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Journal of Pharmacological and Toxicological Methods-Original article

A novel in vivo corneal trans-epithelial electrical resistance measurement device

Running title: A novel corneal TER measurement device

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The authors have no funding or competing conflicts of interest to report.

Abstract

Purpose: To develop a device that is capable of easily measuring corneal transepithelial electrical resistance (TER) and changes in the corneal barrier function.

Methods: We had previously developed an in vivo method for measuring corneal TER using intraocular electrode. This method can be used to precisely measure the decline of the corneal barrier function after instillation of benzalkonium chloride (BAC). In order to lessen the invasiveness of that procedure, we further refined the method for measuring the corneal TER by developing electrodes that could be placed on the cornea and in the conjunctival sac instead of inserting them into the anterior chamber. TER was then calculated by subtracting the electrical resistance, which lacked the corneal epithelial input, from the whole electrical resistance that was measured between the electrodes. Slit lamp examination and scanning electron microscopy (SEM) were used to determine safety of the new device. Corneal TER changes after exposure to 0.02% BAC were determined using the new device as well as SEM and transmission electron microscopy (TEM).

Results: Slit lamp examination before and after exposure of rabbits' corneas to the sensor confirmed safety of the device. SEM examination revealed no difference of the corneal epithelium which exposed to the new device with normal corneas. SEM and

TEM pictures revealed damaged microvilli and tight junctions after instillation of 0.02% BAC. TER change after treatment with 0.02%BAC was similar to those determined by the established anterior chamber method.

Conclusion: We succeeded to develop a less invasive device for corneal TER measurement in vivo in animals. This new device may be applicable in the future for clinical use in humans.

Introduction:

The cornea is one of the few human tissues that always in direct contact with the environment. This fact, together with its transparency, makes the cornea a very special tissue. In particular, the corneal epithelium, which is the outer part of the cornea, acts as a barrier against the daily insults of the external environment. Moreover, and to ensure its transparency, the cornea does not have blood vessels for its nourishment. Nutrients are supplied by diffusion through the epithelium and endothelium layer, ensuring a proper homeostasis (Kinoshita et al., 2001).

Corneal epithelium tight junctions are the most apical intercellular junctions, and play an important role in the establishment and maintenance of the barrier function (Miyoshi and Takai, 2008: Vandenbrouche et al., 2008). Disruption of corneal epithelial barrier function results in ocular irritation (Pflugfelder et al., 2005: Yokoi and Kinoshita, 1995), and increased risk for microbial infections (Alarcon et al., 2011).

The electrophysical properties of a cell or tissue can be determined by passing an electric current through the cell or tissue and measuring the voltage drop and potential difference across the tissue. When the current delivered and the voltage measured were known, the resistance of the tissue can be calculated using Ohm’s law: resistance is equal to the voltage divided by the current (Fukuda and Sasaki, 2012).

The majority of ophthalmic drugs contain adjuvants such as surfactants and preservatives. They are often essential for producing ocular liquid formulation, which solubilize drugs and prevent microbial contamination. Some of these adjuvants act as ocular penetrating enhancers promoting drug penetration through the strong corneal barrier and modifying the physiochemical property of drugs (Saettone et al., 1996: Sasaki et al., 1999). At the same time, however, they destroy the corneal epithelial structure, especially the transcellular integration of superficial cells, which is mainly maintained by tight junctions (Burstein, 1980: Tonjum, 1975). Therefore, investigation of the effect on the cornea by ophthalmic drugs and adjuvants is important.

Corneal transepithelial electric resistance (TER) is maintained by corneal superficial cells with tight junctions between them, which together function as a corneal barrier that is highly resistant against invasion. Measuring changes in TER allows the quantitative and continuous evaluation of the state of the corneal epithelium and its barrier function. The method is suitable for electrophysiologically evaluating corneal toxicity induced by ophthalmic drugs (Donn et al., 1959: Potts and Modrell 1957).

Measurement of corneal TER is a suitable method for evaluating corneal permeability and irritancy quantitatively and continuously. TER reflects the barrier function of the epithelium. Lower corneal TER means more electrical current penetrates through the

damaged superficial cells and tight junctions between them. In addition, it is reported to be a very sensitive test for measuring electrical properties of the cornea (Chetoni et al., 2003: Nakashima et al., 2008).

In vitro experimental systems using cultured cells are often employed to evaluate the barrier function of the corneal epithelium as well as injuries of the corneal epithelium caused by various drugs (Fukuda and Sasaki, 2012: Pellinen and Lokkila, 2009: Yee et al., 2006). These experimental systems provide an excellent means for the objective comparison of the potential for cornea injury among several ophthalmic solutions; however, the extent to which these experimental models reflect the condition of the eyes in vivo remains unclear (Denoyer et al., 2008).

