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Original Article

Characteristics of corneal phospholipidosis induced by topical ocular application of chloroquine and amiodarone in rabbits

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Abstract: Several cationic-amphiphilic drugs such as chloroquine and amiodarone are known to induce phospholipidosis in the cornea by systemic administration. However, the characteristics of ophthalmological and pathological changes when phospholipidosis-inducing drugs are topically applied have not been well studied. This study was conducted to investigate the characteristics of corneal changes caused by topical application of chloroquine and amiodarone to Japanese white rabbits. The changes were evaluated by ophthalmological, histopathological, and ultrastructural examinations. An *in vivo* confocal microscopy was also applied to the chloroquine-treated corneas. In both chloroquine- and amiodarone-treated corneas, diffuse cloudiness was observed by slit-lamp biomicroscopy, and its transparency increased with duration of dosing. Confocal microscopy showed punctate dots in the corneal epithelium. Histopathologically, cytoplasmic vacuolation was found in the corneal epithelium and keratocytes in both chloroquine- and amiodarone-treated eyes. Furthermore, foamy cytoplasm of the corneal endothelium was observed in the chloroquine-treated eyes. Ultrastructural examination showed multi-lamellar inclusion bodies or membrane-like debris in the lysosome-like vacuoles in the cytoplasm of corneal cells, which is a characteristic of the lesions of phospholipidosis. These changes disappeared after a withdrawal period. Continuous dosing of chloroquine resulted in corneal erosion and focal corneal opacity as shown by gross observation and slit-lamp biomicroscopy. Confocal microscopy could detect the corneal changes prior to the appearance of these ophthalmological changes. The present study showed that phospholipidosis caused by ocular administration of chloroquine and amiodarone first induces reversible diffuse corneal cloudiness. Confocal microscopy is a useful method for monitoring induction of corneal phospholipidosis. (DOI: 10.1293/tox.2016-0003; J Toxicol Pathol 2017; 30: 135–143)

Key words: phospholipidosis, amiodarone, chloroquine, cornea, rabbit

Introduction

Phospholipidosis (PLD) is described as an accumulation of intracellular phospholipids and is morphologically characterized by multi-lamellar membrane-like inclusion bodies in the cytoplasm observed by transmission electron microscopy. Drug-induced PLD is a well-recognized phenomenon in nonclinical toxicity studies of some cationic-amphiphilic drugs (CADs) and occurs in various organs and tissues in humans and animals[1, 2]. The cornea is one of the potential targets of PLD induced by the systemic administration of several CADs[3–5]. Corneal PLD in humans is termed drug-induced keratopathy and consists of corneal deposits/cloudiness[3, 6]. Several ophthalmic formulations contain CADs as active ingredients[7, 8], suggesting that topical ocular application of these drugs possesses a potential risk of inducing PLD in the cornea. Indeed, pathological examination of the cornea in rats treated with a topical application of chloroquine or amiodarone showed PLD[9, 10], but detailed morphological follow-up of drug-induced PLD of the cornea has not yet been fully studied. The purpose of this study was to investigate the ophthalmological and pathological characteristics of PLD in the cornea of rabbits after topical ocular application of chloroquine or amiodarone.

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Materials and Methods

Chemicals

Chloroquine and amiodarone, known to induce PLD in the eyes in humans and animals[4, 9–12], were selected as test substances in this study. Chloroquine was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Amiodarone was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Animals and husbandry

For the treatment and care of the rabbits, standard procedures and housing conditions were applied in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All procedures were in accordance with the guidelines for animal experimentation at Senju Pharmaceutical Co., Ltd., and the protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC).

Seven-week-old male Japanese white rabbits (Kbs:JW) supplied by Kitayama Labes Co., Ltd. (Nagano, Japan) were used in this study. The animals were maintained in conventional animal rooms and individually housed in steel cages in an air-conditioned rooms with a temperature of 22°C ± 3°C, 55% ± 10% relative humidity, and a 12-hour light/dark cycle. Each animal was fed with a commercial diet (Lab R Stock, Nosan Corporation, Tokyo, Japan) once daily and supplied tap water *ad libitum*. A wooden block (Bio-Serv, Flemington, NJ, USA) was applied in each animal cage for environmental enrichment.

