FULL PAPER *Clinical Pathology*

A Non-Arthropathic Dose and Its Disposition Following Repeated Oral Administration of Ofloxacin, a New Quinolone Antimicrobial Agent, to Juvenile Dogs

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ABSTRACT. A non-arthropathic dose and disposition of ofloxacin, a potent new quinolone antimicrobial agent, were assessed in male juvenile (3-month-old) dogs, when administered orally at 5, 10 and 20 mg/kg/day once daily for 8 consecutive days. Ofloxacin concentrations in sera and articular cartilages were analyzed by high-performance liquid chromatography (HPLC). Macroscopically, arthropathy characterized by fluid-filled vesicles in articular surface of the humerus and femur was observed in animals receiving 10 and 20 mg/kg/day of ofloxacin, but not in those given 5 mg/kg/day. At 20 mg/kg/day, arthropathy of comparable severity also occurred on day 2. Microscopically, the cavity formation in the middle zone of the articular cartilage was first identified and then necrotic chondrocytes were found numerous around the cavity, followed by appearance of chondrocyte clusters. In pharmacokinetics, peak serum concentration (C_{max}) and area under the concentrations (AUC_{0-24}) were increased in a dose-dependent manner. However, no remarkable differences in these two parameters were noted between a single and repeated treatments, suggesting no accumulation of the drug. The articular ofloxacin concentration 2 hr after treatment was approximately 1.8 (day 2) to 2.0 times (day 8) higher than the serum concentration. Based on these results, a non-arthropathic dose of ofloxacin in male juvenile dogs following an 8-day treatment is considered to be 5 mg/kg/day, and its C_{max}, AUC_{0-24} and articular cartilage concentrations 2 hr after treatment were 3.4 μg/m*l*, 35.1 μg·hr/m*l* and 7.0 μg/g, respectively, under these experimental conditions. Thus, arthropathy due to ofloxacin may be predicted by monitoring serum drug concentration.

KEY WORDS: arthropathy, juvenile dog, non-arthropathic dose, ofloxacin, pharmacokinetics.

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New quinolone antimicrobial agents (quinolones) are widely used in clinical fields because of their broad spectra and bactericidal activity. However, quinolones have been reported to induce arthropathy in juvenile animals such as mice [12], rats [4, 9, 10], rabbits [11], dogs [3, 5, 8, 20], non-human primates [17] and others [1, 2] as a class effect of these derivatives. Among these species, the juvenile dog is thought to be most susceptible to articular cartilage lesions [6, 19, 20].

In our previous report [23], the arthropathic lesion due to ofloxacin was observed only in juvenile dogs, despite the fact that the drug concentration in the synovial fluid and articular cartilage of immature dogs (3-month-old) was equal to or lower than those in mature dogs (18-month-old). Kato and coworkers [10] have indicated that metabolically active immature chondrocytes are more sensitive to the effects of a quinolone, compared with inactive mature cells in the ex vivo study using ^{3}H-thymidine. Moreover, they have demonstrated that the initial target of the drug for the induction of arthropathy is the DNA synthesis of chondrocytes. Supporting this hypothesis, several DNA synthesis inhibitors have been stated to increase in the incidence of cartilage lesions induced by the quinolone in rats [18]. In a more recent investigation using the cultured rabbit chondrocyte at concentrations inducing cartilage lesions, the quinolone inhibited glycosaminoglycan synthesis initially and DNA synthesis and mitochondrial function secondarily [11]. However, the precise mechanism of quinolone-induced arthropathy remains unclear. Meanwhile, there is little information dealing with the pharmacokinetics at a non-arthropathic dose of ofloxacin in juvenile dogs. It is important to provide the safety drug usage. The present study was, thus, carried out to assess a non-arthropathic dose and disposition of ofloxacin in male juvenile dogs receiving repeated oral administration.

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MATERIALS AND METHODS

Animals: Twelve 3-month-old male beagle dogs, weighing 5.2 to 6.3 kg, purchased from BMR Co. (Gifu, Japan) were used in the investigation. After quarantine, each dog was established to be healthy on the basis of physical findings and hematological analytical results. They were individually housed at an environmental temperature of 23 ± 2°C and a relative humidity of 60 ± 20% with a 12 hr light-dark cycle, and were allowed access to a commercial laboratory diet (DM-2: Funabashi Farm, Chiba, Japan) and tap water *ad libitum*. Before blood sampling, the animals were fasted for at least 18 hr with free access to water unless otherwise stated. All dogs were treated humanely, and the study protocol was in accordance with the institutional guidelines of Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan) for use of laboratory animals.

