General considerations in ocular toxicity risk assessment from the toxicologists’ viewpoints

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ABSTRACT — Humans commonly obtain approximately 80% of external information from vision. Since loss of vision markedly decreases quality of life, risk assessments for visual toxicity of new drugs are extremely important. However, the ICH S4 guideline for nonclinical toxicity study of new drugs only indicates a brief instruction for ophthalmologic examinations, and submitted data for drug approval according only to this guideline are not always considered sufficient in light of ocular toxicity risk assessments. The eye is an assembly of many specialized sub-organs which have specific functions, and its integral maintenance of homeostasis plays an important role of visual function. When only a part of integrity of functions is lost, overall function of the eye might be commonly disturbed. Therefore, understanding of anatomy and physiology of these sub-organs may help know mechanisms of observed ocular changes. In ophthalmologic examinations in nonclinical toxicity studies, it is vital to understand the principles and features of each examination. Comparisons of findings between pre and post drug treatment as well as considerations of species differences, strain differences, age differences, and location/degree of abnormalities are essential. In addition, many kinds of spontaneous ocular findings are well known in experimental animals. To differentiate treatment-related changes from spontaneous findings, mastering basic skills for ophthalmologic examinations and taking advantage of collection of background data are necessary. For ocular toxicity risk assessments, while an evaluation of “sight-threatening” effects is most critical matter, “quality of vision” related findings also should be considered. To extrapolate animal data to human, clinical significances of ocular toxicity findings should be evaluated based on considerations for “species differences”, “safety margins”, “reversibility”, and “risk-benefit balance”. In addition, a detailed recording of features of lesions is also important for an appropriate judgment of clinical significance of ocular findings. For preparation of histopathological specimens, careful sampling of organs and suitable selection of fixatives are important. To accurately orient ocular lesions in the specimen for histopathological examinations, securing close communications prior to necropsy among ophthalmologists, gross necropsy pathologists and histopathology technicians should be effective and helpful. It is impossible to detect all ocular changes in histopathological examinations; that is, there is a limitation in histopathological examinations. Therefore, for ocular toxicity risk assessments, comprehensive evaluation with pathological findings as well as other results of various examinations in toxicity studies should be considered. In conclusion, for ocular toxicity risk assessments, integrated judgments from all examination data in nonclinical toxicity studies are required. To achieve appropriate risk assessments which can be extrapolated to human, close communications and sharing of data regarding the eye are most important among toxicologists, clinical sign investigators, histopathology technicians and pathologists.

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INTRODUCTION

Risk assessments for the sensory organs for sight (ophthalmoception), hearing (audioception), taste (gustaoception), smell (olfacoception) and touch (tactioception) are important for new drug developments, but the amounts of published information in the sensory organs are much smaller than those in other organs and tissues. In addition, these sensory organs have many kinds of anatomical and physiological differences among species, and there are smaller number of experts, who can take appropriate risk evaluation. Humans commonly obtain approximately 80% of external information from vision. Since quality of life markedly decreases with blindness, risk assessments for visual toxicity of new drugs are extremely important. In the past, many kinds of drugs and chemicals caused adverse events in humans (Table 1), resulted in big social problems even if not lethal. Humans highly rely on visions for their lives. Therefore, they recognize only a small change related to vision, which leads to accumulate a lot of complaints. Due to this reason, there are a broad array of adverse events related to functional and organ-ic abnormalities of vision including reduction of vision, loss of vision, asthenopia, diplopia, photophobia, eye irri-tation, eye pain, foreign body sensation, ptosis, keratitis, cataract, retinal detachment, cystic macular edema, macular fibrosis, choroidal detachment and choroidal exudate (Japanese Maintenance Organization, 2007).

The repeated dose toxicity study guideline of ICH S4 (Ministry of Health and Welfare, 1999) only indicates a brief instruction of procedure for ophthalmological examinations, such as the number of animals examined, frequency of examinations, etc. Therefore, detailed procedures of examinations should be decided by each company or facility, especially by collaborating with toxicologists as a study director. In addition to procedures of ophthalmological examinations, how appropriately clinical signs and pathological findings are recorded also should be considered essential for adequate ocular toxicity risk assessments.

This article reviews general considerations in ocular toxicity risk assessments from toxicologists’ viewpoints including basics of ophthalmology and principles of examinations, methodology of assessments of ocular findings, and roles of toxicologists and other study personals. This review is based on a workshop entitled “Scientific Viewpoints in Ocular Toxicity Assessment: Departure from Conventional Practice” at the 40th annual meeting of the Japanese Society of Toxicology (Chiba, Japan, 2013).

