). [20] The peeling of the ERM and then of the ILM was performed by fine-tipped forceps. After checking the peripheral retina with scleral indentation, all patients had an air-fluid exchange.

Microperimetry protocol and analysis

We evaluated MS using the Micro Perimeter 1 (MP1) (NAVIS software version 1.7.2; Nidek Technologies) which has an automated eye tracking system. High-contrast areas were chosen for tracking on an infrared image of posterior pole acquired after 5 min of dark adaption. We set the MP1 with threshold strategy 4-to-2, fixation target 2 red cross, 4 apostilbs white background illumination, luminance 0–20 dB, and Goldmann III Stimulus 200 ms. We covered the central 10°, centering the radial grid pattern on the fovea with the exam of 61 stimulus locations. A color image was overlaid onto retinal sensibility points to compare morphologic and functional defects.

Immunohistochemical evaluation

All ILM specimens were formalin-fixed, paraffin-embedded, and serial-sectioned. For each specimen, one section was stained with hematoxylin/eosin to confirm the initial diagnosis and the following two 5-μm serial sections were mounted on poly-L-lysine-coated glass slides and processed for immunohistochemistry (IHC).

Immunohistochemistry was performed with an automated IHC System (Ventana BenchMark XT; Ventana Medical Systems, USA), according to the manufacturer's instructions. The sections were incubated with anti-GFAP (rabbit monoclonal antibody, clone EP672Y, Ventana Medical Systems) and anti-NF (mouse monoclonal antibody, clone MRQ-55 Ventana Medical Systems), as markers of glial and neuronal cells. All sections were counterstained with Mayer's hematoxylin. Slides were evaluated independently by two blinded observers (SS and GI) and the cases with discordance were discussed and resolved by consensus. The expression of these immunohistochemical markers was scored between 0 and 3, by evaluating:

- the strength (intensity) of the staining (0 = absent; 1 = mild; 2 = moderate; 3 = intense);
- the percentage of stained cells (0 = 0; 1 ≤ 10%; 2 = 10–25%; 3 ≥ 25%).

A semi-quantitative evaluation (*H* score) was then obtained by summing the two values for each marker.

Statistical analysis

We converted Snellen values of BCVA into the logarithm of the minimal angle of resolution (logMAR) units for the statistical analysis. Student's *t* tests were used to compare the three groups, two by two, both for visual acuity and *H* scores. *P* values were considered statistically significant if less than 0.05. SPSS Statistics Base version 21.0 (IBM Software) was used to conduct the statistical analysis.

Results

Forty-five eyes from 45 patients with a mean age of 68 ± 7 years (ranging from 51 to 82) were randomly divided into three groups of 15; each group used different dyes for macular peeling. The demographics of the patients are shown in Table 1; there was no statistically significant difference among the three groups at baseline ($P < 0.05$).

The BCVA values at baseline and 1, 3, and 6 months after surgery are shown in Table 2. No significant difference was present at the baseline between the three groups. The difference of BCVA between preop and 6-month follow-up was statistically significant in all groups ($P < 0.001$); the mean improvement in letters (EDTRS at 4 m) at 6-month follow-up was 11.87 (± 7.25) in group 1, 12.27 (± 8.27) in group 2, and 10.87 (± 8.38) in group 3. At 1 and 3 months after surgery, the BCVA was significantly better in group 1 compared with both group 2 (P1-month = 0.03 and P3-month = 0.02) and group 3 (P1-month = 0.04 and P3-month = 0.03). The mean improvement in letters 1 and 3 months after surgery was 10.8 (± 7.6) and 10.87 (± 6.96) in group 1, 6.8 (± 5.57) and 6.67 (± 4.86) in group 2, and 6.73 (± 4.85) and 6.73 (± 4.99) in group 3. There was no statistically significant difference in BCVA between group 2 and group 3 at 1 and 3 months. At 6 months, there was no significant difference between the three groups.

