Enhanced oligonucleotide delivery to mouse retinal cells using iontophoresis

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Purpose: To study the combination of oligodeoxynucleotides (ODNs) intravitreous injection and saline transpalpebral iontophoresis on the delivery of ODNs to photoreceptors in the newborn rd1/rd1 mice.

Methods: Cathodal or anodal transpalpebral iontophoresis (1.43 mA/cm² for 5 min) was applied to eyes of postnatal day 7 (PN7) rd1/rd1 mice immediately before the intravitreous injection of ODNs. The effect of cathodal iontophoresis after ODNs injection was also evaluated. The influence of current intensity (0.5, 1.5, and 2.5 mA) was assayed with cathodal iontophoresis performed prior to ODNs injection. The duration of current-induced facilitation of ODNs delivery to photoreceptors was evaluated for 6 h following iontophoresis. One group of control eyes received cathodal iontophoresis prior to the intravitreous injection of phosphate buffered saline (PBS) or hexachlorofluorescein (Hex). The second control group received ODN or Hex intravitreous injection without iontophoresis. The penetration of fluorescent ODNs in the outer nuclear layer (ONL) was quantified by image analysis of the ONL fluorescence intensity on cryosection microphotographs. Integrity of ODN was assessed using acrylamide gel migration after its extraction from the retina of treated mice. The integrity of retinal structure, 1 and 24 h after iontophoresis, was analyzed using light and electron microscopy.

Results: Transpalpebral anodal or cathodal saline iontophoresis enhanced the penetration of ODNs in all retinal layers. Cathodal iontophoresis was more efficient than anodal iontophoresis in enhancing the tissue penetration of the injected ODN. Photoreceptor delivery of ODN was significantly higher when cathodal saline transpalpebral iontophoresis was applied prior than after the injection. The extent of enhanced tissue penetration decreased in parallel to the increased interval between iontophoresis application and the intravitreous injection. Current of 1.5 mA was safe and optimal for the delivery of ODNs to the ONL. One hour after iontophoresis followed by injection, ODN extracted from the retina of treated eyes remained intact. Histology and electron microscopy observations demonstrated that iontophoresis using the optimal parameters did not induce any permanent tissue alterations or structure damage.

Conclusions: Saline transpalpebral iontophoresis facilitates the penetration of injected ODNs in photoreceptors for at least 3 h. This method may be considered for photoreceptor targeted gene therapy.

Direct iontophoresis enhances the intraocular levels of locally applied drugs, both in experimental models and in patients [1-9]. Different types of devices have been designed to apply the current on the cornea, the sclera, or both, with drug application on the eye surface in containers of various forms and materials. This type of iontophoresis procedure can be qualified as “direct ocular iontophoresis” [1-9]. More recently, direct ocular iontophoresis has also been used to enhance the intratissue and intracellular penetration of oligonucleotides (ODNs) [10-13]. The mechanisms of drug penetration facilitation by iontophoresis include electrorepulsion, electroosmosis and current-induced tissue permeation [14]. Post-ioniophoretic transport of drugs has been described in the skin and results from tissue changes that may persist for a limited period of time after current application [15]. In order to study the penetration of ODNs into photoreceptor cells of newborn rd1/rd1 mice eyes, we have evaluated another procedure which associates electric current application at the eye surface using a saline transpalpebral iontophoresis with intravitreous injection of ODNs. Various conditions of iontophoresis (anodal versus cathodal, current intensity, and time between injection and current application) were evaluated.

METHODS

Animals: C3H/HeN mice homozygous for the nonsense mutation (amino acid position 347) in the β-PDE gene (Janvier, Le Genest, France) were used (36 mice). Mice were maintained in clear plastic cages and subjected to a 12 h:12 h light-dark cycle. Experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmologic and Vision Research and the institutional guidelines regarding animal experimentation in Ophthalmic and Vision Research.

Oligonucleotide: ODN was synthesized and purified by high pressure liquid chromatography by Proligo (Paris, France). A 25 mers phosphorothioate ODN, encoding for the
sense wild type β-PDE gene sequence (25-6x6PS-WTS, 5'-CsCsTsTsCsCsAACCTACGTAGCAAsAsAsAsAsG3') and 5' labeled with hexachlorofluorescein (Hex), was used for histological evaluation of ODN distribution.

