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Original Article

Multi-site study of an *in vivo* phototoxicity evaluation in Sprague-Dawley rats: skin site and sex differences in sensitivity to drug-induced phototoxicity

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ABSTRACT — A standard animal model for phototoxicity evaluation does not appear in any guideline. Sprague-Dawley (SD) rats have been widely used in general toxicity and toxicokinetic studies and can be used in phototoxicity evaluation to reduce animal usage. To standardize phototoxicity procedures of SD rat, we investigated skin site- and sex-related differences in sensitivity to drug-induced phototoxicity at 3 facilities. Six-week-old male and female SD rats were orally administered 30 or 100 mg/kg lomefloxacin and light irradiation 1 hr after dosing; an ultraviolet (UV) irradiation device (10 J/cm^2, UVA) or solar simulator (18 J/cm^2, UVA) was used as light sources. Phototoxic reactions on ventral skin, dorsal skin, and auricle were observed macroscopically at 2, 24, 48, and 72 hr after irradiation. Plasma concentrations of lomefloxacin were also measured in non-irradiated, conscious rats. Skin reaction scores for ventral skin were highest and those of dorsal skin were lowest among the skin sites examined at all dose levels and facilities. Although drug concentrations in plasma were almost similar between sexes or higher in males than females, skin reaction scores appeared higher in females than males for ventral or dorsal skin. A difference in skin reaction scores among facilities was also observed; however, the order of skin sites based on sensitivity was approximately the same. We therefore suggest that appropriate conditions be drafted at each facility as differences in sensitivity to phototoxicity are dependent on skin site or sex in SD rats. Furthermore, we encourage multi-site validation studies to standardize experimental conditions in in-vivo phototoxicity studies.

Key words: Phototoxicity, Sprague-Dawley (SD) rat, Site difference, Sex difference, Lomefloxacin

INTRODUCTION

Drug-induced phototoxicity is an acute tissue response caused by the reaction between light and drugs. A nonclinical phototoxicity evaluation in drug development is important in the prediction of the underlying risk of phototoxicity in humans. The 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) is recommended as an *in vitro* assay in the ICH S10 guideline and has been commonly used to evaluate the phototoxic potential of drugs (ICH, 2013). Although the 3T3 NRU PT generates a low rate of false negative results, the rate of false-positive results is considerably high (Lynch and Wilcox, 2011). *In vivo* methods have also been widely used in the evaluation of

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phototoxicity, as a negative result in an appropriately-conducted *in vivo* phototoxicity study supersedes a positive *in vitro* result as stated in the ICH S10 guideline. Therefore, *in vivo* phototoxicity evaluation sometimes becomes the most important test performed in the nonclinical stage. The methodology of the *in vivo* phototoxicity study, however, has not been standardized in the ICH S10, OECD, or other international guidelines.

Selection of appropriate animal species is crucial when conducting toxicity studies. Mice and guinea pigs are widely used in phototoxicity studies (Nilsson *et al.*, 1993; Matsumoto *et al.*, 2010; Sambuco and Forbes, 1984), and Sprague-Dawley (SD) rats, one of the most common animal species and strains for general toxicity studies, are sometimes used in phototoxicity evaluation (Turnock *et al.*, 2018; Yonezawa *et al.*, 2015). Using SD rats in general toxicity and toxicokinetic studies guarantee the acquirement of information such as tolerability and toxicokinetics that can be used to set dose levels and timing of irradiation in a phototoxicity study, without the need for additional animals to conduct dose-finding studies. SD rat is therefore one of the most rational species for phototoxicity studies when animal welfare is considered.

We successfully validated our ventral skin model in female SD rats using 5 reference drugs (Kuga *et al.*, 2017). Using this model, we found that ventral skin showed significantly higher sensitivity than dorsal skin, and the selection of anatomical sites exposed to light was one of the most important factors that affected sensitivity to drug-induced phototoxicity. Although our model is considered useful for phototoxicity evaluation, it has only been validated in our facility.