BASICS OF EXPERIMENTAL ANIMAL OPHTHALMOLOGY

The eye is an assembly of many specialized sub-organs which have specific functions, and integral maintenance of homeostasis in the eye plays an important role of visual function (Fig. 1). On the other hand, there are extremely many kinds of species differences in the eye (Table 2). The eyelid covers the front surface of the eye, and to replace tear components and to remove foreign body, the eyelid wipes the surface of cornea. Tear film includes the mucin layer at the most inner acting as glue between the cornea and the aqueous layer at the intermediate, the aqueous layer mainly including aqueous components, and the lipid layer at the most outer protecting from evaporation of aqueous components. Integrated homeostasis of each layer of the tear film is necessary for maintenance

Table 1. Drugs and chemicals which have caused ocular adverse events.

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal abnormality</td>
<td>Antimicrobial drug, anesthetic eye drop, contact lens detergents, fluorouracil, cytotoxic, amiodarone, botulinum toxin A, H1 blocker, benzodiazepine, benzalkonium chloride</td>
</tr>
<tr>
<td>Glaucoma</td>
<td>Steroid, docetaxel, paclitaxel</td>
</tr>
<tr>
<td>Cataract</td>
<td>Steroid, busulfan, phenothiazine compound (chlorpromazine), muscarinic inhibitor</td>
</tr>
<tr>
<td>Retinal abnormality</td>
<td>Chloroquine, quinoform, tamoxifen, phenothiazine compound (chlorpromazine and thioridazine), isotretinoin</td>
</tr>
<tr>
<td>Abnormality in optic nerve and retinal ganglion cells</td>
<td>Ethambutol, thinner, methanol, linezolid, ciprofloxacin, fluorouracil, cisplatin, infliximab, adalimumab, imatinib, cyclosporine, tacrolimus, methotrexate, ibuprofen, disulfiram, sildenafil, metronidazole</td>
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</table>
of smoothness of the corneal surface, and corneal superficial layers obtain nutrition from the tear film. There are species differences in frequency of blinking; once per approximately 5-6 min in rodents, and once per approximately 5-6 sec in monkeys and humans. Based on this reason, in the case of ocular instillation drugs, its outflow is comparatively rapid in monkeys and humans. Another example of species differences is the nictitating gland discharging the aqueous components of tear in dogs, while the nictitating membrane is not present in monkeys or humans. The Haderian glands are a well-known example which lead low blinking frequency in several species since the gland remains abundant oily component in the lipid layer.

For receiving light in photoreceptors, tear film, cornea, aqueous humor, lens, vitreous body and retina should be kept transparent. The cornea consists of the corneal epithelium, stroma and endothelium. The corneal epithelium has a potency of regeneration, and slight wounds are rapidly healed. The corneal stroma has regular arrangement of collagen fibers, and this structure makes a transparency of cornea. The corneal endothelium has an enzymatic pump system for dehydration, but it has very limited regeneration potency. Compared with humans, the cornea in rats is half as thick, that in rabbits is approximately 60%, dogs and monkeys are similar, and miniature pigs are thicker.

The aqueous chamber is the space surrounded with the cornea, iris, ciliary body and lens, which is divided to the anterior chamber and posterior chamber by iris, and filled with the aqueous humor. The aqueous humor is secreted from the ciliary body, and excreted via the trabecular or uveoscleral routes. There are species differences in volume, turnover time and outflow route of aqueous humor. The turnover time of aqueous humor is 30-40 min in mice and rats, one hour in monkeys, two hours in humans and rabbits, and three hours in dogs. The main drainage route of aqueous humor in human is the trabecular outflow while it is the uveoscleral outflow in monkeys. The aqueous humor supplies nutrition to cornea and lens, which are avascular sub-organs. The lens plays an accommodation of focus with the ciliary zonule. The vitreous body plays a role of maintenance of eye size, storage of substances and protection for lens and retina from foreign substances. Since there are species differences in vitreous volume, estimated concentration in the vitreous body should be considered in intra-vitreous injection of drugs. Compared to humans, vitreous volume is approximately 75% in minipigs and dogs, half in rabbits and monkeys, and 1% in mice and rats.

