Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.3, No.5, 217-232, 2016

Minireview

Ocular instillation toxicity study: current status and points to consider on study design and evaluation

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(Received July 26, 2016; Accepted August 3, 2016)

ABSTRACT — Ocular instillation toxicity studies (OITSs) are one of general toxicity studies. Yet, OITSs have a unique characteristic that the test article is directly administered as eye drops to the target organ. Compared with general toxicity studies aiming systemic exposure, the study design of OITSs is somewhat distinctive in selecting test species, dosing formulation, administration volume/frequency and ocular examinations. After the administration of eye drops, the exposure level is high in the ocular surface, whereas the bioavailability in the eye balls, especially in the posterior segment, is low. In contrast to the general toxicity studies aiming systemic exposure, the absolute systemic exposure level in OITSs is generally low, while the systemic bioavailability is relatively high. These pharmacokinetic features determine the profiles of local and systemic toxicities in OITSs. Systemic toxicities are more often found in animals of relatively small body size, and are in most cases related with pharmacological actions. Current progress in ophthalmologic imaging technologies enables advanced safety evaluation using imaging biomarkers. Bioanalysis detecting drug levels present in blood in trace amount leads to a detailed safety assessment of systemic toxicity and yields accurate safety margins. Recognizing the peculiar characteristics of OITSs, toxicologists need to propose an appropriate study design and strategy of safety evaluation. Further discussion may be awaited on rationales for testing both sexes, and for conducting separated toxicity studies to evaluate systemic toxicity. This mini-review provides insight regarding current status and points to consider of OITSs.

Key words: Ocular Instillation, General Toxicology, Eyedrop

INTRODUCTION

Ocular instillation toxicity studies (OITSs) are conducted for the development of medical ophthalmic drugs ("eye drop drugs"). Although OITSs are designed as general toxicity studies, they are unique in that unlike those for standard systemic drugs (*e.g.*, drugs to be administered orally or intravenously), eye drop drugs are administered directly to the eyes which are one of the most important sensory organs.

Recently, several excellent reviews and books have been published regarding ocular toxicology including its methodology, safety evaluation and development issues (Attar *et al*., 2013; Onodera *et al*., 2015; Short, 2008; Shibuya *et al*., 2015; Weir and Wilson, 2013). However, the articles focusing on OITSs and related information are generally limited in number.

Eye drop drugs are primarily indicated for diseases of the anterior segment of the eyes; *e.g*., dry eyes, allergy, conjunctivitis or keratitis with/without bacterial infection and control of intraocular pressure, because of the high level of exposure of the ocular surface and the relatively low bioavailability at the posterior segment of the eyes by topical administration (Kumar *et al*., 2011; Patel *et al*., 2013; Urtti, 2006). Recently, drug delivery systems (DDS) are being developed to increase the bioavailability of drugs at the posterior segment of the eyes via topical administration (Gaudana *et al*., 2010; Kang-Mieler *et al*., 2014). From the viewpoint of quality of life, ocular instillation is a beneficial administration method because of its noninvasive feature. In addition, in OITSs, it is possible to use advanced ophthalmological examinations integrated with imaging technologies. Circumstances surrounding OITS are changing.

In this review, we compile information relating to OITSs, and discuss several points. Such information

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would be significant in conducting OITSs, and in considering strategies of safety evaluation for the development of eye drop drugs.

PURPOSE OF AN OCULAR INSTILLATION TOXICITY STUDY

OITSs are repeated-dose general toxicity studies that are primarily conducted for the development of eye drop drugs. There is a guideline for non-clinical local tolerance testing of medical products in the EU (CPMP/SWP, 2001), in which single- and repeated-dose ocular tolerance tests are described. In the USA, the FDA has issued a draft of guideline regarding nonclinical safety evaluation of reformulated drug products and products intended for administration by an alternate route (FDA/CDER, 2008), in which systemic pharmacokinetics (PK) and ocular examinations (slit lamp biomicroscopy, funduscopy, tonometry and histopathology) are described to be conducted when products are instilled into the eye. However, the descriptions in both guidelines are concise, and do not introduce detailed design of OITSs. Overall, there is no definition of OITSs (Attar *et al*., 2013), and this is performed according to the ICH M3 guideline (ICH, 2009) and the ICH S4 guideline in Japan (Ministry of Health and Welfare, 1999), both being generally applied to systemic drugs.

OITSs are often confused with eye irritation tests. Actually, the protocols of preliminary studies or one-/two-week repeated-dose of OITSs are relatively close to those of the eye irritation test (OECD, 2012; Wilhelmus, 2001). Currently, the eye irritation tests are carried out using rabbits for evaluating safety of chemical materials applying to the mucosa or exposing accidentally to humans (Kosaka *et al*., 2015; OECD, 2012). The eye irritation tests are conducted to examine irritant/corrosive responses mainly on the ocular surface. In contrast, OITSs are conducted to evaluate the general toxicity of the medical ophthalmologic drugs; thus, the examinations in OITSs invlove assessment of the whole eye including histopathological examination and the ocular accessory organs as well as assessment of systemic organs (see the section described later). The differences between these two types of studies are summarized in Table 1.

DESIGN OF OCULAR INSTILLATION TOXICITY STUDIES

Animal species

According to the ICH M3 guideline, two animal species need to be selected for general toxicity studies (ICH, 2009). Generally, one is selected from among rodents (*e.g*., rats or mice), and another from among non-rodents (*e.g*., dogs or monkeys). However, rabbits were employed as the first testing species in OITSs during the development of approximately 90% of eye drop drugs approved by the Ministry of Health, Labour and Welfare of Japan (MHLW) during the last decade (2005 to 2016). The next frequent testing species was monkeys (almost all cynomolgus monkeys), followed by dogs (Table 2). Thus, two non-rodent species are usually selected for OITSs. The animal species is selected on a case by case basis, so that some drugs were evaluated using only one animal species (*e.g*., rabbits or cynomolgus monkeys).

Table 1. Differences between ocular instillation toxicity study and eye irritation test.

