Tolerance of Micronized Mineral Pigments for Intrastromal Keratopigmentation: A Histopathology and Immunopathology Experimental Study

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Purpose: To study the tolerance and biocompatibility of mineral pigments for corneal pigmentation histopathologically and immunologically in an experimental animal model.

Methods: Manual intralamellar keratopigmentation was performed in 28 New Zealand white rabbits using micronized mineral pigments. Histopathological examination was performed 3 months after the surgery to determine the level of pigment diffusion, the level of inflammation, and the presence/absence of neovascularization. An immunological analysis was also performed.

Results: No pigment diffusion or changes in color, inflammation, or neovascularization were detected in the treated eyes. Histopathological examination corroborated clinical results regarding inflammation. Pigmented corneas showed a good cosmetic appearance without signs of ocular toxicity. From the immunological perspective, the pigments do not generate an inflammatory response in the rabbit cornea or in vitro.

Conclusions: Micronized mineral pigments could be a valid alternative treatment for corneal pigmentation. Manual intralamellar keratopigmentation technique presented good cosmetic appearance without adverse effects in the treated eyes.

Key Words: corneal tattooing, keratopigmentation, micronized mineral pigments, corneal histopathology, corneal immunopathology

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Keratopigmentation has proven to be effective in the cosmetic restoration of cosmetically disfiguring corneal scars.[1–3] It has also been demonstrated to be an alternative surgical option to correct problems such as glare, photophobia, and monocular diplopia because of iris loss, atrophy, or trauma[1–13] or in cases of disabling light scattering and photophobia caused by aniridia or iris coloboma,[1,7,12–14] showing that this procedure may not only be used in blind eyes for cosmetic but can also be used in sighted eyes for therapeutic purposes.[4–8]

Cosmetic contact lenses, enucleation, or evisceration are the most frequently methods used to improve esthetic appearance in cosmetically unacceptable, painful, and disabled eyes.[9–11] However, there is enough evidence on keratopigmentation to achieve good cosmetic results and adequate patient satisfaction, avoiding extensive and mutilating reconstructive surgery.[3–8,12–14]

Current keratopigmentation practice generally uses indiscriminate pigments and poorly systematized surgical techniques. This is probably the main reason why keratopigmentation, despite its potential applications, is seldom used today. With adequately investigated pigments, jointly from the histopathological tolerance, immune reactivity, and local toxicity perspective, we believe that this technique may offer important applications for therapeutic and cosmetic purposes.

The limited practice and experience in corneal tattooing is mostly because of the unavailability of reliable commercial products, and this is probably related to the lack of systematic studies demonstrating the corneal biocompatibility of these pigments. To our knowledge, there are no previous reports about this topic.

We have previously reported the tolerance and biocompatibility of micronized mineral pigments for keratopigmentation.[4–6,8,15–19] The aim of the present study is to ascertain the tolerance and biocompatibility of the pigments from the histopathological and immunological point of view.

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MATERIALS AND METHODS

Experimental Animal Model

This was a prospective, interventional, nonrandomized, controlled animal experiment using 28 eyes of 14 New Zealand rabbits (average weight 3 kg), 2 control eyes, and 1 pocket eye (in which the intrastromal pocket was created but no pigmentation was performed). Intrastromal corneal tattooing was performed using a manual intralamellar keratopigmentation technique. The study was performed in accordance with the guidelines set for the animal research investigation committees of our institution (Miguel Hernandez University, Spain) and in accordance with the standards of the Association for Research in Vision and Ophthalmology for the use of animals for experimental research (Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research).

Micronized Mineral Pigments

Four different colors of gamma-irradiated micronized mineral pigments were used. The colors used to mimic the natural color of the iris were blue, green, brownish blue, and brownish green (CE mark 0499; Biotec Phocea, France).

Corneal Pigmentation Technique

The animals were anesthetized with intramuscular injections of a 1:1 mixture of ketamine hydrochloride 20 mg/kg (Imalgene 1000; Merial, Lyon, France) and xylazine hydrochloride 4 mg/kg (Xilagesic 2%; Laboratorios Carlier, Barcelona, Spain). In addition, topical anesthesia was applied to the cornea (tetracaine 0.1% and oxibuprocaine 0.4%, Colircusi; Alcon Cusi S.A., Barcelona, Spain).

