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Article *in* Current Eye Research · January 2014

DOI: 10.3109/02713683.2013.865759 · Source: PubMed

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Current Eye Research, Early Online, 1–10, 2014

ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.3109/02713683.2013.865759

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MINI REVIEW

Vital Dyes in Ophthalmology: a Chemical Perspective

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ABSTRACT

Vital dyes have advanced diagnosis and surgical technique in various specialties, including oncology, gastroenterology and ophthalmology. Intra-operative and diagnostic dyes are finding uses in all areas of ophthalmology, including cornea, cataract, retina, glaucoma, orbit and conjunctiva. We provide a summary of current knowledge of the chemical concepts of vital dyes in ophthalmology. We review the properties of dyes, techniques of application, indications and complications in ocular surgery. Vital dyes represent an expanding area of research, and novel dyes deserve further investigation.

Keywords: Chromovitrectomy, indocyanine green, staining, trypan blue, vital dye

INTRODUCTION

Dyes are chemical compounds that bind to various substances in nature to induce color, increasing the visibility of them. Vital dyes are used in the coloration of living cells or other components of tissue, and emerged recently as important and effective surgical adjuvants to enhance visualization of ocular tissues.

In 1993, fluorescein was the first biocompatible dye used in an attempt to stain the anterior capsule by Hoffer and McFarland.[1] Since then, the use of vital dyes as an adjuvant in cataract surgery has been widely reported. Abrams et al.[2] reported in 1978 the first use of vital dye during vitreoretinal surgery and found fluorescein a great aid in vitreous identification. This technique was largely ignored for several decades; however, since 2000 chromovitrectomy has achieved widespread use.

In cataract surgery the blue dye trypan blue (TB) gained widespread use because it stains the anterior capsule and enables easier intra-operative removal of this fine, semi-transparent.[3] The use of vital dyes to stain pre-retinal tissues during vitreoretinal surgery, a procedure known as ''chromovitrectomy'', allows visualization of thin and transparent tissues, such as internal limiting membrane (ILM), epiretinal membrane (ERM) or the vitreous posterior surface. In vitreoretinal surgery, greening and bluish vital dyes such as indocyanine green (ICG) and Brilliant Blue (BriB) also facilitated visualization and removal of pre-retinal membranes as a result of their different affinities to intraocular collagen and cellular elements.[4–7] Vital dyes have also been used in corneal, glaucoma, orbit, strabismus and conjunctival surgery.

Reports in recent years have demonstrated the progressive experience with dyes, whereas laboratory studies have continued to explore the potential of new dyes. Preclinical investigation with reliable methods to analyze possible toxicity of dyes includes functional, histological and ultrastructural and biochemical analysis. Functional anterior segment analysis includes cell culture,[8] specular microscopy and confocal microscopy. Posterior segment analysis includes the use of retinal cell culture, electrophysiological tests and angiographic studies.[9–13]

However, unanswered questions remain regarding how to apply the dyes to achieve best results with less toxicity. This perspective presents the state-of-the-art

Received 22 July 2013; revised 3 October 2013; accepted 6 November 2013; published online 8 January 2014

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information about properties, chemistry, pharmacology and indications of vital dyes.

CHEMICAL CONCEPTS

When vital staining is done in a living organism, it may be called intravital staining. Almost all dyes are organic compounds of the aromatic series and are therefore derivate of benzene (C_6H_6). The six carbon atoms of benzene are joined to form a ring. In order to absorb visible light the aromatic rings must be part of a greater molecule, known as chromogen. The part of the chromogen responsible for the property of color is called a chromophore (Table 1).

The various dyes currently available may be classified according to their pH, solubility, source or staining property. Chemical structures determine the colors, properties and uses of dyes, and provide a rational basis of a classification of these compounds. The capacity of staining depends on many different factors, such as geometry and microtopography of the cells and tissues, or preparation of the specimen.

CHEMICAL GROUPS

Azo Dye

The azo group (–N=N–) is a large class of synthetic organic dyes that is formed when a diazonium ion (diazon component) reacts with either a phenol or an amine (coupling component). The azo bound allow visible light to be absorbed by the dyes. They can be chemically altered, resulting in an enormous range of structural variety commercially available. Most azo dyes have the benzene or naphthalene as the aromatic ring. The aromatic ring carries a wide range of substitute group that determine the color and the dying properties. Azo dye molecules can contain more than one azo linkage, and they can be segregated in groups of monoazo dyes (one azo linkage), and dis, tris and tetra azo dyes (two, three or four azo groups, respectively).