Study design and dosing procedure

Chloroquine was dissolved in a vehicle [physiologic saline or base solution (1.0% hydroxypropyl methylcellulose, 0.5% polysorbate 80, 0.1% sodium citrate, 0.1% sodium edetate, 1.0% D-mannitol, and 0.01% benzalkonium chloride)] at a concentration of 3%, which is known to induce corneal PLD in rats[9]. A 3% chloroquine solution was instilled into the right eye of each animal three times daily (30–50 µL/time) at 3-hour intervals for 7, 14, 30, or 40 days (Table 1). An equal volume of vehicle was instilled into the left eye of each animal in the same way. In addition, a recovery period of 14 days was set for animals receiving 14 days of treatment. Treatment for 30 days resulted in anterior ocular abnormalities in the chloroquine-treated eyes. For observation of the recovery of this change, 2 animals were withdrawn from treatment and assigned as recovery animals (Table 1).

Amiodarone was suspended in a base solution (1.0% hydroxypropyl methylcellulose, 0.5% polysorbate 80, 0.1% sodium citrate, 0.1% sodium edetate, 1.0% D-mannitol, and 0.01% benzalkonium chloride) at a concentration of 3%, which is known to induce corneal PLD in rats[10]. The 3% amiodarone suspension was instilled into the eye of each animal three times daily (50 µL/time) at 3-hour intervals for 60 days.

Ophthalmological examination

The eyes were observed by gross examination and by using a slit-lamp biomicroscope (SL-7F, Nikon Corporation, Tokyo, Japan, or SL-130, Carl Zeiss Meditech Inc, Jena, Germany) before dosing, during treatment on Days 6 or 7, 14, 30, and 40, and at the end of recovery periods. For the staining of corneal erosion, 0.1% fluorescein sodium solution was applied to the eye. Severity and area of corneal cloudiness were scored in accordance with the criteria of the McDonald-Shadduck method[11]. In the animals treated with chloroquine for 7 or 14 days, and those with 14 days of recovery after the 14 days of treatment, the cornea was treated with topical anesthesia (Benoxil® ophthalmic solution, Santen Pharmaceutical Co., Ltd., Osaka, Japan) and analyzed using an *in vivo* confocal microscope (HRT3-RCM combined with the Rostock Cornea Module, Heidelberg Engineering, Heidelberg, Germany) at the end of the dosing or recovery periods.

Table 1. Details of the Dosing Groups

Test substance (concentration)	Dosing days	Recovery days	Number of animals
Chloroquine (3%)	7	-	2
	14	-	2
	14	14	2
	40	-	2
	30	30	1
	30	107	1
Amiodarone (3%)	60	-	2

Histopathological and ultrastructural examinations

At the end of the treatment or recovery periods, the animals were anesthetized with intramuscular injections of ketamine (40 mg/kg) and xylazine (5 mg/kg) and euthanized by exsanguination via the abdominal aorta. Following euthanasia, the eyes were collected from the animals and fixed in 2.5% glutaraldehyde-0.1 M phosphate buffer fixative. The tissues were then embedded in paraffin, and approximately 3 µm sections were prepared and stained with hematoxylin and eosin (H&E) for all animals.

For transmission electron microscopic examination, a part of the cornea was collected from each eye, minced to about 1 mm^2, and placed in 2.5% glutaraldehyde-0.1 M phosphate buffer fixative. After 1 hour, the samples were rinsed with 3% sucrose solution several times and then placed in 1% osmium tetroxide fixative. Following fixation, epoxy resin-embedded tissues were prepared and sectioned, and ultrathin sections were stained with lead citrate and uranyl acetate. In some specimens, examination was performed using a transmission electron microscope (H-300, Hitachi Ltd., Tokyo, Japan).

Results

Ophthalmological examination

In chloroquine-treated eyes, no treatment-related ophthalmologic findings were observed at 7 days of treatment. Diffuse corneal cloudiness was observed in examinations at 14 days of treatment (Table 2 and Fig. 1B) and increased with duration of dosing. The diffuse corneal cloudiness was attenuated at 30 days of recovery and disappeared at 107 days of recovery (Table 2). At 30 and 40 days of treatment with chloroquine, focal corneal opacity, which is a higher degree of cloudiness than the diffuse corneal cloudiness described above, appeared at the center of the cornea (Table 2 and Fig. 1C). In some cases, this finding was associated with corneal erosion (Fig. 1D). Corneal fluorescein staining

Table 2. Summary of the Incidences of Abnormal Corneal Findings in Repeated Topical Ocular Application of Chloroquine and Amiodarone