The retina has photoreceptors (rods and cones) which transduce light (photon) into neuronal signals. The rods play a role of light-vision, and distribute whole of the retina. On the other hand, the cones play a color-vision, and mainly distribute central field of the retina. Only humans and monkeys have the macular, which consists of only...
cones, present at the temporal side of the optic disc. Dogs have the tapetum at the dorsal portion of the fundus, and it commonly shows triangle shape. There are species differences of distribution of the retinal vessels, which enter to the eyes via the optic disc. In rats, dogs, monkeys and humans, vessels are arranged in a radial manner while rabbits have two big vessels to right and left, and guinea pigs have no retinal vessels.

The development of the eye is generally similar among species, and it initiates from differentiation of the neural tube from the embryo epithelium. The optic vesicle exserts from the prosencephalon, and the tip of optic vesicle becomes the optic cup. On the other hand, thickened surface ectoderm becomes the lens placode, and invaginated lens placode interacts with the optic cup and finally forms the lens. The optic cup invaginates further, and outer and inner layer of the optic cup differentiate the retinal pigment epithelium and the retina, respectively.

Table 3 shows embryonic origins of each sub-organ of the eye. Origin of the corneal epithelium and lens is the surface ectoderm. Origins of the iris pigment epithelium, the iridic portion of retina and the retina are the neural ectoderm, and origins of other sub-organs are the mesoderm and endoderm. Species which have long gestation period, such as humans and monkeys, complete retinal development before birth, but species which have short gestation period continue postnatal development of the retina.

As described above, integral maintenance of homeostasis in the eye plays an important role of visual function. The retina has a protective mechanism from increase in temperature and oxidative stress due to excess energy from light and neural activity. The blood-eye barrier at the vascular endothelium and the retinal pigment epithelium protects from exposure of foreign-origin chemicals as the blood-brain barrier does. Since the cornea and lens are avascular, the tear film and aqueous humor supply oxygen and nutrition, and excrete waste products. With disturbance in such mechanism, loss of integrity of homeostasis may result in toxic findings.

In summary, the eye is an assembly of many specialized sub-organs which have specific functions, and integral maintenance of homeostasis in the eye plays an important role of visual function. When only a small part of integrity of functions is lost, overall function of the eye might be disturbed. Therefore, understanding of anatomy and physiology of each sub-organ may help interpret mechanisms of observed ocular changes. In addition, possessing knowledge of species differences of the eye is a prerequisite to interpret ocular findings in nonclinical toxicity studies to extrapolate to humans.

### Table 3. Origins of each sub-organ of the eye.

<table>
<thead>
<tr>
<th>Ectoderm</th>
<th>Mesoderm</th>
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<tbody>
<tr>
<td>Surface ectoderm</td>
<td>Neural ectoderm</td>
</tr>
<tr>
<td>Cornea</td>
<td>○</td>
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<tr>
<td>Iris</td>
<td>○</td>
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<tr>
<td>Lens</td>
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<tr>
<td>Viscous body</td>
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<tr>
<td>Retina</td>
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<tr>
<td>Choroid</td>
<td>○</td>
</tr>
<tr>
<td>Sclera</td>
<td>○</td>
</tr>
<tr>
<td>Conjunctiva</td>
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</table>

**OPHTHALMOLOGICAL EXAMINATIONS IN NONCLINICAL TOXICITY STUDIES**

To evaluate drug effects on the eye, ophthalmological examinations are commonly conducted in nonclinical toxicity studies. Even though a lot of compounds are screened out before clinical trials or marketed due to possibilities to cause ocular abnormalities for the purpose
of preventing adverse events in humans, severe ocular abnormalities including loss of vision can be also found in clinical practices. It is considered difficult to detect cellular changes of the eye only from ophthalmological examinations. Therefore, in order to maximize contributions of results of nonclinical toxicity studies to clinical practices, integration of ophthalmological examinations and histopathological examinations is necessary.

Generally speaking, observations using slit lamp biomicroscope, and direct or indirect ophthalmoscope are considered sufficient as a routine examination in standard repeated dose toxicity studies. Using these tools, morphological changes of the eye are mainly found. Other than those changes, at the same time, functional changes including conditions of tear film, movements of eyelids and neuro-ophthalmic responses should be detected in routine examinations. Pupillary light reflex examinations are often conducted for assessments of visual function in several facilities. When functional abnormalities of the retina are concerned or suspected, an electroretinograms (ERGs) can provide detailed information. In addition, several new technologies for non-invasive morphological examinations including Specular Microscopy for corneal endothelial cells and Optical Coherence Tomography (OCT) for fundus are considered to be a useful tool for nonclinical toxicity assessment.

