
The Combination of Trypan Blue and Brilliant Blue G-Assisted Vitrectomy for Macular Pucker: Histopathological Findings

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Keywords

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Abstract

Purpose: To report on the combined use of trypan blue (TB) and brilliant blue G (BBG) for staining the epiretinal membrane (ERM) and internal limiting membrane (ILM) during vitrectomy and to describe the histopathological findings.

Methods: 10 surgical specimens were removed from 10 eyes with macular pucker during vitrectomy using a commercially available combination of TB and BBG for ERM and ILM staining and peeling. Specimens were evaluated using light and transmission electron microscopy. **Results:** In all cases the combination of TB and BBG was useful for identifying and delineating ERM and ILM. No complications related to the use of the dye were observed during or after surgery. Glial cells were present in all specimens. Hyalocytes were observed in 6 cases and myofibroblasts in 3 of them. In 7 cases native vitreous collagen fibrils were found on the ILM, while in 5 specimens newly formed collagen was present. No clinical evidence of toxicity was observed during the 3-month

follow-up. **Conclusion:** The combined use of TB and BBG appeared to be very useful intraoperatively to improve the visualization of ERM and ILM, thus facilitating their complete removal. Anatomical and histopathological findings demonstrated the safety and the efficacy of this vital dye.

Introduction

The epiretinal membrane (ERM) is the most common type of fibrocellular membrane that proliferates and grows on the inner surface of the retina in the macular area [1, 2]. It is possible to divide the ERM into primary or idiopathic epiretinal membrane (iERM) and secondary ERM [3, 4]. The latter can arise as a result of retinal surgery, laser treatments, trauma, intraocular inflammatory diseases, retinal vascular diseases or retinal detachment. Currently, treatment options are very limited and consist of watchful waiting or vitrectomy surgery [4–6].

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In 1978, Machemer [7] first described the surgical treatment of ERM. This surgery, at first only reserved for secondary ERM, has been later extended to iERM. Nowadays, ERM peeling is the gold standard technique in macular pucker surgery leading to good visual and anatomical outcomes in the majority of cases. Nevertheless, visual acuity improvement is rarely complete, and an ERM recurrence rate of 21% has been reported [8–10]. The unsatisfactory improvement of visual acuity and the recurrence of ERM seem to be correlated with an incomplete removal of the ERM [11, 12].

Therefore, internal limiting membrane (ILM) peeling has been introduced to achieve a deeper cleavage plane and a complete removal of the ERM and to reduce the rate of relapses [5, 6, 13].

The visualization and the peeling of the membranes overlying the retina can be difficult for the surgeon as these structures are not well visualized and are extremely thin and transparent. This may result in a partial removal of the ERM and ILM and, in some cases, may lead to iatrogenic traumas to the retina [14].

To overcome these problems, the use of intraoperative vital dyes to stain the ERM and the ILM, such as trypan blue (TB), indocyanine green (ICG), and brilliant blue G (BBG), has been introduced [15–22].

ICG was the first vital dye used for ILM staining [19]. It was widely used in the past for this purpose, but toxic effects on the retina have been hypothesized [21–26]. It has been reported that high concentrations of ICG may lead to a deeper cleavage plane and consequently to a potential damage to retinal cells [26]. Furthermore, other authors have described an ICG toxic effect on retinal ganglion cells and retinal pigment epithelial cells [19, 22–25].

TB is a vital dye with a good safety profile and a high affinity for the ERM but not for the ILM, making it suitable for ERM peeling but not for ILM peeling [14]. In contrast, BBG is an excellent stainer for visualizing the ILM. Although it has a lower ILM affinity than ICG, it has a better safety profile, proving to be a viable alternative to it [27, 28].

Penha et al. [29] reported an in vitro effect of several vital dyes on toxicity and apoptosis in a human retinal pigment epithelial cell line; overall, the authors observed a better safety profile of BBG with regard to retinal pigment epithelial cells.

In addition, Mansoor et al. [30] demonstrated BBG safety also on 2 other retinal cell lines, the neurosensory retinal cells and the Müller cells.

Recently, the 0.15% TB, 0.025% BBG, and 4.00% polyethylene glycol (PEG) combination (MembraneBlue-Du-

al™, DORC International, Zuidland, the Netherlands) has been introduced for staining both the ILM and ERM [31, 32]. This heavy vital dye is commercially available in Europe.

The aim of this study was to report on the use of MembraneBlue-Dual™ for staining the ERM and ILM during vitrectomy and to describe the histopathological findings.

