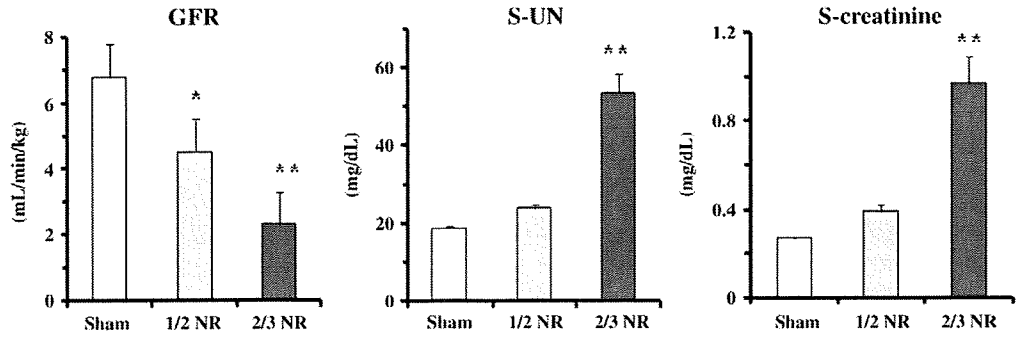

Fig. 2. Glomerular filtration rate (GFR) and serum urea nitrogen (UN) and creatinine concentrations 24 h after operation in male F344 rats subjected to 1/2 or 2/3 nephrectomy (NR). Animals that underwent a sham operation served as the sham control (sham). The GFR value was determined by the three-sample method. Each column and vertical bar represents the mean ± SEM of five animals. $^{*}p<0.05$ and $^{**}p<0.01$ versus the sham control group. S: serum.

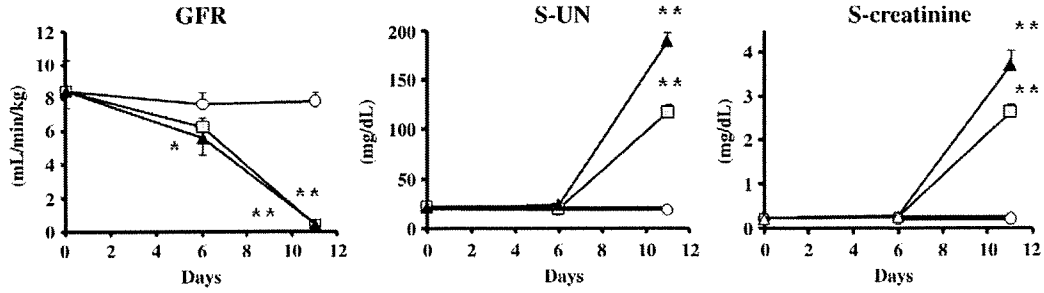

Fig. 3. Glomerular filtration rate (GFR) and serum urea nitrogen (UN) and creatinine concentrations on days 0 (pre-dose), 6, and 11 in male F344 rats treated subcutaneously with gentamicin sulfate (GM, gray squares) at 80 mg/kg/day or puromycin aminonucleoside (PAN, closed triangles) at 15 mg/kg/day for 10 consecutive days. Animals given 0.9% saline solution in the same way served as the control (open circles). The GFR value was determined by the three-sample method. Each column and vertical bar represents the mean ± SEM of 5–7 animals. $^{*}p<0.05$ and $^{**}p<0.01$ versus the saline group. S: serum.

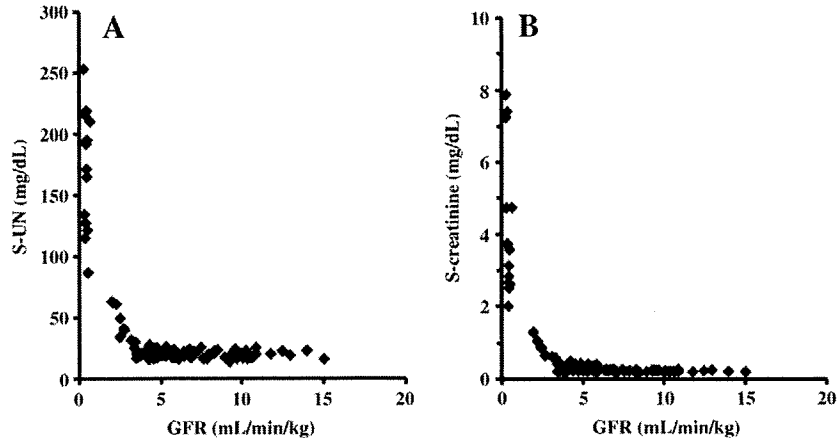

Fig. 4. Relationship between the Glomerular filtration rate (GFR) values versus serum urea nitrogen (UN, A) or creatinine (B) concentrations using data collected from healthy and renal-impaired rats ($n=48$; sample numbers, 125). The GFR value was determined by the three-sample method. S: serum.

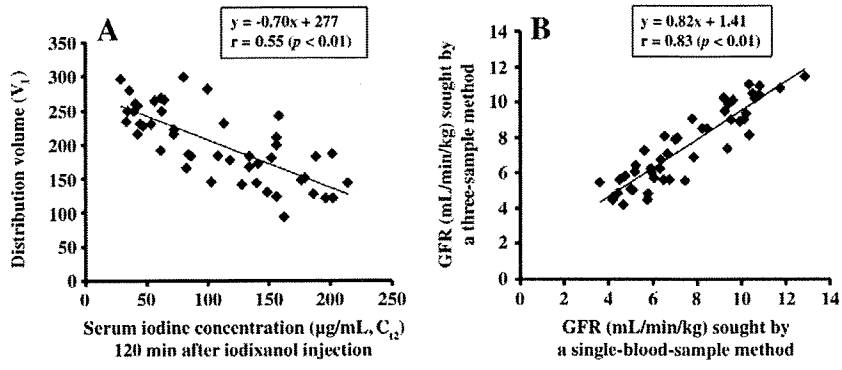

Fig. 5. Correlation between the distribution volumes (V_1) and serum iodixanol concentrations (C_{t_2}) 120 min (t_2) after iodixanol injection (A) and between the GFR values determined by the three-sample method and those determined by the single-blood-sample method (B). The V value in the single-blood-sample method was calculated from $y=-0.70x+277$ ($n=25$; sample numbers, 47).