	Ocular instillation toxicity study	Eye irritation test
Object	Medical ophthalmologic drugs	Mainly for safety evaluation of chemicals
Guideline	ICH S4 and M3	OECD 405
Animal species	Rabbit, monkey, dog, *etc*. (Need rationale for selecting species)	Basically albino rabbit
Dosing duration	Depends on the duration of the clinical trial to be supported	One day (total 21 days including post observation period)
Ocular examination	Whole eye with its accessory tissues Routine optional examinations, sometimes together with ophthalmological imaging tools	Mainly the anterior part of the eye Gross observation or an examination using a slit lamp biomicroscopy
Systemic examination	Basically done including clinical pathological and histopathological examinations	Yes, but limited to clinical sign and gross observation (histopathological examination if necessary)
Evaluation	NOAEL (for focal and systemic toxicities) and target organ/tissue inside/outside of the eyes	Corrosion/irritation potential and its degree

Table 2. Animal species used in ocular instillation toxicity studies of eye drop drugs approved from 2005 to 2016 in Japan.

Species used	Number of Drugs	Rate (%)
Rabbit only	7	33.3
Rabbit and monkey	7	33.3
Rabbit, monkey and dog	4	19.0
Monkey only	2	9.5
Rabbit and dog	1	4.8

This information was obtained from the home page of the Pharmaceutical and Medical Devices Agency (PMDA).

Recently, many articles have been published concerning the comparison and each feature of laboratory animals from the viewpoint of ocular safety evaluation (Atsumi *et al*., 2013; Attar *et al*., 2013; Onodera *et al*., 2015; Shibuya *et al*., 2015; Vézia, 2013). The characteristics of each species are discussed below.

Rabbit

In rabbits, the eyeball is approximately half the weight of the eyeball in humans (Atsumi *et al*., 2013; Samuelson, 2013). The blinking rate of rabbits (every 6 minutes) is considerably lower than that of humans (every 5 seconds) (Vézia, 2013). In addition, the cornea of rabbits is thinner than that of humans (Schoenwald *et al*., 2003; Vézia 2013). These features led us to expect a relatively high exposure level in rabbit eyes after instillation of eye drops compared with human eyes, taking into account the factors influencing corneal permeability of drugs (Maurice, 1995). McDonald and Shadduck (1977) have described that rabbits are relatively sensitive species against ocular irritants when compared with other laboratory animals. Other reasons to use rabbits may include cost of animals and the historically accumulated knowledge from many tested substances.

When employing rabbits for OITS, special care has to be taken to choose pigmented or albino rabbits. Pigmented rabbits present an advantage when evaluating the toxicity of the test article with a melanin-binding profile. As for pigmented rabbits, Dutch-belted rabbits are used in OITSs more frequently than New Zealand white x New Zealand red F1 rabbits (F1 rabbit). Durairaj *et al*. (2012) reported interspecies-differences of ocular melanin content among humans, cynomolgus monkeys, dogs, pigs, F1 rabbit and Dutch-belted rabbits, and the rank order of melanin content differed in each part of the eye among these species. The relationship between melanin-binding and ocular toxicity is described later. It is also noteworthy that the ocular size in Dutch-belted rabbits is smaller than that in New Zealand white or Japanese white rabbits (Atsumi *et al*., 2015).

Monkey

Although the eyeball of cynomolgus monkeys is approximately half the weight of that of humans (Atsumi *et al*., 2013), it has anatomical features very similar to those of humans; *e.g*., relatively small cornea and lens in the eyeball (Short, 2008) and the macula in the fundus (Vézia, 2013). Shibuya *et al*. (2015) recommended the cynomolgus monkeys because their eyes allowed detailed assessment of drug-induced macular lesions in toxicity studies. The blinking rate in cynomolgus monkeys is also similar to that of humans (Vézia, 2013). Cynomolgus monkeys, like in humans, have no nictitating membrane or Harderian glands, both of which are present in rabbits, dogs and pigs (Samuelson, 2013; Vézia, 2013). Cynomolgus monkeys, as well as dogs, are also used in systemic toxicity studies, so that the use of these species in OITSs provides us with toxicological information that can bridge the results obtained from topical and systemic routes in the same species. As for the melanin content in ocular tissues, cynomolgus monkeys have a relatively high concentration in comparison with humans (Durairaj *et al*., 2012). In monkeys, optic nerve degeneration is known to occur in rhesus and cynomolgus monkeys as spontaneous idiopathic optic neuropathy (Fortune *et al*., 2005; Leedle *et al*., 2008).

Dog and pig

The eyeball of purpose-bred beagle dogs weighs approximately 5.5 g, which is about 0.8 times that in humans (Atsumi *et al*., 2013; Samuelson, 2013), while in pigs the eyeball size is nearly close to that in humans (Penha *et al*., 2010; Samuelson, 2013). From a viewpoint of absolute ocular size, these species present an advantage regarding assessment of the topical effect of drugs, because the thickness/length of the tissue is an important factor to decide permeability/distribution when dosing locally (Takeuchi *et al*., 2012). The ocular anatomy of dogs and pigs have relatively the same features; *i.e*., relatively large lens occupying the eyeball in comparison with primates (Atsumi *et al*., 2013; Samuelson, 2013). On the other hand, a tapetum lucidum is present in the choroid in dogs, but not in pigs, rabbits or cynomolgus monkeys (Alina *et al*., 2008; Olliver *et al*., 2004; Vézia, 2013). Currently, these species, especially pigs, are not frequently used in OITSs. However, there are advantages to employ these species in OITSs because of the following reasons; (i) the possibility of mimicking the drug

distribution of humans because of the reason described above, and (ii) lesser drug effects via systemic circulation due to their relatively large body size in comparison with rabbits and cynomolgus monkeys.