Trypan Blue (TB) is a large, very hydrophilic tetrasulfonated anionic azo dye with the formula $C_{34}H_{24}N_6Na_4O_{14}S_4$, and molecular weight of 960.79 Da. Synonyms names are Direct Blue 14, Diamine Blue 3B and Niagara Blue 3B. It has a large planar aromatic system with a lipophilic domain between sulfonated naphthyl end-units. The dye stains the nuclei of damaged and dead endothelial cells in donor corneas, as well as areas of Descemet Membrane (DM) denuded of endothelial cells. TB has been widely used in both vitrectomy and cataract surgery. It is commercially available in a 0.15% concentration for vitreoretinal surgery under the brand name Membrane Blue (DORC International, Zuidland, Netherlands) and as Vision Blue in a 0.06% concentration for cataract surgery (DORC International, Zuidland, Netherlands) (Figure 1). TB as Membrane Blue and Vision Blue comes in a solution containing small amount of sodium salts, 8.2 mg of NaCl, and water. The osmolality of Vision Blue ranges from 257 to 314 mOsm/kg and the pH from 7.3 to 7.6. Low doses of TB do not produce inflammation and corneal toxicity when injected into the anterior chamber.[14] Most studies with TB observed the absence of toxicity for the retina and the RPE.[15] A new use for TB is the visibility of edges of ruptures in surgery of rhegmatogenous retinal detachment.[16] To enhance the TB staining property, this blue dye may be injected into the posterior pole after fluid–air exchange or it may be mixed with glucose at 5–10% to create a heavy TB that is denser than balanced salt solution (BSS).[4,17] Mixing 0.3 ml TB with 0.1 ml glucose 10%, resulting in a 1 mg/ml (0.1%) solution and osmolality of 300 mOsm, is recommended. Current state-of-the-art TB usage recommends blue-dye application mainly for ERM staining.[18,19] Low doses of TB do not produce inflammation and corneal toxicity when injected into the anterior chamber, and no RPE defects or signs of retinal toxicity have been reported in most studies during ERM surgery.

Janus green B (JG) is a lipophilic cationic azo dye of chemical formula $C_{30}H_{31}N_6Cl$ with molecular weight of 511.06 Da. Synonym is Diazine Green S. The most important biological application is histologically to stain mitochondria in living cells. It binds to the disrupted cellular membrane and can be used in cornea for viability testing of yeasts and to assess corneal endothelial cell viability following a toxic insult. JG changes color according to the amount of oxygen present.

Arylmethane Dyes

Arylmethane dyes are so called because they are derived from methane, but in which some of the hydrogen atoms are replaced with aryl rings. They contain one carbon linked to two benzene or naphthalene groups bound to one moiety of N or O and one amino group (diarylmethane or triarylmethane). The variable substitution of rings in the amino group determines further sub classification of this group of dyes, with four recognized families: diarylmethane, aminotriarylmethane, hydroxytriarylmethane (Rosolic acids, Phthaleins and Sulfonphthaleins) and hydroxyaminotriarylmethane.

Gentian violet (GV) is a hexa-N-methylated water-soluble cationic amino triarylmethane dye with cation of moderate size and a slightly non-planar conjugated system. This purple dye has a molecular formula of $C_{25}H_{30}ClN_3$ and molecular weight of 407.98 Da. It is

TABLE 1 Summary of the chemical characteristcs of the dyes most used in ophthamology.