**Iontophoresis and intravitreous injection**: Eyelids of PN7 rdl/rdI mice were opened with a scalpel (Swann Morton, Peynier, France) under topical tetracaine 1% drops anesthesia (Novartis Ophthalmics SA, Rueil Malmaison, France).

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Figure 1. Penetration of oligonucleotides to retinal cells at 1 h after injection of labeled oligonucleotides with or without prior saline iontophoresis. A: PN7 rdl/rdI eye section after intravitreal injection of labeled oligonucleotide (ODNs) without prior saline iontophoresis. B: High magnification of retina from Panel A. C: PN7 rdl/rdI eye section after intravitreal injection of Hex without prior saline iontophoresis. D: Eyeglasses shaped electrode made with aluminum foil and single-use disposable medical grade hydrophilic polyurethane sponge. E: Iontophoresis generator. F: Mouse showing the active eyeglasses shaped electrode and the return electrode connected to the mouse’s neck (arrow). G: PN7 rdl/rdI eye section after intravitreal injection of labeled ODNs with prior cathodal saline iontophoresis. H: High magnification of retina from Panel G. I: PN7 rdl/rdI eye section after intravitreal injection of Hex with prior cathodal saline iontophoresis. J: PN7 rdl/rdI eye section after intravitreal injection of phosphate buffered saline with prior cathodal saline iontophoresis. K: Corresponding phase contrast of Panel J. L: Higher magnification of retina from Panel J. M: Corresponding phase contrast of Panel L. Insets show high magnification of the correspondent picture. ONL represents outer nuclear layer; INL represents inner nuclear layer; GCL represents ganglion cell layer. Scale bars represent 1 mm in A,G,J,K (x2.5), 100 µm in B,C,H,I,L,M (x25), 0.5 cm in D,F, 2 cm in E, and 10 µm in the insets.
Transpalpebral iontophoresis system (patent number FR2830766) was used. Eye glasses shaped aluminum foil and disposable medical grade hydrophilic polyurethane sponge (3.2 mm thick, 1.5x0.7 cm length by width, Optis, Levallois, France), were soaked in phosphate buffered saline (PBS: 0.2 g/l KCl, 0.2 g/l KH₂PO₄, 8 g/l NaCl, 2.16 g/l Na₂HPO₄·7H₂O, pH 7.4) and used as the active electrode (Figure 1D). The electrode covered both closed eyelids of the treated newborn mouse and the return electrode was connected to the mouse’s neck (Figure 1F). An audiovisual alarm indicated any disruption of the electric circuit, ensuring a controlled delivery of the current.

Intraocular injections were carried out with ES TransferTips microcapillaries (Leica, Rueil Malmaison, France) and cut at 2 mm from their extremity, leading to a 60 µm injecting hole. Microcapillaries were linked to a Micro4™ microsyringe pump controller (World Precision instruments, Sarasota, FL). One µl of ODN (500 mM) was injected into the vitreous at a constant pressure of 200 nl/s. The position of the needle was monitored by observation with a dissecting microscope through a glass cover slip placed on the corneal surface. To limit loss of the injected solution and allow the intraocular pressure to equilibrate (as observed by the return of normal iris perfusion), the micropipette needle was left in place for 10 s after the injection before withdrawal.

Treatment protocols: The ODN distribution was evaluated 1 h after the end of the procedure. Anodal or cathodal transpalpebral saline iontophoresis (respectively, positive or negative electrode connected to the eyelids) was performed with a current of 1.5 mA for 5 min (1.43 mA/cm²) before the intravitreous injection of ODN (4 eyes for each experiment). Cathodal iontophoresis was then tested when applied immediately after the intravitreous injection of ODNs (4 eyes). The results derived from this initial set of experiments showed that cathodal iontophoresis performed prior to the intravitreal injection led to the highest ODN penetration in the ONL. This condition was therefore used to evaluate the effect of lower current intensity (0.5 mA for 5 min) or higher current intensity (2.5 mA for 5 min) as well as the duration of current-induced permeation by injecting ODN at various times after iontophoresis (acute, 1, 3 and 6 h, 4 eyes for each experiment). With the optimal conditions defined, the kinetics of fluorescent ODN distribution was evaluated at 1, 6, and 24 h after treatment (4 eyes for each condition). Control animals received intravitreous injection of 1 µl Hex (500 mM, Invitrogen, Cergy Pontoise, France) or PBS with or without previous cathodal iontophoresis (4 eyes for each condition). As additional controls, and to check the integrity of ODN after treatment, 2 mice received intravitreal ODN injection with previous iontophoresis (n=4) and sacrificed at one hour after treatment. ODN integrity, extracted from the retina, was evaluated on acrylamide gel migration. Injections were performed under topical tetracaine 1% drops anesthesia.