No significant correlation with serum iodixanol concentrations was found at 20 μg I/mL or less, and 250 μg I/mL or more (data not shown).

A comparison of the GFR values obtained from the three-sample method with those of the single-blood-sample method yielded a correlation coefficient of 0.83 ($n = 28$; sample numbers, 47), indicating a close correlation ($p < 0.01$, Fig. 5B). The GFR value obtained by the single-blood-sample method in healthy male F344 rats at 6–9 weeks old were 10.1 ± 0.8 mL/min/kg ($n = 18$), indicating no significant difference from the three-sample method.

4. Discussion

According to previous iodixanol pharmacokinetic data (Heglund et al., 1995), an intravenous treatment of male Wistar rats with 200–300 mg I/kg of ^{125}I-iodixanol resulted in a rapid half-life of 25 min in plasma and a total clearance of 8.0 ± 1.9 mL/min/kg ($n = 10$) with less than 1% of the injected dose present in tissues after 24 h. In our work with 1500 mg I/kg of iodixanol, a linear semilogarithmic plot of serum iodixanol concentrations versus time demonstrated the suitability of using a one-compartment model for the clearance calculation (GFR). The GFR values (9.6 ± 0.6 mL/min/kg) in healthy male F344 rats determined by the three-sample method were nearly consistent with the above clearance values. Furthermore, these iodixanol GFR values were in good agreement with our historical data (8.6 ± 1.5 mL/min/kg in Sprague–Dawley rats, $n = 30$, Onodera & Furuhama, 1983) or previously reported data (0.798 ± 0.073 mL/min/100 g in Sprague–Dawley rats, $n = 34$, Gabel et al., 1996) using a standard ^{14}C-inulin clearance, although the experimental conditions were very different. When converting the GFR values from bodyweights (mL/min/kg) to body surface areas (mL/min/m^2, Holt, Rhode, & Kines, 1968) using healthy rat data, the fluctuations (44.2–88.9 mL/min/m^2) in the latter were almost similar to those (7.1–14.9 mL/min/kg) in the former, suggesting that the effect of gains in the bodyweight on GFR calculations can be ignored under the conditions of this study.

In partially nephrectomized rats, the GFR values decreased statistically without and with significant increases in serum UN or creatinine concentrations in 1/2 and 3/4 nephrectomies, respectively, demonstrating no alteration in these parameters following 1/2 reductions in renal mass. Likewise, the GFR values on day 6 in rats treated with GM and PAN showed a tendency to decrease and significantly decreased, respectively, before increases in serum UN and creatinine concentrations. On day 11, severely decreased GFR values along with marked increases in serum items were linked to devastating changes in the renal morphology of both nephropathy groups. Renal histopathological findings brought about by GM or PAM in the present work were well consistent with our previous reports (Furuhama & Onodera, 1986; Onodera & Furuhama, 1983).

Based on cumulative data collected from the preliminary and present studies including healthy and renal-impaired rats, serum UN or creatinine concentrations likely began to increase from the point at which the GFR values decreased to more than 60% of the basal level (9.6 mL/min/kg). The findings of the scatter diagram between the GFR values versus serum UN or creatinine concentrations on the basis of these data resembled those from the standard inulin clearance method (Onodera & Furuhama, 1983).

It has been reported that the formula derived for the GFR calculation with one sample requires that the V value be known, and the accuracy in the V value determines the accuracy in the method (Jacobsson, 1983). Similarly, if the V value of the tracer is known, the plasma disappearance curve can be closely approximated from a single, timed plasma measurement (Harvey et al., 1988). In our study, the V value in an individual animal was determined by substituting the GFR value obtained from the three-sample method and serum iodixanol concentrations at 120 min into Jacobsson's formula. Although the 120 min sample time for rats is shorter than the 300 min for humans (Jacobsson, 1983), this was based on the fact that iodixanol clearance value is 3- to 4-fold higher in rats (Heglund et al., 1995) than in humans (Svaland, Haider, Langseth-Manrique, Andrew, & Hals, 1992). Moreover, the GFR values calculated at 120 min were apparently stable in healthy rats compared to those at 60 or 180 min, because a relatively high concentration at 60 min or an extremely low concentration at 180 min was included. It was essential to have a close relationship between the V values and serum iodixanol concentrations as a prerequisite for a formula to calculate the V value. The formula obtained was valid between 20 and 250 μg I/mL in C_{t_2}, in which the GFR values were calculated as 2.9–13.7 mL/min/kg with the single-blood-sample method. These results implied a margin of error in the GFR values (2.8 mL/min/kg or less) for rats showing severe renal impairment. However, because serum UN or creatinine concentrations evidently increased under these diseased states (about 40 mg/dL and 1.0 mg/dL in Fig. 4A and B, respectively), the clinical significance of the GFR determination may be low. Although a close correlation ($r = 0.83$) existed between the GFR values obtained from the three-sample method and that from the single-blood-sample method, the actual values (10.1 ± 0.8 mL/min/kg) in healthy rats by the latter were somewhat higher than those by the former, as described above. Therefore, when this procedure is applied to pharmacological or toxicological studies in rats, a control group is necessary for each protocol. Further studies are required to collect cumulative background data including differences in strains, genders, and ages. The highlight of this investigation is to be more efficient in animal use.

These results suggest that the single-blood-sample method with a bolus injection of iodixanol, allowing for the repeated use in the same animals, is an expedient procedure without ensuring accurate urine collection, or using radioisotopes.