Materials and Methods

The study adhered to the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects. The protocol was approved by our Departmental Institutional Review Board. This nonrandom, prospective, single-blinded study design was carried out at the University Eye Clinic of Trieste, Italy. Ten patients (6 males and 4 females) with the mean age of 75.2 ± 3.76 years (range 69–80) and suffering from macular pucker were enrolled for ERM and ILM peeling. Four of them were pseudophakic, and 6 were phakic. All 10 patients underwent 25-G pars plana vitrectomy in 1 eye with dye-assisted peeling of the ERM and the ILM. In phakic eyes combined phacovitrectomy was performed.

The best-corrected visual acuity was determined using the Early Treatment Diabetic Retinopathy Study charts with a 4-meter testing distance. The BCVA was converted to logMAR for data analysis.

An optical coherence tomography (Spectral OCT SLO, Optos, USA) was used to measure central retinal thickness (CRT) both in the preoperative and postoperative period.

Statistical Analysis

The variations of visual acuity and CRT after 3 months were analyzed using the Wilcoxon signed-rank test. The possible correlation between the change of visual acuity and the postoperative CRT was evaluated using the Spearman rank correlation coefficient. The level of statistical significance was taken as 5% ($p < 0.05$).

Inclusion Criteria

- Patients suffering from iERM were considered

Exclusion Criteria

- Secondary epiretinal membrane
- Macular hole
- Previous retinal diseases
- Previous vitreoretinal surgery

Surgical Technique

All procedures were performed by a single and experienced surgeon (D.T.). In all patients local anesthesia was used. The surgical technique consisted of a 25-gauge (25 G) 3-port pars plana vitrectomy. Surgeries were performed using the Alcon Constellation Vision System (Alcon Laboratories Inc., Fort Worth, TX, USA). After vitrectomy, induction of posterior vitreous detachment, if necessary, and vitreous removal, a combination of 0.15% TB, 0.025% BBG, and 4.00% PEG (MembraneBlue-Dual™, DORC International, Zuidland, the Netherlands) was injected, once only,

Table 1. Patient demographics, diagnosis and outcomes

Case	Age, years/ gender	Retinal disorder	Surgery	Preop. VA, logMAR	Postop. VA, logMAR	Preop. CRT, µm	Postop. CRT, µm
1	75/M	iERM	PPV + phaco.	0.3	0.1	460	335
2	80/M	iERM	PPV + phaco.	0.5	0.1	395	315
3	69/F	iERM	PPV + phaco.	0.7	0.2	460	265
4	71/M	iERM	PPV + phaco.	0.5	0.2	440	385
5	79/M	iERM	PPV	0.5	0.2	520	370
6	72/F	iERM	PPV	0.5	0.5	360	400
7	77/M	iERM	PPV + phaco.	0.5	0.4	770	520
8	78/F	iERM	PPV	1.0	1.0	330	180
9	79/F	iERM	PPV	0.4	0.1	380	430
10	78/M	iERM	PPV + phaco.	0.5	0.2	495	425

VA, visual acuity; CRT, central retinal thickness; iERM, idiopathic epiretinal membrane; PPV, pars plana vitrectomy; phaco., phacoemulsification.

over the posterior pole to stain the ERM and ILM and was removed after 30 s. The PEG increases the molecular weight and viscosity of MebraneBlue-Dual™, thus allowing effective tissue staining without the need of a prior fluid-air exchange. The stained tissue was removed en bloc (ERM together with ILM) with dedicated intraocular forceps (Eckardt End Gripping Forceps, DORC International, Zuidland, the Netherlands). Surgery was concluded by checking the peripheral retina for possible retinal breaks.

Specimen Preparation

ERM and ILM specimens were obtained from each patient during pars plana vitrectomy, immediately fixed in 2% glutaraldehyde solution, placed into a 0.1-M cacodylate buffer and sent for the transmission electron microscope (TEM) analysis.

The protocol for sample preparation consisted of the following phases:

- repeated washings with 0.1 M cacodylate buffer;
- postfixation with 1% osmium tetroxide;
- dehydration of the tissue through a series of washes with ethyl alcohol at increasing concentrations;
- replacement of ethyl alcohol with propylene oxide;
- treatment with solutions of increasing concentrations of propylene oxide/resin;
- all the specimens were embedded in the epoxy resin (DER 332-732), followed by the polymerization phase at a temperature of 60 °C for 72 h.

Semithin sections (600–1,000 nm) and ultrathin sections (80 nm) were obtained using an ultramicrotome (Leica Ultracut UCT Ultramicrotome, Ithaca, NY, USA).