Sex

OITSs are conducted in both sexes of animals according to the guideline for general toxicity studies (Ministry of Health and Welfare, 1999). The axial length of the eyes differs between sexes in humans (Midelfalt, 1996), rhesus monkeys (Fernandes *et al*., 2003) and rabbits (Bozkir *et al*., 1997). However, these intersex-differences of ocular size are minute. Wagner *et al.* (2008) reviewed gender-based differences in healthy and diseased human eyes, and described that sex hormones influence the lacrimal system, eyelids and blinking, corneal anatomy and symptoms under disease condition across gender in humans. Typically, epidemiologic studies of dry eye have shown a higher ratio in women than in men (Schaumberg *et al*., 2003). It is currently unclear whether apparent intersex-differences exist in the eyes of laboratory animals or not. Further discussion is necessary to decide whether both sexes should be used in OITSs, especially in case that the study is limited to an evaluation of ocular toxicities.

Administration

Dose and concentration

According to the ICH M3 guideline (ICH, 2009), the high dose level in general toxicity studies is set to the maximum tolerated dose (MTD), the maximum feasible dose (MFD) or the doses providing a 50-fold margin of human exposure. This concept for setting the high dose level is applied to OITSs. The high dose level, in some cases, may be determined on the basis of results of preliminary local tolerance studies. In most cases, however, the high dose level in OITSs is set by the MFD derived from reasons inherent to the preparation of the formulation.

Penetration of the test articles through the cornea is largely dependent on the formulation properties (Patel *et al*., 2013; Wai-Yip Lee and Robinson, 2003). The test formulation used in definitive OITSs (*i.e*., GLP studies prior to the first in human trials) is to be the same as or close to the formulation used in clinical trials in order to achieve meaningful risk assessment. The concentration of the test articles is defined mainly by the solubility in the formulation. Other formulation properties (*e.g*., osmolality, pH and excipients) are essential to adjust the formulation. The pH of the eye drop formulation is to be within the physiologically acceptable range. Preservatives need to be selected to avoid any toxicity at a concentration used. For example, benzalkonium chloride, the most common preservative used in ophthalmic preparations, is known to induce corneal alternations when administered topically (Chen *et al*., 2011; Dart, 2003). The concentration of preservatives used in ocular formulations is limited due to toxicities to the eyes (Tu, 2014). Therefore, the high dose level set for OITSs is in most cases decided by the MFD determined on the basis of testing formulations rather than the test article itself.

Instillation volume

Rabbits, which have looser eyelids and a large conjunctival sac, can hold a relatively large volume of eye drops (Vézia, 2013). The instillation volume for rabbits is defined as 0.1 mL in the OECD guideline for the eye irritation test (OECD, 2012). The volume of 0.1 mL is approximately twice the volume of standard eye drops administered in clinical practice (approximately 0.04 mL (Laderer and Harold, 1986)). From our experience, the maximum volume that can be instilled to the eyes varies in other species. For example, the maximal feasible volume is similar in beagle dogs and rabbits, whereas it is less than half that volume in cynomolgus monkeys.

Increasing the instillation volume is not proportional to the increasing ocular exposure (Whiston *et al*., 1993), as the part of the extra volume is lost via the nasolacrimal duct (Chrai *et al*., 1974). Lambert *et al*. (1993) have postulated that 0.01 mL (one-tenth of the recommended test volume) of instillation volume shows a better correlation with human eye irritation experiences in eye irritation tests. Further discussion may warrant appropriate instillation volume in OITSs. Increasing instillation volume does not effectively increase the ocular exposure, but it rather contributes to an increase in systemic exposure that may result in unexpected systemic toxicities. This is discussed in the later section.

Dosing to unilateral or bilateral eyes

It needs to be considered whether the test article is dosed to bilateral or unilateral eyes. Given that there is no particular reason, the test article should be instilled to a unilateral eye from the viewpoint of the animal welfare. When the test article is administered to a unilateral eye, the contralateral eye remains untreated (*i.e*., intact) or it receives the control article (*e.g*., formulation vehicle) serving as a control. However, strictly speaking, the contralateral eye may also be exposed to the test article through the systemic circulation (Maurice, 2002; Regnier, 2013; Wilkie and Latimer, 1991a, 1991b). Therefore, findings of the contralateral eye should be carefully evaluated taking into account a possible exposure to the test article.

Dosing frequency

In standard general toxicity studies of oral or intravenous administration routes, administration is generally carried out once a day while multiple doses are administered daily in OITSs. The frequency of topical instillation in nonclinical settings is basically high when compared to the clinical settings; *e.g*., 4 times/day in nonclinical compared to twice/day in clinical. High dosing frequency in OITSs is intended to obtain higher exposure multiples, especially when the maximum drug concentration is difficult to increase due to physicochemical characteristics of the test article (Attar *et al*., 2013). Chrai *et al*. (1974) reported that a 5 min interval between instillations effectively increases ocular exposure in rabbits. The dosing frequency seems to be 1.5 to twice in many OITSs as in projected clinical usage. Excessive frequent administration may achieve higher exposure level; however, at the same time excessive procedure may restrict animals more often, causing stress and ethical issues.

Examinations for ocular and accessory tissues

Routine/standard ophthalmologic examinations

There is no definitive panel of ocular and systemic examinations in OITSs. The ophthalmologic examination for OITSs is carried out basically according to the guideline to evaluate general toxicology of systemic drugs, and all the anterior segment, ocular media and fundus are examined (ICH, 1999). However, as the drug is administered directly to the eyes, ophthalmologic examinations are more detailed and include more platforms in OITSs. Weir and Wilson (2013) described the following examinations as an example of the standard endpoints for ocular safety in nonclinical ocular toxicity studies; gross observation, slip lamp biomicroscopy, funduscopy, tonometry, electroretinography and histopathology.

On the basis of these principles and our knowledge, we have summarized the ophthalmologic examinations routinely or optionally employed in OITSs in Table 3. The observation and examination of the anterior segment of the eye are of special importance, since the concentration of test article reaches high levels there. In addition to standard gross observation, detailed observation employing a scoring method is highly significant in evaluating the severity of ocular toxicities. For example, the Draize's method scores the gross findings in the cornea, iris and conjunctivae (Draize, 1944). In OITSs, the McDonald Schadduck method (McDonald and Schadduck, 1977) and the Hackett-McDonald method (Hackett and McDonald, 1996), both of which score the findings on the basis of slip-lamp biomicroscopy, are employed frequently. Especially, the latter allows a comprehensive ocular evaluation including the lens and pupil light reflex.