However, studies of the corneal electrical properties performed in *vivo* conditions are quite limited as, only few reported works are available (Biermann et al., 1991: Jurgens et al., 1996: Watanabe et al., 1993), revealing the difficulty of performing these analyses. The last approaches to perform *in vivo* measurements have adapted the existing TER measurement methods for being implemented in living animals (Chen et al., 2012: Fukuda and Sasaki, 2012).

Previously we developed a method of measuring the TER of live rabbit cornea. In that method, the cornea is not damaged by the experimental procedure and the TER is stable

before drug administration. To measure corneal TER, we used a volt-ohm meter which generates ±20uA AC square wave current at 12.5 Hz. Therefore, it was able to measure TER every 0.08s. In addition, TER was monitored with a recorder, which shows TER changes continuously. To our knowledge, no other study has evaluated acute corneal change within few second in vivo (Uematsu et al., 2007). However, the invasiveness of that procedure by inserting one electrode inside the anterior chamber makes it impossible for being used in clinical practice. In order to overcome that problem, in this study we recently developed a less invasive corneal TER measurement method. In previous studies, we demonstrated that benzalkonium chloride (BAC) concentrations between 0.005% and 0.02% immediately caused acute corneal barrier dysfunction (Kusano et al., 2010: Uematsu et al., 2007).

So, our aim in this study is to evaluate acute corneal permeability change after instillation of BAC using a newly developed in vivo less invasive corneal TER measurement method in rabbits.

Materials and Methods:

Chemicals

Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) was obtained from Invitrogen Corp. (Carlsbad, CA, USA). BAC 10% solution (mixed BAC) was obtained

from Wako Pure Chemical, Co, (Osaka, Japan).

Experimental animals

Male white Japanese rabbits (KBT Oriental, Tosu, Japan) weighing 2.5 -3.0kg were individually housed in cages under a controlled temperature (21°C) and humidity (50 ± 5%) and a 12:12 h light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Initiation of the study occurred once the rabbits reached weights of 3.0-4.0 kg, as this was the point where the corneal diameters were of suitable size for experimentation. The rabbits were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

New electrode

The shape of the corneal TER measuring electrodes was designed for clinical use. The device contained a small sensor composed of corneal and conjunctival electrodes covered with conductive gel which is made of 2% agarose and HBSS. The corneal gel is surrounded by an insulator made of soft silicone gel (poly-dimethylsiloxane) which is confirmed as a safe material for human tissue (Figure 1). The surface of the gel and the insulator is designed to have 7.15 mm radius of curvature to fit the corneal surface and prevent leakage of electrical current through the

corneal surface. The base of the sensor was designed so that it could be fixed to the Goldman applanation tonometer mounted on a slit lamp (Figure 1).

Slit lamp examination

For examination of the safety of the corneal sensor, slit lamp examination was performed. The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine and 5 mg/kg xylazine. Corneas were examined using slit lamp and fluorescein staining before and after gently touching the sensor for 5 seconds.

Electron microscopic studies

1. Electron microscopic examination for safety of the sensor

Scanning electron microscopy (SEM) examination

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine and 5 mg/kg xylazine. Sensor was gently touching the corneas for 5 seconds mimicking what happen during measurement of TER by this new device. Normal rabbits' corneas without exposure to the sensor of the new device were used for comparison. After washing the cornea, the rabbits were immediately sacrificed using a lethal dose of intravenous sodium pentobarbital (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). The corneas were carefully excised, fixed in 4% glutaraldehyde in 0.05 M cacodylate buffer for 1 hour and then post-fixed in 1% osmium tetroxide in veronal

acetate buffer containing 0.22 M sucrose. The fixed materials were dehydrated through a series of ethanol washes. Corneas were placed in *t*-butyl alcohol, treated in a freeze-drying apparatus (EIKO ID-2, EIKO, Tokyo, Japan), and then sputter-coated with gold using an auto fine coater (JEOL JFC-1600, JEOL, Tokyo, Japan). After processing, the surface of the corneal epithelium was observed by a scanning electron microscope (Hitachi S2360, Hitachi, Ibaragi, Japan).

2. *Electron microscopic study of corneas exposed to BAC:*

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine and 5 mg/kg xylazine. Some corneas were instilled with 150µl of HBSS as control and some corneas were instilled with 150µl of 0.02% BAC solution. One minute after instillation, the rabbits were sacrificed using a lethal dose of intravenous sodium pentobarbital (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). The part of the corneal epithelium which was touched by the sensor was excised and used for electron microscopic examinations.

A- SEM Examination:

Some corneas were observed using SEM by the aforementioned method.

B- Transmission electron microscopy (TEM) examination:

Some corneas were fixed with 4% glutaraldehyde in 0.05M cacodylate buffer for 1 h,

washed in 0.05M cacodylate buffer overnight, postfixed with osmium tetraoxide in veronal acetate buffer for 1 h, dehydrated in a series of ethanols and embedded in Luveac 812. For light microscopy, semi-thin sections were cut with a Porter-Blum MT2 microtome and stained with 1% toluidine blue to select the site for electron microscopy. Ultrathin sections were cut with a Porter-Blum MT2 microtome, stained with uranyl acetate and lead citrate and examined with transmission electron microscope (Hitachi H300, Hitachi ,Ibaragi, Japan).