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References

Aspelin, P., Aubry, P., Fransson, S. -G., Strasser, R., Willenbrock, R., & Berg, K. J. (2003). Nephrotoxic effects in high-risk patients undergoing angiography. *New England Journal of Medicine, 348*, 491–499.

Bröchner-Mortensen, J. (1972). A simple method for the determination of glomerular filtration rate. *Scandinavia Journal of Clinical Laboratory Investigation, 30*, 271–274.

Brown, S. C. W., & O'reilly, P. H. (1991). Iohexol clearance for the determination of glomerular filtration rate in clinical practice: Evidence for a new gold standard. *Journal of Urology, 146*, 675–679.

Darling, I. M., & Morris, M. E. (1991). Evaluation of "true" creatinine clearance in rats reveals extensive renal secretion. *Pharmaceutical Research, 8*, 1318–1322.

Furuhama, K., & Onodera, T. (1986). The influence of cephem antibiotics on gentamicin nephrotoxicity in normal, acidotic, dehydrated, and unilaterally nephrectomized rats. *Toxicology and Applied Pharmacology, 86*, 430–436.

Gabel, R. A., Ranaei, R. A., & Kivlighn, S. D. (1996). A new method of measuring renal function in conscious rats without the use of radioisotopes. *Journal of Pharmacological and Toxicological Methods, 36*, 189–197.

Groth, S., & Aasted, M. (1981). ^{51}Cr-EDTA clearance determined by one plasma sample. *Clinical Physiology, 1*, 417–425.

Guesry, P., Kaufman, L., Orloff, S., Nelson, J. A., Swann, S., & Holliday, M. (1975). Measurement of glomerular filtration rate by fluorescent excitation of non-radioactive meglumine iothalamate. *Clinical Nephrology, 3*, 134–138.

Harvey, J. N., Jaffa, A. A., Loadholt, C. B., & Mayfield, R. K. (1988). Measurement of glomerular filtration rate and renal plasma flow in the diabetic rat by the single-injection isotopic technique: Effects of altered distribution volume of ^{51}Cr-EDTA and ^{125}I-hippuran. *Diabetes Research, 9*, 67–72.

Hatanaka, S., Kondoh, M., Kawarabayashi, K., & Furuhama, K. (1994). The measurement of gastric emptying in conscious rats by monitoring serial changes in serum acetaminophen level. *Journal of Pharmacological and Toxicological Methods, 31*, 161–165.

Heglund, I. F., Michelet, Å. A., Blazak, W. F., Furuhama, K., & Holtz, E. (1995). Preclinical pharmacokinetics and general toxicity of iodixanol. *Acta Radiologica, 36*, (Suppl. 399), 69–82.

Holt, J. P., Rhode, E. A., & Kines, H. (1968). Ventricular volumes and body weight in mammals. *American Journal of Physiology, 215*, 704–715.

Jacobsen, P. B., Blindheim, L., & Skotland, T. (1995). Bioanalytical methods for iodixanol and their application to studies on metabolism and protein binding. *Acta Radioloica, 36,* (Suppl. 399), 61–66.

Jacobsson, L. (1983). A method for the calculation of renal clearance based on a single plasma sample. *Clinical Physiology, 3,* 297–305.

Japanese Association for Laboratory Animal Science. (1987). Guidelines for animal experimentation. *Experimental Animal, 3,* 285–288.

Kishimoto, M., Yamada, K., Tsuneda, R., Shimizu, J., Iwasaki, T., & Miyake, Y. (2008). Effect of contrast media formulation on computed tomography angiographic contrast enhancement. *Veterinary Radiology & Ultrasound, 49,* 233–237.

Kishimoto, M., Yamada, K., Watanabe, A., Miyamoto, K., Iwasaki, T., & Miyake, Y. (2007). Comparison of excretory urographic contrast effects of dimeric and monomeric non-ionic iodinated contrast media in dogs. *Journal of Veterinary Medical Science, 69,* 713–715.

McCullough, P. A., Bertrand, M. E., Brinker, J. A., & Stacul, F. (2006). A meta-analysis of the renal safety of isosmolar iodixanol compared with low-osmolar contrast media. *Journal of the American College of Cardiology, 48,* 692–699.

Onodera, T., & Furuhama, K. (1983). Determination of inulin and PAH clearance in different types of nephropathy rats. In A. W. Hayes, R. C. Schnell, & T. S. Miya (Eds.), *Development in the science and toxicology* (pp. 443–446). Amsterdam, Netherlands: Elsevier.

Sampaio-Maia, B., Serrao, P., Guimaraes, J. T., Vieira-Coelho, M. A., & Pestana, M. (2005). Renal dopaminergic system activity in the rat remnant kidney. *Nephron Experimental Nephrology, 99,* e46–e55.

Svaland, M. G., Haider, T., Langseth-Manrique, K., Andrew, E., & Hals, P. A. (1992). Human pharmacokinetics of iodixanol. *Investigative Radiology, 27,* 130–133.

Tarloff, J. B., Goldstein, R. S., & Hook, J. B. (1989). Strain differences in acetaminophen nephrotoxicity in rats: Role of pharmacokinetics. *Toxicology, 56,* 167–177.

Thomsen, H. S., & Hvid-Jacobsen, K. (1991). Estimation of glomerular filtration rate from low-dose injection of iohexol and a single blood sample. *Investigative Radiology, 26,* 332–336.

Zager, R. A. (1987). Exogenous creatinine clearance accurately assesses filtration failure in rat experimental nephropathies. *American Journal of Kidney Diseases, 10,* 427–430.