Mice were sacrificed by an intraperitoneal lethal dose of pentobarbital (6 g/100 ml; Ceva Santé Animale, Libourne, France). Their eyes were enucleated and processed for the various tests as described below.

Evaluation and quantification of fluorescent oligonucleotide distribution: At various times after treatment (1, 4, 6, 8, and, 24 h), the eyes were enucleated, rinsed in PBS and embedded with Tissue-Tek optimal cryotechnique compound (Bayer Diagnostics, Puteaux, France) for cryo-sectioning. Sections (10 µm) were fixed in 4% paraformaldehyde (Merck Eurolab, Strasbourg, France) for 5 min at room temperature. They were then washed in PBS, counterstained for 2 min with 1:3000 DAPI (Sigma-Aldrich, Saint-Quentin Fallavier, France), and then washed again in PBS, before being mounted in Gel Mount (Microm Microtech, Francheville, France). For localization of labeled ODN, sections were examined under a fluorescent microscope (Aristoplan, Leica), and images were captured using a digital SPOT camera (Optilas, Evry, France) with a constant exposure time of 3 s for 2.5 times magnification.
tion) or 0.8 s (for 25 times magnification).

Five sagittal sections throughout each eye were photographed for fluorescence quantification (three pictures per section, n=15 values for each eye). All pictures were taken with a 25x objective and a similar exposure time of 0.8 s. Intensity of the outer nuclear layer (ONL) fluorescence was quantified by using the luminosity feature of Photoshop. Tissue and background regions were manually selected. Mean pixel brightness was determined for each region by using the “Histogram” imaging feature. Differences in background levels among images were controlled by dividing the mean brightness level per pixel of the tissue by region the background region from each section image.

Oligonucleotide integrity: Neural retinas were dissected 1 h after intravitreous injection of Hex-labeled ODNs with prior cathodal saline iontophoresis (1.5 mA for 5 min, n=4). Pools of two retinas were placed in 500 µl of digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS) containing 0.5 mg/ml of proteinase K and incubated for 90 min at 56 °C. After incubation, the DNA samples were extracted once with phenol (1:1 v/v) and once with chloroform/isoamylalcohol (1:1 v/v; 24:1 v/v). The samples were then extracted once with isobutanol (1:2 v/v) and once with diethyl ether (1:1 v/v), precipitated with ethanol/ammonium acetate (1:2 v/v; 1:3 v/v), dried, resuspended in 20 µl TE buffer (10 mM Tris, pH 8; 1 mM EDTA, pH 8) and treated with DNAse1. Ten µl of each sample was denatured with 2.5 µl formamide (90% formamide, 0.05% bromophenol blue) and then run for electrophoresis on 12% denaturating polyacrylamide gel. Fluorescent Hex ODNs were visualized and photographed (Typhoon, Amersham).

Structure analysis of the retina: For structure analysis of the whole ocular globe, eyes treated with cathodal 1.5 mA iontophoresis for 5 min prior to intravitreous injection were studied. One, 6 and 24 h after the intravitreous injection, mice were sacrificed; their ocular globes were enucleated, fixed and studied (4 eyes for each condition). Cryosections at the optic nerve level were counterstained with hematoxylin and eosin, examined using a photonic Aristoplan microscope (Leica, Rueil Malmaison, France), and photographed using a digital SPOT camera (Optilas). In order to assess potential tissue changes associated with the treatment procedure, transmission electron microscopy (TEM) was performed. For this purpose, 4 eyes received iontophoresis followed by ODN injection, another 4 eyes received ODN injection without iontophoresis, and 4 other eyes received iontophoresis alone. At 1 and 24 h after treatment (2 eyes per time) the mice were sacrificed, their eyes enucleated and fixed in 2.5% glutaraldehyde of cacodylate buffer (Na 0.1 M, pH 7.4). After 1 h, globes were dissected at the limbus, the posterior eyeball post-fixed for 3 h and cut in four parts. Tissues were post-fixed in 1% osmium tetroxide in cacodylate buffer (Na 0.1 M, pH 7.4) and dehydrated in graduated ethanol solution (50, 70, 95, 100%). The tissues were then included in epoxy resin and oriented. Semithin sections (1 µm), obtained with an ultramicrotome Reichert Ultracut E (Leica), were stained by toluidin blue. Ultrathin sections (80 nm) were contrasted by uranyl acetate and lead citrate and observed with an electron microscope JEOL 100CX (JEOL, Tokyo, Japan) under 80 kV.