	Dosing					Recovery		
Days	6/7	14	30	40	60	14	30	107
Findings (Dosing period)						(14)	(30)	(30)
Chloroquine								
Number of animals examined	10	8	4	2	-	2	2	1
Gross observation								
Focal corneal opacity	0	0	2	1	-	0	2	1
Slit-lamp biomicroscopy								
Corneal cloudiness	0	5	4	2	-	0	2	1
Severity	0	(1)	(2–3)	(2–3)	-	0	(1–2)	0
Area of cloudiness	0	(4)	(4)	(4)	-	0	(4)	0
Corneal fluorescein staining	0	0	2	1	-	0	0	0
Corneal neovascularization	0	0	0	2	-	0	2	0
Amiodarone								
Number of animals examined	2	2	2	2	2	-	-	-
Slit-lamp biomicroscopy								
Corneal cloudiness	0	2	2	2	2	-	-	-
Severity	0	(1)	(2)	(2–3)	(2–3)	-	-	-
Area of cloudiness	0	(4)	(4)	(4)	(4)	-	-	-

Values in brackets: McDonald-Shadduck scores. Severity of corneal cloudiness: 0, normal cornea; 1, some loss of transparency; 2, moderate loss of transparency; 3, underlying structure are just barely visible. Area of cloudiness: 0, normal cornea with no area of cloudiness; 4, 76–100% area of stromal cloudiness. -: Not examined.

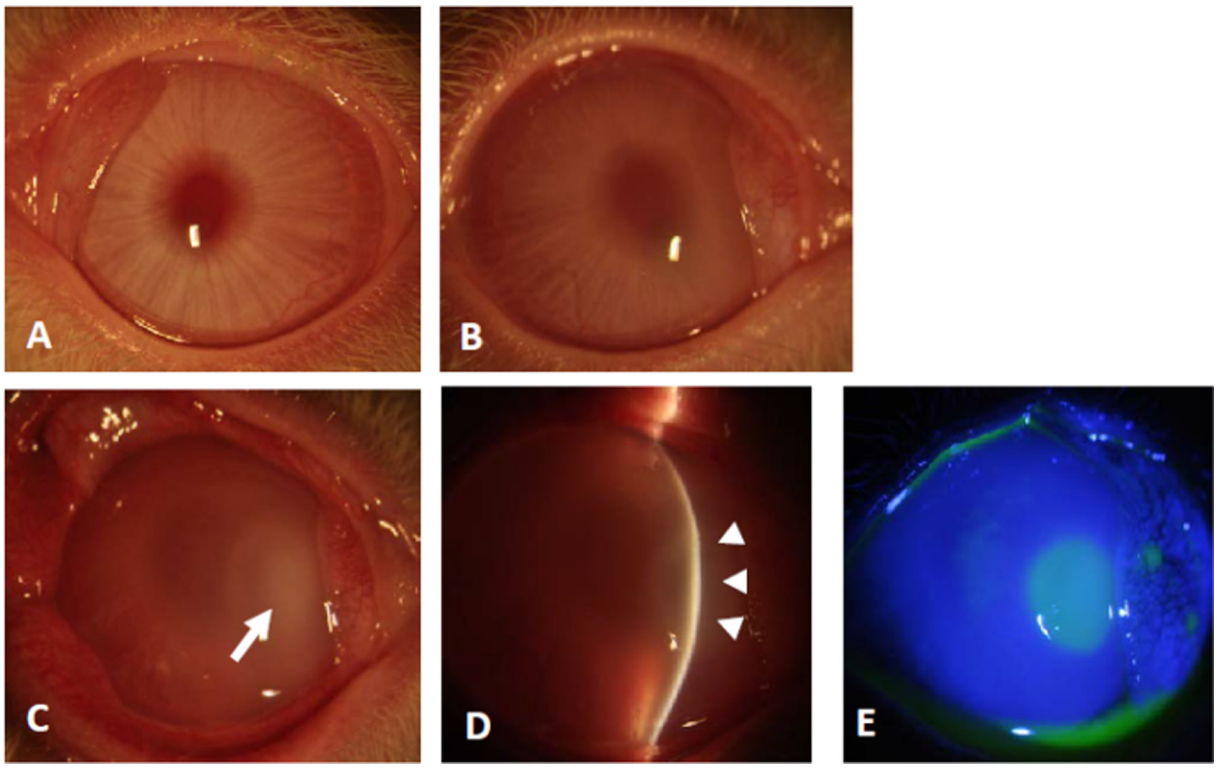

Fig. 1. Representative photographs of corneal changes of chloroquine-treated eyes in rabbits observed by a slit-lamp biomicroscope. No abnormal findings can be seen in the vehicle-treated eye (A). Diffuse corneal cloudiness appears in the eye at 14 days of treatment (B). At 30 days of treatment, this change increases in severity, and severe focal cloudiness (arrow) also appears (C). A narrow slit beam reveals corneal erosion (arrowheads) at the site of focal corneal opacity (D). Corneal erosion is also detected by fluorescein staining (E).