Therefore, in the present study, we validated this SD rat model with the cooperation of individuals at 3 facilities, Takeda Pharmaceutical Company Limited (Takeda), Kaken Pharmaceutical Company Limited (Kaken), and LSI Medience Corporation (LSIM), where the same protocol was employed at the facilities. This protocol focused on the effects of skin site (ventral skin, dorsal skin, or auricle) and sex on sensitivity to lomefloxacin-induced phototoxicity. We selected lomefloxacin as the reference drug for use in this multi-site study as it is an established phototoxic drug in both humans and animals (Oliveira *et al.*, 2000; Adachi *et al.*, 2015; Shimoda *et al.*, 1993). Study conditions in each facility conformed to the ICH S10 guideline.

MATERIALS AND METHODS

Animals

Crl:CD(SD) rats (age, 5 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and acclimated to the laboratory environmental conditions for 1 week. On the day of dosing, rats were 6 weeks old and prior to dosing, they were divided into 3 groups: vehicle group, 30 mg/kg lomefloxacin group, and 100 mg/kg lomefloxacin group. Each group had 5 or 6 animals per sex in each facility. In addition, 6 males and 6 females were included for a toxicokinetic evaluation without light irradiation in Takeda.

The animals were housed in an animal room that was maintained between 20ºC and 26ºC. The animals were placed in individual rat cages and granted free access to feed and water.

The research project was approved by the Institutional Animal Care and Use Committees of Shonan Research Center (Takeda Pharmaceutical Company Limited), Kaken Pharmaceutical Company, and Kumamoto Laboratory (LSI Medience Corporation). The study also conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Drug administration and light irradiation

Lomefloxacin hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was suspended in 0.5 w/v% methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan; Wako Pure Chemical Industries Ltd., Osaka, Japan) solution. After rats were weighed, they were given an oral administration of a 5 mL/kg suspension once. Hair was removed from the back and abdomen of the rodents using an electric clipper at Takeda and LSIM, and an electric shaver followed by an electric clipper at Kaken. An hour following lomefloxacin administration, rats were irradiated under anesthesia. A mixture of medetomidine (0.15 mg/kg), midazolam (2.0 mg/kg), and butorphanol (2.5 mg/kg) was injected intramuscularly 15 min prior to light irradiation at Takeda and LSIM, and pentobarbital (50 mg/kg) injected intraperitoneally immediately before irradiation at Kaken. Animals were covered with aluminum foil to outline irradiation and non-irradiation sites. Animals were then irradiated using a solar simulator (SXL-5009V, Seric., Ltd., Japan) or ultraviolet (UV) irradiation device with UVA light source (FL20S BL/DMR; Toshiba Medical Systems Corporation, Tokyo, Japan) and UVB light source (TL20W/12RS; Philips, Amsterdam, Netherlands). The study design and conditions, including light and anesthesia in each facility, are summarized in Table 1. The irradiation dose of UVB was less than half of the mean erythema dose previously determined at Takeda or Kaken. After light exposure, atipamezole (0.06 mg/kg) was injected intraperitoneally to aid in animal recovery from anesthesia at Takeda and LSIM. Skin reaction was

Table 1. Difference in study conditions among the testing facilities.

	Takeda	Kaken	LSIM
Light source	Solar simulator (SXL-5009V)	UV irradiation device (FL20S BL/DMR and TL20W/12RS)	UV irradiation device (FL20S BL/DMR)
UVA irradiance	18 J/cm^2	10 J/cm^2	10 J/cm^2
UVB irradiance	0.36 J/cm^2	0.016 or 0.031 J/cm^2*	None
Visible light	Available	None	None
Hair remover	Clipper	Clipper and shaver	Clipper
Anesthetics	MMB (i.m.)	Pentobarbital (i.p.)	MMB (i.m.)

* Male ventral skin and female dorsal skin were irradiated for 0.016 J/cm^2. Other skin sites were irradiated for 0.031 J/cm^2.
MMB: Mixture of medetomidine, midazolam, and butorphanol.

Table 2. Criteria for scores in the evaluation of skin reaction (Draize Criteria).