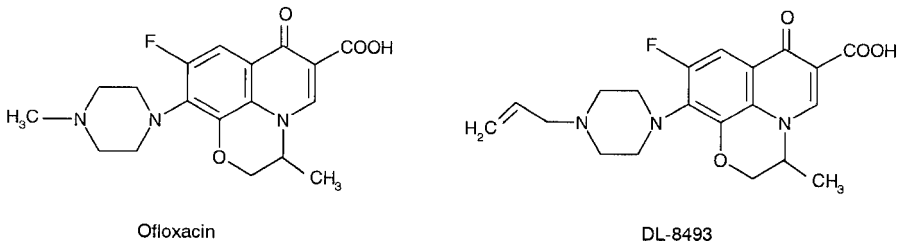

Fig. 1. Chemical structure of ofloxacin and DL-8493 as an internal standard.

Drugs and experimental design: Ofloxacin or DL-8493 (Fig. 1), an internal standard for HPLC, was synthesized at Daiichi Pharmaceutical Co., Ltd. The powdered ofloxacin contained in a sixteenth ounce gelatin capsule (Toropac Inc., East Hanover, NJ, U.S.A.) was orally administered to the dogs at 5, 10 and 20 mg/kg/day once daily for 8 consecutive days. This treatment duration was set on the basis of the ordinary indication of quinolones in human medicine to avoid the appearance of resistant bacteria. The first day of treatment was regarded as day 1 in this study. On days 1 and 7, blood samples (0.5 m*l*) were withdrawn from the cephalic vein immediately before and 1, 2, 3, 6 and 24 hr after treatment. The dogs (n=3) were sacrificed by exsanguination 2 hr following the last treatment on day 8 under anesthesia with concurrent iv administration of xylazine (2 mg/kg) and ketamine (40 mg/kg). In the 20 mg/kg/day group, three further animals were sacrificed 2 hr following the second administration on day 2 in the same manner. The dosage levels used in the present investigation were selected from previously published reports [22, 23]. Sera separated from the blood and articular cartilage samples were frozen at –30°C until assay.

Assessment of arthropathy: Clinical signs and body weights were recorded throughout the experiment, and hematological analysis was performed before commencement of the treatment and at sacrifice. Macroscopic examination focused on the articular cartilages in the humerus and femur. The regional area of these articular cartilages was highly susceptible to quinolone-induced arthropathy in juvenile dogs [3, 20]. Assessment was made first for fluid-filled vesicle formation or local erosion in the articular cartilages, after which the surface layers of hyaline cartilage in the right humerus and femur were scraped off without contaminating bone tissues for determination of the drug concentration. Next, the proximal and distal portions of both the left humerus and femur were excised, fixed in 10% buffered formalin solution, and decalcified in 25% formic acid-sodium citrate solution. Subsequently, they were trimmed into 4-mm thick slabs, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin (HE) for histopathological examination.

Ofloxacin assay: The ofloxacin concentration was assayed by the HPLC method described previously [23]. Briefly, the serum sample (50 μl) was diluted with 0.5% KH_2PO_4 (pH 2.9) to yield an appropriate concentration within the detectable limit. The articular cartilage sample (50 mg) was added to 1N NaOH (500 μl), and incubated at 60°C for 2 hr for extraction of ofloxacin. An aliquot of each sample (120 μl) was transferred into a disposable glass tube, and 0.5% KH_2PO_4 (960 μl, pH 2.9) and DL-8493 solution (0.5 μg/m*l*, 120 μl) as the internal standard were added to them. The resulting mixture (1,000 μl) was applied to Waters Oasis Extraction Plate (96-well plate, Waters, Milford, MA, U.S.A.) using a gentle suction, and ofloxacin and DL-8493 were eluted from the plate with methanol (1,000 μl). The eluate was evaporated to dryness with a centrifugal evaporator. The residue was dissolved in tetrahydrofuran (THF, Wako Pure Chemical Industries, Ltd., Osaka, Japan)-0.5% KH_2PO_4 (6:94, v/v, 200 μl), and an aliquot (20 μl) was automatically injected onto a HPLC system consisting of a separation module (Waters 2690, Waters, Milford, MA, U.S.A.) with a fluorescence detector (Waters 474, Waters, Milford, MA, U.S.A.) at an excitation wavelength of 300 nm and an emission wavelength of 500 nm. A reversed phase column (Symmetry C18, 3.5 μm particle size, Waters, Milford, MA, U.S.A.) with a guard column (Symmetry C18, Waters, Milford, MA, U.S.A.) was used, and the column temperature was kept at 40°C in a column oven. THF-0.5% KH_2PO_4 was used as the mobile phase at a flow-rate of 1 m*l*/min. Validation study showed interference-free and linear plots over a concentration ranges of 10 to 10,000 n*g*/m*l*. The assay limit of the drug concentration in sera and cartilages was 20 and 40 n*g*/m*l*, respectively.