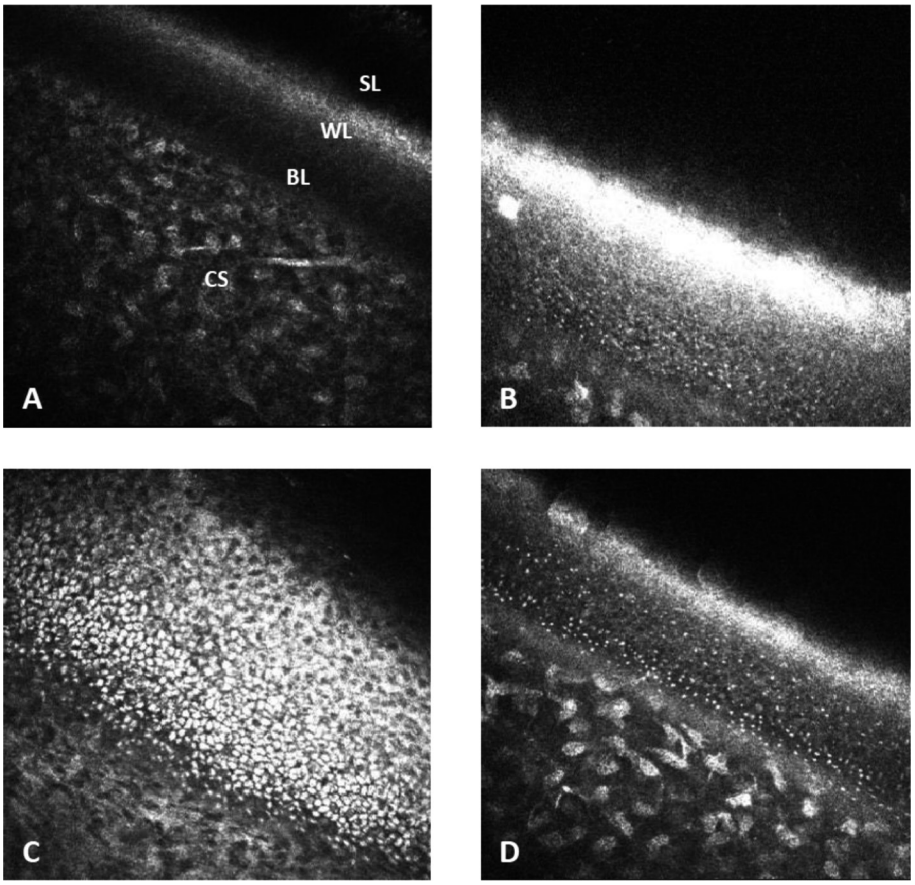

Fig. 2. Representative photographs of *in vivo* confocal microscope sections in the rabbit corneas treated with topical ocular application of chloroquine or its vehicle. No abnormal findings can be seen in the vehicle-treated eye (A). At 7 days of treatment with chloroquine, punctate dots can be seen predominantly on the basal cell layer of the corneal epithelium (B). Following 14 days of treatment, punctate dots increase in size and number (C). The number of these dots is decreased by treatment withdrawal (D). SL, superficial layer; WL, wing-cell layer; BL, basal layer; CS, corneal stroma.

revealed corneal erosion at the site of focal corneal opacity (Table 2 and Fig. 1E). Corneal neovascularization was observed in some corneas (Table 2). Focal corneal opacity was still observed after 107 days of recovery.

In amiodarone-treated eyes, no treatment-related ophthalmologic findings were observed at 7 days of treatment. Diffuse corneal cloudiness was observed in examinations at 14 days of treatment and increased with duration of dosing (Table 2).

In the *in vivo* confocal microscopy (CM), punctate dots were observed on the basal layer of the corneal epithelium after 7 days of chloroquine treatment (Fig. 2B). In the cornea after 14 days of treatment, punctate dots increased in the number, and the size of the dots was intensely enlarged (Fig. 2C). Withdrawal of treatment for 14 days resulted in the size of each dot becoming smaller than the size of the dots in the cornea treated with for 14 days (Fig. 2D).