Corneal TER measurement in vivo in Rabbits

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine (Ketalar, Sankyo, Tokyo, Japan) and 5mg/kg xylazine (Celactal, Bayer HealthCare, Osaka, Japan). One electrode was placed in the conjunctival sac and the corneal sensor was gently touched the cornea for 5 seconds. Corneal TER was measured using a volt-ohm meter (EVOMX, World Precision Instruments, Sarasota, FL, USA) that generates a ±20μA AC square wave current at12.5Hz. The electrical current used in this study meets electrical safety standards specified by IEC60601-1 (second edition) for the safety of medical electrical equipment (International Electrotechnical Commission, 2005). Furthermore, it is known that an electric current of 25 μA has no adverse effects such as necrosis or apoptosis on cells (Kojima et al., 2014). In the

preliminary examination, we determined that the electrical resistance between electrodes without corneal epithelium is 222 ohm cm^2. Therefore, the TER value was defined as “measured TER - 222” ohm cm^2. The schema of the electrical pass way in this method is shown in Figure 2. Measurements of the corneal TER were made within a 5 second period by gently touching the sensor to the ocular surface before and one minute after instillation of 150μl of 0.02% BAC or HBSS as a control. After obtaining the TER of the cornea before and after the exposure to 0.02%BAC or HBSS, results were then calculated as a percentage of the pre-exposure TER value (100%). In this corneal TER study we used six corneas for each group.

Statistical analysis

All results were expressed as the mean ± standard error. Statistical comparisons were performed using an analysis of variance followed by a Tukey test for the TER measurements. Values of $p < 0.05$ were considered to indicate statistical significance.

Results:

Safety of the sensor to corneal epithelium

The slit lamp examinations showed intact corneal surface with negative fluorescein staining before touching the sensor (Fig. 3A, B). There was no change in corneal epithelium after touching the sensor (Figure. 3C, D)

Scanning electron microscopic examinations revealed that there were no morphological changes noted in the corneal epithelium after touching the sensor (Figure 4d, 4e, 4f) as compared with normal corneas (Figure 4a, 4b, 4c). The superficial cells of the cornea of both groups were intact with normal microvilli.

Corneal morphological change by BAC

Control corneas exposed to HBSS were intact in SEM examinations (Figure 5a). On the other hand degenerated microvilli were observed after exposure to 0.02% BAC (Figure 5c). TEM pictures revealed well-formed tight junctions and intact microvilli in control corneas (Figure 5b). On the other hand tight junctions were loosened and microvilli were shortened in the 0.02% BAC corneas (Figure 5d).

Corneal TER measurement in vivo in Rabbits

The mean corneal TER for the live rabbits used in this study was 877 ± 189 ohm cm^2. Immediately after exposure to 0.02% BAC, TER decreased to 34 ± 12% of the pre-exposure value. There was a significant decrease in the corneal TER after exposure of the cornea to 0.02% BAC solution ($P <0.01$). Figure 6 shows the TER changes that occurred after corneal exposure to 0.02% BAC.

Discussion:

Many methods have been used to evaluate corneal irritation and permeability induced

by ophthalmic drugs. Ocular irritability is conventionally tested according to modified procedure of Draize by scoring the degree of damage to rabbit eyes (Draize et al., 1944: York and Steiling, 1998). Alternative methods include evaluation of toxicity in cultured ocular cells (Kruszewski et al., 1997), direct confocal microscopic analysis (Furrer et al., 2000), and various other approaches using isolated animal corneas (Monti et al., 2002: Prinsen, 1996). Corneal drug permeability has been evaluated by diffusion experiment in vitro (Sasaki et al., 1999). The epithelial barrier function in humans has been examined by measuring the permeability of fluorescence (Gobbels and Spitznas, 1989: Ishibashi et al., 2003: Joshi et al., 1996).

Drug toxicity must be rapidly evaluated because topically instilled drugs become rapidly diluted with tears (Jordan and Baum, 1980). However, ocular surface changes are difficult to be elicited within a short period using the previous described methods.

Because TER is a sensitive, reliable, and versatile test of barrier function, it is used to study the integrity of tissues and cell sheets such as the intestinal epithelium and Madin–Darby canine kidney cells (Balda et al., 1996: Blikslager et al., 2007: Moyes et al., 2007). Furthermore, TER is also a useful indicator of corneal toxicity (Chetoni et al., 2003: Rojanasakul and Robinson, 1990: Rojanasakul et al., 1990).

In general, TER reflects the barrier function of epithelium, with lower corneal TER

values indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and tight junctions existing in the epithelium. Thereafter, the corneal toxicity of ophthalmic drugs was investigated by measuring TER in isolated corneas in vitro. However, the isolated cornea is difficult to handle because it can easily become damaged during experimental procedures and by hydration with buffers, and the TER can be unstable at the start of experiments.