Group	Characteristic property	Dyes/comercial names	Molecular weight (Da)	Molecular formula	Osmolarity	Indication in ophthalmology
Azo	Benzene or Naphthalene as the aromatic ring	Trypan Blue (Membrane Blue; Vision Blue)	960.79	$C_{34}H_{24}N_6Na_4O_{14}S_4$	0.15% concentration for vitreoretinal surgery; 0.06% concentration for cataract surgery	Vitreoretinal and cataract surgery
		Janus green B/Diazine Green S	511.06	$C_{30}H_{31}N_6Cl$		Acess corneal endothelial cell viability
Arylmethane	Carbon linked to two benzene or naphthalene groups	Gentian violet	407.98	$C_{25}H_{30}ClN_3$	0.001–2%	Anterior capsule visualization and marker of the cornea and conjunctiva
		Bromophenol Blue	670	$C_{19}H_{10}Br_4O_5S$	0.02–2%	Vitreoretinal surgery and Anterior capsule visualization
		Patent blue (Blueron)	582	$C_{27}H_{31}N_2NaO_6S_2$	0.24%	Vitreoretinal and cataract surgery
		Brilliant Blue (Acid Blue or Coomassie Brilliant Blue, Brilliant Peel)	854	$C_{47}H_{48}N_3S_2O_7Na$	0.25%	Vitreoretinal surgery
		Light Green	792	$C_{37}H_{34}N_2Na_2O_9S_3$	10–20% in water, 0.2–4.0% in ethanol and insoluble in xylene	
		Fast Green	765.89	$C_{37}H_{34}N_2Na_2O_{10}S_3$	6% in water, 0.5% in ethanol	
Cyanine dyes	One or more methine group	Indocyanine Green	775	$C_{43}H_{47}N_2NaO_6S_2$	0.5%	Vitreoretinal and cataract surgery
		Infracyanine Green	775	$C_{43}H_{47}N_2NaO_6S_2$	0.5%	Vitreoretinal and cataract surgery
Thiazine dyes	One ring of four carbon, one nitrogen and one sulfur atom	Methylene blue	319	$C_{16}H_{18}C_lN_3S$		
		Toluidine blue	305	$C_{15}H_{16}N_3SCl$	1%	Ocular surface neoplasia
Xanthene dyes	Two aryl rings are bridged by an oxygen atom	Fluorescein Sodium	332.31	$C_{20}H_{12}O_5$	2%	Ocular surface, fluorescein angiography, Vitreoretinal and cataract surgery
		Rose Bengal	1017.64	$C_{20}H_2Cl_4I_4Na_2O_5$	0.5–1.0%	Ocular surface diagnostic
		Rhodamine 6G	442.54	$C_{28}H_{31}N_2O_3Cl$	0.0002–0.02%	Vitreoretinal and cataract surgery

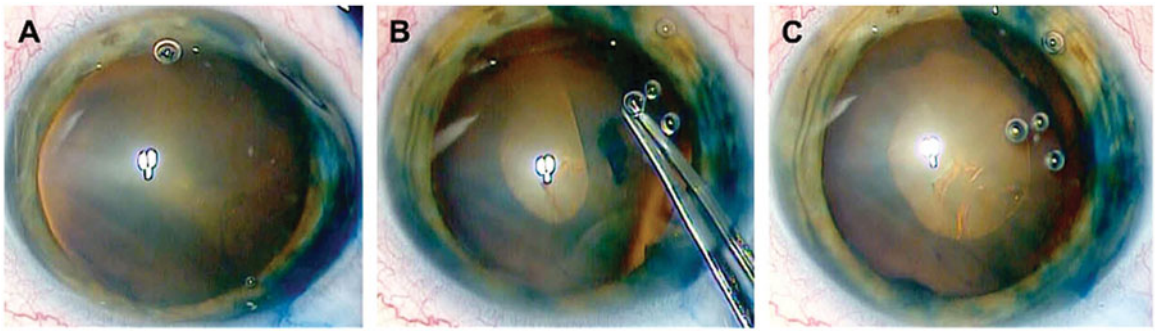

FIGURE 1 Intra-operative anterior capsule staining with trypan blue in cataract surgery. (A) Intracamerular injection of 0.06% trypan blue stains the anterior capsule greatly in a dark-bluish color. (B) The surgeon may identify the edge of the capsulorrhexis as the blue anterior capsule is contrasted to the uncolored lens cortex and nucleus. (C) At the end of the curvilinear capsulorrhexis maneuver, further steps may be executed during the emulsification surgery.

also known as crystal violet or methyl violet and changes from yellow to blue-violet according to pH value. In ophthalmology GV has been applied for anterior capsule visualization and as a marker of the cornea and conjunctiva at concentration of 2%.[20] GV is proposed to mark the peripheral stromal surface containing DM and endothelium in cases of DLEK, Descemet's stripping and automated endothelial keratoplasty.[21] Anterior capsule staining with GV in humans was first presented in 1998.[22] GV may be particularly advantageous for its low cost, but first its safety should be validated in a study with a larger cohort. Possible complications associated with the use of GV are corneal endothelium toxicity at concentration of 0.5% or higher.[23]