The semithin sections were stained with toluidine blue and analyzed using light microscopy. These sections allowed for selecting the useful fields for ultrastructural examination with TEM.

The single sections were put on a special mesh of a few millimeters in diameter. Finally, they were stained with uranyl acetate and lead citrate, and were observed and photographed using TEM (Phillips EM 208 transmission electron microscope operating at an accelerating voltage 100 kV).

Results

The functional and anatomical results of this series of patients are shown in Table 1.

All patients were followed up for 3 months. The mean visual acuity improved statistically and significantly from 0.54 logMAR (± 0.19 standard deviation (SD)) before surgery to 0.30 logMAR (± 0.28 SD) at the last follow-up visit. The postoperative best-corrected visual acuity improved significantly ($p = 0.011$) in all patients (0.24 logMAR ± 0.16 SD).

CRT showed a significant ($p = 0.012$) postoperative reduction (from 450 to 362.50 ± 94.90 µm). The mean reduction of CRT was 98.50 ± 95.77 µm.

The Spearman correlation coefficient ($p = 0.019$) did not show any correlation between visual acuity improvement and CRT reduction.

During surgery the posterior vitreous detachment appeared to be already present in all patients. Therefore, the surgically induced detachment of the posterior vitreous was not necessary.

In all cases the combination of TB and BBG was useful for identifying and delineating the ERM and ILM, in order to allow a complete en bloc removal. In all patients the staining of both the ERM and ILM was very effective. No complications or side effects related to the use of the dye were observed during or after surgery. Intraocular residual of dye on the underlying retina was not found. At the latest follow-up, no biomicroscopic clinical evidence and optical coherence tomographic findings of residual membranes or recurrence were observed.

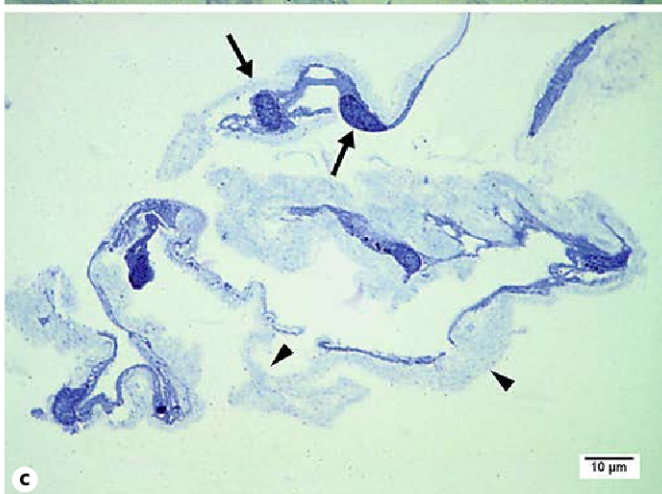
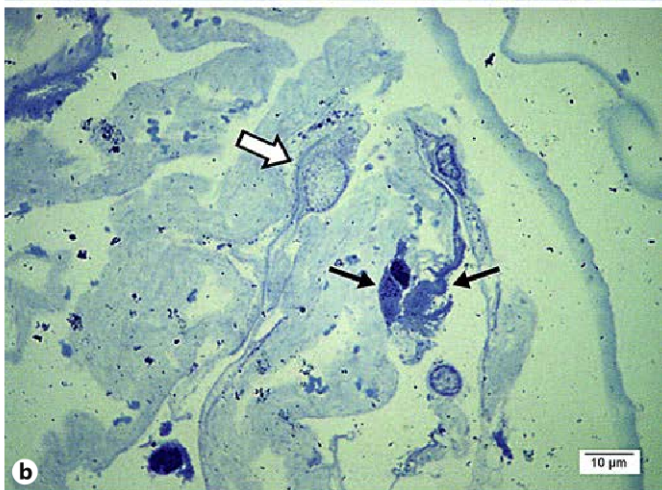
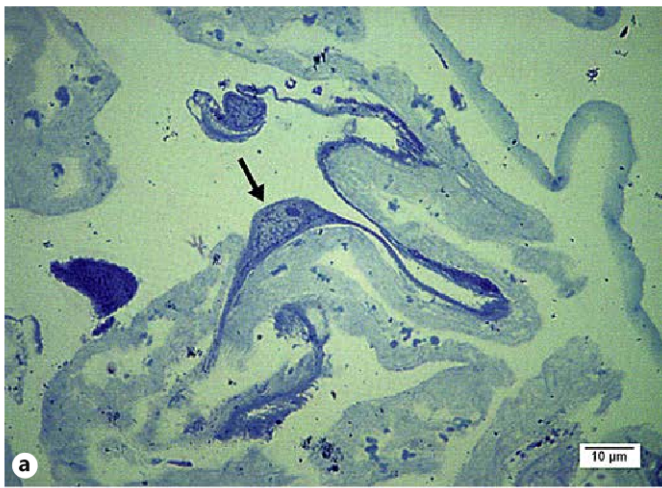