Score for erythema
0; No erythema
1; Very slight erythema
2; Well defined erythema
3; Moderate to severe erythema
4; Severe erythema (beet redness) to slight eschar formation (in-depth injuries)
Score for edema formation
0; No edema
1; Very slight edema (barely perceptive)
2; Slight edema (area well defined with evident raising)
3; Moderate edema (raised approximately 1 mm)
4; Severe edema (raised more than 1 mm and extended beyond area of exposure)

observed at 2, 24, 48, and 72 hr after the end of light irradiation, using the Draize method (Table 2; Draize, 1959). Prior to observation, the criteria of skin reaction scores were harmonized among staff at each facility to ensure an equal evaluation was performed at the facilities.

Skin reaction evaluation

Skin reaction scores (erythema and edema formation) for individual animals were summed for each site and time point, and mean score and total mean score calculated using the following equation:

Mean skin reaction score = Total of erythema and edema scores / Number of animals tested.

The sum of mean skin reaction score at all time points was defined as "Total mean skin reaction score". Based on the results from the study, a statistical analysis was not deemed suitable; therefore, such analysis was not performed.

Toxicokinetics

Plasma concentration of lomefloxacin was determined to investigate the differences between sexes or dose levels without light irradiation at Takeda. Blood samples (approximately 0.25 mL) were withdrawn from the external jugular vein of conscious animals (n=3/sex/dose) at 0.5, 1, 2, 4, 8, and 24 hr after dosing. To obtain plasma, blood samples were centrifuged at 17400 × g for 1 min under a chilled condition (4°C). The plasma samples were transferred to 1.5 mL tubes and stored at -20°C or lower until analysis. The plasma concentrations of lomefloxacin were determined using liquid chromatography/tandem mass spectrometry (LC/MS/MS) and the toxicokinetic parameters (maximum observed concentration [C_{max}], the time to reach C_{max} [t_{max}], and area under the concentration-time curve from the 0 to 24 hr [AUC_{24}]) calculated. All the samples were precipitated with acetonitrile containing the internal standard (IS). The precipitated sample was centrifuged for 5 min at 21,600 × g. The supernatants were diluted with 0.01 mol/L ammonium formate containing 0.2% (v/v) formic acid and then injected onto a LC/MS/MS system. The LC/MS/MS system was a LC-30AD pump (Shimadzu, Kyoto, Japan) with a triple quapole mass spectrometry detection API4000 (AB Sciex, Framingham, MA) equipped with turbo ion spray ionization source in the positive ionization mode. The chromatographic separation was achieved with a reverse phase

(C18) column (Kinetex XB-C18, 2.6 μm, 2.1 x 50 mm, Phenomenex, Torrance, CA) at 50°C. The mobile phase, consisting of 0.01 mol/L ammonium formate containing 0.2% (v/v) formic acid (mobile phase A), and acetonitrile containing 0.2% (v/v) formic acid (mobile phase B), was delivered at a flow rate 0.7 mL/min. The analyte was eluted using a flowing gradient condition: mobile phase B was increased from 10% to 90% within 0.3 min, and this condition was maintained for 0.7 min. It was then set back to 10% within 0.01 min and maintained for 0.6 min for re-equilibration. Detection was accomplished by multiple reaction monitoring in positive ionization mode (SRM m/z = 352.0 → 308.0 for Lomefloxacin, m/z = 250.3 → 116.3 for Alprenolol [IS]). Analyst software version 1.6.2 was used for data acquisition and and processing. The concentration of Lomefloxacin in each sample was back calculated using a calibration curve generated from a set of calibration standards.

RESULTS

Skin phototoxicity assessment

The results obtained from the observation of skin reactions are shown in Figs. 1 and 2. Skin score was highest in the ventral skin and lowest in the dorsal skin under almost all conditions; an exception occurred at Kaken with males exposed to 100 mg/kg. In this group, the auricle was more sensitive than the ventral skin.