Pharmacokinetic analysis: Peak serum ofloxacin concentration (C_{max}), time of C_{max} (t_{max}) and half-lives ($t_{1/2}$) were calculated on the basis of serum drug concentrations obtained from at 1, 2, 3, 6 and 24 hr after treatment on days 1 and 7 using a PAG-CP software model (ASMedica Co., Osaka, Japan). The area under the concentration versus time curve from 0 to 24 hr (AUC_{0-24}) was also sought using the linear trapezoidal rule with extrapolation.

Statistical analysis: Quantitative data are represented as the mean ± standard deviation (SD). Statistical significance of values was analyzed by paired *t*-test (SAS system, SAS Institute Inc., Cary, NC, U.S.A.). A *P* value of less than 0.05 was regarded as significant.

RESULTS

Assessment of arthropathy: Mild abnormal gait behavior

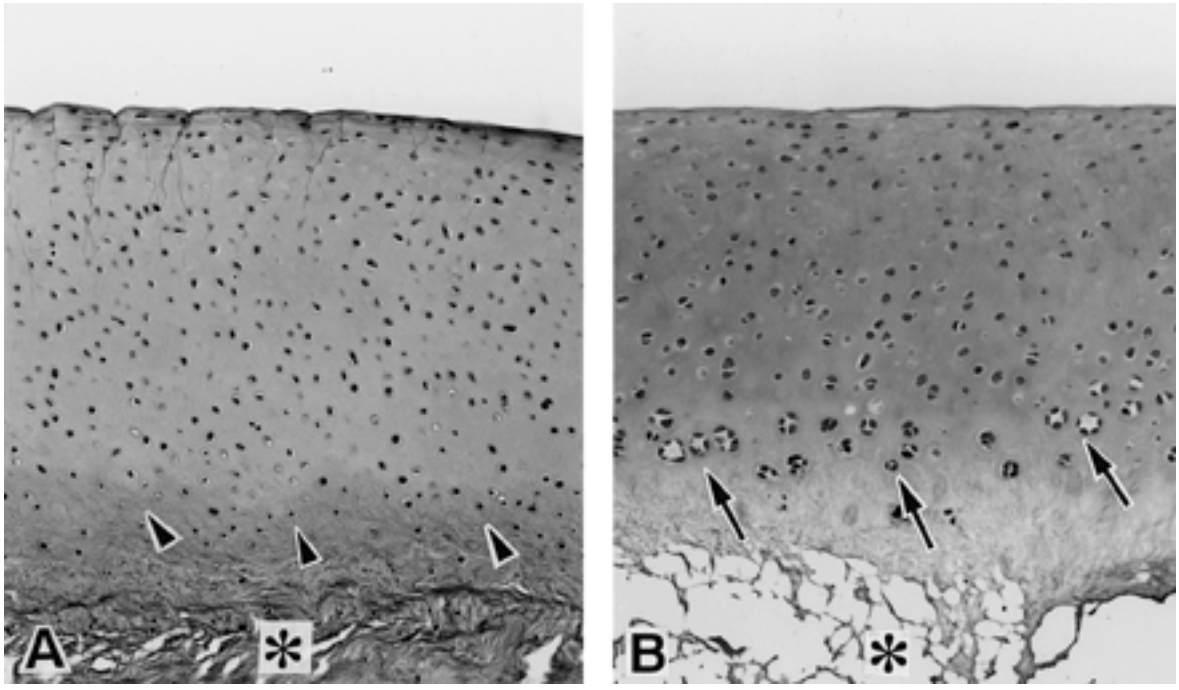

Fig. 2. Cavity formation (asterisk) in the middle zone of the femoral articular cartilage of male juvenile dogs treated orally with 20 mg/kg/day of ofloxacin for 8 consecutive days. Dogs were sacrificed 2 hr after treatment on days 2 and 8 under anesthesia with concurrent iv administration of xylazine (2 mg/kg) and ketamine (40 mg/kg). Numerous chondrocyte necrosis (arrowhead) was observed around the cavity on day 2 (A). The appearance of chondrocyte clusters (arrow) was seen in the matrix with increased staining intensity on day 8 (B). HE stain. × 67.

Table 1. Microscopic findings in male juvenile dogs treated orally with ofloxacin for 8 consecutive days

			Dose (mg/kg/day)	5	10	20	
			Days of sacrificed	Day 8	Day 8	Day 2	Day 8
Portion		Findings	n	3	3	3	3