Fig. 1. Light micrographs. Original magnification $\times 100$. **a** Single layer of leveled cells; nucleus (arrow). **b** Cells with an oval or flattened heterochromatin nucleus and dark cytoplasm (black arrows). Cells with clearer cytoplasm and a round euchromatin nucleus (white arrow). **c** Single layer of cells with oval nucleus and dark cytoplasm (arrows) that lie on a layer of extracellular material, light colored and cell-free, corresponding to the ILM (arrowheads).

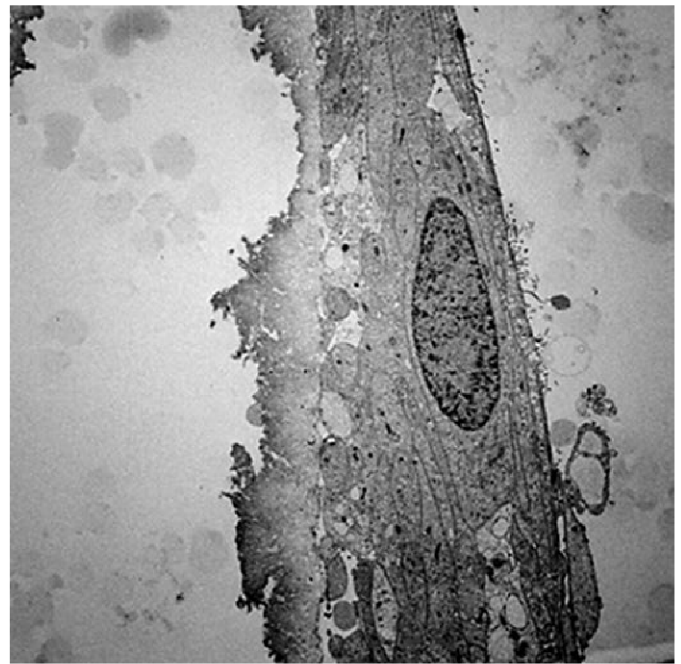


Fig. 2. Transmission electron micrograph. Polarized cells, with a fusiform shape, an elongated nucleus and with the presence of microvilli on 1 of the 2 plasma surfaces. Original magnification $\times 1,000$.

Histopathological Findings

All specimens were analyzed using light microscopy and TEM.

All specimens analyzed with light microscopy showed the presence of both ERM and ILM. In light microscopy-acquired images the ERM appeared to be flat in some samples and coiled in others due to the sample preparation procedure. The ERM was constituted of a single layer of leveled cells (Fig. 1a). The aspect of these cells was not the same in all samples but some differences were found in most of them. Several cells had an oval or flattened heterochromatin nucleus and a dark cytoplasm, while a smaller part of them had a clearer cytoplasm and a round euchromatin nucleus (Fig. 1b).

These cells lay on a layer of extracellular material, light colored and with uniform appearance, cell-free, corresponding to the ILM (Fig. 1c).

TEM showed the presence of both ERM and ILM. In most of the samples, ERMs were made up of a single layer of cells lying on a thick layer of collagen. Only in 1 sample was a multiple-layer cell distribution found. Cells of the ERM appeared very different in shape. The main cell type was represented by polarized cells, with a fusiform shape, an elongated nucleus and microvilli on 1 of

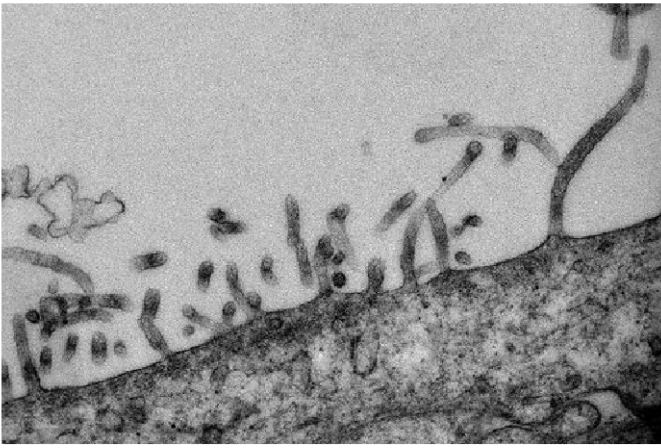


Fig. 3. Transmission electron micrograph. Detail of microvilli (finger-like protrusions of the membrane with a 100-nm diameter). Original magnification $\times 13,000$.