Skin score was observed to increase with dose levels. At 30 mg/kg, skin reactions were not observed in the dorsal skin of both sexes at Takeda and LSIM; however, slight erythema was observed at Kaken. At 100 mg/kg, all skin sites had skin reactions in both sexes at all facilities.

In the ventral and dorsal skin, the skin reaction score appeared higher in females than males; however, no clear sex-related difference was observed in the auricle.

The Kaken had the highest score for almost all conditions used when compared to the other facilities. For the abdomen in both sexes and the auricle in females at 100 mg/kg, higher scores were obtained at LSIM than Kaken. Takeda had the lowest score in all conditions, except male abdomen at 100 mg/kg.

Time course changes for the mean skin reaction score are shown in Figs. 3-6. In the ventral or dorsal skin, skin reactions always initiated at 2 hr after irradiation, except in cases that lacked findings. For the auricle, skin reaction began 24 hr after irradiation at 30 mg/kg in both sexes at Takeda and LSIM, and at 100 mg/kg in males at Takeda. Skin reaction occurred at 2 hr with 100 mg/kg administered to both sexes at Kaken and LSIM, and in females at Takeda.

In the control group or non-irradiated area, skin reactions were not observed at any site, time point, or facility (data not shown).

Toxicokinetics

The toxicokinetic parameters are shown in Fig. 7 and summarized in Table 3. The mean C_{max} and AUC_{24} dose-dependently increased in both sexes. In addition, evident sex differences in C_{max} and AUC_{24} at 30 mg/kg were not observed, and C_{max} and AUC_{24} in males tended to be higher than in females at 100 mg/kg.

DISCUSSION

The main purpose of this study was to clarify the effects of skin site and sex on sensitivity to drug-induced phototoxicity. We demonstrated that the ventral skin of

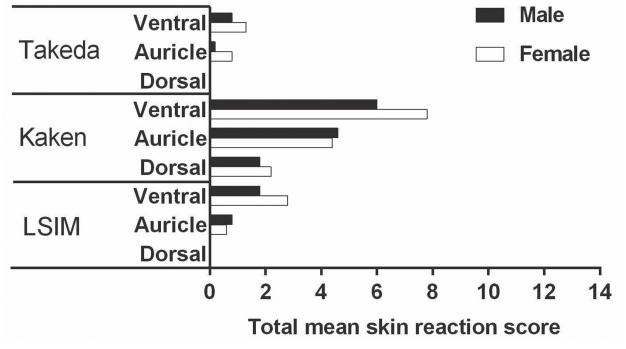

Fig. 1. Total mean skin reaction scores (sum of mean skin reaction score at all time points) when 30 mg/kg of lomefloxacin is administered.

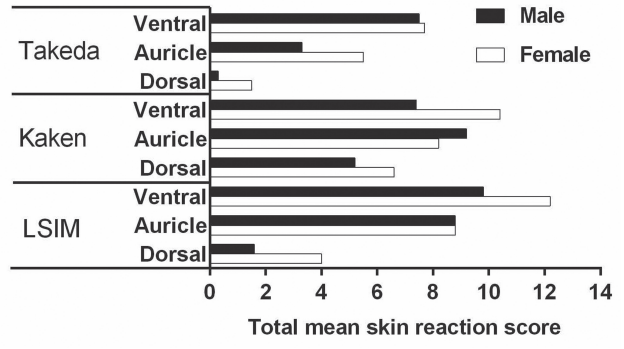

Fig. 2. Total mean skin reaction scores (sum of mean skin reaction score at all time points) when 100 mg/kg of lomefloxacin is administered.