We previously described a corneal TER measurement system in vivo using custom-designed thin stick electrodes and a volt-ohm meter could measure the barrier function of the intact cornea in rabbits. That design more accurately reflects the clinical instillation of ophthalmic drugs and gives us a relevant data about the acute corneal toxicity of some eye drops (Kusano et al., 2010: Onizuka et al., 2014: Uematsu et al., 2007).

Although that previously described corneal TER measurement system in vivo was very informative and reliable method, but still invasive. It can’t be used clinically for human being studies because of intraocular electrode. In order to lessen the invasiveness of the procedure, we further refined the method for measuring the corneal TER by developing electrodes that could be placed on the cornea and in the conjunctival sac instead of inserting them into the anterior chamber. TER was then calculated by subtracting the

electrical resistance, which lacked the corneal epithelial input, from the whole electrical resistance that was measured between the electrodes.

Ophthalmic preparation is used not only to treat ocular surface disorders but also for intraocular diseases such as glaucoma. Preservatives are a major component of the ophthalmic preparations, providing antimicrobial activity and preventing decomposition of the active drug in multi-dose bottle. As a soluble antimicrobial agent and surfactant, BAC is the most commonly used preservative in ophthalmic solutions for its apparently good safety/efficacy profile (Baudouin et al., 2010).

BAC is a major surfactant in addition to the preservatives added to ophthalmic drugs and is invasive to the cornea. Its effect is so strong that it destroys not only the surface of corneal superficial cells but also the tight junctions between them. Corneal TER is mostly composed of electric resistance of superficial cell membranes and tight junction, therefore destruction of the corneal surface leads to increased current permeability through the cornea, which is represented as TER decrease (Uematsu et al., 2007).

In our formerly established method, we also demonstrated that one minute exposure to 0.02% BAC immediately caused acute corneal TER decrease to 20-30% (Kusano et al., 2010: Onizuka et al., 2014).

In this study, we checked the reliability and the efficacy of this new less invasive

corneal TER measurement method by measuring the corneal TER changes after application of 0.02%BAC in rabbits. The results of this new less invasive method agreed with those of formerly established anterior chamber method. Also, SEM examinations revealed no harmful effect of this device on the corneal epithelium integrity as compared with control corneas.

As the concentration of the drug after topical instillation decreases rapidly with the tears in the conjunctival sac and with the secreting tears especially induced by drug stimulation, it is important to evaluate the effect of agents in a short period, preferably within 1 min after drug instillation. In this study, as the previous one, TER was measured by a volt-ohm meter every 0.08 s and monitored continuously with a recorder. Therefore, the time course of TER change in a short period was clearly observed. So, it preserves the advantage of the previous device in determination of acute corneal TER changes after application of eye solutions.

The only concern about this less invasive technique, in our point of view, is the variation of TER before treatment. This problem we faced in the previous invasive technique. Variation of TER before treatment should exist as an individual difference between corneas. So, to produce reliable data, the conditions of the experimental setting need to be identical and variation in TER is solved by converting the data to

percentages.

Development of a new corneal TER measurement device made it possible to measure safely and with good reliability the TER changes of the corneal animals in vivo. This new device may be applicable in the future for clinical use in humans for the first time to measure TER of the human cornea and it will open the gate to investigate safely and with great reliability the direct effect of different eye drops treatment on the corneal epithelium.

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Figure Legends

Figure 1.

The sensor is composed of corneal and conjunctival electrodes covered with conductive gel. The corneal conductive gel is surrounded by an insulator made of soft silicone gel (poly-dimethylsiloxane). The surface of the gel and the insulator is designed to have 7.15mm radius of curvature to fit the corneal surface and prevent leakage of electrical current through the corneal surface.

Figure 2.

Corneal TER was measured by passing electrical current through corneal epithelium after the sensor was gently touched to the cornea. Electrical resistance except corneal epithelium is 222 ohm cm^2.

Figure 3.

Slit lamp photographs of corneas before (a, b) and after (c, d) the touching of the new sensor. There was no epithelial defect or fluorescein staining noted in the corneal epithelium after being touched with the new sensor.

Figure 4.

SEM photographs of normal corneal epithelium (a, b, c) and corneal epithelium after the touching of the new sensor (d, e, f). Magnifications of the photographs are x 300 (a, d),

x 2000 (b, e), and x 12,000 (c, f). The superficial cells (asterisk) of the normal corneas and corneas exposed to new device were normal in appearance with normal microvilli (arrow heads).

Figure 5.

SEM and TEM photographs of corneal epithelium of control group (a, b) and 0.02% BAC group (c, d). Control corneas exposed to HBSS were intact with normal microvilli and tight junction (arrow). On the other hand microvilli were degenerated (arrow heads) and shortened (asterisk), and tight junctions were loosened (arrow) in the 0.02% BAC corneas.