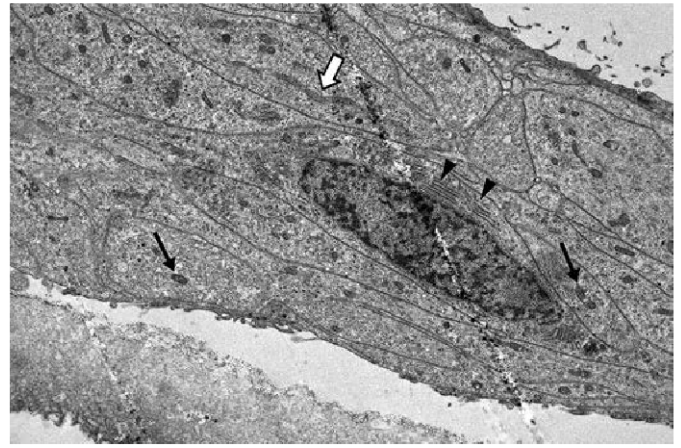


Fig. 4. Transmission electron micrograph. Multiple layer cell distribution: cells contained many mitochondria (black arrows), some of them with a tubular shape (white arrow), many cisterns of granular endoplasmic reticulum within their cytoplasm (arrowheads). Original magnification $\times 2,500$.

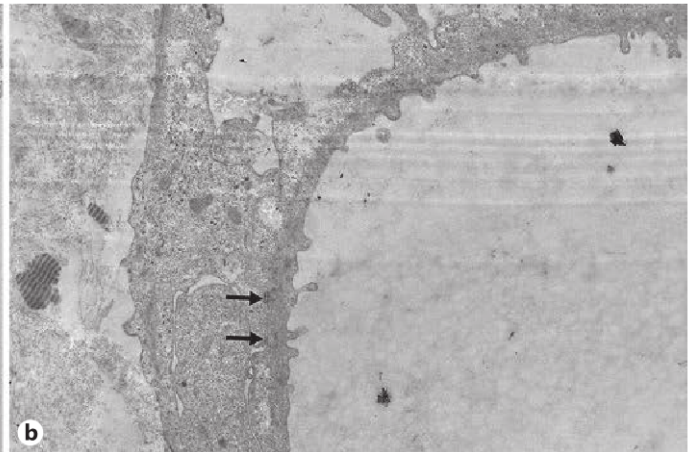
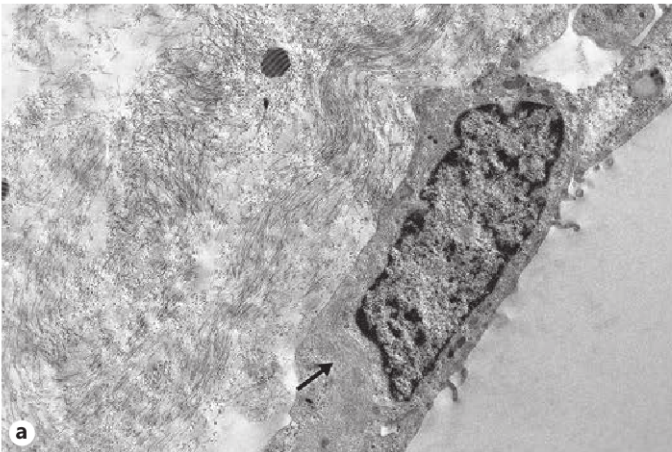


Fig. 5. Transmission electron micrographs. **a** Filaments of 10 nm in thickness, ascribable to intermediate cytoskeleton components (arrow), typical of glial cells. **b** Cell interconnection by intercellular junctional complex (arrows), typical of glial cells. Original magnification $\times 3,200$.

the 2 plasma surfaces (finger-like protrusions of the membrane with a 100-nm diameter). This type of cells was similar to the glial ones (Fig. 2, 3).

Several cells contained many mitochondria, some of them with a tubular shape, many free ribosomes and cisterns of granular endoplasmic reticulum within their cytoplasm that is a typical feature for an active secretory stage (Fig. 4). Other cells showed filaments averaging 10 nm in diameter, ascribable to intermediate cytoskeleton components, typical of the glial cells (Fig. 5a). Some of

these cells were interconnected by intercellular junctional complexes, another feature of glial cells (Fig. 5b).