Table 3 shows the data regarding macular sensitivity preoperatively and at 1, 3, and 6 months after surgery. There was no statistically significant difference between baseline and

Table 1 Demographic findings

	Group 1	Group 2	Group 3	*P* G1vsG2	*P* G1vsG3	*P* G2vsG3
Number of patients	15	15	15	1	1	1
Age, mean (SD), y	66 (7.72)	69 (6.01)	67 (6.99)	0.43	0.75	0.76
Male/female	8/7	6/9	7/8	0.16	0.33	0.33
Eye right/left	9/6	7/8	7/8	0.49	0.43	1
Phakic/pseudophakic	4/11	3/12	4/11	0.33	1	0.33

SD standard deviation, *y* years

Table 2 Best-corrected visual acuity results: mean values (SD)

BVCA	Group 1	Group 2	Group 3	*P* G1vsG2	*P* G1vsG3	*P* G2vsG3
Baseline						
Snellen	20/62	20/68	20/66	0.6	0.69	0.84
logMAR	0.49 (0.19)	0.53 (0.18)	0.52 (0.19)			
1 month						
Snellen	20/39	20/50	20/49	0.03	0.04	0.64
logMAR	0.29 (0.13)	0.4 (0.15)	0.39 (0.13)			
3 months						
Snellen	20/38	20/50	20/49	0.02	0.03	0.75
logMAR	0.28 (0.12)	0.4 (0.13)	0.39 (0.13)			
6 months						
Snellen	20/37	20/40	20/39	0.52	0.32	0.89
logMAR	0.27 (0.13)	0.3 (0.12)	0.29 (0.12)			

BCVA best-corrected visual acuity, *G1* group 1, *G2* group 2, *G3* group 3, *logMAR* logarithm of the minimum angle of resolution, *SD* standard deviation

6 months between the three groups, whereas MS was significantly better in group 1 than both group 2 (P1-month = 0.04 and P3-month = 0.04) and group 3 (P1-month = 0.02 and P3-month = 0.04) at 1 and 3 months after surgery. The differences between group 2 and group 3 were not significant 1 and 3 months after surgery. The MS at 6 months was significantly better than at baseline in each group ($P < 0.05$).

Histopathological examination of the hematoxylin/eosin-stained sections confirmed that all the specimens were appropriate, consisting of ILM. The results of immunohistochemical evaluation of GFAP and NF in the three groups are resumed in the Table 4. One representative sample from each group is shown in Fig. 1. GFAP was detected in 8 of 15 (53%) specimens in group 1, 14 of 15 (93%) in group 2, and all specimens in group 3. NF was detected in 8 of 15 (53%) specimens in group 1 and 100% of specimens in group 2 and group 3.

The GFAP and NF expression of each group was compared using *H* scores (Table 5). Group 1 had a lower expression of GFAP and NF than dyes without lutein (PGFAP < 0.001 and PNF < 0.001), whereas there was no statistical difference between group 2 and group 3 for either markers (PGFAP = 0.26 and PNF = 0.66).

Discussion

Pars plana vitrectomy with ERM-ILM peeling is the treatment of choice for removal of the epiretinal tissue for symptomatic patients. [21] The aim of double peeling (ERM and ILM) is to release tangential traction generated by incomplete PVD or posterior hyaloid completely. [22] However, this surgical procedure results in trauma to the retina, inducing mechanical injury of Müller cells. [12, 17] Electron microscopic evaluation has shown degenerated and necrotic Müller cell processes on peeled ILM specimens [23] and injury to macular Müller cells has been associated with the delayed recovery of the b wave of focal macular electroretinography, even at 6 months after ILM peeling. [24] After iERM peeling, early and late inner retinal changes in the macula have been described as swelling of the arcuate retinal nerve fiber layer and a dissociated optic nerve fiber layer defect, respectively. [25] These temporary physiological alterations are evident in regions with a greater density of Müller cells and do not alter the functional results. [25] In the presence of increased GFAP, vitrectomy with ILM peeling leads to intraretinal alterations and consequent damage of the macroglia and microcirculation. [10, 26] Furthermore, ILM peeling may also induce a contraction of