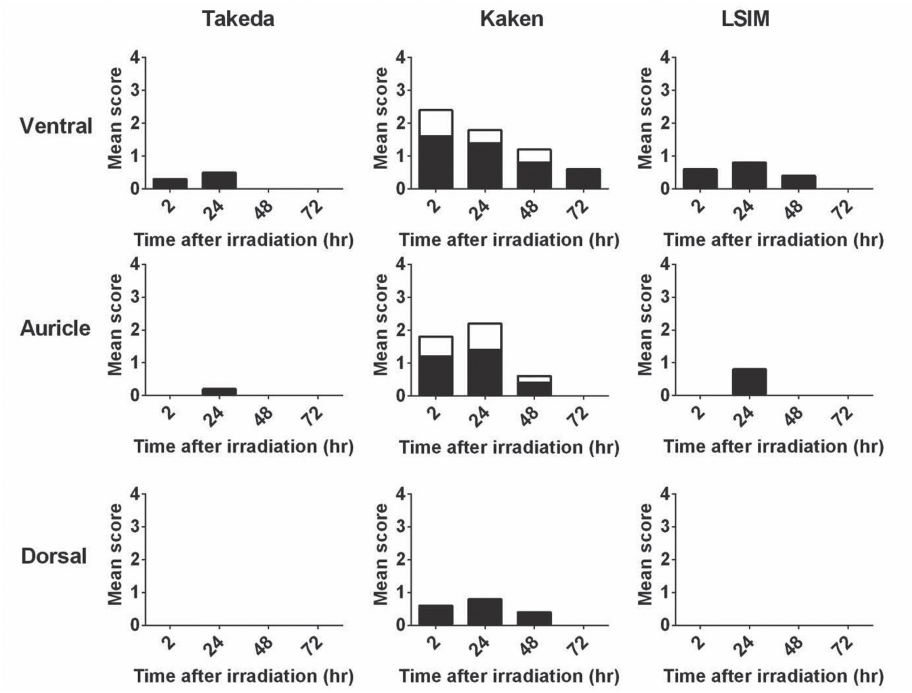

Fig. 3. Mean skin reaction scores in males after dosing with 30 mg/kg of lomefloxacin. Black and white bars indicate scores of erythema and edema, respectively.

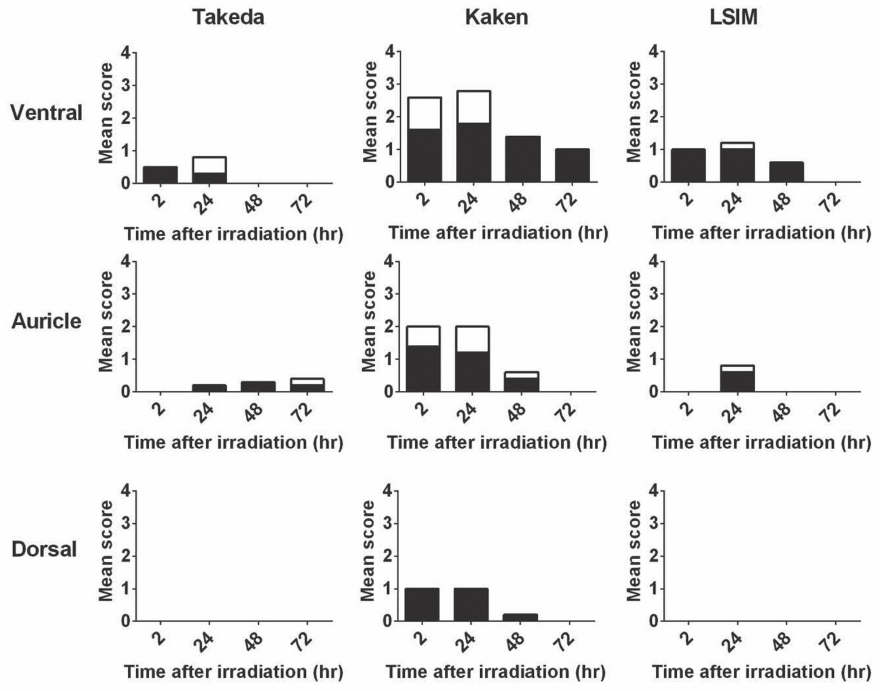

Fig. 4. Mean skin reaction scores in females after dosing with 30 mg/kg of lomefloxacin. Black and white bars indicate scores of erythema and edema, respectively.