A great number of nonpolarized cells was detected. They were characterized by an irregular surface with numerous finger-like protrusions and with numerous secretory granules; these cells were suggested to be hyalocytes derived from the hematopoietic monocyte/macrophage lineage (Fig. 6).

In some specimens myofibroblast-like cells were observed. This cluster of cells was characterized by fusiform

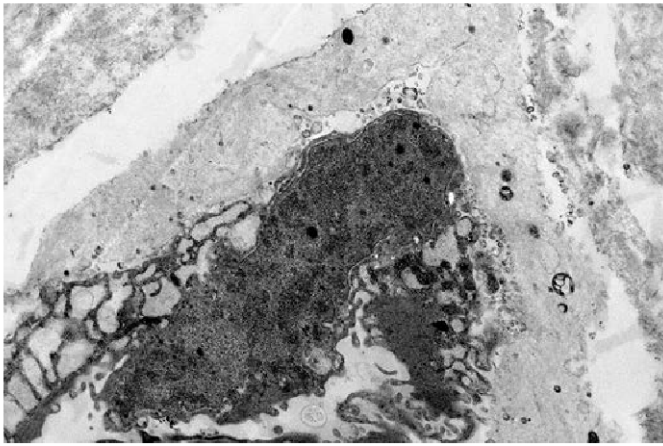


Fig. 6. Transmission electron micrograph. Hyalocyte detail (non-polarized cells characterized by an irregular surface with numerous finger-like protrusions and with numerous secretory granules). Original magnification $\times 3,200$.

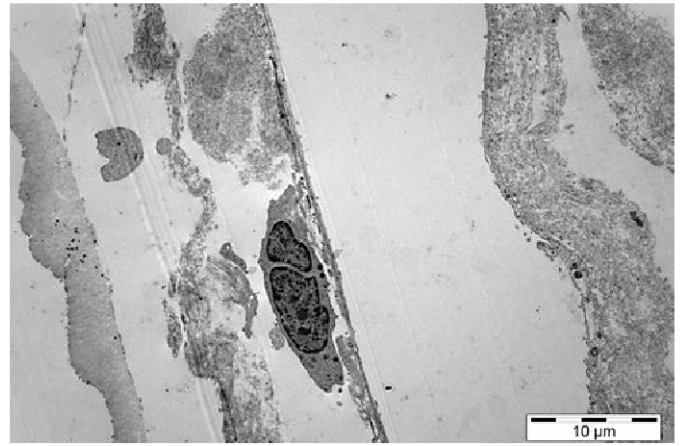


Fig. 7. Transmission electron micrograph. Myofibroblast-like cells (cells characterized by fusiform and irregular nuclei and by the absence of finger-like protrusions). Original magnification $\times 1,000$.

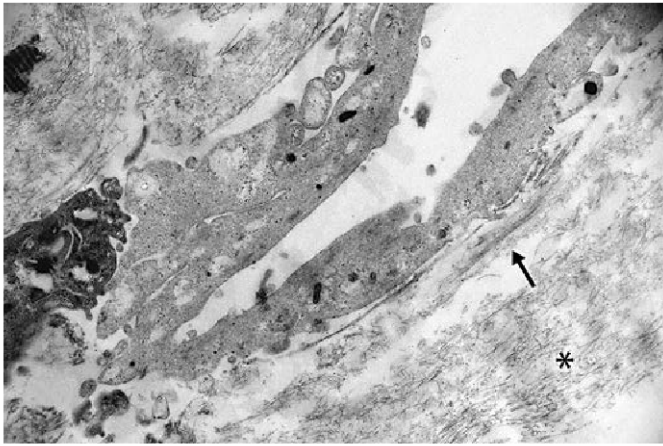


Fig. 8. Transmission electron micrograph. Collagen layer between the cells and the ILM. Native vitreous collagen (asterisk) and newly formed collagen (arrow). Original magnification $\times 4,000$.

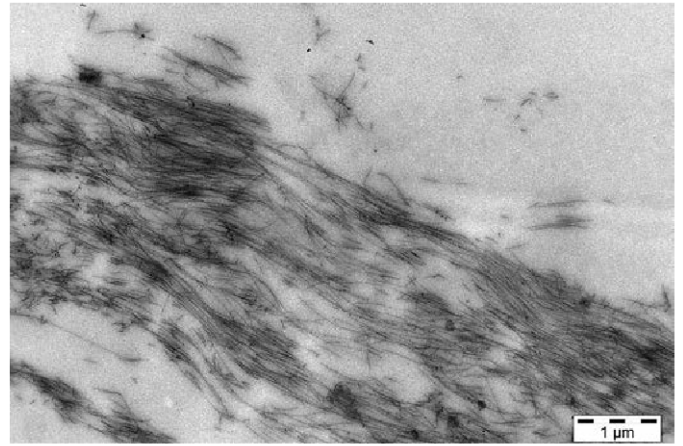


Fig. 9. Transmission electron micrograph. Native vitreous collagen detail. Original magnification $\times 6,300$.

and irregular nuclei and by the absence of finger-like protrusions (Fig. 7).