Histopathological and ultrastructural examinations

In the chloroquine-treated eyes, the initial change after 7 days of treatment was the appearance of cytoplasmic vacuolation in the corneal epithelium, which occurred predominantly in the basal layer (Fig. 3A). These vacuoles were enlarged and occasionally contained eosinophilic inclusion bodies following 14 days (Fig. 3B) and 40 days of treatment (Figs. 3D–3F). Vacuoles were found not only in the basal cells but also in the wing cells and the superficial cells. Vacuolation of keratocytes was also present (Figs. 3B, 3D, 3E). In addition, the cytoplasm of the corneal endothelium had a foamy appearance (Fig. 3F). In the ultrastructural examination, the formation of vesicles containing multi-lamellar inclusion bodies, which is a characteristic of PLD, was found in the cytoplasm of the corneal epithelium (Figs. 4A, 4B), keratocytes (Figs. 4C, 4E), and corneal endothelium (Figs. 4D, 4F). Inflammatory cell infiltration and corneal neovascularization were found in the corneal stroma in the eyes treated for 40 days (Table 3). In the eyes after recovery for 14 days followed by treatment for 14 days, vacuoles were observed only in the corneal epithelium (Fig. 3C). These vacuoles were still observed in the corneal epithelium and keratocytes after recovery for 30 days followed by treatment for 30 days but were not detected after recovery for 107 days (Table 3).

In the amiodarone-treated eyes, vacuolation in the

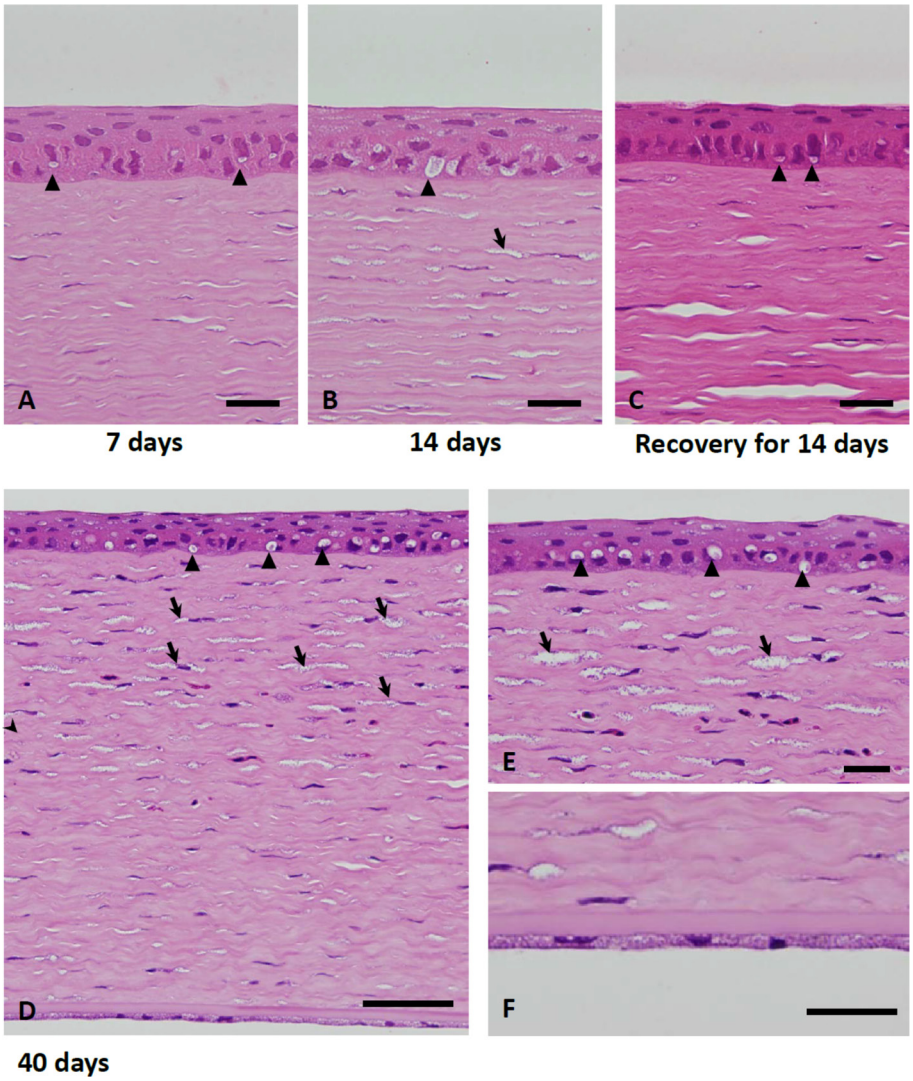

Fig. 3. Histopathological changes of rabbit corneas treated with topical ocular application of chloroquine. In the corneal epithelium, vacuolation (arrowheads) appears, predominantly in the basal cells, with treatment for 7 days (A) and increases in size and number with treatment for 14 days (B) and 40 days (D, E). In the eyes after recovery for 14 days followed by treatment for 14 days, vacuoles decrease in number and size (C). Vacuolation also appear in keratocytes of the corneal stroma (arrows) with treatment for 14 days (B) and 40 days (D, E). In addition, the cytoplasm of the corneal endothelium has a foamy appearance (F). H&E staining, magnification bar = 50 µm (D), 20 µm (A–C, E, F).