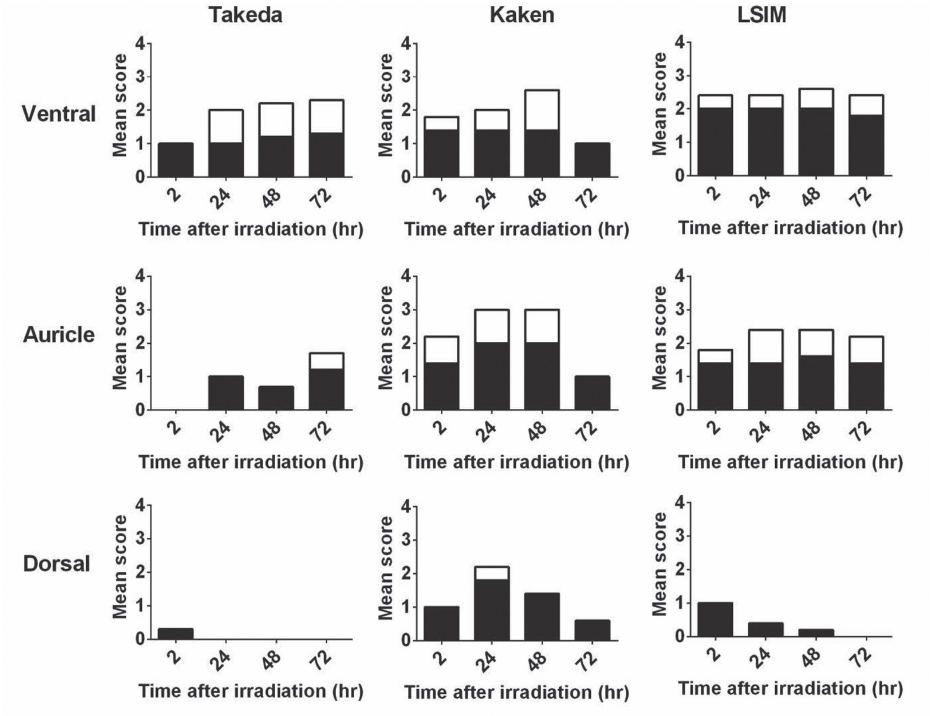

Fig. 5. Mean skin reaction scores in males after dosing with 100 mg/kg of lomefloxacin. Black and white bars indicate scores of erythema and edema, respectively.