Observations for the vitreous and fundus are carried out as routine examinations using appropriate ophthal-

Table 3. Ophthalmological examinations used routinely or optionally in ocular instillation toxicity studies.

Category	Methods	Testing segment of eye
Routine examination	Gross observation	Conjunctiva and cornea
	The McDonald Schadduck method or the Hackett- McDonald method, using a slit lamp biomicroscopy	Conjunctiva, cornea, lens and/or iris
	Ophthalmoscopy	Cornea, lens, vitreous body and retina
	IOP using tonometry	Iridocorneal angle (function), *etc.*
	Measurement of ffERG	Retina (function)
Optional examination	Counts of blinking rates	Conjunctiva and cornea (irritation feeling)
	Schirmer test, a test using a phenol red thread and tear break up time	Lacrimal gland and tear film (function)
	Corneal confocal microscopy	Cornea
	Pachymetry	Corneal thickness
	Specular photomicroscopy	Cornea (endothelium)
	Observation using a Laser flare-cell meter	Anterior chamber
	Measurement of mfERG	Retina (function)
	Fluorescein fundus angiography	Retina and choroid (blood vessel)
	Optical coherence tomography (OCT)	Retina

moscopy. Direct and indirect ophthalmoscopies are available for fundus observations (Munger and Collins, 2013). Regardless of taking photographs, it is highly recommended to sketch the findings as records and keep them together with the detailed description of findings.

Intraocular pressure (IOP) is a parameter of functional alteration in the iridocorneal angle. Tonometry is a method addressed in the FDA guideline (FDA/CDER, 2008). The IOP is measured by means of applanation tonometry, rebound tonometry or pneumotonograph in non-clinical toxicity studies (Munger and Collins, 2013). Several cautions have to be taken when using a tonometry in OITSs. Firstly, it is necessary to acclimatize the animals in order to obtain accurate IOP values. Without training prior to the measurement, the IOP values are usually extremely high, introducing biases regarding drug effects. Secondarily, the procedure to measure IOP insults the corneal epithelium in some degree, because the apparatus is attached to the corneal surface. Examination groups and order of examination must be appropriately designed, so that excessive stress to the eye and its influence concerning interpretation of other results are avoided.

Functional changes in the retina can be detected by full-field electroretinography (ffERG). Ideally, the method recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) is applied, since it can identify changes in cone and rod responses in ffERG (Marmor *et al*., 2009). However, this method is not suitable for dealing with a large number of animals within a limited period of time. Then, it might be practical to plan the ISCEV method as a follow-up examination conducted only when ERG abnormalities are detected. There are also some cautions regarding ffERG. For example, corneal or lens opacity interferes with exposure of the retina to light, and anesthesia affects the results of ffERG, as reported by Penha *et al*. (2010).

Optional examinations

As tear film examinations, Schirmer test and phenol red thread test, in which calibrated strips of a non-toxic filter paper or cotton thread treated with the pH indicator phenol red, respectively, is placed in the conjunctival sac, are applied for quantitative analyses of tear volume (Featherstone and Heinrich, 2013). Other clinical examination for tear film is tear break up time, which was reported in dogs (Moore *et al*., 1987) and in rabbits (Wei *et al*., 2013). These tests deliver information on the functional status of lacrimal glands. They also provide, together with blinking rates, indirect information on the presence of corneal stimulation of test articles.

Recent progress of imaging technology enables us to detect changes that may be indiscernible in routine ophthalmological examinations. Corneal confocal microscopy captures detailed changes in the corneal epithelium (Guthoff *et al*., 2009). For the corneal endothelium, morphological changes and density can be monitored by specular photomicroscopy (McCarey, 2008). Optical coherence tomography (OCT) is a powerful tool for retinal observations (Gabriele *et al*., 2010). All these modalities can be applied to animals in OITSs, and bring about fine images the resolution of which approaches to histopathology.

Other examinations of the anterior segment of the eyes, which are often integrated into OITSs, are pachymetry for quantitative measurement of corneal thickness (Donaldson and Hartley, 2013) and the laser flare-cell meter for quantitative measurement of flare in the anterior chamber (Ladas *et al*., 2005).

For the posterior segment of the eyes, multifocal electroretinography (mfERG) provides information on the corresponding electrophysiological response of the retina with regional abnormalities (Kellner *et al*., 2000; Ver Hoever *et al*., 2013). Permeability of the blood vessels and new vessels are observed with fluorescein fundus angiography in the retina and choroid (Donaldson and Hartley, 2013).

Kontadakis *et al*. (2014) introduced in a booklet that cytokines and growth factors in tear film are candidates as biomarkers of ocular surface diseases. Serum cytokines are also reported to be biomarkers of age-related macular degeneration (Mo *et al*., 2010). Yet, there still are limited numbers of biomarkers available for noninvasive monitoring of drug-induced ocular toxicities. The ocular imaging technologies we have already described may in turn play an alternative role in its screening and investigation of ocular toxicities in both animals and humans.

Histopathological examinations for the eyes and ocular accessory organs

Histopathological examination is one of the essential elements of OITSs. Recent reviews have also stressed the importance of histopathological examination in the evaluation (Onodera *et al*., 2015; Shibuya *et al*., 2015). In OITSs, test articles reach the highest exposure level not only in the corneal epithelium but also the conjunctiva and drainage route including the mucosa of nasolacrimal duct and nasal cavity. Therefore, these tissues need to be carefully examined with particular attention. The lacrimal glands, Harderian glands (when present), nasal cavity and nictitating membrane (when present) are also to be subjected to the histopathological examination. To achieve accurate histopathological evaluation, quality of the his-

tological slides has to be critically taken into account. In this regard, there are several papers that describe the technical considerations in detail (Schafer and Render, 2013a; Onodera *et al*., 2015; Shibuya *et al*., 2015).

OCULAR EXPOSURE

The standard concentration of test articles in OITSs varies from 0.001 to 10% (from 0.01 to 100 mg/mL). After ocular instillation, the eye drop is diluted with tears, and the test articles may be trapped to some extent by tear films especially for those having a high protein binding ratio (Regnier, 2013). Nevertheless, the tissues covering ocular surface (*e.g*., conjunctiva and corneal epithelium) still are exposed to a very high concentration of test article.