Table 3. Summary of the Incidences of Histopathological Findings in the Corneas Treated with Topical Ocular Application of Chloroquine and Amiodarone

Findings	Chloroquine						Amiodarone
Dosing days	7	14	14	40	30	30	60
Recovery days			14		30	107	
Number of animals examined	2	2	2	2	1	1	2
Corneal epithelium							
Vacuolation	2	2	2	2	1	0	2
Corneal stroma							
Vacuolation, keratocyte	0	2	0	2	1	0	2
Inflammatory cell infiltration	0	0	0	1	0	0	0
Neovascularization	0	0	0	0	1	0	0
Corneal endothelium							
Foamy cytoplasm	0	1	0	2	1	0	0

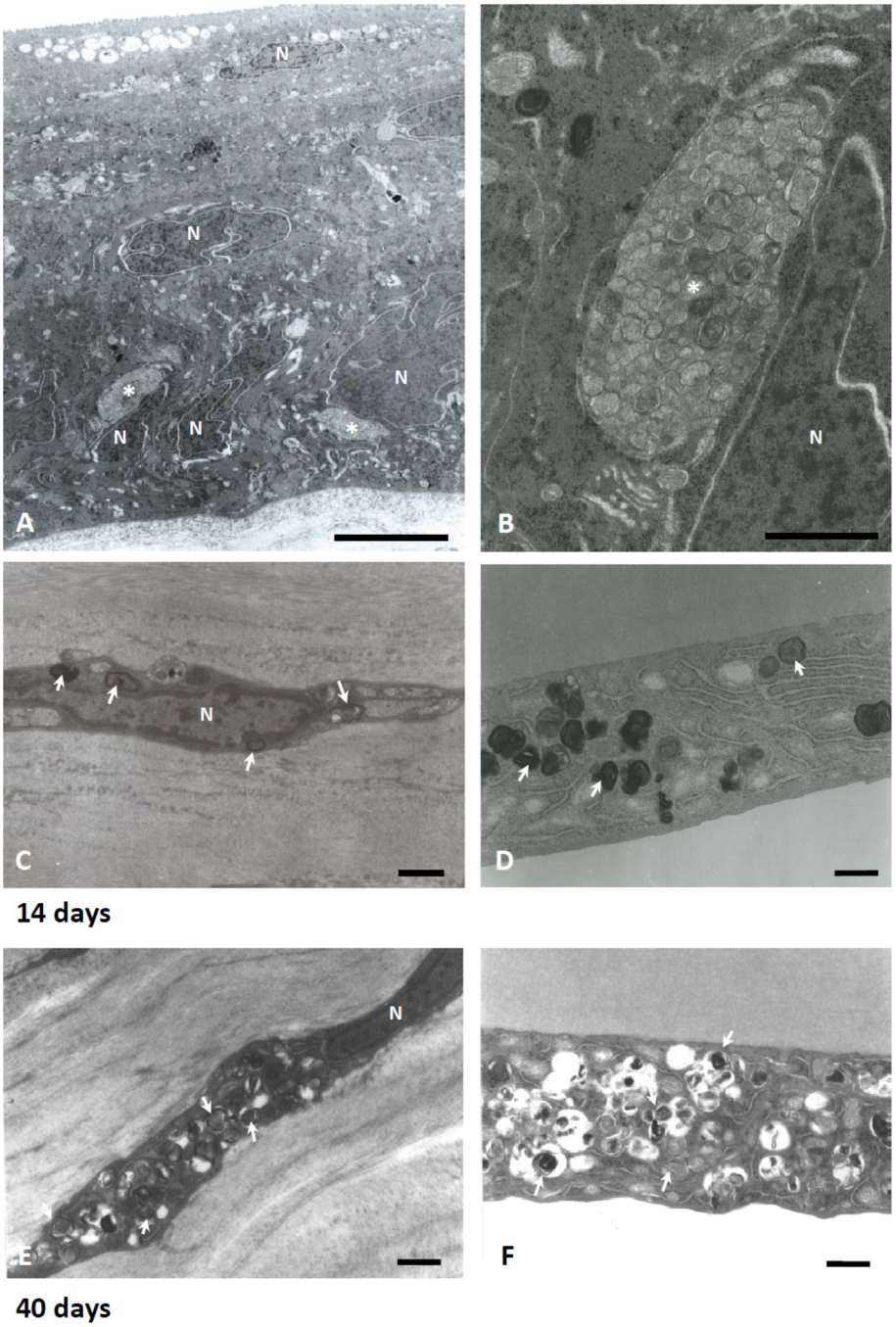

Fig. 4. Ultrastructural changes of rabbit corneas treated with topical ocular application of chloroquine. Figure 4 shows corneas treated for 14 days (A–D) and 40 days (E–F). In the corneal epithelium, lysosome-like vesicles containing numerous electron-dense multi-lamellar bodies (asterisks) appear in the cytoplasm (A, B). In the corneal stroma and endothelium, multi-lamellar bodies (arrows) appear in the cytoplasm (C, D) and increase in number with 40 days of treatment (E, F). Transmission electron micrograph, magnification bar = 5 µm (A, C, E), 1 µm (B, D, F). N, nucleus.

corneal epithelium and keratocytes was observed (Figs. 5A, 5B). Ultrastructural examination revealed membrane-like debris and/or liquid-like materials containing vacuoles in the corneal epithelium (Figs. 5C, 5D) and numerous multi-lamellar inclusion bodies in the keratocyte cytoplasm (Fig. 5F).

Discussion

In the chloroquine-treated corneas of rabbits, histopathological examination revealed that cytoplasmic vacuolar/foamy changes first appeared in the corneal epithelium, and subsequently appeared in the corneal stroma and endothelium after repeated dosing of the eyes. The affected cells