Table 3 Preoperative and postoperative macular sensitivity: mean (SD)

MS, dB	Group 1	Group 2	Group 3	*P* G1vsG2	*P* G1vsG3	*P* G2vsG3
Baseline (SD)	8.93 (2.33)	8.83 (2.4)	8.73 (2.15)	0.33	0.25	0.65
1 month (SD)	10.4 (2.41)	9 (1.77)	8.87 (2.05)	0.04	0.02	0.33
3 months (SD)	11.07 (2.53)	9.97 (2.13)	9.73 (2.17)	0.04	0.04	0.45
6 months (SD)	11.43 (2.51)	11.53 (2.47)	11.57 (3.26)	0.57	0.67	0.93

dB decibel, *G1* group 1, *G2* group 2, *G3* group 3, *MS* macular sensitivity, *SD* standard deviation

Table 4 Mean values (SD) of glial fibrillary acidic protein and neurofilament protein scores in groups 1, 2, and 3

	GFAP		NF	
	% cells +	Intensity	% cells +	Intensity
Group 1	0.60 (0.63)	0.73 (0.88)	0.53 (0.64)	0.67 (0.9)
Group 2	2 (0.85)	2.07 (0.8)	2 (0.76)	2 (0.85)
Group 3	2.13 (0.99)	2.47 (0,.92)	2.07 (0.7)	2.13 (0.74)

GFAP glial fibrillary acidic protein, *NF* neurofilament protein, SD standard deviation

Müller cells, thus reducing macular volume, damaging the basement membrane of Müller cells. [17] The iatrogenic mechanical damage of Muller cells can be evaluated by the amount of GFAP and NF present on the specimens of tissue peeled. [12, 19]

Chromovitrectomy has gained popularity because it minimizes trauma to the underlying neuroretinal tissue due to a better visualization of ERM, ILM and vitreous; nevertheless, the dyes can themselves cause intraretinal damage. [27] Indocyanine green was the first vital dye used to stain the ILM. Several studies have shown potential ICG toxicity in terms of damage to the photoreceptors and RPE cells, reduction of RPE cell viability, RPE and optic nerve atrophy, loss of epiretinal cellular integrity, cellular toxicity, perimetric defects, and worse functional results than eyes without ICG-stained ILM. [20, 28, 29] However, low concentrations of ICG (0.5% or less) should be relatively safe, minimizing potential ICG toxic effects on the retina. [20, 27] In this study, we used ICG 0.05%.

Trypan Blue stains the ERM but not the ILM, due to its strong binding with degenerated cellular elements; a concentration of 0.15% has been shown to be relatively safe. [27]

Brilliant Blue has high affinity for the ILM and has been introduced as a safer alternative for ICG. [20] Several studies demonstrated that ICG induces apoptosis in retinal cells due to the upregulation of the pro-apoptotic protein Bax and the downregulation of the anti-apoptotic protein Bcl-2. [28–31] In contrast, it has been suggested that BBG, in addition to not inducing apoptosis in retinal cells, [31] might have a protective effect on retinal tissue due to upregulation of Bcl-2 [28, 29] and acting as a P2RX7 antagonist, preventing induced apoptosis in photoreceptors in vitro. [29, 30] During surgery, dyes can migrate into the subretinal space; subretinal injection has led to histological and functional retinal damage using ICG and TB (greater for ICG), but not BBG. [20, 32] However, various in vitro toxic effects have been reported for BBG, such as necrosis of RPE cells and decreased cell viability of retinal ganglion cells. [29]

When GFAP is overexpressed, as in the presence of ERM, the intraretinal adhesion between Müller cells and the epiretinal tissue is stronger, and this adhesion further increases in the presence of ICG or BBG, with a more pronounced effect for ICG. [33] Both dyes lead to a significant increased biomechanical stiffness of the stained ILM, possibly due to the binding of the dyes to extracellular matrix proteins of the ILM and specific tissue–dye interactions. [33, 34] This results in easier ILM removal and larger fragments but, on the other hand, an increased risk of morphological damage during the surgical maneuver. [12, 34]