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FULL PAPER *Toxicology*

Clinicopathological Aspect of Dysglycemia in Naive and Diabetic Rats induced by the Fluoroquinolone Antibacterial Gatifloxacin

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Running Head: DYSGLYCEMIA DUE TO GATIFLOXACIN

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ABSTRACT. To ascertain the clinicopathological process underlying dysglycemia induced by the fluoroquinolone antibacterial gatifloxacin (GFLX), we orally administered 100 or 300 mg/kg/day to male clinically healthy (naive) or spontaneous type II (diabetic) Goto-Kakizaki rats for 15 days (days 1 to 15). Treatment of naive rats with GFLX led to decreased blood glucose concentrations at 100 mg/kg/day on day 1. In diabetic animals, markedly increased blood glucose concentrations were noted from 100 mg/kg/day on day 3, and all of the animals given 300 mg/kg/day died or were killed because of moribund conditions by day 9. In a glucose tolerance test, serum insulin concentrations decreased significantly in naive rats receiving 300 mg/kg/day. Microscopically, cytoplasmic vacuolations of the pancreatic islets were observed in naive rats receiving 300 mg/kg/day, and congestion and/or hemorrhage were additionally noted in diabetic rats given 100 mg/kg/day or more. In toxicokinetics with 100 mg/kg/day, $AUC_{0\text{-}8\ hr}$ values for GFLX were higher in diabetic rats than in naive rats, and relatively high serum GFLX concentrations at 8 hr post-dose and extraordinarily high pancreatic GFLX concentrations were also observed in diabetic rats. These results demonstrate that hypoglycemia or hyperglycemia induced by GFLX is associated with higher distribution and retention of GFLX in the pancreas, leading to disturbed insulin secretion.

KEY WORDS: blood glucose, diabetic rats, gatifloxacin, pancreas, toxicokinetics

Despite fluoroquinolone antibacterials (quinolones) regarded as being generally safe, this derivative class has been recognized to exhibit occasionally serious adverse reactions, and thereby some were withdrawn from the market [1, 12 18]. Most recently, the quinolone gatifloxacin (GFLX) was withdrawn voluntarily from the U.S. and Japanese markets due to disturbed blood glucose homeostasis. Briefly, GFLX was reported to cause dysglycemia (hypoglycemia or hyperglycemia) with much higher frequencies in the retrospective human surveillance [3, 15], and particularly diabetes mellitus was considered to be one of the risk factors [6]. In nonclinical investigations, many studies have reported dysglycemia as a consequence of quinolone administration in mice [8] and rats [9] as well as in *in vitro* studies [13, 17, 23]. As for GFLX, Saraya *et al.* [17] stated that it stimulated insulin secretion and inhibited β-cell ATP-sensitive K^+ (K_{ATP}) channel currents in a dose-dependent manner in mouse pancreatic islets. Yamada *et al.* [22] reported that GFLX acutely stimulated insulin secretion associated with hypoglycemia, and chronic GFLX exposure decreased islet insulin content by inhibiting insulin biosynthesis related to hyperglycemia in mouse pancreatic islets. Tomita *et al.* [19] reported that GFLX induced insulin oversecretion in the short-term, and decreased insulin productivity or increased insulin disintegration in the long-term using the insulin-secreting cell line HIT-T15. Based on the *in vivo* studies, Ishiwata *et al.* [10] reported that serum glucose concentrations decreased and increased in normal and streptozotocin-induced diabetic rats, respectively, given a single intravenous injection of GFLX at 50 mg/kg, with elevations in both serum insulin and epinephrine (adrenaline) concentrations. However, few reports have dealt with the clinicopathological process of hypoglycemia or hyperglycemia induced by GFLX after repeated oral administration using the identical animals.

Therefore, we examined periodic blood glucose concentrations, serum insulin concentrations, pancreatic morphology and toxicokinetics for serum GFLX levels with pancreatic concentrations in clinically healthy (naive) Wistar rats and spontaneous type II (diabetic) Goto-Kakizaki (GK) rats given orally GFLX for 15 consecutive days. GK rats are known to evoke hyperglycemia, low insulin secretion, glycosuria and glucose intolerance with significant thickening of the glomerular basement membrane at 12 weeks of age [21], with impaired pancreatic K_{ATP} channels [20].

MATERIALS AND METHODS

All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [11] and also approved by the Animal Experimental Ethics Committee of Iwate University (Morioka, Japan).

Animals and housing conditions: Male Wistar and spontaneous type II diabetic GK rats at 10 weeks of age were obtained from Japan SLC, Inc. (Shizuoka, Japan), and studies began after a 10-day acclimation period. Wistar rats are the parent strain for GK rats [5]; the former were regarded as "naive rats", and the latter as "diabetic rats" for the present study. The animals were housed in an air-conditioned facility (a temperature of 22 ± 3°C, a relative humidity of 55 ± 25%, and lighting at 8:00 AM to 8:00 PM with a 12-hr light cycle), and fed commercial rodent chow (MEQ, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*.

Chemicals: GFLX purchased from Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan) was used after grinding the tablets in a mortar. All other chemicals and reagents were of the highest grade available from commercial sources, unless otherwise stated.

Treatment: Naive and diabetic rats were orally administered GFLX by gavage at a dose of 100 or 300 mg/kg/day once daily for 15 consecutive days (days 1 to 15), and killed 8 hr after the final treatment on day 15. GFLX was suspended at a concentration of 1-3% in a 0.5% methylcellulose (MC) aqueous solution, and dosage levels were expressed in terms of the free base. The animals given 0.5% MC solution alone (10 m*l*/kg) in the same way served as the vehicle control. The first day of the dosing was regarded as day 1 for this study. The GFLX dose, treatment period, and termination time used were selected on the basis of results from our previous report [4]. In brief, when GFLX was administered orally to male naive Sprague-Dawley rats at a dose of 30, 100, or 300 mg/kg/day for 2 weeks, no morphologic change in the pancreas was observed at doses of up to 100 mg/kg/day, but moderate vacuolations in the pancreatic islets were seen at 300 mg/kg/day. A fluctuation in blood glucose concentrations in rats given 100 mg/kg/day or more was grasped 2 hr after treatment, but not 24 hr later.