Pigmented or epithelial cells were not observed in any sample.

In 3 cases cells were leaning directly on the ILM with very few collagen fibrils interposed, while in 7 cases a layer of native vitreous collagen (NVC) and a minor quantity of newly formed collagen between the cells and the ILM was found (Fig. 8, 9).

Native vitreous collagen was composed by collagen fibrils with a diameter ranging from 10 to 16 nm and regular arrangement of collagen fibrils, whereas newly formed

collagen was constituted by fibrils with a more than 16-nm and irregular arrangement of collagen fibrils. In some samples a fibrous long spacing collagen, embedded in the NVC, was observed. The fibrous long spacing collagen consisted of broad bands of electron-dense material, which were interrupted by electron-lucent areas containing fibrillar material with a periodicity of 80–100 nm, being the typical periodicity of collagen fibers, usually 64 nm (Fig. 10). The ILM appeared as a layer of filamentous and amorphous material, ultrastructural features of basal laminae. In all the analyzed samples, the ILM was characterized by a smooth vitreous surface and an undulating

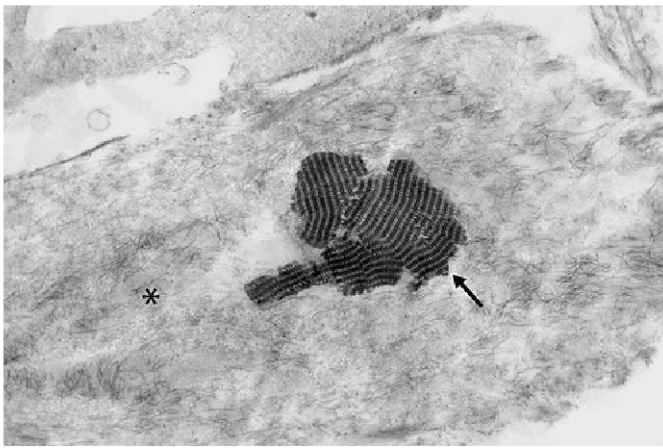


Fig. 10. Transmission electron micrograph. Fibrous long spacing collagen (arrow), embedded in the native vitreous collagen (asterisk). Original magnification $\times 5,000$.

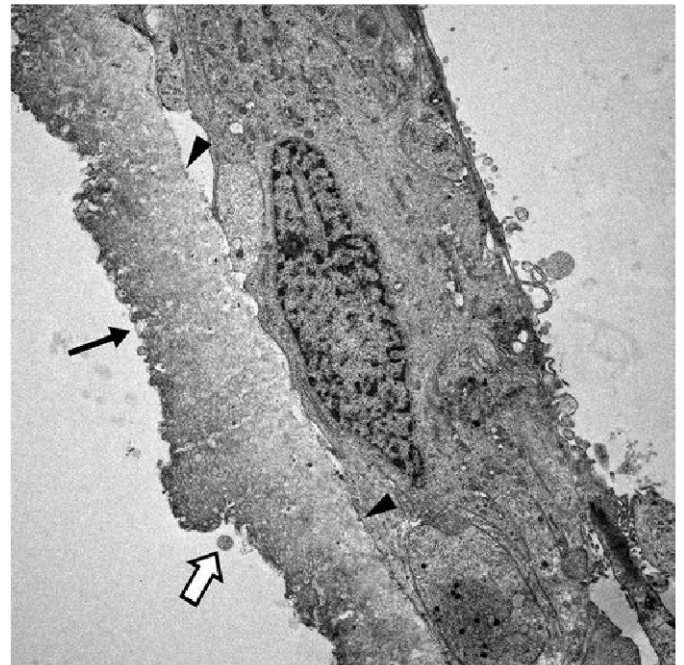


Fig. 11. Transmission electron micrograph. ILM characterized by a smooth vitreous surface (arrowheads) and an undulating retinal surface (black arrow). A small rounded cellular fragment (retinal debris) at the retinal surface of the ILM (white arrow). Original magnification $\times 1,600$.