Bromophenol Blue (BPB), $C_{19}H_{10}Br_4O_5S$, is a hidroxy triarylmethane dye also known as bromphenol blue or tetrabromophenolsulfonphthalein. BPB has a molecular weight of 670 Da, maxima light absorption at 598 nm and color changing from yellow to blue between pH 3.0–4.6. Staining of the anterior capsule of lens in cataract surgery was demonstrated at concentration of 0.2% in enucleated porcine eyes.[24] BPB can be an alternative for chromovitrectomy, once BPB can promote enhanced ILM coloring and identification. Comparison of six biological stains (Light Green Yellowish, E68, Chicago Blue, Rhodamine, Rhodulinblau-basic and Rhodulinblau-basic 3) revealed that BPB stained the epiretinal membrane (ERM) and ILM better, and induced neither *in vitro* damage on ARPE-19 nor primary RPE-cell proliferation at concentrations of 0.2% and 0.02%.[24,25] Moreover, BPB at concentrations of 1% and 2% promoted enhanced ILM coloring and identification.[24,25] Pre-clinical studies revealed that BroB induced neither *in vitro* damage on ARPE-19 nor primary RPE-cell proliferation at concentrations of 0.2% and 0.02%. Further human clinical data should elucidate the best indication of BPB in chromovitrectomy and its safety in comparison with other current dyes available.

Patent Blue (PB) is a hydrophilic anionic triarylmethane dye with the chemical formula of $C_{27}H_{31}N_2NaO_6S_2$ and a molecular weight of 582 Da. Also known as Patent Blue Violet or Sulfan Blue, PB has its maximal absorption under 410 and 635 nm in water. This dye is orange under acid conditions and blue in alkali. PB has been certified in Europe since 2003 for capsule staining during cataract surgery in a concentration of 0.24% under the brand name of Blueron (Geuder) and has been applied off-label during vitreoretinal surgery.[26] Our clinical data[27] revealed that patent blue might be as an appropriate vital dye for coloring the glial ERM from various causes in a similar manner to trypan blue. There is conflicting data regarding the retinal toxicity of PB. In one study PB induced only mild and reversible retinal toxicity,[12] whereas RPE cells exposed *in vitro* to PB showed no toxicity.[27]

Brilliant Blue is a blue anionic aminotriarylmethane chemical compound with chemical formula of $C_{47}H_{48}N_3S_2O_7Na$ and molecular weight of 854 Da. Also named Acid Blue or Coomassie Brilliant Blue. Animal and human data on the use of BriB during vitreoretinal surgery and for anterior capsule staining have been described, resulting in its approval for intraocular use in Europe in 2007 under the brand name of Brilliant Peel (Fluoron/Geuder, Heidelberg, Germany). Anterior capsule visualization for capsulorrhexis can be accomplished with BriB injection in an isoosmolar solution at concentrations of 0.25 mg/ml or higher (Figure 2). No damage has been observed in rat eyes including apoptotic cell death or degeneration of corneal endothelial cells in the long-term observation period.[28] The safety profile of BriB in chromovitrectomy was investigated in preclinical experiments[29] with no significant retinal pathologic changes observed in animals with light and electron microscopy after low-dose BriB injection. BriB promotes appropriate ILM staining in an isoosmolar solution of 0.25 mg/ml for ERM and macular holes treatment in humans, and no clinical signs of toxicity have been observed in long-term

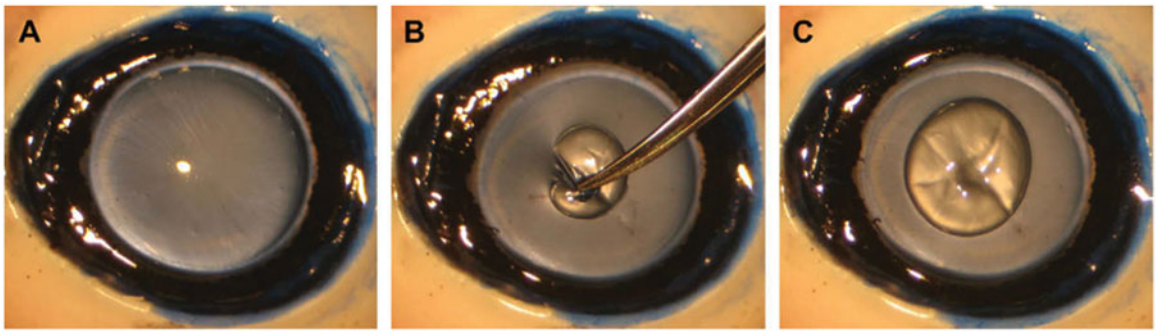

FIGURE 2 Anterior capsule staining with brilliant blue in porcine eyes. (A) Intracamerular injection of 0.5% brilliant blue may color the anterior capsule. (B) The capsulorrhexis may be performed after the purple-bluish stained capsule contrasted with the underlying unstained lens material. (C) The fast and successful capsulorrhexis enables the following steps of the cataract surgery.