Laboratory tests and pancreatic pathology: Blood was withdrawn repeatedly from the tail vein by a previously mentioned procedure under conscious conditions [7]. Glucose concentrations were measured

periodically using whole blood (approximately 2.5 μl) 2 hr after each treatment on days 1, 3, 5, 7, 10, 12, and 14 using a G checker (Sanko Junyaku Co., Ltd., Tokyo, Japan) with glucose oxidase as a substrate. On day 5, serum insulin concentrations 2 hr after treatment were determined by commercially available ELISA kits (Levis rat insulin kit, Shibayagi Co., Ltd., Gunma, Japan). In a glucose tolerance test, a 10% glucose solution was intravenously administered at 5 ml/kg (0.5 g/kg) to both naive and diabetic rats at pre-dose on day 13, and blood samples (2.5 μl and 0.4 ml) were collected from the tail vein 60 and 5 min later for measuring blood glucose and serum insulin concentrations, respectively. These sampling times were chosen based on the result of the previous study [4] to avoid excessive stresses due to repeated blood collections. On day 15, the surviving animals were euthanized by exsanquination under ether anesthesia immediately after the terminal blood samplings for toxicokinetics. Following necropsy, the pancreas of each animal was excised, fixed in 10% buffered formalin, embedded in paraffin wax, cut at 3-μm thickness, stained with hematoxylin and eosin (H-E), and histopathologically examined. The histological examination was done in a blinded manner.

Toxicokinetics for GFLX: All animals were fasted for 15 hr before the experiment, but with free access to water. On day 15, blood (0.5 ml) was collected via the tail vein or cervical vein of surviving animals just before administration of GFLX (0 hr), and 0.5, 1, 2, 4, and 8 hr after the last treatment; sera were separated, and stored at -80°C until assayed. A part of the pancreas (approximately 0.1 g) excised at necropsy was weighed, mixed with 0.1 M phosphate buffer (Calbiochem, Darmstadt, Germany) at a ratio of 1:9, and homogenized with a disperser mixer (Nagashima Keiki, Saitama, Japan). After centrifugation (4°C, 3000 rpm, 10 min), the supernatant was stored at -80°C until assayed. Quantification of GFLX in serum and pancreatic specimens was carried out by an agar diffusion bioassay method as outlined in a previous report [16] with *Bacillus subtilis* ATCC 6633 (Remel Co., Lenex, KS, USA) as a test organism. The standard was prepared at known concentrations (0.625, 1.25, 2.5, 5, and 10 μg/ml) of GFLX, and included in each plate to compensate for any plate-to-plate variation. Zones of inhibition were measured with a digital micrometer caliper (CD-15CPX; Mitutoyo Co., Kanagawa, Japan), and the results from the standard were used to calculate the concentration in each sample. A linear relationship existed between the zone of inhibition and the logarithm of GFLX concentration. Peak serum GFLX concentration (*Cmax*), time of *Cmax* (*Tmax*), and

half-lives ($t_{1/2}$) were calculated on the basis of serum GFLX concentrations obtained 0, 0.5, 1, 2, 4, and 8 hr after treatment using PAG-CP software (ASMedica Co., Ltd., Osaka, Japan). The area under the concentration curve versus time from 0 to 8 hr ($AUC_{0\text{-}8\,hr}$) in serum was sought using the linear trapezoidal rule with extrapolation.

Statistical analysis: Quantitative data are expressed as the mean ± standard deviation (SD) of the group. Statistical evaluation was occasionally performed by one-way ANOVA, and differences between treatment and vehicle control groups were analyzed by Dunnett's test (among three groups) or Student's t-test (between two groups). A probability level of $p < 0.05$ indicates statistical significance.

RESULTS

Periodic Blood Glucose Concentrations: When measured blood glucose concentrations as a basal level under the fed conditions (n = 15) at pre-study, the values in naive and diabetic rats were 117.4 ± 5.3 mg/dl and 262.0 ± 48.5 mg/dl, respectively.

In naive rats given GFLX at 100 mg/kg/day, significantly decreased blood glucose concentrations were seen on days 1, 3, 5, 7, and 12. At 300 mg/kg/day, decreased blood glucose concentrations were seen only on day 1 (Fig. 1A). A significant increase in serum insulin was noted only at 100 mg/kg/day (Fig. 1C).

In diabetic rats receiving GFLX at 100 mg/kg/day, markedly increased blood glucose concentrations were observed from days 3 to 14 (Fig. 1B) with decreased insulin concentrations (Fig. 1D). Although animals given 300 mg/kg/day displayed extremely high blood glucose concentrations from day 3 (Fig. 1B), one, two, and two animals died or were killed because of moribund conditions on days 7, 8, and 9, respectively. Toxic signs in these animals included reduced bodyweights, decreases in spontaneous activities, abdominal breathing, hypothermia, ptosis, weakness, and/or abdominal posture. Serum insulin concentrations at 300 mg/kg/day decreased to the qualification limit (approximately 0.04 ng/ml, Fig. 1D).

Glucose Tolerance Test: In a test performed at pre-dose on day 13, blood glucose increased markedly in naive rats given GFLX at 300 mg/kg/day (Fig. 2A) or in diabetic rats administered GFLX at 100 mg/kg/day (Fig. 2B). Serum insulin concentrations decreased significantly in naive rats receiving GFLX at 300

mg/kg/day (Fig. 2C). In diabetic rats given GFLX at 100 mg/kg/day, however, no statistical decrease was observed because of a large interindividual variation and an extremely low concentration in the corresponding control group (Fig. 2D). The test can not be carried out in diabetic rats given 300 mg/kg/day because of being killed by day 9.

Pancreatic Pathology: Cytoplasmic vacuolations of the pancreatic islets were microscopically observed in naive rats given GFLX at 300 mg/kg/day or in diabetic rats receiving 100 mg/kg/day or more, and congestion and/or hemorrhage in the islets or peri-islets with yellowish-brown pigmentation were additionally noted in diabetic rats (Table 1). Hypertrophic pancreatic isles were seen, and their cell nuclei were compressed by clear vacuoles or vacuoles containing eosinophilic homogenous substances. These vacuolated cells were mainly observed in the central area of the islets, and remaining intact cells were compressed by affected cells (Fig. 3B). The cells in the marginal area of the islets which seems to be α-cells were unaffected in morphology (Fig. 3C).