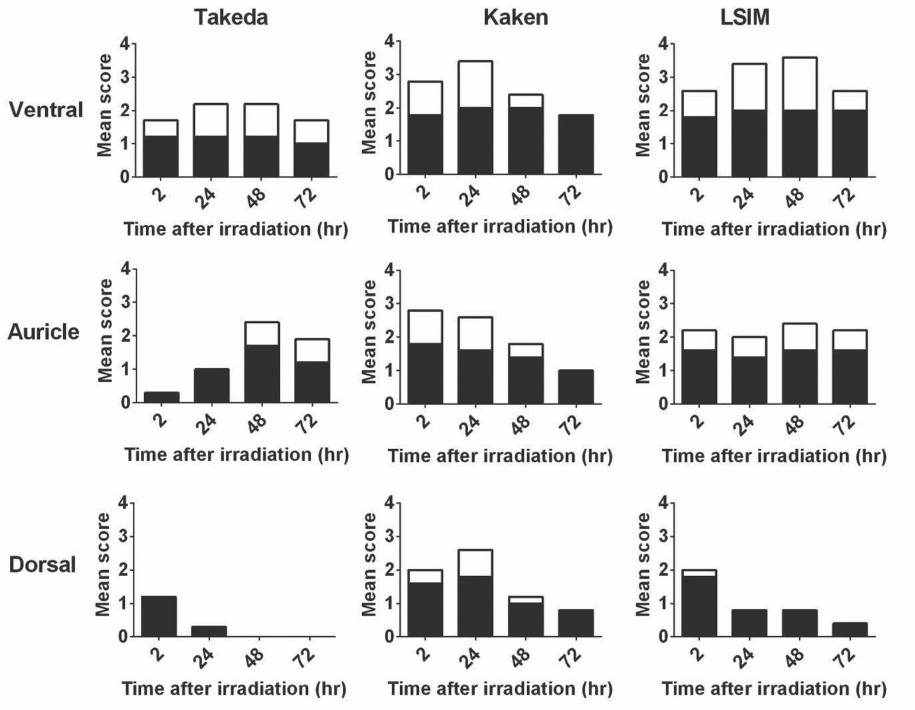

Fig. 6. Mean skin reaction scores for females after dosing with 100 mg/kg of lomefloxacin. Black and white bars indicate scores of erythema and edema, respectively.

Table 3. Toxicokinetic parameters for lomefloxacin.

	Male (N = 3)			Female (N = 3)		
Dose (mg/kg)	t_{max} (hr)	C_{max} (ng/mL)	AUC_{24} (ng·hr/mL)	t_{max} (hr)	C_{max} (ng/mL)	AUC_{24} (ng·hr/mL)
30	0.8 (0.3)	2260 (210)	8200 (516)	0.7 (0.3)	2690 (700)	7810 (635)
100	1.0 (0.0)	7320 (1020)	34700 (6110)	1.0 (0.0)	4790 (304)	25200 (2040)

Mean (S.D.)

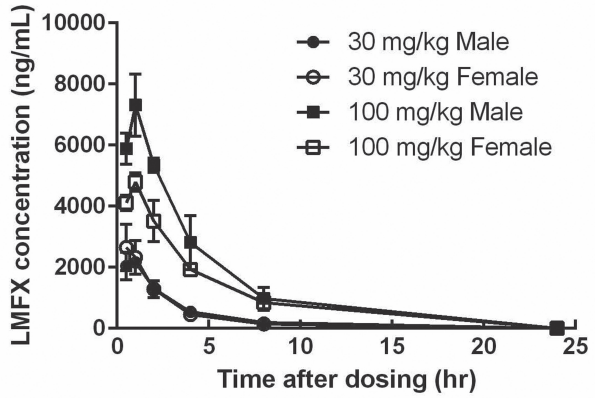

Fig. 7. Time course changes for lomefloxacin (LMFX) in rat plasma (Mean ± S.D.).

females exhibited the highest sensitivity while male dorsal skin had the lowest sensitivity, regardless of the study conditions utilized.

Evidently, sensitivity in phototoxicity differed among skin sites in the SD rats used in this study, a result aligning with our previous study (Kuga *et al.*, 2017). Erythema response to light irradiation differs among skin sites in humans and may be a result of differences in the barrier function of each skin site (Olson *et al.*, 1966). The skin is composed of two layers, epidermis and dermis, with the epidermis mainly consisting of two layers, the stratum corneum and viable epidermis. The outer two layers, stratum corneum and viable epidermis, have been recognized as the main barriers to UV and visible light (Bruls *et al.*, 1984). UV-induced erythema response correlates with thickness of the stratum corneum and viable epidermis (Waterston *et al.*, 2005). The dermis contains many blood vessels, and drug concentration in this layer may be higher than in the outer layers. The function of the dermis as a UV barrier, however, has not been well described. For drug-induced phototoxicity, the severity of erythema is dependent on both plasma drug concentration and the dose of irradiated UVA (Ljunggren *et al.*, 1981). In our previous study using SD rats, ventral skin showed higher sensitivity to drug-induced phototoxicity than dorsal skin; thus, we concluded that site variation in sensitivity to drug-induced phototoxicity mainly correlated with skin thickness rather than tissue drug concentration. This is because the stratum corneum and dermis in the ventral skin were thinner than those in the dorsal skin; drug concentration was almost comparable between the dorsal and ventral skin (Kuga *et al.*, 2017). A sufficient dose of UV that reaches drugs in blood or tissue is critical to the induction of a phototoxic reaction. In addition, the dose of UV passing through the skin barrier may inversely correlate with skin thickness. The onset and peak time point of skin reactions also differed among skin sites. Detectability of auricle at 2 hr was relatively low and skin scores at 100 mg/kg were highest at 2 hr in the dorsal skin in 2 companies. This result suggests that care should be exercised when selecting time points for skin observation.