Humerus	Proximal	Cavity		0	3[a)]	3	3
		Chondrocyte clusters around the cavity		0	3	0	3
	Distal	Cavity		0	0	1	3
		Chondrocyte clusters around the cavity		0	0	0	0
Femur	Proximal	Cavity		0	3	3	3
		Chondrocyte clusters around the cavity		0	3	0	3
	Distal	Cavity		0	2	2	3
		Chondrocyte clusters around the cavity		0	1	0	3

Dogs were sacrificed 2 hr after treatment on day 2 and 8 under anesthesia with concurrent administration of xylazine (2 mg/kg) and ketamine (40 mg/kg). The left humerus and femur were examined. a) Number of animals showing changes.

and decreased locomotor activity were observed only in 2 of 3 animals receiving 20 mg/kg/day of ofloxacin between days 7 and 8. Neither abnormal findings of body weight gain nor alterations of blood analyses were noted in any of the dogs throughout the experimental periods. At termination, multifocal fluid-filled vesicles in the articular cartilage with increased synovial fluid were macroscopically observed in animals receiving 10 and 20 mg/kg/day of ofloxacin, but not in those receiving 5 mg/kg/day. Vesicles were seen bilaterally or sometimes unilaterally in the articular cartilage. Additionally, partial erosion of the vesicle and its fragments in synovial fluid were sporadically noted. In animals sacrificed 2 hr after the second administration of 20 mg/kg/day, arthropathy was also observed to the same extent as that seen at scheduled sacrifice on day 8. Microscopically, cavity formation in the middle zone of the articular cartilage was observed in dogs receiving 10 and 20 mg/kg/day ofloxacin (Fig. 2 and Table 1). Essentially the same