Table 2. Ultrastructural features of idiopathic epiretinal membrane (ERM)

Case	ERM/ILM en bloc	Distribution of cells	Glial cells	Hyalocytes	Myofibroblasts	NVC	NFC	FLSC
1	+	monolayer	+	+	-	-	-	-
2	+	monolayer	+	-	+	+	+	-
3	+	monolayer	+	+	-	+	+	+
4	+	monolayer	+	+	-	+	+	+
5	+	monolayer	+	+	-	+	-	-
6	+	monolayer	+	-	+	-	-	-
7	+	monolayer	+	-	-	+	+	-
8	+	monolayer	+	-	+	+	+	+
9	+	multilayer	+	+	-	-	-	-
10	+	monolayer	+	+	-	+	-	-

ILM, internal limiting membrane; NVC, native vitreous collagen; NFC, newly formed collagen; FLSC, fibrous long spacing collagen.

retinal surface. Some small rounded cellular fragments, probably corresponding to retinal debris, were found at the retinal surface of the ILM (Fig. 11).

The morphological features of our samples are summarized in Table 2.

Discussion

The surgical removal of epiretinal macular membranes is the gold standard in the macular pucker surgery [13]. ILM removal has been shown to be beneficial in

terms of final visual outcome and reduction of ERM recurrence [6–9]. Gandorfer et al. [22] demonstrated that the ERM removal alone did not lead to a complete separation of the fibrocellular tissue from the macula. Several studies showed that ILM peeling was associated with a lower rate of recurrence, and ILM peeling was considered essential to remove the scaffold for further cell proliferation [5, 6, 13]. The intraoperative use of vital dyes for visualization of the ERM and ILM has been introduced to allow a complete and safe removal of the membranes during the surgery procedure [15–22, 32]. The purpose of the present study was to report on the combined use of TB and BBG for staining ERM and ILM during vitrectomy and to describe the histopathological findings.

In most of the samples analyzed using TEM, the ERM cells were separated from the ILM by a thick layer of NVC. This finding has already been found in previous studies supporting the hypothesis of Sebag [33, 34], who hypothesized that an abnormal posterior vitreous detachment was a possible cause of the ERM formation. According to Sebag, an abnormal split of the posterior vitreous cortex would occur with a forward displacement of the

vitreous body leaving the outer layers of the cortex, containing hyalocytes, attached to the macula.

Moreover, in some samples fibrous long spacing collagen embedded in the NVC was found. It has been considered an intermediate step of the fibrillar collagen degradation process, and it may represent a modeling process of the premacular cortex vitreous.

Similarly to other studies reported in the literature [35, 36], ERM cells of our specimens, both those separated from the ILM and those tightly attached to the ILM, appeared to derive from hyalocytes or glial cells and to a lesser extent from myofibroblasts. The ultrastructural analysis of Schumann et al. [35, 36] has already shown glial cells and hyalocytes as the main cellular population observed in the ERM. Kampik et al. [37] focused on the importance of glial cell and hyalocyte interactions in the ERM formation too.

In our study the combined use of TB and BBG appeared to be very useful intraoperatively to improve the visualization of the ERM and ILM, thus facilitating their easier complete removal during macular pucker surgery. PEG increases the molecular weight and viscosity of the dye, allowing for an effective tissue staining without any need of a prior fluid-air exchange. None of the surgical procedures required a fluid-air exchange to obtain an effective staining of the targeted area, avoiding fluid-air exchange-related complications. In addition, residual staining or dye remnants were not detected. No obvious structural damage related to the membrane peeling procedure was found during the 3-month follow-up. No complications relating to the use of the dye were observed both during and after surgery.

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Schumann et al. [36] reported that the structural evidence of retinal debris at the ILM can be used as an indicator for retinal damage. From the ultrastructural analysis of our specimens only some very small cellular debris were found on the retinal surface of the ILM, without any intraocular residual of dye on the underlying retina, thus suggesting a good safety profile of MembraneBlue-Dual™.

Only few studies in the literature reported the behavior and the efficacy of MembraneBlue-Dual™ in vitreoretinal surgery [31, 32]. In addition, there are no related histopathological findings.

Concerning clinical and histopathological findings of our preliminary study, the combined use of TB and BBG appears hopeful in macular pucker surgery.

This study has some limitations including the small number of specimens evaluated and the lack of a control group for comparison. Moreover, further studies are needed to better investigate MembraneBlue-Dual™ long-term safety and effectiveness. An immunohistochemical analysis may better identify the origin of the cells involved in ERM formation and its pathogenesis.

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Disclosure Statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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