Manual intralamellar keratopigmentation, also called intralamellar corneal staining, was performed by the same surgeon in 27 eyes (1 eye was used as control). The center of the cornea was marked with a caliper and the pupil size determined by an RK optic zone marker (Katena, New York). One free-hand radial incision to midstromal depth was performed with a 45° knife from the limbus to the border of the marked pupil at the 12-o'clock position (Sharpoint; Surgical Specialties Corporation, Reading, PA). From the radial incision, the cornea was dissected intralamellarly in the same plane with a MiniCrescent knife (Sharpoint; Surgical Specialties Corporation) and then with a helicoidal dissector (pigtail or spiral corneal dissector CPK; Epsilon, Irvine, CA) 180° clockwise and 180° counterclockwise intrastromally and circumferentially along the route of the pupil margin (leaving the pupillary area intact).

The desired color was injected inside the intrastromal tunnel in 26 eyes with a 30-gauge irrigation cannula (except in the control eye and in the pocket eye, where the tunnel was created but no pigment was injected). No corneal sutures were used. Antibiotic prophylaxis was topically applied using Ciprofloxacin hydrochloride 3 mg/mL (Oftacilox, Alcon) and Chloramphenicol ointment (OftalmolosaCusi, Alcon) twice a day for 7 days. Cyclopentolate hydrochloride 10 mg/mL (Colircusi cycloplegic; Alcon) was also applied topically twice a day for 3 days. Additional prophylactic actions were taken to avoid infection using intraoperative antibiotic and gamma radiation of the mixtures as preoperative sterilization of the pigments.

Postoperative Experimental Clinical Evaluation

The follow-up period after the surgery was 3 months. The eyes were examined under a slit lamp by an independent observer. At each examination, the degree of inflammation was quantified using an anterior segment inflammation scale measuring conjunctival injection (0–3), corneal haze (0–3), corneal neovascularization (0–3), and epithelial defect (in millimeters). Other parameters documented included the presence of edema, pigment diffusion, or changes in the color and/or texture of the pigmentation (Table 1).

Histopathological Study

The histopathological analysis was performed by the Histology Unit of the Valencia University Pathology Department, Spain. The eyes were enucleated and placed in Davidson liquid for 48 hours and then placed in 70% ethanol until tissue processing. They were embedded in paraffin and sectioned in microslides with a microtome. Sections were stained with hematoxylin–eosin to determine the presence and distribution of inflammatory cells (lymphocytes, macrophages, and neutrophils) surrounding the pigment. Any inflammatory infiltrate was described as acute or chronic on the basis of its cellular composition. The presence/absence of neovascularization was also documented.

Morphometric Analysis of the Pigment Depth

The morphometric image analysis was performed using the program Image ProPlus 7.0 from Media Cybernetics. The pigment depth was measured in all cases.

Immunopathology Study

This analysis was performed using 2 different tests: leukocyte common antigen (LCA) immunostaining to prove the immunological tolerance of the pigments in the rabbit cornea; and second in vitro, using isolated human mononuclear cells mixed with different pigment colors, to observe if there was any inflammatory response.

TABLE 1. Anterior Segment Inflammation Scale

Clinical Examination		Histological Study	
Conjunctival injection	0–3	Inflammation	0–3
Corneal haze	0–3	Neovascularization	0–3
Neovascularization	0–3	Pigment diffusion	Yes/No
Epithelial defect	Yes/No		
Pigment diffusion	Yes/No		
Changes in color	Yes/No		

LCA Immunostaining

This type of staining reveals the presence of leukocytes in the treated cornea. LCA is a type I transmembrane protein that is present in various forms in all differentiated hematopoietic cells except erythrocytes and plasma cells that assists in the activation of those cells (a form of co-stimulation). It is expressed in lymphomas, B-cell chronic lymphocytic leukemia, hairy cell leukemia, and acute nonlymphocytic leukemia. In our study, rabbit bone marrow was used as a positive control to compare the positive and the negative staining results. One histological slide of each rabbit was examined and stained with this marker and compared with the bone marrow of the rabbit.

In Vitro Study Using Isolated Human Mononuclear Cells

Isolated human mononuclear cells were obtained from human blood using ficoll. This is a hydrophilic polysaccharide used to separate blood in layers after centrifuging, where monocytes and lymphocytes form a buffy coat layer just under the plasma layer. This buffy coat contains mononuclear cells.