Toxicokinetics for GFLX: A significant difference in serum GFLX concentrations was seen between naive rats given 100 and 300 mg/kg/day (Fig. 4A). Meanwhile, high serum GFLX concentrations remained even at 8 h post-dose in diabetic rats compared to naive rats (Fig. 4B). In GFLX at 100 mg/kg/day, serum *Cmax* was lower in diabetic rats than in naive rats, and $t_{1/2}$ and $AUC_{0\text{-}8\,hr}$ values for GFLX were higher in diabetic rats than in naive rats. Moreover, high pancreatic GFLX concentrations were observed in diabetic rats (Table 2).

DISCUSSION

GFLX has been reported to largely affect insulin secretion [17, 19, 22], and to inhibit pancreatic K_{ATP} channel currents, especially via the pore-forming subunit Kir6.2 [17]. Pancreatic K_{ATP} channels are crucial in the regulation of insulin secretion. Closure of the channels depolarizes the β-cell membrane, opening the voltage-dependent calcium channels to allow calcium influx. The resultant intracellular Ca^{2+} increase triggers exocytosis of insulin granules [2]. Despite such extensive information, the clinicopathological process of GFLX-induced hypoglycemia or hyperglycemia remains unclear with respect to changes in the

time- and dose-dependency in *in vivo* studies.

Oral treatment of naive Wistar rats with GFLX at 100 mg/kg/day exhibited decreases or tendencies to decrease blood glucose concentrations throughout the study periods with no significant change in serum insulin concentration (on day 5) or pancreatic pathology (on day 15). At 300 mg/kg/day, decreased blood glucose concentrations were noted only on day 1, but slight to mild cytoplasmic vacuolations of the pancreatic islets were observed. No difference in serum glucose fluctuations was seen between Wistar rats, a parent strain for GK rats, utilized in the present study and Sprague-Dawley rats used in the previous investigation [4]. In the glucose tolerance test, neither concentrations of blood glucose nor serum insulin were seen in naive rats given GFLX at 100 mg/kg/day, indicating no changes in glucose homeostasis via insulin. In contrast, significantly decreased serum insulin concentrations after the glucose load in naive rats given 300 mg/kg/day were noted with markedly increased blood glucose concentrations, encouraging disturbed insulin secretion. These findings implied that the functional reserve (mass) for insulin biosynthesis/secretion via blockage in pancreatic K_{ATP} channels of naive rats given 100 mg/kg/day remained sufficiently, whereas a subtle disturbance in insulin secretion along with possibly accelerated biosynthesis in part, allowing for apparently "normal" blood glucose and serum insulin concentrations without the glucose load, in naive rats receiving 300 mg/kg/day may exist on day 3 and thereafter. Alternatively, further investigations are required to resolve the latter matter.

In diabetic animals given 100 mg/kg/day or more, markedly increased blood glucose concentrations were noted from day 3. Additionally, at 300 mg/kg/day, all animals died or were killed because of moribund conditions such as reduced bodyweights, hypothermia, weakness, and/or abdominal posture by day 9. This event may be attributed to the severe dehydration and/or hemoconcentration related to hyperglycemia, presumably leading to mortal depressions in peripheral blood circulations. Cytoplasmic vacuolations of the pancreatic islets with congestion/hemorrhage observed in diabetic animals given 100 mg/kg/day or more may reflect a direct action of GFLX on pancreatic β-cells. According to previous immunohistochemical approaches [4], vacuolated islets in naive Sprague-Dawley rats given oral GFLX at 300 mg/kg/day for 2 weeks were stained positively for insulin, demonstrating them to be pancreatic β cells. By electron microscopy, insulin secretory granules evidently decreased in the vacuolated islets. Although

alterations in pancreatic α-cells were not histopathologically detected, further studies including immunohistochemical approaches are necessary to elucidate the fluctuation of glucagon which plays a pivotal role in blood glucose regulations in conjunction with insulin.

On the basis of fluctuations of blood glucose concentrations in naive and diabetic rats given GFLX, our nonclinical results resembled somewhat previous *in vitro* and clinical profiles showing that hypoglycemic episodes were seen after an initial single exposure [19], and that most hyperglycemic episodes were seen in patients with diabetes mellitus more than 2 days after the start of administration [3].

According to previous Pharmacokinetic data from naive Sprague-Dawley rats treated orally with quinolone derivatives at 300 mg/kg/day for 2 weeks [4], pancreatic drug concentrations with a rank order (from the highest to lowest) were GFLX (128 μg/m*l*) > lomefloxacin (69 μg/m*l*) > levofloxacin (39 μg/m*l*), although no difference was observed in serum drug concentrations (12 to 15 μg/m*l*) among the quinolones tested. GFLX was considered to be predominantly distributed in the pancreas relative to other quinolones. Accordingly, our toxicokinetics added support to the above viewpoints that a large volume of distribution of GFLX in the pancreas may play a pivotal role in the high occurrence of dysglycemia, although there were differences in the detective procedures or experimental conditions. Actually, only GFLX was withdrawn from the market because of life-threatening consequence and hospitalizations, despite the fact that quinolone derivatives display potential dysglycemia more or less as a class effect [1, 8, 14]. In GFLX at 100 mg/kg/day, elevations in $AUC_{0\text{-}8\ hr}$ values for GFLX and pancreatic GFLX concentrations were observed in diabetic rats compared to naive rats. It was considered that possibly depressed peripheral blood circulations in diabetic rats would cause severe hyperglycemia as a consequence of enhancing the distribution and retention of GFLX in the pancreas. This is well consistent with reported clinical experiences involving GFLX, which has been associated with causing mortal hyperglycemia in patients with diabetes mellitus [6]. In diabetic rats given 100 mg/kg/day, the half-life increase with dose as does the *Cmax* would be attributed to dehydration and/or hemoconcentration due to hyperglycemia.