In the eye of laboratory animals used in nonclinical toxicity studies, many kinds of spontaneous abnormalities have been observed and these findings should be appropriately differentiated from treatment-related changes. For example, while drug-induced corneal insult and cataract have been well known, spontaneous opacities of the cornea (Fig. 2) and lens (Fig. 3) are also often observed in albino rats (Inagaki and Kuno 2001; Taradach et al., 1981; Shibuya et al., 2001a, 2001b). Especially, in Wistar rats, severe corneal opacities are in very high incidence (Hashimoto et al., 2013; Okamura et al., 2011). Incidence of these spontaneous findings in rats varies by ages, product lots or housing environments. Rabbits, commonly used in nonclinical studies for ocular drug development, are well known to have differences of spontaneous abnormalities among breeds (Holve et al., 2011). In terms of retinal spontaneous abnormalities, the retinal atrophy is one of classic ones, which is macroscopically observed as a hyper reflectivity of fundus. Although several drugs

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**Fig. 2.** Slit lump photograph of the cornea in Fischer 344 rat shows punctate multifocal crystalline opacities at the intrapalpebral region.

**Fig. 3.** Slit lump photographs of the lens in Sprague-Dawley rats. Focal lens opacities are observed in the anterior cortex (A) and the nuclear (B). Two white curve lines are light reflection from the surfaces of cornea (more remarkable line) and lens.
induce retinal atrophy, a spontaneous liner hyper reflectivity of fundus is frequently observed in albino rats (Fig. 4) (Hubert et al., 1994). Diffused hyper reflectivity of fundus due to light-induced damage is often occurred in albino rats (Fig. 5), and investigators should confirm housing conditions including light intensity, distance between animal and source of light, and duration of lighting (Wasowicz et al., 2002; De Vera Mudry 2013). In addition to the common knowledge in nonclinical studies, zinc pyrithione- and several macrolide antibiotics-induced tapetum specific abnormalities have been known (Cloyd et al., 1978; Massa et al., 1984; Fortner et al., 1993).

Detailed recordings of lesions are essential for ocular toxicity assessment and useful to distinguish toxicity findings from spontaneous ones. When it comes to lens opacity, for example, its incidences are usually different by location. Since lens has a variety of microanatomy among the cortex vs nucleus, anterior vs posterior, and others, descriptions about location will be helpful to evaluate a cause and progress of findings.

Since pupillary light reflex is used as an indicator of retinal function, loss of pupillary light reflex often indicates loss of retinal function. However, even when almost all of the retinal functions are damaged and vision is lost, slow pupillary light reflex caused by photosensitive melanopsin in retinal ganglion cell may be remained (Markwell et al., 2010). Also, even when retinal function is intact and vision is remained, there is a case where pupillary light reflex is negative or weakened due to damage of the efferent nerve and pupillary sphincter. Occasionally, mydriasis does not occur in dark conditions, which might be related to abnormality of trigeminal reflex and/or inflammation mediator due to iritis. Based on these reasons, findings should be recorded not only with positive or negative in reflex, but also with quickness and degree.

Electroretinograms (ERGs) and pattern visual evoked potentials (P-VEPs) are useful indicators to objectively grasp functional changes in the case of extremely slight or no organic abnormalities. ERGs are indicators of retinal function. In the field of toxicology, full-field ERGs are recorded in animals. In usual cases, five responses are included in full-field ERGs; rod response, maximal response, oscillatory potentials, cone response and flicker response (Fig. 6). Rod responses are originated from rod function working in the scotopic condition while cone response and flicker response are from cone func-

Fig. 4. Fundus photograph in Sprague-Dawley rat shows linear hyper reflectivity of fundus (Black arrows).

Fig. 5. Fundus photographs in Sprague-Dawley rat show diffused hyper reflectivity of fundus due to excessive light exposure (A) and normal fundus (B). Irregular surface of the retina and narrowing retinal vessels are also observed.
Oscillatory potentials are from amacrine cells. Maximal responses are a mixture of cone and rod function. With full-field ERGs, toxic effects of compounds on rods and cones could be separately evaluated. Sasaki et al. (2006) showed using full-field ERGs toxic effects of NaIO₃ in dogs were expressed first in rods followed by cones.