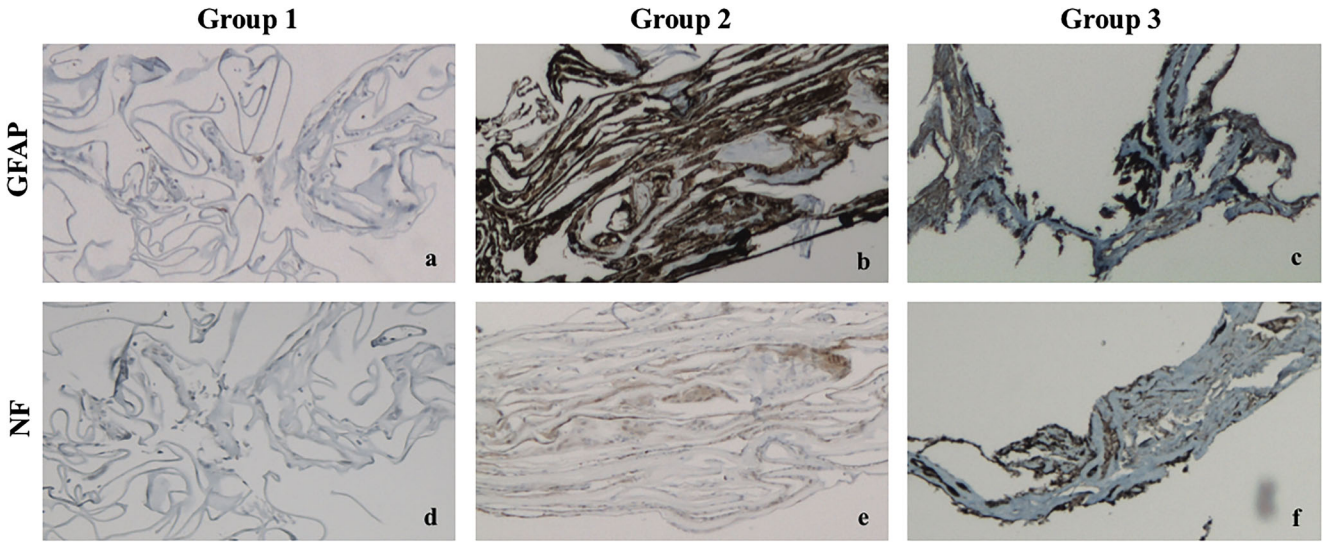

Fig. 1 Immunostaining of peeled internal limiting membranes of 3 representative patients, one from each group 1a, 1d: sample from patient of group 1: the immunohistochemical expression of GFAP (a) and NSE (d) was weak and only focal; 1b, 1e: sample from patient of group 2: strong and diffuse signal was observed for both GFAP (b) and NF (e); 1c, 1f: sample from patient of group 3: moderate, discontinuous immunostaining was found for both GFAP (c) and NF (f)

Table 5 *H* score of glial fibrillary acidic protein and neurofilament protein in groups 1, 2, and 3

	Group 1	Group 2	Group 3	*P* G1 vs G2	*P* G1 vs G3	*P* G2 vs G3
H score GFAP	1.33 (1.4)	4.07 (1.22)	4.6 (1.68)	$P < 0.001$	$P < 0.001$	0.26
H score NF	1.2 (1.52)	4 (1.31)	4.2 (1.21)	$P < 0.001$	$P < 0.001$	0.66

G1 group 1, *G2* group 2, *G3* group 3, *GFAP* glial fibrillary acidic protein, *NF* neurofilament protein, *SD* standard deviation

The dyes with combination of TB and BBG aimed to take advantage from the different staining properties of these two dyes. In this study, we chose two different combined dyes, among those commercially available. In group 1, we used TB 0.15% + BBG 0.05% + lutein 2%. The resulting dye is heavier than balanced saline solution, leading to a more comfortable injection. [35] Lutein is a lipophilic pigment and it is considered a dye; however, it has been reported that the addition of lutein did not improve the ILM staining of BBG. [35] In group 2, we injected a combination of TB 0.15% + BBG 0.025% + PEG 3350 4%. The carrier PEG 3350, a polyether compound, is considered inert and makes the dyes heavier without toxic effects on the retina. [36]