It is concluded that the animals possessing normal pancreatic functional mass for insulin biosynthesis, secretion, or degradation cause hypoglycemia to occur despite plenty of insulin stores, while the animals with low insulin secretion due to diabetes mellitus evoke hyperglycemia despite depleted insulin stores

when repeatedly administered oral GFLX. Furthermore, dysglycemia induced by GFLX is associated with higher distribution and retention of GFLX in the pancreas, leading to disturbed insulin secretion.

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REFERENCES

1. Andersson, M. I. and MacGowan, A. P. 2003. Development of the quinolones. *J. Antimicorb. Chemother.* **51**: S1, 1-11.
2. Ashcroft, F. M. and Rorsman, P. 1989. Electrophysiology of the pancreatic beta-cell. *Prog. Biophys. Mol. Biol.* **54**: 87-143.
3. Frothingham, R. 2005. Glucose homeostasis abnormalities associated with use of gatifloxacin. *Clin. Infect. Dis.* **41**: 1269-1276.
4. Furuhama, K. 2007. Toxicology assessment of fluoroquinolones: a case study. pp. 571-588. *In*: Nonclinical Drug Safety Assessment: Practical Considerations for Successful Registration (Sietsema, W. K. and Schwen, R. eds.), FDAnews, Virginia.
5. Goto, Y., Kakizaki, M. and Masaki, N. 1975. Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proc. Jpn. Acad.* **51**: 80-85.
6. Haerian, H., McHugh, P., Brown, R., Somes, G. and Solomon, S. S. 2008. Gatifloxacin produces both hypoglycemia and hyperglycemia: a retrospective study. *Am. J. Med. Sci.* **335**: 95-98.
7. Hatanaka, S., Kondoh, M., Kawarabayashi, K. and Furuhama, K. 1994. The measurement of gastric emptying in conscious rats by monitoring serial changes in serum acetaminophen level. *J. Pharmacol. Toxicol. Meth.* **31**: 161-165.
8. Hori, S., Kizu, J. and Kawamura, M. 2006. Effect of fluoroquinolones on plasma glucose levels in

fasted and glucose-loaded mice. *J. Infect. Chemother.* **12**: 109-111.

9. Ishiwata, Y., Itoga Y. and Yasuhara, M. 2006. Effect of levofloxacin on serum glucose concentration in rats. *Eur. J. Pharmacol.* **551**: 168-174.

10. Ishiwata, Y., Sanada, Y. and Yasuhara, M. 2006. Effects of gatifloxacin on serum glucose concentration in normal and diabetic rats. *Biol. Pharm. Bull.* **29**: 527-531.

11. Japanese Association for Laboratory Animal Science. 1987. Guidelines for animal experimentation. *Exp. Anim.* **3**: 285-288

12. Lode, H. and Rubinstein, E. 2003. Adverse effects. pp. 407-419. *In*: Quinolone Antimicrobial Agents (Hooper, D. C. and Rubinstein, E. eds.), 3rd ed., ASM Press, Washington DC.

13. Maeda, N., Tamagawa, T., Niki, I., Miura, H., Ozawa, K., Watanabe, G., Nonogaki, K., Uemura, K. and Iguchi, A. 1996. Increase in insulin release from rat pancreatic islets by quinolone antibiotics. *Br. J. Pharmacol.* **117**: 372-376.

14. Owens, Jr. R. C. and Ambrose, P. G. 2005. Antimicrobial safety: focus on fluoroquinolones. *CID* **41**: S144-S157.

15. Park-Wyllie, L. Y., Juurlink, D. N., Kopp, A., Shah, B. R., Stukel, T. A., Stumpo, C., Dresser, L., Low, D. E. and Mamdani, M. M. 2006. Outpatient gatifloxacin therapy and dysglycemia in older adults. *N. Engl. J. Med.* **354**: 1352-1361.

16. Salgado, H. R., Lopes, C. C. and Lucchesi, M. B. 2006. Microbiological assay for gatifloxacin in pharmaceutical formulations. *J. Pharm. Biomed. Anal.* **40**: 443-446.

17. Saraya, A., Yokokura, M., Gonoi, T. and Seino, S. 2004. Effects of fluoroquinolones on insulin secretion and β-cell ATP-sensitive K^+ channels. *Eur. J. Pharmacol.* **497**: 111-117..

18. Takasuna, K., Chiba, K. and Manabe, S. 2009. Pre-clinical QT risk assessment in pharmaceutical companies: issues of current QT risk assessment. *Biomol. Therap.* **17**: 1-11.

19. Tomita, T., Onishi, M., Sato, E., Kimura, Y. and Kihira, K. 2007. Gatifloxacin induces augmented insulin release and intracellular insulin depletion of pancreatic islet cells. *Biol. Pharm. Bull.* **30**: 644-647.

20. Tsuura, Y., Ishida, H., Okamoto, Y., Kato, S., Sakamoto, K., Horie, M., Ikeda, H., Okada, Y. and Seino,

Y. 1993. Glucose sensitivity of ATP-sensitive K^+ channels is impaired in beta-cells of the GK rat. A new genetic model of NIDDM. *Diabetes* **42**: 1446-1453.

21. Yagihashi, S., Goto, Y., Kakizaki, M. and Kaseda, N. 1978. Thickening of glomerular basement membrane in spontaneously diabetic rats. *Diabetologia* **15**: 309-312.

22. Yamada, C., Nagashima, K., Takahashi, A., Ueno, H., Kawasaki, Y., Yamada, Y., Seino, Y. and Inagaki, N. 2006. Gatifloxacin acutely stimulates insulin secretion and chronically suppresses insulin biosynthesis. *Eur. J. Pharmacol.* **553**: 67-72.

23. Zünkler, B. J.and Wos, M. 2003. Effects of lomefloxacin and norfloxacin on pancreatic β-cell ATP-sensitive K^+ channels. *Life Sci.* **73**: 429-435.