follow-up period.[29,30] Novel solvents are being studied recently in combination with BriB in other to improve tissue coloration, such as addition of Deuterated Water (BriB+D_2O) and association of BriB and PB. Further studies should elucidate the role and safety of BriB in cataract and corneal surgery, and in long term follow-up no sign of toxicity was found in human eyes.[31]

Light Green (LG) has a molecular formula of $C_{37}H_{34}N_2Na_2O_9S_3$, and a molecular weight of 792 Da. It is soluble at 10–20% in water, 0.2–4.0% in ethanol and insoluble in xylene. LG is a diaminotriphenylmethane, with the amino groups both being benzylated. Routine LG staining includes collagen fiber stains in histopathology, especially Masson's trichrome, the Papanicolaou cytological polychrome stains and the Twort stain for microorganisms in tissue sections. Experimental studies showed safe profile of LG to stain the anterior capsule of lens, vitreous and ILM with satisfactory staining of these structures in the concentration of 0.5%.[32] LG showed no cell no toxic effect on ARPE-19 and primary RPE cell proliferation at concentrations of 0.2% and 0.02%.[24]

Fast Green (FG) has a molecular formula of $C_{37}H_{34}N_2Na_2O_{10}S_3$, is orange in acids, green under neutral conditions and deep blue in alkali. FG is soluble at 6% in water, 0.5% in ethanol and insoluble in xylene. FG is a diaminotriphenylmethane, both amino groups being benzylated. The very hydrophilic anion is large, with a large aromatic system, which is markedly non-planar due to an ortho-sulfonate substituent. Experimental studies showed safe profile of FG to stain the anterior capsule of lens and vitreous with satisfactory staining of these structures in concentration of 0.5%, but only faint retinal ILM staining.[32]

Cyanine Dyes

Cyanine dyes are part of a larger group called polymethine dyes, which has one or more –CH= (methine) group. Cyanine dyes are highly colored, organic compounds first synthesized over a century ago. They have been mostly used as sensitizers in photography or textile dyeing.

Indocyanine Green is a tricarbocyanine anionic vital dye with a molecular formula of $C_{43}H_{47}N_2NaO_6S_2$ and mass of 775 Da. The cyanine agent has amphiphilic properties that allow it to bind to both cellular and acellular elements in living tissues.[33] Maximum absorption is 775 nm in water and fluorescence maximums of ICG is within the near-infrared range at 835 nm. ICG may have affinity for the matrix components of the ILM, such as collagen fibers and laminin.[34] ICG is useful in retinal angiography, as it improves visualization of choroidal tissues. In ocular surgery its use remains off-label despite widespread popularity. ICG commercial products contain from 4% to 5% iodine, which represents both residues of the dye synthesis process and the iodine in the molecular formula. It is recommended that the green dye initially be diluted in distilled water before further dilution in saline solution, because of a higher risk of precipitation in saline.

Indocyanine green is as a useful agent for DLEK, as it may be used to stain the donor corneal stroma disk transplanted to the host anterior chamber, enhancing visualization of the tissue interface.[35] Another application of this dye is to improve anterior lens capsule visualization using concentrations ranging from 0.125% to 0.5%.[22] However, ICG staining of anterior segment tissues has not gained much popularity, and there is evidence showing that ICG is harmful to anterior segment structures.[33]

Clinical data showed that ICG-guided ILM peeling is facilitated in many diseases[36,37] (Figure 3), although potential clinical toxic effects of ICG on the retina are being suggested, and options to avoid toxicity are being studied.[38] The mechanisms of toxicity are unclear, but is postulated that ICG could migrate to subretinal space causing retinal damage,[39,40] RPE changes,[41,42] visual field defects,[43–47] and optic nerve atrophy.[5,6,48] Animal studies have evaluated the potential retinal toxicity of subretinal and intravitreal