We evaluated the iatrogenic mechanical damage to Müller cells by the amount of GFAP and NF present. The dye where lutein was added had lower expression of GFAP and NF on the peeled ILM. This suggests an association between the presence of neuronal and glial cells and the type of dye used. Comparing the ultrastructure of ICG- and BBG-peeled ILM, Brockmann et al. [37] found more frequent and larger cellular fragments on the BBG-peeled ILM, suggesting that the BBG might induce a more pronounced alteration of retinal cell layers. Sousa-Martins et al. [16] compared the mode of interaction between the retina and TB, ICG, BBG, triamcinolone acetonide, or lutein-based dyes. All dyes revealed an affinity with the ILM. The lutein-based dyes showed a physical interaction with membrane models of human ILM and ERM, whereas all the other dyes tested showed a stronger chemical interaction leading to stronger adhesion to retinal tissue. Moreover, ICG and triamcinolone resulted in an alteration of the membrane models, with the higher effect in terms of disintegration of the membrane, whereas no significant membrane disorganization was found with lutein-based dyes. [16] The addition of lutein to vital dyes may result in reduced adhesion to retinal tissue leading to less iatrogenic damage during macular peeling. Moreover, we also compared the three groups in terms of functional outcomes. Both BVCA and MS were significantly better at 1 and 3 months after surgery in group 1, where a lutein-based dye was used. This faster functional recovery supports the immunohistochemical evidence of reduced mechanical damage.

At 6 months follow-up, we did not find any functional difference between the groups; however, significant functional differences have been reported after peeling of diabetic ERMs with an increased perifoveal capillary-free zone. Such findings could be explained by the impaired diabetic perifoveal capillary plexus, which is more sensitive to surgical damage induced in the Müller cells by ILM peeling. [10]

Our histological findings show that in the presence of preoperative Müller cell damage (e.g., in diabetic retinopathy), it is important to choose a dye for peeling that induces the least iatrogenic intraretinal damage.

In addition to the mechanical damage, retinal phototoxic injury also plays an important role during vitreoretinal procedures. [38] Intraoperative light exposure causes phototoxic damage to RPE that could be enhanced by vital dyes, photosensitizing substances that pass through retinal layers. [20] Endo-illumination induces the decomposition of vital dyes, increasing levels of free radicals and creating photoproducts that are harmful to retinal cells. The risk of phototoxicity to the neuroretina could be higher for light greater than 450 nm, which only occurs in the dye-stained retina due to the higher absorption of photons emitted by the intraoperative light source. [20, 38] In addition, the presence of dyes in the subretinal space could enhance the damage to the RPE after exposure to various light wavelengths. [39] In human RPE cells exposed to ICG, illumination was found to play a significant role in mediating cell toxicity. [40] A slim possibility of phototoxicity similar to ICG has been described for BBG. [27] Lutein has been recently associated with anti-oxidant and blue light-filtering properties. Lutein could have a protective antioxidant effect, acting as a scavenger of free radicals generated during inflammatory processes as well as during surgery and inhibiting various proinflammatory intracellular pathways, such as membrane lipoperoxidation. [41–43] Moreover, lutein has been shown to absorb blue light at wavelengths around 450 nm with a consequent potential protective effect against phototoxicity from intraoperative light sources. [42]

The strength of this study is the evaluation of both functional and structural results; moreover, we performed two functional tests, visual acuity, and microperimetry, to obtain more accurate functional outcomes. However, this is a pilot study with some limitations, such as a small sample size and relatively short follow-up period; further studies will be necessary.

In conclusion, this is the first study demonstrating that adding lutein may be advantageous in terms of less

intraoperative iatrogenic peeling-induced macular damage and the consequent faster functional recovery after surgery.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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