On the other hand, the pigments were also prepared and 2 mixtures were prepared:

1. Control: 1 mL of balanced salt solution (BSS) + 175 μL of black pigment.
2. Experimental compound: 1 mL of BSS + 50 μL of Brown pigment + 50 μL of White pigment + 25 μL of Blue pigment + 50 μL of Green pigment.

The buffy coat containing the mononuclear cells was mixed separately with the pigment mixtures A (control) and B (experimental compound) in a culture medium for 16 hours to observe if there was any inflammatory response of these cells. This inflammatory response was determined by analyzing the production of the cytokines by BD Cytometric Bead Array. The cytokines measured were tumor necrosis factor (TNF) alpha, interleukin (IL-8), IL-6, and IL-1b.

RESULTS

Histopathological Study

The corneal epithelium was intact, with no hyperplasia or atrophy in the 28 eyes; normal thickness was maintained (between 3 and 5 cells). Bowman membrane was not examined because rabbits do not have this structure despite the great similarity with the human eye. Keratocytes around the pigment were larger than elsewhere with an eosinophilic cytoplasm containing small thin pigmented granules. The nucleus was discretely bigger with increased chromatin and small or nonidentifiable nucleolus. There was no fibrosis in the corneal stroma, and collagen fibers maintained their wavy shape. Type I collagen was predominant followed by type III.

Stromal extracellular matrix remained intact. Macrophages were not found, and a defined, continuous, and homogeneous layer of pigment was found in the midstroma of all eyes, with no signs of pigment diffusion. The cohesiveness of the pigment was demonstrated, although in some areas it formed denser clusters, which did not affect the final cosmetic appearance. The edges of the pigmented areas were clearly defined, without dispersion. Peripheral corneal nerves were normal. There were no histological changes in the endothelial layer or Descemet membrane in tattooed or control eyes.

Hematoxylin–Eosin Staining

Microscopic examination using hematoxylin–eosin staining revealed blackish bands, parallel to the corneal surface, of variable length and thickness, which seem to be located between the stromal collagen lamellae (Figs. 1A–D). Sometimes, pigment granules are also observed inside some stromal cells, which can be located at different distances from the pigmented area.

Because of the strong black discoloration of the pigments seen microscopically, it is not possible to secure the intra- or extracellular location, but in our opinion, it is mainly located extracellularly between the collagen fibers, and probably inside cells (fibroblasts or stromal keratocytes), which sometimes have an increased size.

Regardless of the color used intrastromally, all pigments showed the same blackish color microscopically. Only in samples pigmented blue or bluish brown, small precipitates with blackish blue were observed (Fig. 1D). No inflammatory cells were observed (lymphocytes, macrophages, or neutrophils) after stromal pigmentation. The absence of neovascularization was also documented in all cases.

Masson Trichrome Staining

Regarding the organization of the collagen fibers, no major changes were seen in their lamellar arrangement using the Masson trichrome staining (Fig. 2A). However, the lamellar collagen pattern could be altered in some areas (Fig. 2B, arrow). These specific changes in the collagen lamellar arrangement were seen near the pigmented area. It was common to observe several "swirls" in some histological sections, which may have irregular lines and pigmentation spots inside (Fig. 2B). No pigment diffusion was observed in any case.

Morphometric Analysis

Morphometric analysis was also performed to determine the exact location of the pigment expressed as a percentage, relative to the entire thickness of the cornea, as compared with stromal thickness. A representative picture of the measurements performed is shown in Figure 3. The morphometric analysis showed variable pigment depth in each case, probably related to the fact that the technique used was manual intrastromal keratopigmentation (compare Fig. 1A and B).

Immunopathology Study

LCA Immunostaining

Nonspecific immunostaining was not observed in the histopathological section of any rabbit cornea. It is possible to appreciate in Figure 4A that the staining is negative, concluding that the pigments did not arouse any inflammatory reaction in this preparation, whereas in the rabbit bone marrow used as a positive control, immunoreactive cells were detected (arrows in Fig. 4B).