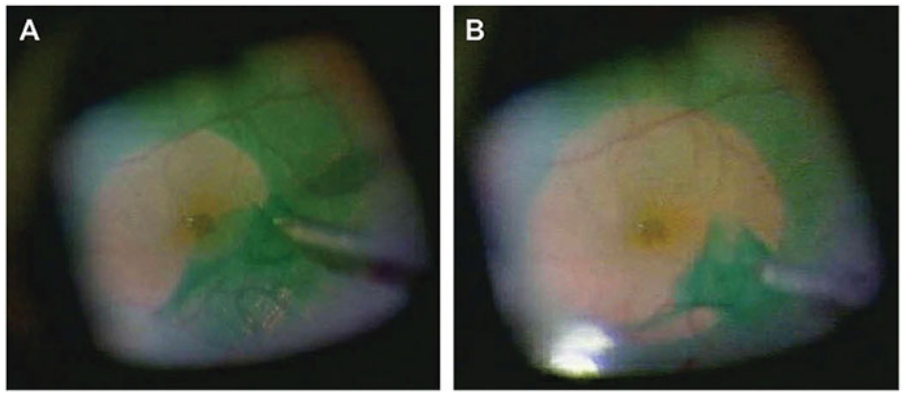

FIGURE 3 ILM-staining with ICG in macular hole surgery. (A) The green-stained ILM is easier to view and peel, since the green cyanine dye changes the biomechanics of the fine ILM. (B) Continuous removal of the ILM enables assurance that all antero-posterior and tangenctial traction is removed, although some dye may remain in the eye when the dye is injected onto the posterior pole.

ICG injections using ERG and histological findings to show a concentration-dependent retinal toxicity.[49] ICG also caused cytotoxicity to cultured human RPE cells,[42,50,51] retinal ganglion cells,[52,53] and Muller cells in a dose- and time-dependent manner *in vitro*.[54] Because of the numerous reports about ICG retinal toxicity and the existing alternative, ICG should generally not be used in retinal surgery. Some clinical side effects of ICG-assisted chromovitrectomy include RPE defects, visual field defects and optic nerve atrophy.

Infracyanine Green (IfCG) is a green dye with the same chemical formula and similar pharmacologic properties as ICG. IfCG dye possesses two pharmacologic differences when compared to ICG. First, IfCG contains no sodium iodine, which must be added to ICG during the dye synthesis. Second, the presence of the sodium iodine in the ICG solution necessitates dilution in water, resulting in a hypotonic solution of 248–275 mmol/kg. The iodine-free IfCG binds with high affinity to the acellular ILM, but not to epiretinal membranes (a cellular tissue).[55] Several clinical investigations have shown positive results with IfCG application with little or no retinal toxicity.[56,57]

Thiazine Dyes

Thiazine Dyes have a planar dibenzothiazine heterocyclic chromophore and are organic chemical compounds with one ring of four carbon, one nitrogen, and one sulfur atom that may generate various molecules that act as dyes, tranquilizers and insecticides. The thiazine dyes used in biology and medicine are always a small conjugated system, mostly strong bases and hydrophilic.[58] The presence of nitro or carbonyl substituents results in less basic dye.

Methylene blue (MB) is a heterocyclic aromatic diaminothiazine dye with molecular formula of $C_{16}H_{18}C_lN_3S$ and a molecular weight of 319 Da. The cation is weakly hydrophilic. MB is used in ophthalmology for guiding layered excision of certain cutaneous carcinomas, for the removal of orbit dermoid cysts and to facilitate identification of the adipose pocket during blepharoplasties. Severe corneal endothelial decompensation with bullous keratopathy was reported after unintentional use of MB for capsule staining during cataract surgery.[59]

Toluidine blue (ToB) is a metachromatic blue dye of chemical formula $C_{15}H_{16}N_3SCl$ and a weight of 305 Da that is used frequently for histologic staining. The use of 1% TB eye drops is an efficient method for the clinical diagnosis of ocular surface squamous neoplasia and premalignant lesions (Figure 4). Nevertheless, the intensity of the staining does not correlate with the degree of malignancy of these tumors.[60] In ophthalmic surgeries ToB toxicity limits its application intraocularly.[61]

Xanthene Dyes

The term xanthene is applied to a yellow organic heterocyclic compound of chemical formula is $C_{13}H_{10}O$. Xanthene dyes can be considered as derivate of diaryl or triarylmethanes in which two aryl rings are bridged by an oxygen atom. Fluorescein, eosin and rhodamine are derived from xanthene. Xanthene dyes fluoresce yellow to pink or bluish to red. This class of dyes is divided into various subgroups based on ionicity or lipophilicity/hydrophilicity.[62] The chromophore of aminoxanthene dyes are cationic and that hydroxyxanthenes anionic.