Sex-related differences in sensitivity to drug-induced phototoxicity have not been previously reported. We demonstrated that the ventral and dorsal skin in females tended to be more sensitive to phototoxicity than in males, although drug concentration in plasma was higher in males than females in the 100 mg/kg group. This result suggests that sex-related differences in sensitivity may be associated with morphological factors rather than drug exposure; thus yielding skin site differences. The epidermis and dermis of the dorsal skin of male mice appear slightly thinner and thicker, respectively, than in female mice (Azzi *et al.*, 2005). Unlike the ventral or dorsal skin, evident sex-related differences in phototoxic reactions in the auricle were not observed, indicating negligible sex-related differences in skin thickness of the auricle.

Although the criteria for skin reaction scores were comprehensively shared with the staff at each facility prior to the study's initiation, large differences in skin reaction scores were found among the facilities. This difference may have been a result of three inconsistent factors in the experimental conditions: light irradiation, hair removal method, and anesthesia. First, the difference in light condition was considered to be the greatest influencer on reactions occurring on the skin. For almost all skin sites tested, the scores obtained at Kaken were the highest of the 3 facilities. The UVA light condition was the same between Kaken and LSIM; however, Kaken irradiat-

ed both UVB and UVA light. Lomefloxacin absorbs both UVA and UVB (Zhao *et al.*, 2010); thus, UVB light was also considered to enhance the phototoxic effect of lomefloxacin. Takeda's light source also included light from the UVB-region and a higher UVA dose was administered at this facility than at the other 2 facilities; skin reaction scores obtained at Takeda were the lowest of the 3 facilities for almost all conditions used. Since the spectral distribution of the solar simulator used at Takeda completely differed from the UV irradiation devices used at Kaken and LSIM, the intensity of light sources required to induce skin reactions should not be characterized using only each UV light dose. Indeed, mean erythema dose for UVB light was much lower at Kaken than Takeda based on previous studies (Kuga *et al.*, 2017; Yonezawa *et al.*, 2015). Solar simulators are recognized as the ideal light source for phototoxicity evaluations as they are similar to sunlight. UV irradiation devices have often been used in phototoxicity studies to simplify the experimental conditions. One should, however, exercise caution when comparing different light sources used in phototoxicity evaluation. Although only the light source used at Takeda included visible light, the presence of visible light was not considered to be related to the inter-site differences in the results as lomefloxacin does not absorb in the visible-light region (Zhao *et al.*, 2010). Second, hair removal procedures may also affect the skin's sensitivity to phototoxicity. The hair remaining on the skin can act as sunscreen, while excessive contact following hair removal may damage the stratum corneum, which is an important barrier to light irradiation. For removal, Takeda and LSIM only used electric clippers, while Kaken used electric clippers and then an electric shaver. The electric shaver was demonstrated to be more invasive than the electric clippers, and may have enhanced the sensitivity of the skin. Therefore, this may have caused the increased sensitivity observed in the dorsal and ventral skin at Kaken when compared to the other 2 facilities. Despite this occurrence, the method used at Kaken should not be viewed as "too invasive" as no skin reaction was observed in the vehicle control group. As hair was not removed from the auricle in any of the facility used, the difference in sensitivity among the facilities was not relevant to the difference in the hair removal method employed, at least for the auricle. Third, the difference in the anesthetics used may change the drug kinetic parameters, leading to differences in skin reaction. This may have transcended despite specifying any effects of anesthetics on toxicokinetics as blood samples were withdrawn from conscious animals.

In conclusion, we demonstrated skin site- and sex-related differences in sensitivity to lomefloxacin-induced phototoxicity in SD rats housed at all 3 facilities. To standardize the experimental conditions for *in vivo* phototoxicity study to achieve high relevance to humans, we encourage further multi-site validation studies using reference drugs with known positive or negative effects on phototoxicity in humans.

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

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