Compared with ERGs which are indicators in retina, P-VEPs are those in visual pathway unlimited to retina (Fig. 7). Therefore, P-VEPs can be an indicator to detect damage of visual pathway other than that of retina while ERGs cannot detect such damage. With P-VEPs, toxic
effects of compounds on visual pathway could be evaluated. Sasaki et al. (2003, 2004) demonstrated P-VEP can detect toxic effects of ethambutol in rats. While these electrophysiological indicators are useful in the field of toxicology, there are difficult cases where small focal abnormalities in the retina or visual pathway cannot be detected. Also, for these examinations in animals, immobilization of animals with animal positioner or anesthesia is necessary and it is an area for improvement in order that these indicators are incorporated in toxicity studies as a common examination.

The main purpose of ophthalmology examinations includes in-life morphological and functional investigations of eyes during antemortem period, and it is difficult to retain permanent samples like histopathological specimens at necropsy for in-life examinations. Therefore, ophthalmological examinations should be conducted by well-educated and -trained ophthalmologists with appropriate examination skills in order to record all findings accurately. In addition, results from ophthalmology examinations should be informed to histology sample technicians, pathologists and toxicologists so that ocular findings can be sufficiently shared for subsequent histopathological examinations.

From these viewpoints, in ophthalmologic examinations in nonclinical toxicity studies, it is vital to understand principles and features of each examination. Comparisons of findings between pre and post drug treatment as well as considerations of species differences, strain differences, age differences, and location/degree of abnormalities are essential. In addition, to encourage further appropriate ocular toxicity risk assessments, ophthalmologists should share results of ophthalmological examinations with other study personals.

CLINICAL SIGNIFICANCES IN ANIMAL OCULAR FINDINGS AND RISK ASSESSMENTS

As described above, recently the ophthalmological examination skills of Japanese researchers have been improved, but they still need further improvement, especially for data handling and process of ocular toxicity risk assessments.

In the past, many kinds of drugs and chemicals (e.g. methanol, chloroquine, quinoform, and others) caused adverse events in humans (Table 1) and resulted in big social problems (Li et al., 2008; Nakao, 2008; Hosotani, 2008; Mimura, 2008). Since blindness deteriorates quality of life, a critical matter in ocular toxicity risk assessments is whether or not effects are “sight-threatening”, the same as whether or not “life-threatening” in other risk assessments.

On the other hand, ocular abnormalities, even if they do not cause blindness, may result in a reduction of “Quality of Life (QOL)”, or what we call a reduction of “Quality of Vision (QOV).” As described above, clinical patients often complain about a reduction of QOV. Human eyes have sophisticated functions including quick automatic focus, automatic exposure control, shaking adjustment, three dimensional vision, night-vision, target tracking and others. The establishment of these functions needs highly integrated control of visual information processing, muscle motion (extraocular muscles and muscles related to eyelids, iris and lens) and the afferent and efferent nervous responses. Even if only a part of these functions is affected, humans would feel a reduction of QOV. It is difficult to predict QOV-related findings in human from nonclinical toxicity studies, since there are many kinds of species differences between human and laboratory animals and QOV-related findings are commonly based on patient’s subjective complaints, which are not able to be detected in animals. However, a part of QOV-related findings in human like abnormalities in muscle motion and nerve can be observed in ophthalmological examinations in animals in the same manner. It is important to consider these limits and potential for extrapolation from animals to humans in conducting ocular toxicity risk assessments.

For risk assessment in humans, clinical significances of ocular toxicity findings should be evaluated. Generally speaking, since there are common principles in risk assessments among organs, “species differences”, “safety margins”, “reversibility”, and “risk-benefit balance” are also considered in evaluating clinical significances of ocular toxicity.

The eye has extremely many kinds of “species differences”, and it is vital to recognize that prediction of human ocular toxicity is very difficult from the results of nonclinical toxicity studies. On the other hand, toxicity changes in animals do not always mean human risk. For example, toxicity findings in the Harderian gland cannot be extrapolated to human, since there are no Harderian glands in human. Therefore, we have to appropriately judge if ocular findings in animals can be extrapolated to human.

High dose treatment of chloroquine caused severe ocular adverse effects, but it has been still used in overseas, because there are safety margins secured with lower dose level in human for malaria than toxic dose level of chloroquine. Based on this fact, we could say that considering dose-relationship and NOAEL are useful for ocular toxicity risk assessment.