Figure 6.

TER changes after instillation of HBSS and 0.02% BAC. After instillation of HBSS, TER was 107 ± 13% of the pre-exposure value. After instillation of 0.02% BAC, TER decreased to 34 ± 10%. There was a significant difference between two groups ($P < 0.01$).

Figure 1.

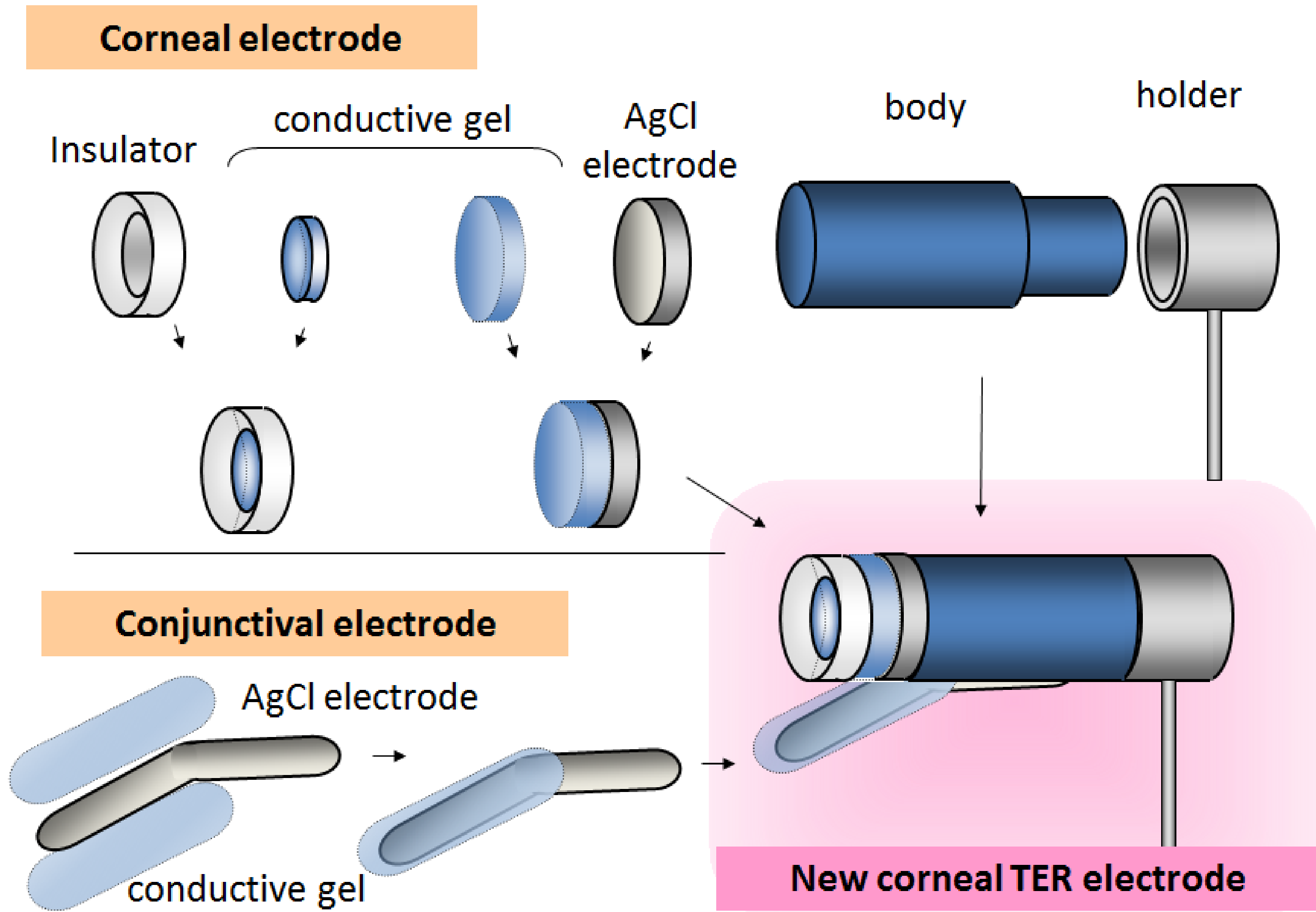
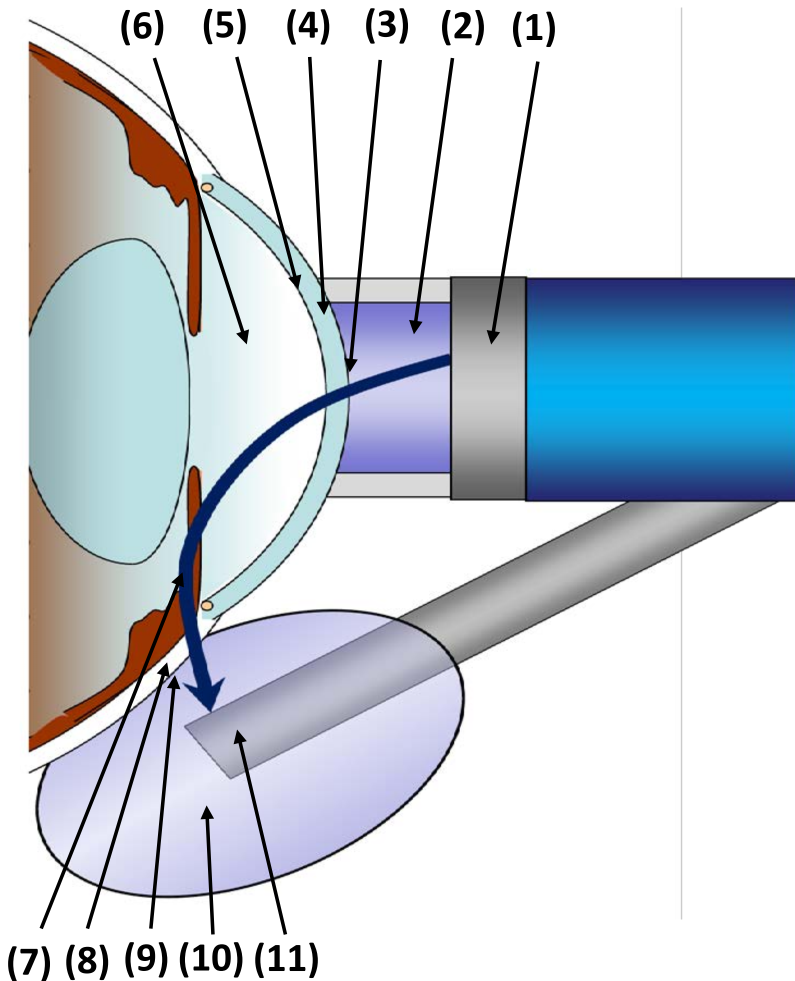