Statistics: Results were expressed as means±SD and compared using the analysis of variance (ANOVA) test with post hoc Fisher test. A p<0.05 was considered significant.

![Figure 3: Analysis of the iontophoresis intensity on the penetration of oligonucleotides.](image)

Figure 3. Analysis of the iontophoresis intensity on the penetration of oligonucleotides in the outer nuclear layer. Relative fluorescent intensities in outer nuclear layer (ONL) were represented by histograms expressed as mean±SD (vertical bars). Fluorescence in the ONL shows a significant increase of intensities when using cathodal saline iontophoresis at 1.5 mA as compared to cathodal saline iontophoresis at 0.5 mA (*p<0.05).

![Figure 4: Analysis of the iontophoresis effect duration on the oligonucleotides delivery to the outer nuclear layer.](image)

Figure 4. Analysis of the iontophoresis effect duration on the oligonucleotides delivery to the outer nuclear layer. Cathodal saline iontophoresis was performed immediately, 1 h, 3 h, or 6 h before the intravitreous injection of labeled oligonucleotides (ODNs) in PN7 rd1/rd1 mice. One hour after the injection, the penetration of ODNs in outer nuclear layer (ONL) was quantified. The relative fluorescent intensities in ONL were represented by histograms expressed as mean±SD (vertical bars).
RESULTS

Enhanced oligonucleotide delivery to retinal cells of rd1/rd1 mice: One h after the intravitreal injection of Hex-labeled WTS ODN in rd1/rd1 PN7 mice, without applied current, fluorescence was observed in the nuclei of the ganglion cell layer (GCL) and in the most superficial nuclei of the inner nuclear layer (INL; Figure 1A,B, inset). No fluorescence was detected in the ONL (Figure 1A,B). One hour after intravitreal injection of the fluorochrome (Hex) alone, diffuse fluorescence was observed in the vitreous and in cells of the INL but without specific accumulation in the cell nuclei (Figure 1C, inset).

When saline iontophoresis (Figure 1D-F) was applied and immediately followed by injection of the labeled ODN, intense fluorescence was observed in all retinal layers (Figure 1G,H). Specific localization to the retinal cell nuclei was seen in these cases (inset Figure 1G). When saline iontophoresis was applied and followed immediately by the injection of the fluorochrome (Hex) alone (without ODN), diffuse fluorescence was observed in all retinal layers (Figure 1I). However, this fluorescence was not accumulating specifically in the cell nuclei (inset in Figure 1I). No fluorescence was observed in the retinal layers of PBS-treated eyes (Figure 1J,L) or non-injected control eyes (with or without saline iontophoresis, data not shown; 4 eyes for each condition).

ODN integrity was confirmed by acrylamide gel electrophoresis of the DNA extracted from retinas of treated eyes 1 h after iontophoresis followed by injection (data not shown).

Effect of iontophoretic parameters on oligonucleotide penetration: We have chosen to perform iontophoresis prior to ODN injection in order to limit the risk of infection and of potential reflux of ODNs from the globe by mechanical pressure of the probe on the eyelids. Cathodal or anodal iontophoresis (1.5 mA for 5 min) prior to the intravitreal injection of ODNs showed that the application of current enhanced ODN penetration when compared to injection without iontophoresis (p<0.05) or no treatment (Figure 2). Furthermore, prior cathodal saline iontophoresis significantly enhanced ODN penetration in the ONL cells when compared to anodal saline iontophoresis (p<0.001). Performing cathodal saline iontophoresis immediately prior to the intravitreal ODN injection significantly enhanced the ODN penetration in ONL when compared to application of cathodal iontophoresis immediately following ODN injection (p<0.001). From all tested conditions, cathodal iontophoresis performed immediately prior to ODN injection yielded the highest ODN penetration in the ONL of treated mice eyes. The latter condition was therefore used to evaluate further parameters.