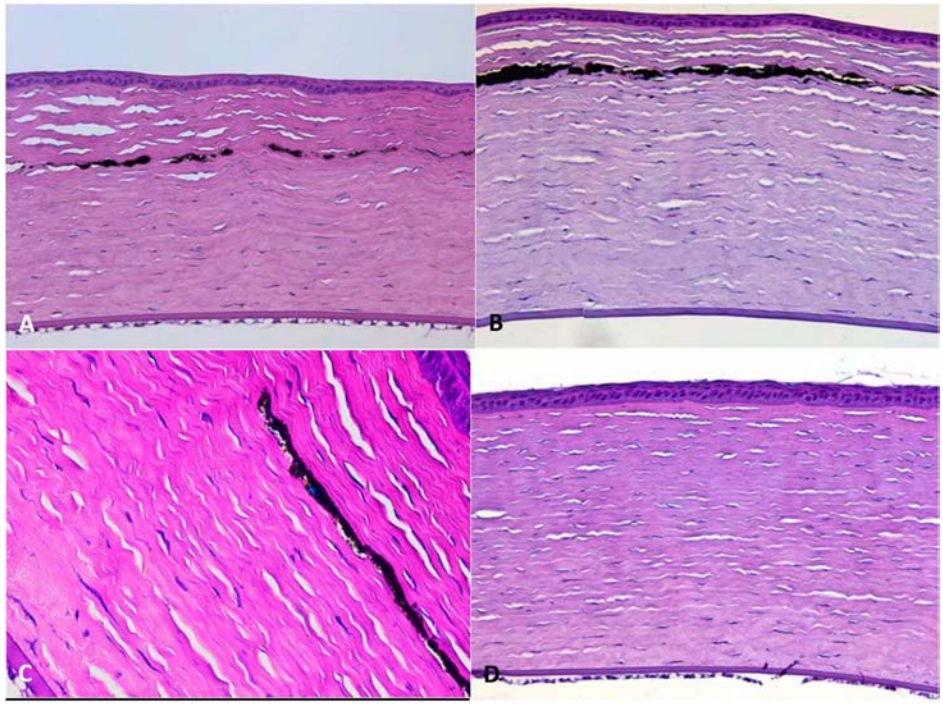

FIGURE 1. A–D, Hematoxylin–eosin staining. A–C, Blackish bands, parallel to the corneal surface, of variable length and thickness, which seem to be located between the stromal collagen lamellae. D, Hematoxylin–eosin staining of a control eye.

In Vitro Study of Pigments and Isolated Human Mononuclear Cells

The result of this study is observed in Figure 1. As mixture A (control) did not initiate an inflammatory reaction in vivo, it was observed, as expected, that the mixture B (different pigment colors) did not initiate an inflammatory reaction in vivo.

The cytokines measured were TNF alpha, IL-8, IL-6, and IL-1b. There were no TNF alpha, IL-6, and IL-1b cytokines detected in the experimental mixture B. The only cytokine detected in the problem mixture was IL-8, and the level of response (pg/mL) was much lower than that in the control mixture.

Experimental Clinical Evaluation

The degree of inflammation was quantified using an anterior segment inflammation scale. One week after the procedure, 7 eyes had a minimal degree of conjunctival injection (grade 1: in 6 eyes, grade 2: in 1 eye), 5 eyes had an epithelial defect coinciding with the radial incision area, 3 eyes had corneal haze (grade 1). The eyes were also examined to detect the presence of neovascularization from the limbus toward the center of the cornea, and no cases with such characteristics were found during the clinical follow-up. The health of these 28 eyes was satisfactory during the first and second month. The favorable postoperative status was also observed in the last review 3 months after the surgery (Fig. 5). By that time, all corneas were clear, even in those in which no pigment was applied or pocket eyes. The pigmented corneas showed a good cosmetic appearance without changes in color or dispersion of the pigments.

DISCUSSION

Keratopigmentation has proven to be effective in the cosmetic correction of cosmetically disfiguring corneal scars.[1–3,16–19]

In the present investigation, the histopathological examination has revealed pigmented granules to be present