In contrast, the bioavailability of drug in the aqueous humor is generally less than 10% (Kumar *et al*., 2011; Patel *et al*., 2013; Urtti, 2006). Several authors have described the factors influencing the ocular absorption of eye drop drugs (Fraunfelder, 2008a; Gunda *et al*., 2008; Regnier, 2013). Chemical properties which affect ocular permeability are as following: hydrophilicity or lipophilicity, ionization or unionization, molecular weight, drug concentration in the formulation, the additives contained in the formulation *etc*. Upon instillation, the drug is distributed to each part of eyes. The way of distribution is simply interpreted as two routes: (1) corneal route and (2) conjunctival/scleral route (Attar *et al*., 2013; Regnier, 2013). Recently, from a view point of DDS to the retina/choroid, three routes were postulated; (1) (trans-corneal) trans-vitreous route, (2) (trans-corneal) uvea-scleral route and (3) (trans-scleral) periocular route (Gadek and Lee, 2011). The distribution of each drug depends on its physiochemical property. These are shown in Fig. 1.

Biological conditions of animals may modify ocular toxicities. Absorption into the cornea basically depends on the concentration of drugs because the gradient of drug concentration is a limiting factor of absorption (Regnier, 2013). Thus, changes in tear volume, either test article-related or not (*e.g*., effect of anesthesia), modulate the absorption ratio of drug. It is also known that drugs are drained from the ocular surface by blinking (Maurice, 1995; Vézia, 2013), indicating that a change in blinking rates might modulate the severity of ocular toxicity. Besides, loss of the integrity of the corneal epithelium (*e.g*., corneal injury) would allow easy entrance of hydrophilic drugs into the eye (Johnson *et al*., 1995), suggesting that once the corneal epithelium is damaged, toxicity of test articles is exacerbated due to increasing ocular exposure.

Recently, there have been several papers on transporters in the eyes (Attar and Shen, 2008, Nakano *et al*., 2014; Zhang *et al*., 2008). Transporters are identified in many tissues of the eyes including cornea, iris-ciliary body and retina/choroid. Zhang *et al*. (2008) compared differenc-

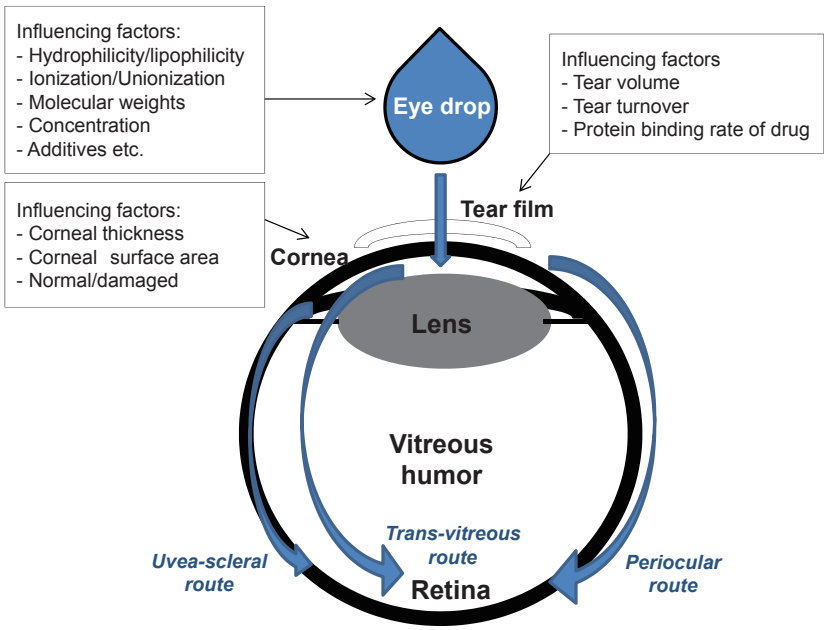

Fig. 1. Factors influencing ocular absorption of drug.

es in transporter gene-expression levels across ocular tissues and species (rabbits, dogs, cynomolgus monkeys and humans). In human ocular tissues, the main efflux transporter is multidrug resistance protein 1 (MRP1), and the main uptake transporters are peptide transporter 1 (PEPT1), organic cation transporter 1 (OCT1), organic cation transporter, novel type 1 (OCTN1) and organic cation transporter, novel type 2 (OCTN2) (Zhang *et al*., 2008). Further investigation is awaited to clarify the significance of transporters on ocular PK of drugs and interspecies-specificities.

In the posterior segment of the eyes, the exposure level after instillation of test articles is much low due to a number of anatomical and functional barriers in the retina, especially in the posterior retina (Gaudana *et al*., 2010). Despite this, some eye drops are known to exert pharmacological actions in the retina after instillation of drugs such as brimonidine, difluprednate, dexamethasone *etc*. (Gaudana *et al*., 2010; Kang-Mieler *et al*., 2014). A study done using nipradilol suggested that diffusion from posterior periocular tissues across the posterior sclera is the main route for local penetration of the instilled drug to reach the posterior retina-choroid (Mizuno *et al*., 2009). Recently, DDS to the posterior segment of the eyes via topical administration are under development. The safety evaluation for the posterior segment of eyes will become important as drugs to be delivered to the posterior segment of the eye are develped.

There are several papers on drug metabolism in ocular tissues (Attar *et al*., 2005, 2013; Nakano *et al*., 2014; Zhang *et al*., 2008). A number of cytochrome P450 (CYP) families are detected in the ocular tissues as listed in Table 4; whereas their activities or gene-expression levels are considerably lower in ocular tissues than in organs that play a role of drug metabolism such as the liver. As other drug-metabolizing enzymes, Attar *et al*. (2005, 2013) introduced oxidoreductase (aldehyde oxidase, ketone reductase, cyclooxygenase and monoamine oxidase), hydrolytic enzymes (aminopepidase, acetylcholinesterase, butyrylcholinesterase, carboxylesterase, phosphatase, aryl sulfatase, N-acetyl-β-glucosaminidase and β-glucuronidase) and conjugating enzymes (arylamine acetyltransferase and glutathione S-transferase). Some of these enzymes are involved in the metabolism for drugs topically administered (Attar *et al*., 2013). However, little information is available regarding differences across species and ocular tissues.