Figure 5. Penetration of oligonucleotides to retinal cells at different timea after injection with prior saline iontophoresis. Hex-labeled oligonucleotides (ODNs) in red of PN7 rd1/rd1 eye sections at 1 h (A), 4 h (B), 6 h (C), 8 h (D) and, 24 h (E) after treatment (cathodal saline iontophoresis immediately prior to intravitreous injection). DAPI staining in blue of Panels A-E is shown in corresponding middle Panels F-J. Double staining with DAPI in blue and Hex-labeled ODNs in red is shown in corresponding lower Panels K-O. Inset shows a high magnification picture of the outer nuclear layer (ONL) with double staining. INL represents inner nuclear layer, GCL represents ganglion cell layer. Scale bars represent 100 µm in A-E (x25); the scale bar in the inset represents 10 µm.
Effect of current intensity: Iontophoresis with 0.5 mA significantly decreased the ODN penetration in ONL when compared to 1.5 mA (p<0.05), showing that ODN penetration in the ONL depends on the amount of applied current (Figure 3). Increasing the current intensity to 2.5 mA (for 5 min) induced electric skin burns and pain (data not shown). This condition was therefore not further studied.

Duration of postiontophoretic facilitation of ODN penetration: The postiontophoretic enhancement of ODN penetration decreased with the increased interval between iontophoresis and injection. Facilitation of ODN penetration in the ONL was highest with the shortest interval, during at least 3 h, and vanished when ODN injection was performed 6 h after saline iontophoresis (Figure 4). These results demonstrate that the iontophoresis facilitation of intraretinal penetration is temporary.

Kinetics of oligonucleotide distribution in the retina: Using optimal treatment parameters (cathodal iontophoresis 1.5 mA for 5 min prior to intravitreous injection), we observed most intense ONL fluorescence 1 h after the ODN injection, decreasing rapidly at later times (Figure 5). Indeed, already at 4 and 6 h after treatment, we noted that fluorescence was decreasing in the outer retina (Figure 5B,C). At 8 h, ODN fluorescence remained only in the GCL (Figure 5D), and no fluorescence is observed in the neuroretina at 24 h (Figure 5E). This kinetic suggests that Hex-labeled ODNs may be rapidly degraded after their retinal penetration.

Light and electron microscopy observations: The ocular gross histology structure was not affected by the iontophoresis application. No lesion or cell damage was detected at 1 h (Figure 6A,D), 6 h (Figure 6B,E), or 24 h (Figure 6C,F) after treatment.

Analysis of semi-thin sections revealed that 1 h after application of saline iontophoresis, internuclear spaces within the INL and ONL were increased. Linear enlargements can be followed from the outer rows of the INL up to the external limiting membrane. This localization is suggestive of retinal Müller glial cell (RMG) prolongations. Note that nuclei in the INL and the ONL have normal structures and do not show any signs of apoptosis or necrosis (Figure 7D,E). No such changes were observed in untreated control retinas or in eyes without current application (Figure 7A-C). Twenty four h after iontophoresis application, internuclear spacing was no longer observed and the ONL had regained a normal architecture (Figure 7G).

TEM analysis showed that in eyes receiving injection without electric current applied or in untreated control eyes, the retina retained a normal structure without any detectable changes (Figure 8A,B). Eyes analyzed 1 h after iontophoresic current application demonstrated enlargement of RMG prolongations (Figure 8C, arrow) with normal integrity of the photoreceptor nuclei. At 24 h after iontophoresis, treated eyes were found to no longer have enlargement of RMG prolongation (Figure 8E). Thus the observed RMG changes induced by the electric current were temporary and it was evident that no permanent ultra-structure change was induced by the optimal iontophoresis parameters used in this study. Particularly, no alteration of photoreceptors could be detected.