Fluorescein is a xanthene fluorophore with a weak acidic hydroxyxanthene, small size, chemical structure of $C_{20}H_{12}O_5$ and molecular weight of 332.31 Da. The vital dye in water has a very high fluorescence with an absorption maximum at 490 nm at pH 9 and excitation at 494 nm and emission maximum of 521 nm. Fluorescein may be conjugated with over 50 different salts or derivatives, including Fluorescein

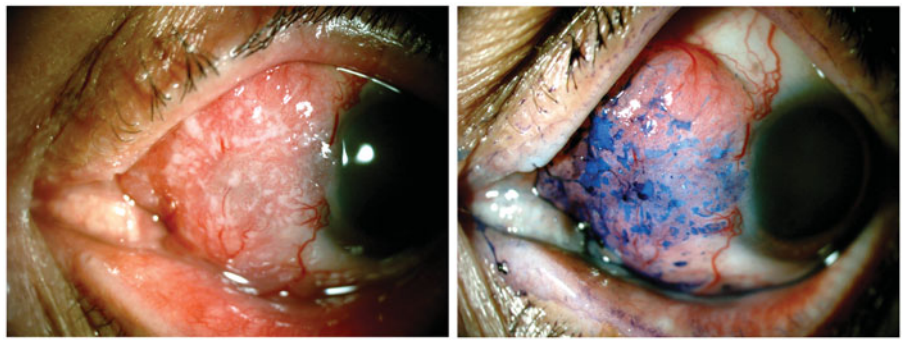

FIGURE 4 Clinical appearance Anterior Biomicroscopy of a patient with ocular surface squamous neoplasia without dye (left image) and evidencied with 1% Toluidine Blue (rigth image). The lesion is elevated with a gelatinous appearance. Note the feeder vessels.

Sodium (FS) and Fluorescein Diacetate (FD). In ocular surgery the xanthene compound has been shown to stain the vitreous gel either in the form of FS or FD.[63,64] Fluorescein is used extensively as a diagnostic tool, such as evaluation of the ocular surface and fluorescein angiography.

The primary purpose of FS staining in cornea is to detect epithelial defects and to assist in the diagnosis of erosions, corneal abrasion and keratitis as it only enters damaged cells at the ocular surface.[65] FS staining is a tool to evaluate the status of the precorneal tear film with respect to tear break-up time (BUT) and contact lens fitting, to evaluate tear volume and clearance, to measure corneal epithelial and endothelial permeability, and to detect aqueous humor flow and leakage. FS was first described to stain the anterior capsule in 1993.[1] The main use of FS in chromovitrectomy is vitreous staining, because of the capacity of improving visualization of clear vitreous fibers. In regards to the safety of FS, corneal endothelial cytotoxicity with FS in concentrations up to 10% have not resulted in loss of cellular integrity or disruption of organelles or cell lysis.[66]

Fluorescein Diacetate stains non-viable corneal endothelial cells.[67] Its uptake and metabolism by these cells is an active process, and duration of staining is only ~40 min.[68] Therefore, care should be taken to avoid false-negative results.

Rose Bengal (RB) is an acidic hydroxyxanthene of large overall size with chemical structure $C_{20}H_2Cl_4I_4Na_2O_5$, molecular weight of 1017.64 Da. RB has an absorption maximun of 548 nm in aqueous alkali and when excited in the green emits at 567 nm. In ophthalmology, RB is used in various ocular surface disorders since its first reported use on the eye in 1914.[69] RB in either 0.5% or 1.0% solution stains desquamated epithelial cells and evaluates keratoconjunctivitis sicca (KCS), epibulbar neoplasia, herpetic corneal epithelial dendrites and various forms of superficial punctate keratitis. Safety testing showed no toxicity to corneal cells, but damage on glial cells exposed to RB.[70]