Figure Legends

Fig. 1. Blood glucose (upper) and serum insulin (lower) concentrations in naive (A and C) and diabetic (B and D) rats treated orally with gatifloxacin (GFLX) at a dose of 100 (gray square and column) or 300 (filled triangle and column) mg/kg/day for 15 consecutive days. The animals given the 0.5% methylcellulose (MC) solution in the same way served as the respective vehicle controls (open diamond and column). Blood glucose concentrations were measured 2 hr after GFLX treatment on days 1, 3, 5, 7, 10, 12, and 14, and serum insulin concentrations were determined 2 hr after GFLX treatment on day 5. Diabetic rats given GFLX at 300 mg/kg/day died or were killed because of moribund conditions by day 9. Each point (or column) and vertical bar represents the mean ± SD of five animals. The number of animals used is given in parentheses. BQL: blow the qualification limit. Significant difference from the vehicle control group by Dunnett's test: $*p < 0.05$.

Fig. 2. Blood glucose (upper) and serum insulin (lower) concentrations in a glucose tolerance test at pre-dose on day 13 in naive (A and C) and diabetic (B and D) rats treated orally with gatifloxacin (GFLX) at a dose of 100 or 300 mg/kg/day for 12 consecutive days. The animals given the 0.5% methylcellulose (MC) solution in the same way served as the respective vehicle controls. Blood glucose and serum insulin concentrations were measured 60 and 5 min, respectively, after a bolus injection of 10% glucose (0.5 g/kg) using ELISA. Since diabetic rats given GFLX at 300 mg/kg/day died or were killed because of moribund conditions by day 9, this test was not performed. Each column and vertical bar represents the mean ± SD of five animals. ND: no data. Significant difference from the vehicle control group by Dunnett's test or Student's t-test: $*p < 0.05$.

Fig. 3. Histopathological findings of the pancreatic islet in diabetic rats treated orally with 0.5% methylcellulose (MC) solution (A) and gatifloxacin (GFLX) at 300 mg/kg/day (B and C) for 15 and 8 days, respectively. The animal given GFLX at 300 mg/kg/day was killed on day 9 because of moribund conditions. Note hypertrophic pancreatic isles with clear vacuoles (B, arrowhead) or

vacuoles containing eosinophilic homogenous substances. These vacuolated cells were mainly observed in the central area of islets, and remaining intact cells were compressed by affected cells. The α-cells in the marginal area were morphologically unaffected (C, arrows). × 100, H-E stain

Fig. 4. Serum gatifloxacin (GFLX) concentrations following oral administration at a dose of 100 (gray square) or 300 (filled triangle) mg/kg/day to naive (A) and diabetic (B) rats for 15 days. Blood samples were collected just before (0 hr) and 0.5, 1, 2, 4, and 8 hr after GFLX treatment on day 15, except for the GFLX 300 mg/kg/day group in which two surviving rats were examined on day 9 (the values show the average). The number of animals used is given in parentheses. All animals were fasted for 15 hr before the experiment, and serum GFLX concentrations were determined by a bioassay method with *B.subtilis* ATCC 6633. Each point and vertical bar represents the mean ± SD of five animals. Significant difference from naive rats given GFLX at 100 mg/kg/day by Student's t-test: $*p < 0.05$, $\dagger p < 0.05$.

Table 1. Histopathological findings of the pancreas in naive and diabetic rats treated orally with gatifloxacin (GFLX) at 100 or 300 mg/kg/day for 15 days

Animals	Dosage level (mg/kg/day)	n	Vacuolation in the islets					Congestion/hemorrhage in the islets/peri-islets		
			-	±	+	++	+++	-	±	+
Naive rats	Vehicle control	5	5	0	0	0	0	5	0	0
	GFLX 100	5	5	0	0	0	0	5	0	0
	GFLX 300	5	0	2	3	0	0	5	0	0
Diabetic rats	Vehicle control	5	5	0	0	0	0	5	0	0
	GFLX 100	5	0	2	3	0	0	0	1	4
	GFLX 300[a]	4	0	0	0	1	3	0	0	4

One animal was not examined histopathologically because of severe postmortem changes. Animals given the 0.5% methylcellulose (MC) solution served as the respective vehicle controls. The histopathological score was as follows. -: negative/not present; ±: slight/very few; +: mild; ++: moderate; +++: marked/severe. [a]One, two, and two animals died or were killed on days 7, 8, and 9, respectively, and four animals were microscopically examined.

Table 2. Pharmacokinetic parameters and pancreatic drug concentrations on day 15 in naive and diabetic rats treated orally with gatifloxacin (GFLX) at 100 or 300 mg/kg/day for 15 days

Animals	Dosage level (mg/kg/day)	n	*Cmax* (μg/m*l*)	*Tmax* (hr)	*t1/2* (hr)	$AUC_{0\text{-}8\ hr}$ (μg·hr/m*l*)	Pancreatic GFLX concentrations (μg/g)
Naive rats	GFLX 100	5	20.5 ± 1.8	1.40 ± 0.55	2.37 ± 0.29	87.9 ± 4.4	27.6 ± 4.2
	GFLX 300	5	38.8 ± 4.4*	1.80 ± 0.45	3.64 ± 0.84*	206.9 ± 17.8*	56.7 ± 10.1*
Diabetic Rats	GFLX 100	5	16.5 ± 1.2†	2.00 ± 0	7.72 ± 2.66†	101.5 ± 7.8†	58.5 ± 10.2†
	GFLX 300[a]	2	30.8	5.00	90.1	208.6	85.8

Pancreas specimens were collected 8 hr after the final treatment. Values show the mean ± SD. Significant difference from naive rats given GFLX at 100 mg/kg/day by Student's t-test (†$p < 0.05$). [a]Determination of serum GFLX concentrations in diabetic rats receiving GFLX at 300 mg/kg/day was carried out on day 9 (n = 2) because of moribund conditions.