DISCUSSION
Our results show that the application of transpalpebral saline iontophoresis enhances ODN penetration into photoreceptors.
Figure 7. Semithin eye sections at different times after treatment. Semithin section of control untreated PN7 rd1/rd1 eye section (A). PN7 rd1/rd1 eye section at 1 h after intravitreous injection of labeled oligonucleotide (ODNs) without prior saline iontophoresis (B) or with prior cathodal saline iontophoresis (D). Higher magnifications showing retina structures from eyes shown in Panels B,D are shown in Panels C,E, respectively. PN7 rd1/rd1 eye section at 24 h after intravitreous injection of labeled ODNs without prior saline iontophoresis (F) or with prior cathodal saline iontophoresis (G). Arrow represents vacuoles. RPE represents retinal pigment epithelium cells; ONL represents outer nuclear layer; INL represents inner nuclear layer. Scale bars represent 50 µm in A,B,D,F,G (x25) and 25 µm in C,E.
of newborn mice eyes. The facilitation and enhancement of penetration were associated with the intensity of the current. In this study, we determined that a 1.5 mA (1.43 mA/cm²) current applied for 5 min is efficient and safe for newborn mice eyes. No structural damage of the treated eyes was observed, and the normal architecture of the retina was preserved using these iontophoresis parameters in newborn (PN7) mice. For direct ocular iontophoresis, the active electrode is in contact with the drug solution and electrorepulsion is thought to facilitate the penetration of drug in ocular tissues [16]. However, electrorepulsion is responsible only for a part of the current effect, as facilitated diffusion of noncharged molecules can also be achieved [17]. Electroosmosis is another mechanism of drug penetration acting through a flow process (vol/distance/time). Electroosmosis-induced drug penetration is particularly important for larger molecules [14,18,19]. Increased “passive” permeability for a limited period of time after the application of current was also observed in the skin [15,20]. In these experiments, it was demonstrated that the slow recovery of skin impedance following iontophoresis was due to the movement of ions in response to electric field and that the resulting postiontophoretic enhanced-diffusion was not associated with damage to the skin barrier [21]. Application of saline iontophoresis before the local instillation of phenylephrine increased the observed vasoconstriction effect of this drug [22]. Most of the studies elucidating the different mechanisms of iontophoresis drug penetration and facilitation have been conducted on the skin. The results obtained by our present study show that saline iontophoresis also influences the permeability of intraocular tissues to charged molecules such as ODNs. It is not clear why iontophoresis prior to intravitreous injection is more efficient than if the current is applied after injection. One hypothesis is that when iontophoresis is applied after intravitreous injection, potential extraocular diffusion of the injected ODNs due to the mechanical pressure may be responsible for this observed phenomenon. Additional experiments using different conditions and parameters are being carried out to provide more clues regarding these results.

Figure 8. Ultrathin eye sections observed by transmission electron microscopy at different times after treatment. Ultrathin sections of control untreated PN7 rd1/rd1 eye section from ONL (A,F) to RPE (K). PN7 rd1/rd1 eye sections at 1 h after intravitreous injection of labeled oligonucleotides (ODNs) without prior saline iontophoresis, from ONL (B,G) to RPE (L) or with prior cathodal saline iontophoresis, from ONL (C,H) to RPE (M). PN7 rd1/rd1 eye sections at 24 h after intravitreous injection of labeled ODNs without prior saline iontophoresis, from ONL (D,J) to RPE (N) or with prior cathodal saline iontophoresis, from ONL (E,I) to RPE (O). Arrow represents vacuoles. RPE represents retinal pigment epithelium cells; IS represents inner segments; ONL represents outer nuclear layer. Scale bars represent 5 µm.
The ODNs used in this study were negatively charged and had a molecular weight of 7591 g/mol. These characteristics allow them to penetrate through the internal limiting membrane. However, the exact mechanisms responsible for the transport from the inner retina to the photoreceptor cells remain poorly understood. Such transport is probably highly regulated and does not follow passive diffusion. Simple direct vitreous injection of ODNs does not lead to their penetration into photoreceptor nuclei. On the other hand, when saline iontophoresis is performed with the intravitreal injection of ODNs, labeling of the ONL is observed. This labeling may reflect internalization. In our study, the only changes observed in the retina using semithin histology were enlarged linear spaces observed from the outer rows of the INL up to the external limiting membrane at one hour after saline iontophoresis. The localization of these spaces may indicate that changes have occurred in the retina in certain ocular diseases. Bull Soc Ophtalmol Fr 1965; 102:1825-9.


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