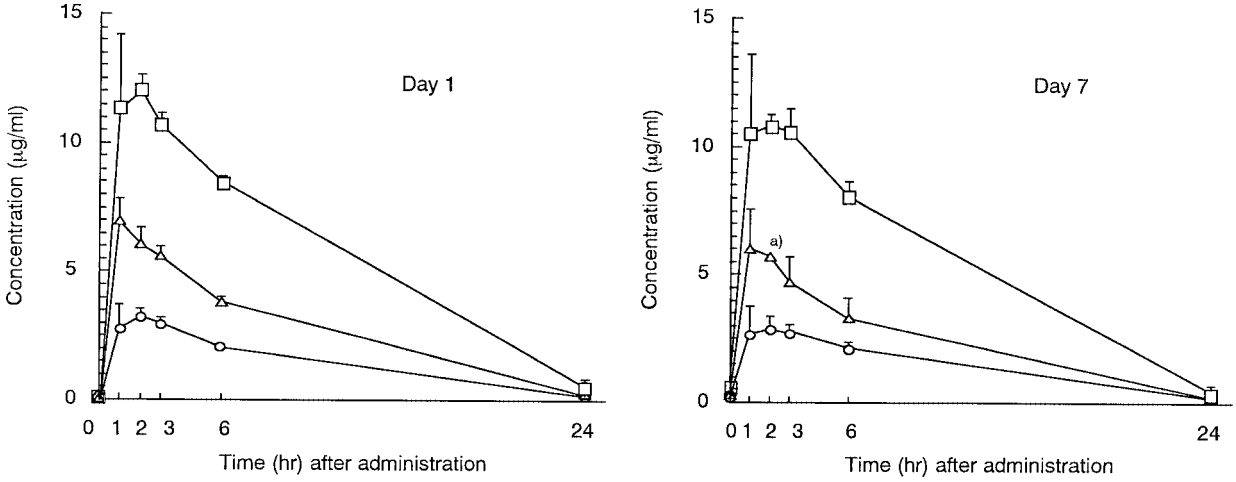

Fig. 3. Serum drug concentrations in male juvenile dogs treated orally with ofloxacin for 8 consecutive days. Serum ofloxacin concentrations were analyzed by HPLC. Data are represented as the mean ± SD of 3 animals. 5 mg/kg/day (—○—), 10 mg/kg/day (—△—), 20 mg/kg/day (—□—). a) n=2.

Table 2. Pharmacokinetic parameters of ofloxacin in male juvenile dogs treated orally with the drug for 8 consecutive days

Dose (mg/kg/day)	Days of blood collection	n	Parameters			
			C_{max} (μg/m*l*)	t_{max} (hr)	$t_{1/2}$ (hr)	AUC_{0-24} (μg·hr/m*l*)
5	Day 1	3	3.4 ± 0.2	1.7 ± 0.6	5.2 ± 0.6	34.7 ± 0.4
	Day 7		3.3 ± 0.6	2.0 ± 1.0	5.2 ± 0.4	35.4 ± 3.1
10	Day 1	3	6.8 ± 0.8	1.0 ± 0.0	4.3 ± 0.8	65.9 ± 3.3
	Day 7		6.0 ± 1.5	1.0 ± 0.0	4.7 ± 0.5	57.0 ± 2.4
20	Day 1	3	12.1 ± 0.7	1.7 ± 0.6	4.8 ± 0.4	139.2 ± 3.7
	Day 7		11.5 ± 1.2	2.0 ± 1.0	4.5 ± 0.5	131.7 ± 10.2

Data are represented as the mean ± SD of 3 animals.

lesions were noted in dogs receiving the second administration of 20 mg/kg/day. Necrotic chondrocytes and unmasked collagen fibers were seen in the edematous area around the cavity. The cavity occasionally contained tissue debris, and the cartilage constituting the outer wall of the cavity protruded into the joint space and was sometimes detached from the articular surface. On day 8, however, chondrocyte clusters around the cavity were frequently observed, and its regional matrix exhibited an increase in staining intensity. The proximal portions of the humerus and femur had the highest incidence of arthropathy among these articular cartilages examined. In addition, no microscopical changes in the epiphysial plate were observed in all animals treated with ofloxacin.

Pharmacokinetic analysis: The C_{max} and AUC_{0-24} increased in a dose-dependent manner from 5 mg/kg/day of ofloxacin. However, no remarkable differences in these two parameters were noted between a single (day 1) and repeated treatments (day 7, Fig. 3). At 5 mg/kg/day (Tables 2 and 3), the C_{max}, t_{max}, AUC_{0-24} and articular drug concentration were 3.3 to 3.4 (mean: 3.4) μg/m*l*, 1.7 to 2.0 (1.9) hr, 34.7 to 35.4 (35.1) μg·hr/m*l* and 5.6 to 8.6 (7.0) μg/g, respectively. The articular ofloxacin concentration 2 hr after treatment was increased in a dose-dependent fashion, and was approximately 1.8 (Day 2) to 2.0 times (Day 8) higher than the corresponding serum concentration (Table 3). In the 20 mg/kg/day group, there was no significant difference in articular ofloxacin level between days 2 and 8.