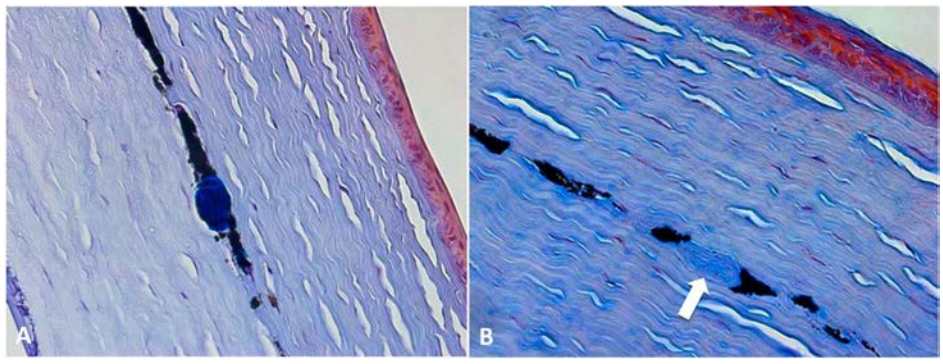

FIGURE 2. A, Masson trichrome staining showing no major changes in the collagen lamellar arrangement. B, Masson trichrome staining showing "swirls" in some histological sections (arrow).

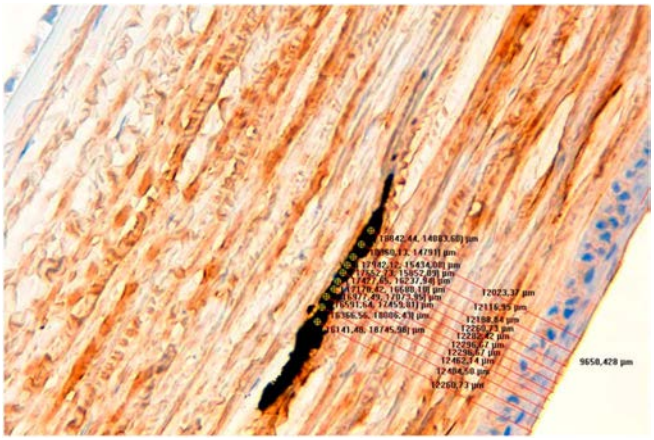

FIGURE 3. Image analysis. Representative picture of the measurements performed to determine pigment depth. Program used: Image ProPlus 7.0.

inside the keratocytes when the pigment used is a nonmetallic dye, unlike what occurred in patients treated with metallic pigments.[1] It is known that keratocytes can actively ingest and retain particles of nonmetallic dyes in their cell membranes for a very long period of time.[1] Corneal fibroblasts can ingest injected ink particles by endocytosis in a period of 3–4 days, and those particles can be stored for at least 6 months.[20] In previous reports, and in accordance with these experimental findings, India ink particles have been described to be clinically present, both intracellular and extracellular, as observed in the present experimental investigation, in some patients.[21]

Ultrastructural studies comparing metallic pigments with nonmetallic pigments demonstrated the presence of intracellular granules inside the keratocytes in the nonmetallic pigment and intracellular and extracellular granules after the metallic pigment tattoos with platinum chloride.[1,22] As per its observation, the absence of extracellular particles in nonmetallic tattoo suggests that endocytosis of organic substances by the human corneal fibroblasts is more permanent and stable than the endocytosis of metallic dyes, which easily are found located extracellularly. Thus, there are differences between human corneas and other species such as the rabbit concerning the intracellular or extracellular location and distribution of the pigments according to their chemical profile, either metallic or nonmetallic.[1]

Phagocytosis by fibroblasts is the consequence of the corneal protective reaction from injuries and damages caused by nontoxic foreign materials.[20] Assuming that metallic corneal pigments are more toxic than organic ones, it is reasonable to expect a larger amount of cellular debris in the extracellular matrix of the cornea when using metallic corneal tattooing substances.[20] This particular histopathological characteristic could also induce low-grade inflammation, which is relevant as it may be followed by subsequent softening and melting of the corneal stroma, as has been previously described.[23]

In this investigation, we have confirmed that corneal tattooing using micronized mineral pigments as the ones used