Melanin affinity is also a factor modulating the toxicological feature of drugs in the eyes. It has been reported that the pharmacological effects of drug are milder in pigmented animals than in albino animals (Salazar *et al*., 1976; Cheruvu *et al*., 2008). In the case of a drug binds to melanin, the content of melanin may decrease its free-concentration at the target site, resulting in a decreases of its pharmacological effect (Regnier, 2013). In turn, a melanin-bound drug may form a reservoir from which it is gradually released, prolonging thereby the pharmacological effect of the drug even after withdrawal of the administration (Urtti, 2006). Leblanc *et al*. (1998) described that melanin binding of drugs itself is not predictive of ocular toxicity. Thus, the profile of ocular toxicity of melanin-binding drug depends on the toxicity of the drug, melanin-binding manner and duration of exposure including during the withdrawal period.

OCULAR TOXICITY AND SAFETY ASSESSMENT

Toxicities of test articles in eye drop formulations are classified into primary-, off-target- and chemical-related effects. Toxicities basically occur in the anterior segment of the eyes, because the concentrations of test articles are high at the site of topical administration. Therefore, toxicities are most often observed in the conjunctiva, cornea, iris and the tissues/organs surrounding the eyes such as eye lids, skin and lacrimal glands. Indeed, adverse events have been reported on the ocular surface with eye drop drugs launched to the market (Fraunfelder *et al*., 2008a, 2008b).

Cornea, conjunctive and eyelids

Test articles subjected to OITSs for supporting clinical trials are selected among those that show less cytotoxicity, based on preliminary tests such as *in vitro* cytotoxicity tests. In addition, their formulations are also adjusted to those used in clinical trials in humans. Therefore, no overt toxic findings such as chemical burn or corrosion are expected in OITSs.

Hyperemia is a finding frequently observed in OITSs, as well as in clinical settings for topical medication (Fraunfelder *et al*., 2008a). If the test article has pharmacological action of vasodilation, hyperemia will be transient and not accompanied by histopathological changes. If hyperemia is a sequela of tissue injury, edema and histopathological inflammatory changes will be present (Schafer and Render, 2013a).

Corneal opacity disturbs vision, and is toxicologically of great importance. Its reversibility should be carefully investigated by setting a study with an appropriate period of recovery. Reversibility may vary from site to site in the damaged cornea. If the damage is limited to the surface epithelium and center of the cornea, opacity will likely

Table 4. Cytochrome P450 in ocular tissues.

Family	Isozyme	Species (identified tissue)	Ref.
CYP1	CYP1A	Rat (cornea) Mouse (uveal tissue under induction; there is a difference between strains.)	Sugamo *et al*. (2009) Zhao and Shichi (1995)
	CYP1B1	Human (trabecular meshwork) Human (anterior uveal tract)	Stoilov *et al*. (1997) Stoilov *et al*. (1998)
CYP2	CYP2A6	Human (cornea and iris-ciliary body) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2B1/2	Rat (cornea and retina)	Tanaka *et al*. (2002)
	CYP2B2	Rat (lens)	Nakamura *et al*. (2007)
	CYP2C	Mouse (cornea, ciliary body, lens and retina)	Tsao *et al*. (2001)
	CYP2C8	Human (cornea and retina/choroid tissue) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2C9	Human (cornea) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2C11	Rat (cornea and retina)	Tanaka *et al*. (2002)
	CYP2C19	Human (cornea) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2D6	Human (cornea, iris-ciliary body and retina/choroid) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2E1	Human (cornea and retina/choroid) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP2J	Mouse (samples were whole eyes excepting optic nerve.)	Xie *et al*. (2000)
CYP3	CYP3A	Rabbit, dog and human (conjunctiva, cornea, choroid-retina, iris-ciliary body and lacrimal glands)	Attar *et al*. (2005)
	CYP3A1	Rat (lens)	Nakamura *et al*. (2007)
	CYP3A4	Human (cornea and iris-ciliary body) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
	CYP3A5	Human (cornea) Human (corneal epithelium)	Zhang *et al*. (2008) Kölln and Reichl (2012)
CYP4	CYP4B1	Human and rabbit (conjunctivae, cornea, choroid-retina, iris-ciliary body and lacrimal glands)	Attar *et al*. (2005)
	CYP4V2	Human (retina)	Nakano *et al*. (2014)

disappear, since corneal epithelium can regenerate within one week in rabbits (Pfister, 1975) and humans (Hanna *et al*., 1961). In contrast, if the stem cells of the corneal epithelium (*i.e*., the limbus) are damaged at the limbus, the recovery of corneal opacity may be compromised (Kruse, 1994).

Corneal opacity can be caused by edema, cell infiltration and deposits of the test articles or mineral *etc.* in the corneal stroma. Stromal edema often occurs subsequent to damage of the corneal endothelium, as the corneal endothelium plays an important role in maintaining the balance of fluid in the cornea. In rabbits, the corneal endothelium keeps a regenerative capacity; however, that is not the case in adult primates or dogs (Attar *et al*., 2013). Thereby, reversibility in the rabbit corneal endothelium should be carefully evaluated for its relevance in humans. In any case, once stromal tissue is damaged, recovery requires a relatively long period of time to recover its transparency due to slow remodeling of stromal tissue. Stromal injury may be repaired with fibrous tissues leading in this case to incomplete reversibility of transparency (Ashby *et al*., 2014).

Eye drop drugs frequently label cautions of allergic reactions (Fraunfelder *et al*., 2008a, 2008b). It is usually difficult in OITSs to judge whether inflammatory findings in the ocular surface are related with allergic reaction

or not. Sensitization hazards may be evaluated by other non-clinical platforms such as the local lymph node assay done in mice (OECD, 2010).