Reversibility of ocular toxicity findings can be predict-
ed not only from results of the recovery study with withdrawn period. Since possibilities of regeneration in several cells or sub-organs (e.g. corneal endothelial cells and retina) are limited, toxicity findings for these cells or sub-organs are considered irreversible. Since corneal endothelial cells play an important role of pump, extensive damage of corneal endothelial cells causes irreversible corneal opacity due to edema. Retina plays a role of phototransduction, and retinal changes, even if slight, may cause “sight-threatening” effects. Cataract is defined as a disease with opacities in lens due to denaturation and aggregation of lens protein, and lens opacity is never disappeared once occurred.

“Risk-benefit balance” is considered as a very important factor for ocular toxicity risk assessments. Anti-hypoglycemia drugs cause hyperglycemia in normal animals, and sometimes results in cataract. However, in the case of patients with hypoglycemia, the risk of hyperglycemia will not extremely low since the drug is withdrawn when blood glucose increased higher than normal level. In the case of “sight-threatening” diseases for which there is not any other effective option, high risks even for blindness might be acceptable within risk-benefit balance, like the clinical trial for age-related macular degeneration by implantation of the iPS-derived retinal pigment epithelium sheet (Kamao et al., 2014). At this moment, subjects of this advanced therapeutic procedure are only limited to the patients for whom any other treatment method, including anti-VEGF drugs and photodynamic therapy, are not effective. In the case of drugs which cause ocular toxicity, if benefits clearly outweigh risks, development and/or launch of drugs might be acceptable. For example, steroid is well known to frequently cause glaucoma and cataract (Mimura, 2008; Kashiwagi, 2008; Gupta and Wagner, 2009), but these are essential drugs for various diseases. Since periodical examinations (including intraocular pressure measurement, fundus examination and slit lamp biomicroscopy) can enable to detect glaucoma and cataract at the early stage, it is considered beneficial and acceptable to use steroids. From the point of view, if ocular toxicity findings are observed in nonclinical toxicity studies during drug development process, but when benefits outweigh risks, a careful monitoring in clinical trials enables to continue the drug development.

Detailed recordings of ocular findings are also a critical part in ophthalmological examinations in nonclinical toxicity studies. Concise and precise descriptions of features of lesions (location, size, severity, shape, color, and others) are necessary for a judgment of clinical significance of ocular findings, including speculation of mechanisms, relationship with drug treatment, differentiation from spontaneous changes, possibility of abnormalities in related tissues, effects on vision and QOL, reversibility, and others. For example, corneal opacity is one of commonly observed findings, and detailed description helps discussion on its clinical significance for aforementioned reason. Causes of corneal opacity include “crystalline deposition”, “edema of corneal stroma”, “trauma”, “scar”, “neovascularization”, “pigmentation”, and others. “Crystalline deposition” is a deposition of minerals such as calcium in a sub-epithelium of cornea (Figs. 2 and 8), and is spontaneously observed in high incidence in Wistar, Sprague-Dawley and Fischer 344 rats (Shibuya et al., 2001a, 2001b; Inagaki and Kuno, 2001; Williams, 2002). In the case of calcium deposition, opacities are sharply marginated, often observed at the interpupillary region, and increased with age. A cause of “edema of corneal stroma” is dysfunction of enzymatic pump in the corneal endothelial cells, and margin of opacity is unclear. “Traumatic” corneal opacity is mainly caused by fighting, and shows a linear shape. “Scarring”, “neovascularization” and “pigmentation” is frequently observed in the repairing process from damage on the cornea, or related to chronic irritation to corneal surface. Abnormalities of tear film components may relate to chronic irritation, and detailed examinations of secretion tissues of tear film components are necessary. To reflect features of corneal opacity, detailed descriptions with adjectives should be recorded. If just only “corneal opacity” is recorded, it is difficult to make a judgment for clinical significance.

For ocular toxicity risk assessments, while an evaluation of “sight-threatening” effects is most critical mat-
ter, QOV-related findings also should be considered. To extrapolate to human, clinical significances of ocular toxicity findings should be evaluated in consideration of “species differences”, “safety margins”, “reversibility”, and “risk-benefit balance”. In addition, detailed recordings of features of lesions are also important for a judgment of clinical significance of ocular findings.