Figure 2.



Electrical current pass way

(1) Corneal electrode

(2) Conductive gel

(3) Corneal epithelium

(4) Corneal stroma

(5) Corneal endothelium

(6) Aqueous humor

(7) Uvea

(8) Sclera

(9) Bulbar conjunctiva

(10) Conductive gel

(11) Conjunctival electrode

Electrical resistance except corneal epithelium (3) is 222 ohm cm^2.

Figure 3.

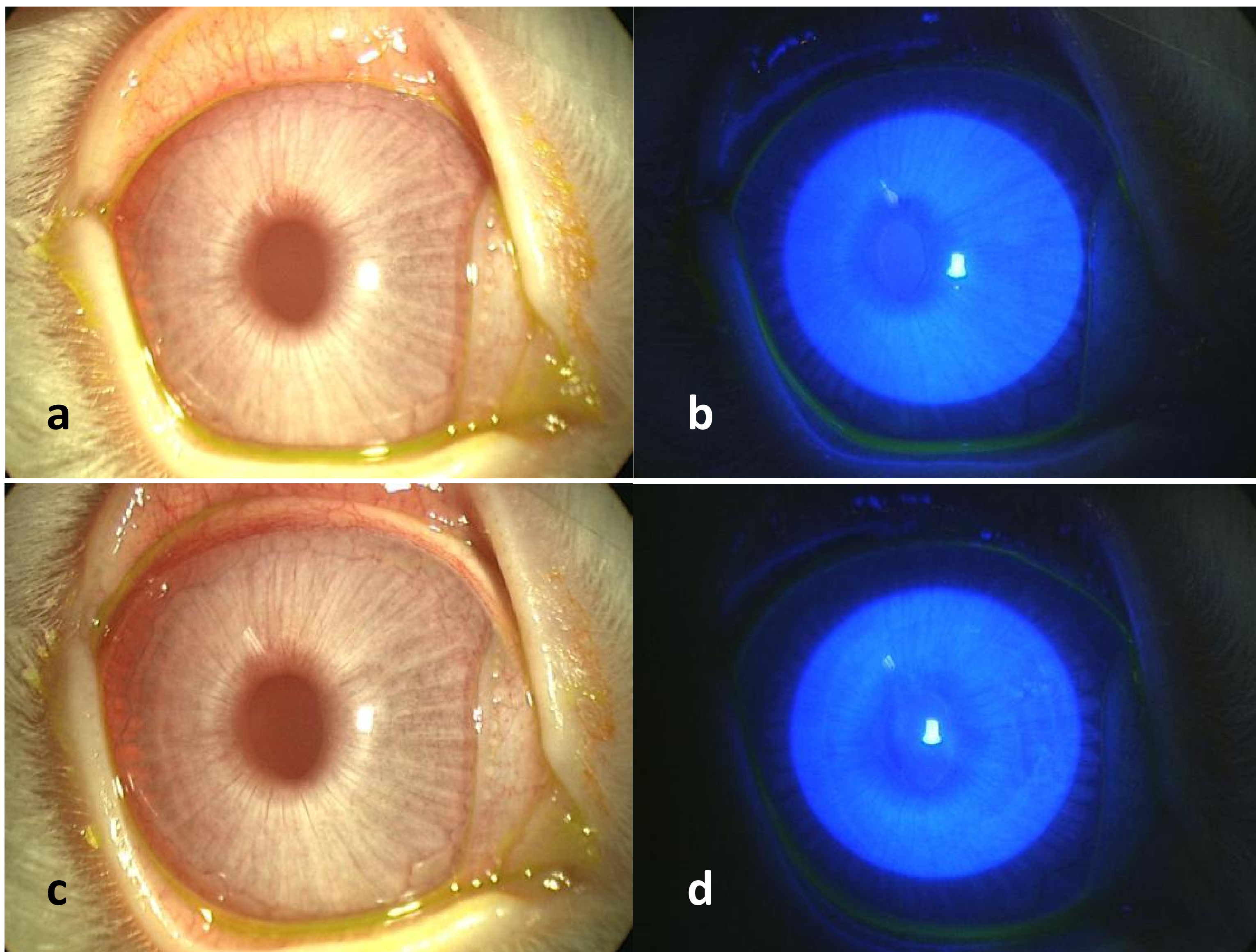
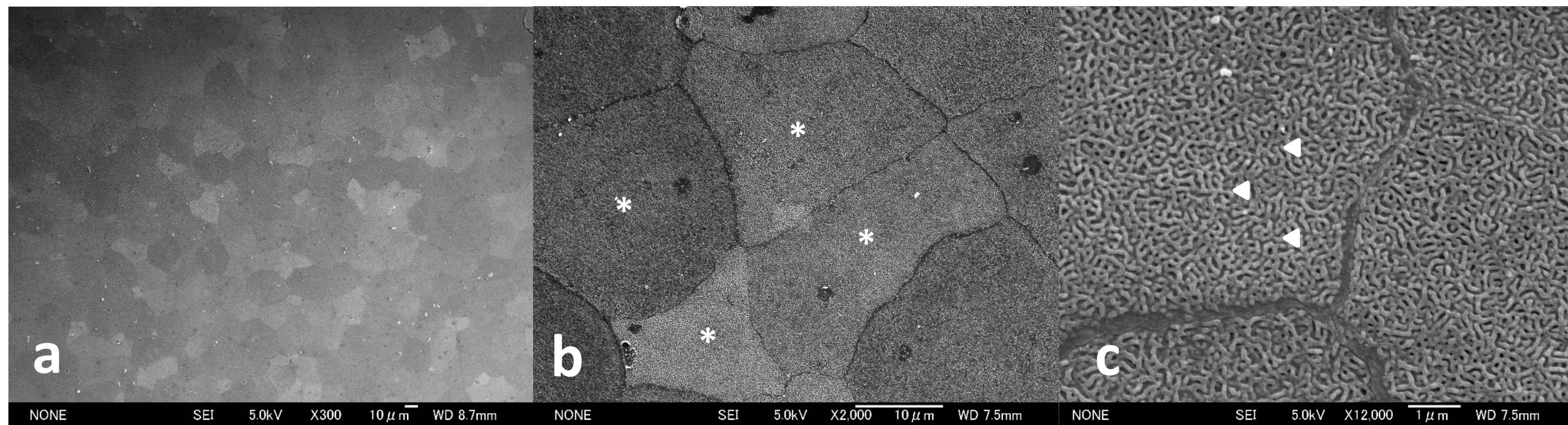


Figure 4.