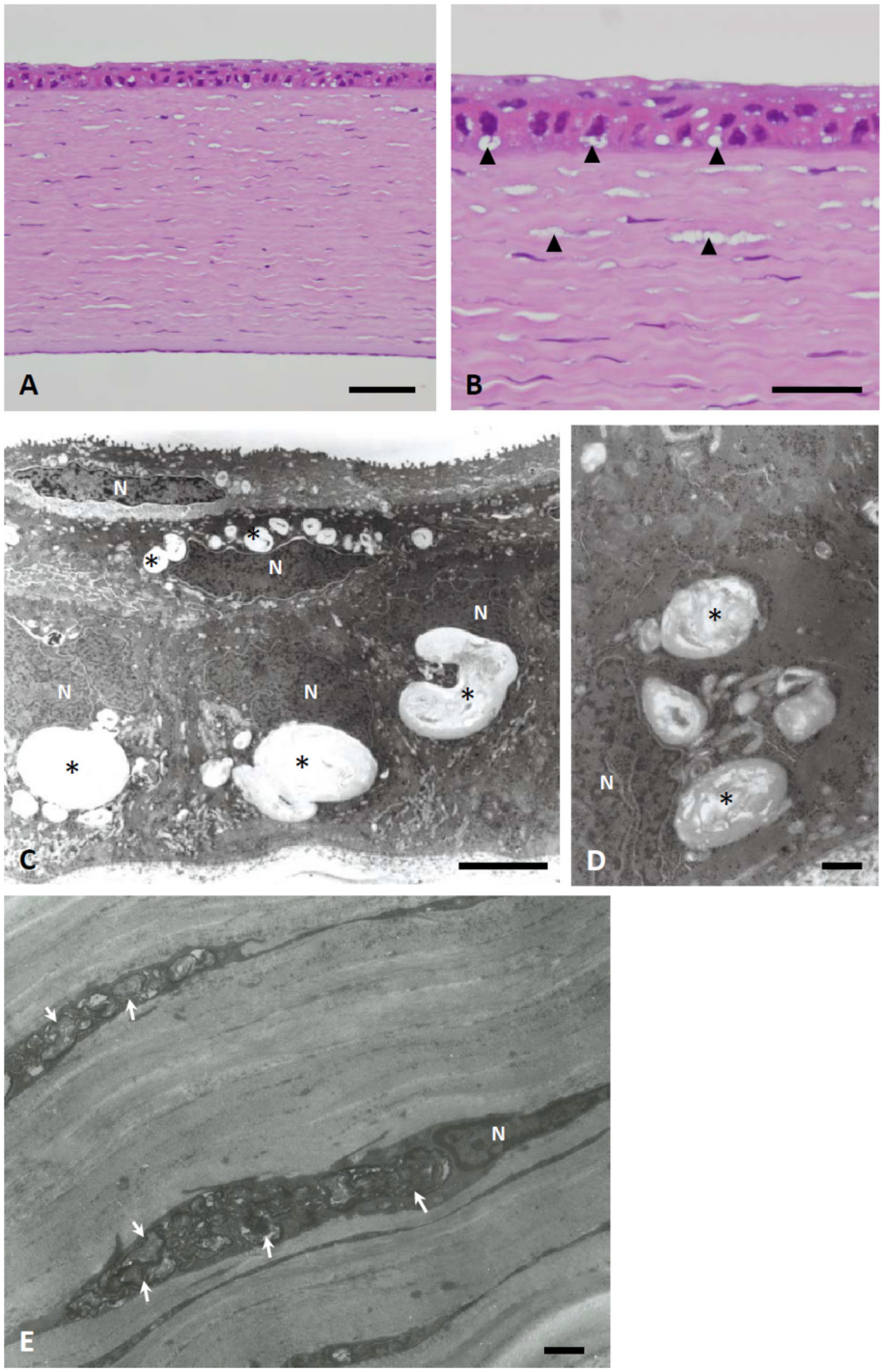

Fig. 5. Histopathological and ultrastructural changes of rabbit corneas with topical ocular application of amiodarone for 60 days. Vacuolation is found in the cytoplasm of corneal epithelium and stroma (arrowheads) (A, B). Ultrastructurally, affected epithelial cells have enlarged cytoplasmic vacuoles (asterisks), and the vacuoles contain liquid-like materials (C, D). In the corneal stroma, irregularly shaped multi-lamellar bodies (arrows) are filled with keratocyte cytoplasm (E). H&E staining, magnification bar = 50 µm (A), 20 µm (B). Transmission electron micrograph, magnification bar = 5 µm (C), 1 µm (D, E). N, nucleus.

were found predominantly on the surface side of the cornea, which was exposed to drugs more than the inner side. Ultrastructurally, these cytoplasmic changes consisted of formation of multi-lamellar inclusion bodies in lysosome-like vacuoles, which is a characteristic of PLD[1, 2]. The number and size of these lamellar bodies in affected cells increased with duration of dosing with chloroquine. Similarly, enlarged cytoplasmic vacuoles were also observed in the corneal epithelium and keratocyte of the amiodarone-treated corneas. Ultrastructural examination detected multi-lamellar inclusion

bodies in the keratocyte cytoplasm, as well as in the cells of chloroquine-treated corneas. In the corneal epithelium, cytoplasmic vacuoles containing membrane-like debris and/or liquid-like materials were observed but did not appear lamellar bodies inside, which was morphologically different from the observations for the keratocyte. These results indicate that topical exposure to chloroquine or amiodarone induces PLD in the corneas of rabbits.

Cytoplasmic vacuolation could not be detected by means of the gross observation or the slit-lamp biomicroscopy, but it was found extensively in the corneal epithelium and keratocytes of the corneas with diffuse corneal cloudiness. These results suggest that accumulation of cytoplasmic vacuoles leads to the appearance of corneal cloudiness, presumably due to the decrease of transparency. In humans systemically treated with CADs such as chloroquine and amiodarone, one of the most common symptoms in