DISSCUSION

To ensure safety in usage of quinolones it is important to estimate the non-arthropathic dose and pharmacokinetics of the drug, with the pathological lesion in the articular cartilage being a target tissue. In the present study, arthropathy was observed only in animals receiving 10 and 20 mg/kg/day of ofloxacin, but not in those given 5 mg/kg/day. These findings demonstrate that a non-arthropathic dose of ofloxacin in male juvenile dogs following an 8-day treatment is considered to be 5 mg/kg/day. Clinical behavior and pathological findings of arthropathy seen in our study were comparable to the previous data on the known quinolones [3, 5,

Table 3. Articular cartilage concentrations of ofloxacin in male juvenile dogs treated orally with the drug for 8 consecutive days

Dose (mg/kg/day)	Days of sacrifice	n	Humerus (μg/g)		Femur (μg/g)	
			Proximal	Distal	Proximal	Distal
5	Day 8	3	6.6 ± 0.6	5.6 ± 0.4	7.1 ± 1.4	8.6 ± 3.8
10	Day 8	3	10.7 ± 0.5	8.7 ± 1.8	9.2 ± 1.2	10.9 ± 2.7
20	Day 2	3	18.8 ± 1.9	18.4 ± 0.9	19.3 ± 0.6	19.9 ± 1.8
	Day 8	3	15.6 ± 3.4	15.1 ± 0.9	18.1 ± 1.9	16.9 ± 0.7

Data are represented as the mean ± SD of 3 animals.

8, 20]. Meanwhile, the pharmacokinetic analysis revealed a linear relationship between absorption following oral administration and tissue distribution. Ofloxacin concentrations in sera and articular cartilages obtained in the 20 mg/kg/day group were also compatible with those of the previous report [23]. At the non-arthropathic dose (5 mg/kg/day), the C_{max}, AUC_{0-24} and articular cartilage concentrations were 3.4 μg/m*l*, 35.1 μg·hr/m*l* and 7.0 μg/g, respectively. According to numerous data on antimicrobial activity of ofloxacin [7, 15, 16, 21], the majority of gram positive and gram negative bacteria were stated to be inhibited by the drug at concentrations of 1.56 μg/m*l* or less *in vitro*. The serum ofloxacin concentration attained in dogs given 5 mg/kg/day thus seems to be good enough to exert antibacterial activity.

In dogs receiving 10 mg/kg/day, while arthropathy occurred in the proximal humeral portions and the femoral portions at which the mean drug concentration was 10.3 μg/g, no such lesion observed in the distal humeral portion at which the mean drug concentration was 8.7 μg/g. Interestingly, the mean drug concentration in all articular cartilage portions examined of dogs given 5 mg/kg/day that showed no arthropathy was 7.0 μg/g. From these data, the critical drug concentration in cartilage for the induction of arthropathy was estimated to be approximately 9.0 μg/g. At the point of C_{max} (t_{max}), the articular ofloxacin concentration was approximately 1.8 (day 2) to 2.0 times (day 8) higher than the serum concentration. Based on the information, the threshold of serum drug concentration for causing arthropathy was considered to be about 4.5 μg/m*l*. Therefore, the induction of arthropathy due to ofloxacin may be predicted by monitoring its serum level. In a toxicokinetic study of norfloxacin, a quinolone derivative, in juvenile animals [13], chondrotoxicity was reported to depend on the magnitude and duration of exposure to the drug. A minimal arthropathic dose of norfloxacin in dogs receiving a 7-day oral treatment was thought to be 50 mg/kg/day, while the articular drug concentration 1 hr after treatment was 6.9 to 9.9 μg/g. Taken together with these data, the sufficient penetration of the drug into the articular portion was postulated to be essential for the induction of arthropathy, although norfloxacin possessed a low bioavailability by gavage.

In animals receiving 20 mg/kg/day, when the articular drug concentration was compared between days 2 and 8, no evidence of drug accumulation was found. Similarly, there were no remarkable differences in pharmacokinetic parameters, such as C_{max} and AUC_{0-24}, between a single (day 1) and repeated treatments (day 7). Since arthropathy seen on day 2 was comparable in severity with that found on day 8, repeated administration of ofloxacin was not considered to enhance the arthropathic lesion. This fact was supported by the presence of chondrocyte clusters including regeneration of the articular cartilage on day 8. Okazaki *et al.* [14] have reported pharmacokinetics of ofloxacin in dogs differ from that in rats, suggesting species difference. This implied that there was species specificity even in the arthropathic dose of the drug [9, 10].

In conclusion, the present data suggest a safe dosage level of ofloxacin against the induction of arthropathy in juvenile dogs, and emphasize that the arthropathy may be avoided by monitoring serum drug concentration.

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