**OCULAR PATHOLOGICAL FINDINGS AND RISK ASSESSMENTS**

General information on drugs and antemortem study data play important roles in appropriately evaluating pathological finding in gross and histopathological examinations. Useful general information of drugs includes pharmacodynamics, mechanisms of action, target organ/molecules, distribution and melanin binding. Albino rodents are commonly used in nonclinical toxicity studies. Several drugs are known to show significant affinity to melanin and the retinal pigmented epithelium is one of targets for systemically dosed drugs with melanin affinity (Mecklenburg and Schraermeyer, 2007). On the other hand, there are several drugs which bind to melanin without ocular effects (Leblanc et al., 1998). Therefore, it is important to accumulate background data of both albino and pigmented rodents for appropriate ocular risk assessments of drugs with or without melanin affinity (Imawaka et al., 1997; Shibuya et al., 1997). Prior to necropsy, pathologists should know overall visual conditions of individual animals from clinical sign data and from ophthalmological examinations, including size and symmetry changes of the eyes, conditions of the eye lids, abnormal gait and avoidance of obstruction. Abnormal gait and avoidance of obstruction may relate to loss or reduction of vision as well as abnormalities in four limbs or central nervous system. To identify or to estimate affected regions in histological study, pathologists should obtain records of locations (eyelids, conjunctiva, cornea, aqueous chamber, lens, vitreous body, retina, optic disc, choroid, vascular system and others) in ophthalmological examinations, and/or information of functional changes from electro-physiological examinations (ERGs, VEPs and others).

Appropriate preparation of pathological specimen by minimizing damage of specimen, and appropriately selecting fixatives are necessary for accurate pathological evaluation. Even if ring-tipped forceps is used, direct pinching of the eyeball itself often makes a tractive load to several weak parts, and cause artificial damage in optic disc and peripheral retina, lens luxation, or others. In order to appropriately collect the eyeball from animals, educations of its anatomical features in laboratory animals to the technicians who conduct gross necropsy are necessary. While the eyeballs generally are collected at the end of process of necropsy, earlier collection and immediate dip to the fixative are preferable, since several sub-organs of the eye (retina, optic nerve or others) belong to the nerve system which is more vulnerable to deterioration with time.

Table 4 shows commonly used fixatives for the eyeball. Advantages and disadvantages of the fixatives for each sub-organ in the eye are different. Using the same fixative among studies is useful in collecting background data and comparing study data with background data. However, fixatives more appropriate for sub-organs should be selected for histopathological examination, if needed. The eyeball is an assembly of sub-organs with different hardness. For example, the retina, optic nerve, or others belonging to the nervous system are very soft and delicate, although the lens, sclera, cornea, or others are very hard and fibrous. Since appropriate dipping timing in the fixative is different among sub-organs, preliminary experiments for fixatives condition are necessary for each animal species. Fixation in unnecessarily longer duration generally makes difficult preparation of specimens, like, sectioning of hard tissues such as the lens, and ends up causing damage not only in the sub-organ itself but also in the adjacent tissues.

Orientations of ocular lesions are based on the records of ophthalmological examinations and clinical signs. Prior to necropsy, securing close communications among ophthalmologists, gross necropsy pathologists and histopathology technicians should be effective and helpful.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Cornea</th>
<th>Iris/ciliary body</th>
<th>Lens</th>
<th>Retina</th>
<th>Immuno-histochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davidson solution</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Mixed solution of formalin and glutaraldehyde</td>
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<td>10% neural buffered formalin</td>
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<td>4% glutaraldehyde</td>
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<td>Bouin solution</td>
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</table>

Table 4. Commonly used fixatives for the eyeball.
To reflect location of ocular lesions accurately on the histological slides, markings should be put on the sample using an oil-based felt pen or Indian ink (Fig. 9). If a part of eyelid skin remained with the eyeball, the direction of the eyeball can be identified easily. Histopathological examinations are commonly done with the sagittal section of eyeball. Before embedded in paraffin blocks, quarter to one-third of the eyeball is removed and then embedded in larger cups to avoid deforming of the eyeball. Haematoxylin and eosin staining is commonly selected for histomorphological examinations for the eyeball, but other specific staining methods or histoimmunohistochemical staining methods should be considered for a detailed observation of lesions.