Rhodamine dyes are aminoxanthene, which carries a carboxylic acid substituent on a pendant phenyl ring. Due to their good photochemical and photophysical properties, they are widely used in modern far-field optical microscopy and nanoscopy.[71] In acid solutions the dye forms a lopophilic cation, while is strongly alkaline solutions an anion is present. It is soluble in 2% in water and 1.8% in ethanol. In ophthalmology, the major used derivate is the Rhodamine 6G, with a molecular formula of $C_{28}H_{31}N_2O_3Cl$ and molecular weight of 442.54 Da. The absorption/emission spectral range is 530/556 nm, respectively, after the dye proper dilution. The intraocular toxicity of R6G is dose-dependent, and it has been studied *in vitro* in ARPE-19 cells by colorimetric testing, where it showed significant toxicity at doses of 0.2% and higher.[24] Although the use of this vital dye in ophthalmology is not frequent, the Rhodamine 6G, was shown to strongly stain the lens capsule,[24] and ILM in Rhesus monkey.[72]

Natural Stains

Alizarin Red (AR) is part of anionic anthraquinone dyes. It changes of color according to pH, from yellow to red in the range 3.6–6.5 and from orange to violet at pH 9.4–12. Considerable batch variation occurs with respect to colors and pH ranges. AR is soluble 7.7% in water and 0.2% in ethanol. One application of AR in microscopy includes counterstaining donated corneas after vital staining with trypan blue.

Lissamine Green (LG) is an aminoxanthene carrying two sulfonate substituents. Under neutral conditions this dye is a hydrophilic anion. Both the free acid form and the sodium salt are available. Its synonyms include acid green S, wool green S or C, and fast light green. LG is used as a caries detector dye with subsequent inspection of staining and observation of live cells. It is soluble in acetone, dimethylformamide, ethanol and methanol. LG has a staining profile nearly identical to that of Rose Bengal,[73] but the fist is significantly less irritating.[74] Given the better patient

tolerance and non-toxic effect of LG, it appears to be a better dye than RB in evaluating ocular surface disorders.[65]

Lutein ($C_{40}H_{56}O_2$) is a lipophilic pigment and based on its molecular contents, it belongs to class of the xanthophyll family (contains oxygen), one of the two major carotenoid families. Lutein contains 40 carbon atoms hence known as tetraterpenoids. The biochemical structure of lutein is in the form of an alternate conjugated double bonds and single bonds along the polyene chain terminated by oxygen containing rings on either side. The chemical reactivity of lutein is attributed to the presence of a conjugated polyene chain which is highly reactive and electron-rich system.[75] Upon oxidation the resultant lutein degradation products have the polyene chain with a varied length and end group such as aldehyde and ketone which might render these products as highly reactive compounds.[76] Together with zeaxanthin, both are considered dyes and are associated with the prevention of age related maculopathies, due to its antioxidant effect and their exclusive distribution in the macula.

FINAL REMARKS

The clinical importance of the ''vital dyes'' was not realized until in 1882, when fluorescein dye was used to outline breaks in corneal epithelium. Since then, numerous artificial dyes have been used to detect ocular surface epithelial pathology. Various dyes are used today, and each has unique properties that are beneficial for a specific use. There is general agreement that, in cataract surgery, vital dyes enable much better visualization of the anterior capsule, although some issues remain.[77]

Various dyes are used today in corneal diseases, and each has unique properties that are beneficial for a specific use. Rose bengal has the unique property of evaluating the protective status of the preocular tear film. Fluorescein penetrates intercellular space, and staining indicates increased epithelial permeability. Lissamine green B stains devitalized cells. Recent exploration into the interaction of different dyes to tear film layer and corneal epithelial cells have clarified the mechanism of actions of these dyes and enhanced the understanding of the underlying pathogenesis of various ocular surface disorders.

Chromovitrectomy facilitated the visibility of structures and pre-retinal membranes. ICG had an important role as a pioneer in the use of ILM for removal in chromovitrectomy, but more research is needed to assess its safety profile. The exact safety profile of different dyes in chromovitrectomy has not yet been established, and the current state-of-the-art chromovitrectomy should be performed using dyes at concentrations and volumes as low as possible. Future studies should clarify the safety and optimal indications of novel dyes in ophthalmology.

There are many dyes available in ophthalmology, and we believe that it is possible and necessary to investigate novel and specific vital dyes. The field of vital dyes offers great opportunities for research in ophthalmology.

DECLARATION OF INTEREST

The authors report no conflicts of interest

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