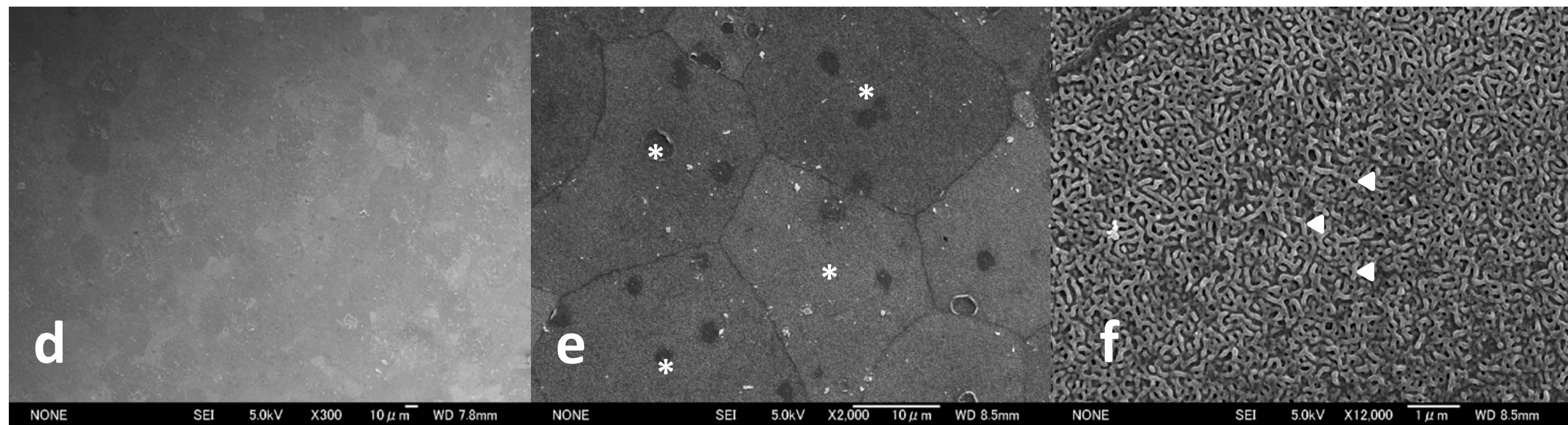


Figure 5.

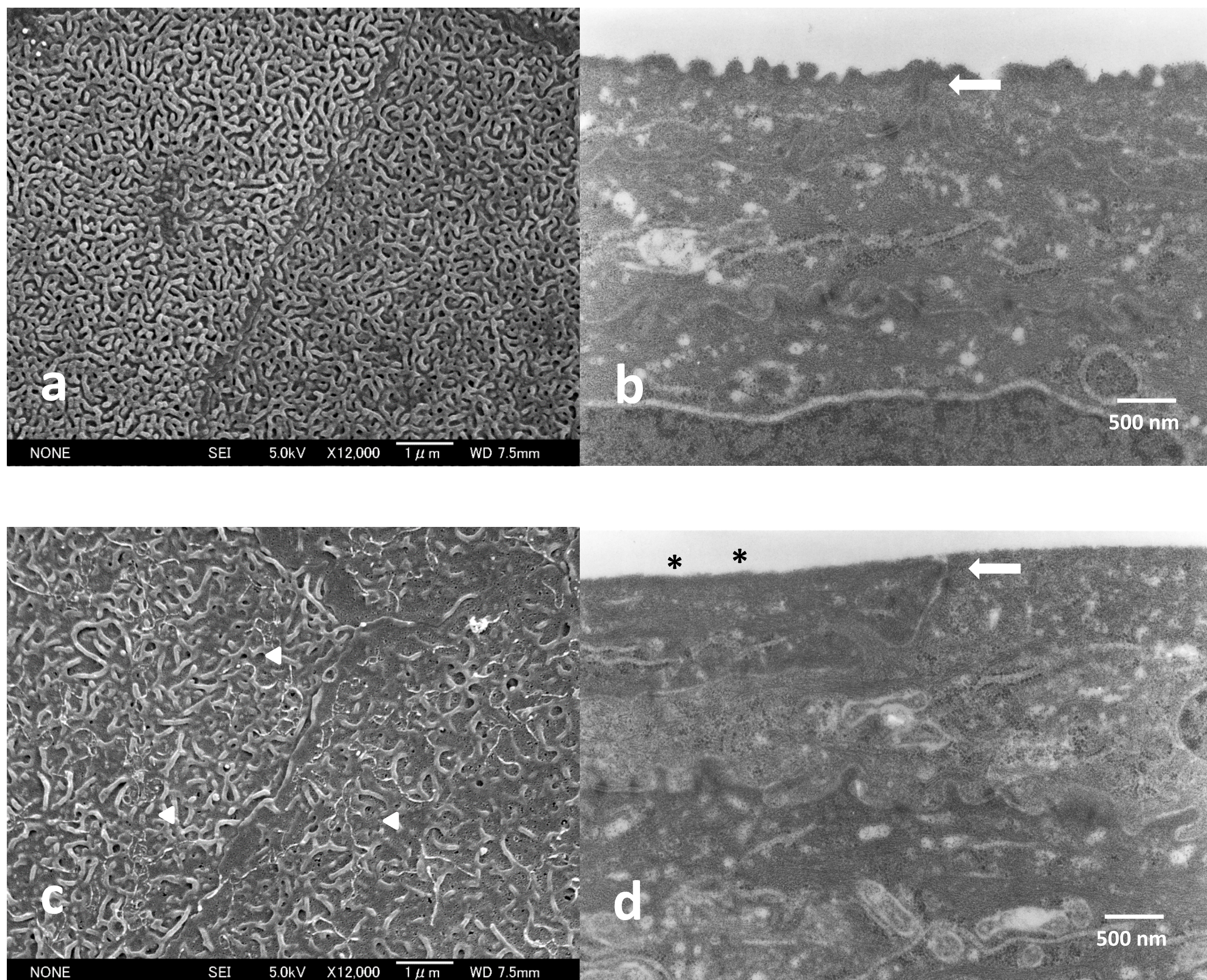


Figure 6.