Oral treatment with cationic amphiphilic drugs is known to cause phospholipidosis in the cornea in animals (Drenckhahn *et al*., 1983) and in humans (Mäntyjärvi *et al*., 1998). Bockhardt *et al*. (1978) reported that amiodarone induced lipidosis-like alternation in rats via ocular instillation. We also demonstrated phospholipidosis in the cornea of rabbits when treated with eye drops (Yamagiwa *et al*., 2015). If phospholipidosis is not associated with corneal opacity and remains within the degree of histopathological diagnosis, this microscopic finding is likely of low toxicological significance. Corneal phospholipidosis induced in humans by amiodarone is reversible and associated with little visual impairment (Dart, 2003; Davidson and Rennie, 1986).

Decreases in lacrimal secretion result in keratoconjunctivitis sicca (Schafer and Render, 2013a). Several eye drop drugs are known to induce dysfunction of the lacrimal systems (Fraunfelder *et al*., 2008b). Integrity of the tear film should be taken into account when evaluating corneal toxicity.

Uvea

IOP is influenced by primary pharmacological actions (*i.e*., glaucoma therapeutic agent), off-target pharmacological effects, malfunction or damage of tissues following inflammatory changes (Schafer and Render, 2013a). An elevation of IOP, when prolonged, may cause symptoms of glaucoma, followed by functional ocular damage, even of vision.

Mydriasis or miosis can be caused by pharmacologically primary or off-target effects of the drugs. These effects are basically transient. Drugs, which dilate the pupil or limit pupil constriction, yield a shallow anterior chamber and a narrow filtration angle (Hadjikoutis *et al*., 2005). These structural alterations may possibly lead to IOP elevation (Schafer and Render, 2013a). Corticosteroids are reported to induce IOP elevation in humans, whereas this phenomenon is not well reproduced in animals (Attar *et al*., 2013).

There are eye drop drugs which alter the iris color. Levobunolol, a β-blocker to treat glaucoma, is reported to cause depigmentation of the iris in humans (Doyle and Liu, 1999). Schafer and Render (2013a) described that depigmentation of the uvea may occur as a result of inflammatory changes. On the other hand, prostaglandin analogs cause the hyperpigmentation of the iris due to an increase in melanin synthesis (Schafer and Render, 2013a). This hyperpigmentation is permanent in human patients (Fraunfelder *et al*., 2008b).

Lens

The anterior aspect of the lens faces the chamber filled with aqueous humor, so that the lens epithelium is directly exposed to drugs present in the chamber. In humans, lenticular opacities are reported to be caused by topical drugs such as anticholinesterase, pilocarpine and acetylcholine (Fraunfelder *et al*., 2008b). In experiments using laboratory animals, lenticular opacities are found to be caused through multiple mechanisms of action; abnormalities of sugar-, protein-, lipid- and electrolyte-metabolisms (Schafer and Render, 2013b). Some of these lenticular opacities are transient in animals and humans. However, restoration of injured lenticular tissues is considerably slow (Hanna *et al*., 1961). Schafer and Render (2013b) described that the cataracts induced by glucocorticoids in humans is difficult to produce in animals.

Retina

A number of drugs and chemicals cause various types of retinal toxicity (Schafer and Render, 2013b). Retinal toxicities basically arise in experimental and clinical settings, when drugs are administered systemically, whereas they are infrequent when administered topically. This is most likely due to the low bioavailability of drugs in case of topical administration, especially in the posterior segment of the eye including the retina. However, as described above, some eye drop drugs are reported to exert pharmacological actions in the posterior segments of eyes (Gaudana *et al*., 2010; Kang-Mieler *et al*., 2014). Therefore, ophthalmological examination of the retina, including imaging technology tools to detect slight retinal changes, becomes to be important in OITSs.

Idiosyncratic adverse drug reactions

Idiosyncratic adverse drug reactions (IDRs) are known as lethal side effects in humans. These reactions are mainly observed in the skin, liver and bone marrow, and are considered to be immune mediated (Uetrecht and Naisbitt, 2013). According to the description by George *et al*. (2014), Steven-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare in case of topical preparations. However, there have been cases of TEN with eye drops of dorzolamide/timolol/latanoprost (Flórez *et al*., 2005) and SJS with eye drops of sulphamethoxazole (Wu and Chen, 2015). Safety assessment of the IDR should be considered in future non-clinical studies (Uetrecht and Naisbitt, 2013).

Local and systemic NOAELs

In OITSs, the no observed adverse effect level (NOAEL) should be separately defined for local and systemic effects. Lewis *et al*. (2002) have proposed in local administration studies that two NOAELs be established, one on the basis of local (administration site) effects and another on the basis of systemic effects. Local and systemic toxicities may not be basically interrelated in OITSs. However, when the physical condition is compromised in animals, ophthalmological findings could be modified by systemic toxicities.

In silico prediction of ocular exposure and risk assessment of ocular toxicity

It is impractical to measure drug exposure levels in human ocular tissues. Even though PK can be monitored in ocular tissues in laboratory animals, a number of animals need to be allocated to analyze PK in the eye. Recent advances in computational simulation may overcome this dilemma. For example, Tojo (2004) reported a PK model for ocular drug delivery. *In silico* drug penetration was reported in the posterior eye tissues after topical instillation in rabbits (Shikamura *et al*., 2011). In addition, Ueda *et al*. (2010) reported an ocular diffusion model of antimicrobial drugs in normal and diseased eyes. These simulation models and *in silico* prediction are useful tools to estimate ocular exposure levels in animals and humans, and will be the future challenges of technology.

SYSTEMIC TOXICITY IN OCULAR INSTILLATION TOXICITY STUDY

Examinations for systemic organs

Assessment of systemic toxicities in OITSs is basically the same as in general toxicity studies of drugs administered via systemic routes. Weir and Wilson (2013) described the following examinations as systemic endpoints in ocular toxicity studies; clinical observation, body weight, food consumption, clinical pathology, necropsy, organ weights, histopathology, toxicokinetics (TK) and immunogenicity (biologics). Based on our knowledge and experiences, systemic examinations in the in-life stage (excluding ophthalmologic examinations described in the previous section) are summarized in Table 5. Among those, ECG and blood pressure measurements are routinely performed in general toxicity studies of systemic drugs using monkeys and dogs. These endpoints are to be integrated into OITSs, since cardiovascular effects are common side effects of eye drop drugs (Izazola-Conde *et al*., 2011; Labetoulle *et al*., 2005; Lama, 2005; Shiuey and Eisenberg, 1996).