Differentiation of artifacts from toxicity findings is very important in histopathological examinations for the eyeball. As mentioned above, specimens are damaged by various exogenous factors, such as postmortem changes and preparation process of specimen. Man-made artifacts include lack of the optic disc, invagination of optic nerve into inside the eyeball, partial lack of the corneal epithelium, lens luxation-related fold formation of the peripheral retina. Retinal detachment and degeneration of the retinal cells might occur due to postmortem changes. Fixative condition may cause roughness of the corneal stroma, vacuolization of the lens cortex, mal-staining of the lens nucleus, retinal detachment, atrophy of the retinal cells and cytoplasmic vacuolization of the retinal cells. By comparing with other sub-organs in the eye and by confirming whether or not there are related concurrent changes, most artifacts can be differentiated. Artifacts due to fixative conditions usually can also be differentiated based on comparison of treated animals with control ones or specimens which are prepared with a different product lot of fixatives.

In addition, differentiation of spontaneous changes from toxicity findings is also important, since various spontaneous ocular changes are well known in rodents, commonly used in nonclinical toxicity studies. Strain-specific or age-related histological ocular lesions in the cornea, lens and retina have been reported (Shibuya, 2001a, 2001b; Ban et al., 2008). Severity and incidence of those ocular changes should be recorded as historical control data in each facility. It certainly helps differentiate treatment-related changes from spontaneous changes.

The following are points to consider in judging if the findings are treatment-related: bilateral vs unilateral; dose-relationship; the same findings in control animals; incidence comparison with control animals; progression with time; reversibility; and similar findings in other organs.

Since pathological evaluations are conducted at the final stage of toxicity study and can provide information of tissue and/or cellular level changes, they commonly have a big impact on risk assessments. However, it is impossible to detect all ocular changes only by histopathological examinations. Even if every possible effort is made, small local abnormalities are difficult to be found, and functional ocular toxicity cannot be detected even in elaborate microscopic or electron microscopic examinations. Therefore, there is a limitation in histopathological examinations. From the point of view for ocular toxicity risk assessments, a comprehensive evaluation with pathological findings and other results in toxicity studies should be considered.

Fig. 9. Standard sampling methods of the eyeball and marking of ocular lesions.
CONCLUSIONS

The eye is an assembly of many specialized sub-organs which have specific functions, and integral maintenance of homeostasis in the eye plays an important role of visual function. When only a part of integrity of functions is lost, overall functions of the eye might be commonly disturbed. Therefore, understanding of anatomy and physiology of these sub-organs may help know mechanisms of observed ocular changes. In addition, knowledge of species differences in the eye is a principle to extrapolate ocular findings in nonclinical toxicity studies to those in humans, and taking advantage of collection of historical background data is necessary.

In ophthalmologic examinations in nonclinical toxicity studies, it is vital to understand principles and features of each examination. Ophthalmological examinations should be conducted by well-educated and trained ophthalmologists with appropriate examination skills, and all findings should be recorded accurately during examinations on the spot. Comparisons of findings between pre and post drug treatment as well as considerations of species differences, strain differences, age differences, and location/degree of abnormalities should be essential.

For ocular toxicity risk assessments, while an evaluation of “sight-threatening” effects is most critical matter, QOV-related findings also should be considered. To extrapolate to human, clinical significances of ocular toxicity findings should be evaluated in consideration of “species differences”, “safety margins”, “reversibility”, and “risk-benefit balance”. In addition, concise and precise recordings of features of lesions are also important for a judgment of clinical significance of ocular findings.

For preparation of histopathological specimens, careful sampling of organs and appropriate selection of fixatives are important. To accurately orient ocular lesions in the specimen for histopathological examinations, securing close communications prior to necropsy among ophthalmologists, gross necropsy pathologists and histopathology technicians are effective and helpful. Since it is impossible to detect all ocular changes only by histopathological examinations, there is a limitation in histopathological examinations. Therefore, for ocular toxicity risk assessments, comprehensive evaluations with pathological findings and other results of various examinations in toxicity studies should be considered.

The eye has very important physical functions, and it is a complicated organ. Ideally, integral ocular toxicity risk assessments should be conducted by the specialists such as “ocular toxicologists”, who have widespread knowledge of anatomy, physiology, and pathophysiologic of the eye both in laboratory animals and in humans as well as sophisticated skills of ophthalmological examinations. In addition, such specialists ideally understand a methodology for toxicology science. Since the number of such specialists is very small, development of them should be accelerated. In the current situation, toxicologists as Study Director play a role in judging ocular toxicity risk assessments without asking any help of ocular toxicology specialists. To enhance appropriate ocular toxicity risk assessments, sharing of data regarding the eye and closely communicating one another among toxicologists, clinical sign investigators, histopathology technicians and pathologists are the most important agenda.

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

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Toxicologists’ viewpoints in ocular toxicity risk assessment

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