drug-induced keratopathy is corneal deposits[3, 4]. These corneal deposits are most likely due to the induction of corneal PLD, since affected corneas have the appearance of lamellar bodies in the corneal epithelial cell cytoplasm in an ultrastrctural examination[6, 12, 13]. These corneal findings manifest as linearly or dendritically distributed grayish punctate opacities in the inferior cornea, are considered to affect visual acuity rarely, and are reversible by withdrawal of the drug being used[3, 4]. In this study, the corneal ophthalmological change induced by PLD was also reversible, but the area of the change was relatively large as compared with that in humans as reported previously. This result implies that excessive PLD induction by topical ocular application of CADs, as well as by the systemic treatment, also affects visual function. Focal corneal opacity was still evident at the end of the recovery period, but this finding was considered to be related to ocular inflammatory changes possibly due to the ocular irritation of chloroquine.

In the corneal epithelium with light microscopic and ultrastructural changes, the appearance of punctate dots was detected by CM. This finding was found prior to the appearance of diffuse corneal cloudiness. Transmission electron microscopy is the most reliable and definitive tool for the diagnosis of PLD[2]; however, it is applied only after the termination of dosing in nonclinical toxicity studies. In the human clinical examination, there are some reports indicating that CM enables detection of the induction of PLD by systemic treatment with CADs[14–18]. The results of this study also suggest that CM has the potential to be used for non-invasive monitoring for the diagnosis of PLD of the cornea.

In summary, the results of our study revealed that topical application of chloroquine and amiodarone induced PLD in rabbit corneas, and corneal cloudiness appeared with the induction of PLD. CM could detect the corneal cellular changes prior to the appearance of corneal cloudiness, suggesting that the *in vivo* confocal microscope appears to be a potential imaging tool for the detection of corneal PLD.

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