Histopathological examinations of systemic organs, which are the same as those done in general toxicity studies using systemic administration routes, are of importance to evaluate systemic organ toxicities in OITSs. In a case where the NOAEL has already been established together with appropriate TK data in a separated toxicity study using a systemic administration route, the histopathological examination of systemic organs in OITSs may not be highly significant.

Consideration of systemic exposure regarding toxicity

Systemic exposure levels are relatively low with eye drop drugs in comparison with those administered systemically. For example, when a 1% eye drop formulation is instilled to both eyes 3 times daily (assuming a drop size of 40 μL/time, and a body weight of 60 kg), the dose level is calculated to be 0.04 mg/kg/day. The systemic bioavailability is relatively high for eye drop drugs (above 75% (Reginer, 2013) and from 20% to 100% (Chastain, 2003)) in comparison with their intraocular bioavailability. A large part of eye drops is drained into the nasolacrimal duct and reach the nasal mucosa. During this

Table 5. Systemic examinations in the in-life stage (except for ophthalmologic examinations) in ocular instillation toxicity studies.

Category of examination	Examinations and comments
Basal parameters	Clinical signs (including mortality), body weight and food intake are usually recorded. Water intake is added if needed.
Clinical pathology	Hematology and clinical chemistry are usually investigated, as long as there is no special reason. Urinalysis can also be included in the list of examinations. Regarding urinalysis, the duration of sampling needs to be considered, since eye instillation is usually repeated several times in a day.
Physical parameters	ECG, blood pressure and body temperature can be examined in OITSs the same as in studies using systemic routes in monkeys and dogs. In a study using rabbits, ECG and blood pressure are not usually evaluated, probably due to limited knowledge of the relevance for humans and poor background data in rabbits.

drainage process, the eye drop drugs are absorbed into the systemic circulation. Lee *et al*. (1993) reported that at least 50% of systemically absorbed drug reaches the blood stream from the nasal mucosa. Thus, the kinetics of ophthalmologic drugs instilled in the eyes is similar to that of drugs injected intravenously, both being less influenced by first pass metabolism (Lama, 2005). An investigation using timolol showed that systemic bioavailability and cardiopulmonary effects were similar between ocular instillation and intravascular routes in humans (Korte *et al*., 2002).

TK is highly important in evaluating systemic toxicities in OITSs. In addition, TK provides information for interpreting ocular findings in control eyes when the drug is instilled to the unilateral eyes. At present, bioanalytical technology for measuring drug concentration is under development, and this technology enables measurement of drug concentrations as little as 10 pg/mL or less. This allows an accurate evaluation of PK profiles in both OITSs and human clinical trials, and establishes appropriate safety margins for systemic toxicity.

Systemic toxicity and safety assessment

Systemic toxicities or side effects are rare events in non-clinical and clinical studies of eye drop drugs. In humans, systemic side effects are seen with glaucoma therapeutic agents on the cardiovascular and central nervous systems (Izazola-Conde *et al*., 2011; Labetoulle *et al*., 2005; Lama, 2005; Shiuey and Eisenberg, 1996). As the dose level of eye drop drugs is not adjusted for body size, the population of small body size (*e.g*., children) is at a relatively high risk of experiencing systemic side effects. In children, there have been cases of systemic side effects induced by eye drops of atropine (Princelle *et al*., 2013), cyclopentolate (Pooniya and Pandey, 2012) and mydriatic agents (Labetoulle *et al*., 2005).

The systemic dose levels per body weight are considerably higher in OITSs than in a clinical setting, because the weights of rabbits and cynomolgus monkeys used for OITSs are approximately 3 to 4 kg. The daily systemic dose levels are 7.5 to 50 times higher in OITSs than in humans, even when using the same concentration as in the formulation (Table 6). Giving a higher concentration of drug or setting more frequent administration, the systemic dose levels in OITSs would fulfill the recommended high dose levels (mean exposure margin 50 times the clinical dose) in the ICH M3 guideline (ICH, 2009). Therefore, in some cases, OITSs are considered appropriate to assess systemic toxicities of drugs used in human clinical trials. Overall, integrating the evaluation of systemic toxicities and systemic TK into OITSs can exclude separated systemic toxicity studies from the package of non-clinical studies. This approach would meet the concept of three Rs of animal ethics (ILAR, 2011). Discussion may be awaited on the rationales for conducting separated toxicity studies to evaluate systemic toxicity.

CONCLUSION

This mini-review is intended to introduce the OITSs and to draw attention to some points that should be considered for these peculiar studies. OITSs have a unique feature in that administration takes place directly in the eyes. Toxicologists conducting this study need to carefully establish the study design and strategy for safety evaluation, taking into account all the characteristics of eye drop drugs.

ACKNOWLEDGEMENT

We are grateful to Drs. Haranosono Y., Kozai S., Kawamura A. and Ohtori A. of Senju Pharmaceutical Co., Ltd., for advices on ocular drug delivery systems and pharmacokinetics during the preparation of this manuscript.

Conflict of interest---- The authors declare that there is no conflict of interest.

Table 6. Relative systemic dose levels in human clinical trials and ocular instillation toxicity studies.

	Species			
	Human (adult)	Cynomolgus monkey	Dog	Rabbit
Body weight	60 kg	4 kg	10 kg	3 kg
Administration volume (time/eye)	40 µL (one drop)	30 µL	50 to 100 µL	50 to 100 µL
Administration to eye	Both eyes	One eye	One eye	One eye
Instillation frequency per day (vs. human as 1)	x 1	x 2	x 2	x 2
Multiples of systemic daily dose (vs. human as 1)	x 1	x 11.3	x 7.5 to 15	x 25 to 50

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