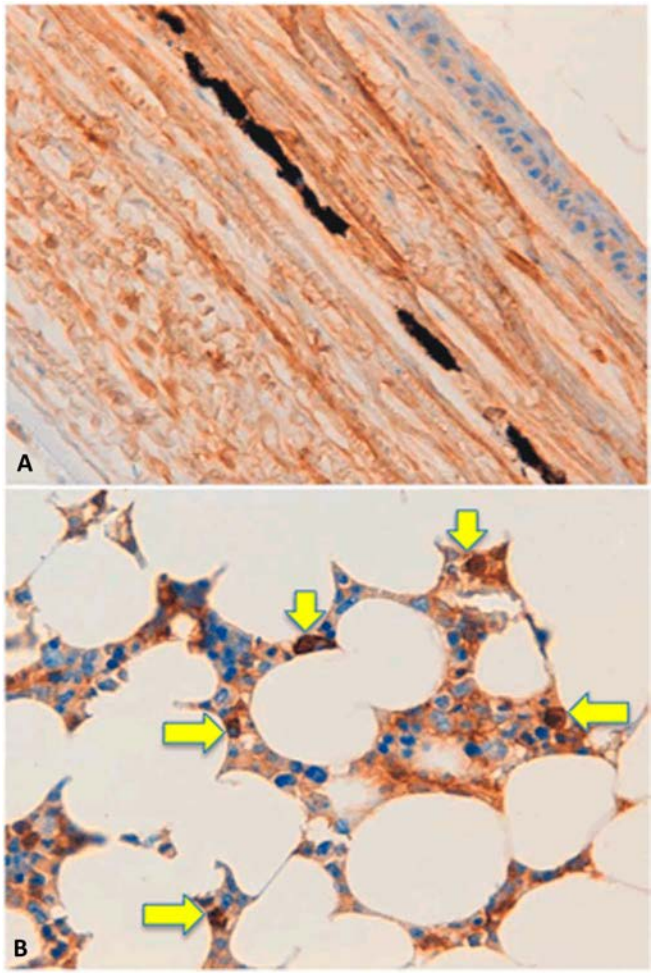

FIGURE 4. A, Negative LCA immunostaining. No inflammatory reaction is observed. B, Positive LCA immunostaining in the rabbit bone marrow, used as a positive control. Immunoreactive cells were detected (arrows).

in this experimental animal model is a safe procedure. From the histopathological perspective, our results show that the used pigments do have corneal tolerance and biocompatibility, not showing evidence of local toxicity and confirm previous experimental reports published on this topic.[15–19] We believe that the keratopigmentation technique may offer important applications from therapeutic and cosmetic perspectives, as suggested by previous reports. This is also, to the best of our knowledge, the first study that demonstrates the coincident histopathological and immunopathological in vitro and in vivo tolerance of the experimental cornea to these pigments.

Concerning the surgical technique, the intrastromal keratopigmentation technique maintains the integrity of the corneal epithelium basement membrane. Damage to this basal membrane, whether mechanical, chemical, or traumatic, is considered a potential cause of recurrent corneal epithelial erosions.[24] If the corneal epithelium is removed leaving an intact basement membrane, the epithelium regenerates within 48 hours and strong adhesion to the underlying stroma is

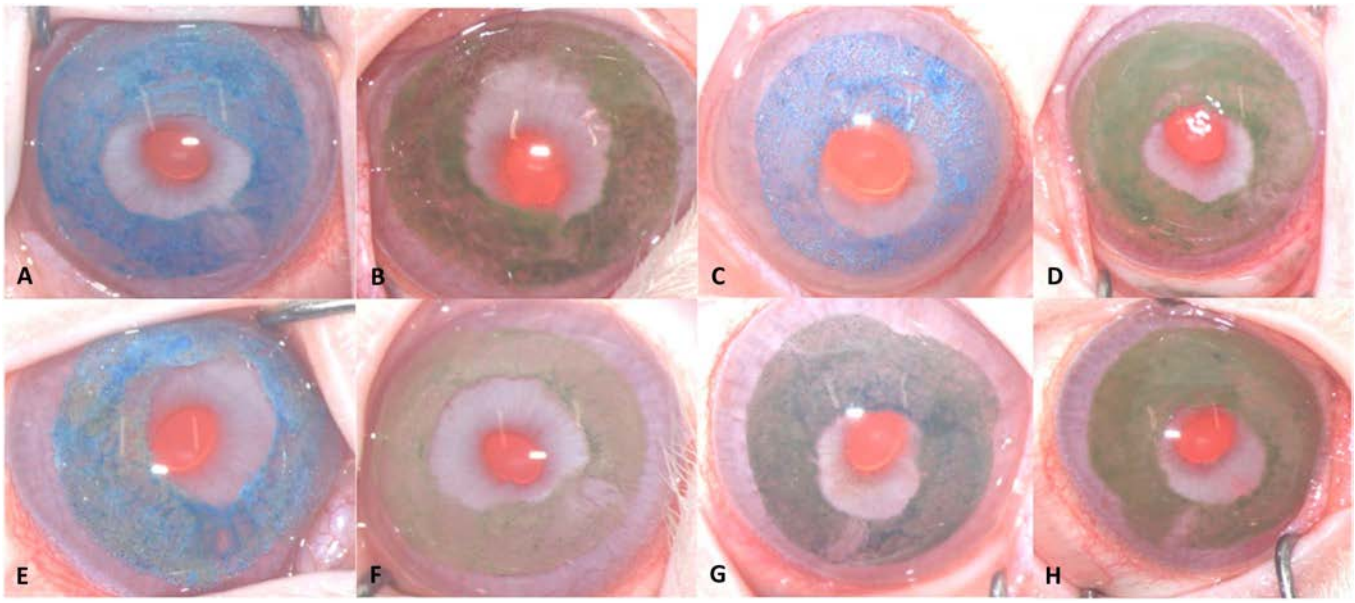

FIGURE 5. Clinical appearance after keratopigmentation with different colors. A and C, Blue. B and D, Green. E and G, Brownish blue. F and H, Brownish green.

achieved in 7 days. However, if the basement membrane is also coincidentally removed, then epithelial adhesion requires up to 8 weeks.[24] In all treated eyes in this investigation, we observed no complications associated with the surgical technique and probably this is because, as per the surgical technique used, it did not affect the basal membrane. The corneal architecture was not affected and the intrastromal tunneling technique was shown to be advantageous in this regard, not only because the epithelium was not affected but also because the pigment was retained in the tunnel, avoiding dispersion and reducing its inflammatory potential, not contacting the ocular surface, providing good results for both cosmetic and therapeutic use. This finding may have important clinical implications and explain the lack of complications found in previous clinical reports on this topic.[4–6,15–19,25]

Femtosecond-assisted keratopigmentation has also been shown to be a useful technique to create the lamellar pockets for keratopigmentation and to facilitate the clinical procedure in the human eye, achieving predictable configuration and distribution of the pigments in the stroma.[4,6,7]

Micronized mineral pigments present an additional advantage over other natural pigments because their particle size is reduced by micronizing procedures. The small particle size diminishes the chances of developing a foreign body reaction against the pigment intruded into the corneal stroma.[15,17–19] Corneal pigments used in the past caused some complications.[2] There were no experimental studies like the one reported here to show their safety. Therefore, experimental studies, such as the one reported herein, are necessary to avoid those problems before the general use of a pigment of any chemical profile in clinical practice. Some of the complications described for corneal tattooing are underpigmentation, overpigmentation, discoloration, pigment migration, accidental perforation, healing problems, and uveitis.[26] These complications were not observed in our experimental study. There were no local signs of toxicity, and we believe that pigment components have no toxic potentials.

Another major advantage of the micronized mineral pigments is the wide range of colors available.[17–19] In this study, we have used several colors (Figs. 1, 2) and no inflammatory response was detected histopathologically or immunologically. This is very convenient in the clinical use of keratopigmentation as the main clinical goal is to accurately mimic the natural color of the patient's eye to obtain the best possible cosmetic result.[26] The mixing of different pigments to obtain a matching color can make keratopigmentation a laborious and time-consuming procedure. Probably in the future, micronized mineral pigments as the ones used in this investigation might be mixed and prepared in monodose vials adequately sterilized and ready to use during the surgery.[17,18]

The safety of the technique, tolerance, and biocompatibility of the intrastromally located micronized mineral pigments used in keratopigmentation have been demonstrated experimentally and for the pigments here used, from the clinical, histopathological, and immunological point of view. Although intrastromal keratopigmentation technique has shown good results in this study, the conclusions of this study should be limited to the intrastromal use of the pigments described here. In therapeutic keratopigmentation, not all corneal scars may be amenable to intrastromal lamellar pigmentation and might require different or more superficial techniques to cover the affected area. Further investigations to ascertain the safety of the pigments used for keratopigmentation when superficial techniques are used warrant further attention.

In conclusion, no evidence of stromal inflammation or neovascularization was found in corneas colored using the micronized mineral pigments used in the present investigation

when they were injected intrastromally. Such findings further confirm that adequately selected pigments injected intrastromally are a viable alternative for cosmetic and therapeutic uses of keratopigmentation techniques and confirm recent clinical reports on this topic.

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