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## *In vivo* and *in vitro* chondrotoxicity of ciprofloxacin in juvenile rats<sup>1</sup>

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**KEY WORDS** ciprofloxacin; knee joints; articular cartilage; glycosaminoglycans; cell survival; toxicokinetics; juvenile rats

### ABSTRACT

**AIM:** To study the relationship between chondrotoxicity and toxicokinetics of ciprofloxacin (CPFX). **METHODS:** Rats, 4-week old, were treated with CPFX 0, 400, 800, and 1200 mg/kg ig once daily on seven consecutive days. The knee joint cartilage was examined histopathologically. The concentration of CPFX in venous blood and knee joint cartilage samples were determined by a microbioassay using *Escherichia coli* 44102. The effects of CPFX on proliferation of chondrocytes and secretion of soluble proteoglycans were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 1,9-dimethylmethylene blue (DMB) assay, respectively. **RESULTS:** Cartilage was severely lesioned after treatment with CPFX 800 or 1200 mg/kg for 7 d, such as matrix swelling and loss of chondrocytes. The thickness of cartilage was significantly decreased compared with the control group. The maximum serum concentration ( $C_{max}$ ), the area under the plasma concentration-time curve ( $AUC_{0-\infty}$ ), and concentration in cartilage was  $16.3 \pm 2.1$  mg/L,  $97.2 \pm 12.3$  mg·h·L<sup>-1</sup>, and  $13.4 \pm 2.8$  μg·g<sup>-1</sup> and  $21.8 \pm 2.5$  mg/L,  $143.1 \pm 22.3$  mg·h·L<sup>-1</sup>, and  $20.3 \pm 3.5$  μg·g<sup>-1</sup> after oral administration of CPFX 800 or 1200 mg/kg on d 1, respectively. The data on d 6 were similar with that on d 1. CPFX inhibited proliferation of chondrocytes and the secretion of soluble proteoglycans. **CONCLUSION:** CPFX concentrations in serum and cartilage could provide a better basis for risk assessment.

### INTRODUCTION

Quinolones (QNs) were widely used in clinical practice owing to their wide spectrum antibacterial activity and high degree of bioavailability. They were not approved for use in children and adolescents due their toxic effects on joint cartilage of immature animals<sup>[1]</sup>. So QNs was a second-line drug following the failure of

a well established antibiotic treatment<sup>[2]</sup>.

The extraordinary age-related drug toxicity of QNs was demonstrated in juvenile dogs, rats, rabbits, guinea pigs, and marmosets. However, the relationship between the adverse clinical effects of these drugs and lesions in target tissue of laboratory animals has not been clarified<sup>[3,4]</sup>.

A rational comparison or extrapolation of chondrotoxic potentials between different species should be based on kinetics. As very few toxicokinetic studies in immature animals have been published, reasonable extrapolations of the toxicological results are still limited. Because there exists significant differences in the kinetics of drugs in rodents and humans, it makes no sense

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to compare the doses applied in toxicological experiments in rats with those used therapeutically in humans. Instead, concentrations achieved in plasma or the target tissue represented a more reliable basis for such comparisons<sup>[5]</sup>.

Kato *et al* reported that levofloxacin inhibited glycosaminoglycan synthesis initially and DNA synthesis and mitochondrial function secondarily at actual arthropathic concentrations in cultured rabbit chondrocytes but that these changes were reversible and not enough to kill the cells<sup>[6]</sup>. Egerbacher *et al* reported that enrofloxacin and ciprofloxacin hydrochloride inhibited the proliferation of cultured canine and equine chondrocytes and decreased their ability to adhere to the culture dish<sup>[7]</sup>.

Ciprofloxacin (CPFX) is widely used in the clinic, while it is not clear at what concentrations in plasma and target tissue it will induce the lesions of articular joints. So in this paper we studied the kinetics of CPFX in plasma and determined its concentration in joint cartilage of juvenile rats and the related mechanisms.

## MATERIALS AND METHODS

**Materials** Male 4-week old SD rats were provided by the Experimental Animal Center of Fudan University and housed under controlled conditions (temperature  $21\pm 1$  °C and lighting 8:00-20:00) with food and water *ad libitum*. CPFX was provided by the He-nan Institute for Drug Control. HAM's F-12 and fetal calf serum were supplied by Gibco. Trypsin, collagenase, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and chondroitin sulfate C were from Sigma. 1,9-Dimethylmethylene blue (DMB) was obtained from Polysciences. Image Analysis System was purchased from Shanghai Shenteng Information Technology Co.

**Kinetics** Rats were randomly assigned to 4 groups (each including 5 animals). CPFX at 400, 800, and 1200 mg/kg body weight was administered *ig* once daily on seven consecutive days. CPFX was suspended in 0.5 % sodium carboxymethylcellulose (CMC). Rats in control group were *ig* 0.5 % CMC. Venous blood was collected from an incised tail vein just before and at 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 12 h after the dosing on d 1 and d 6. Serum was separated and stored at -20 °C until assay.

**Histopathology** Four hours after final dosing the rats were killed. The left knee joint was removed, fixed in 10 % formalin, and stored. Cartilage was obtained

from the femoral condyle of the right knee joint, weighed and homogenized in saline. After centrifugation, the supernatant was stored at -20 °C until assay.

The joint samples were decalcified and embedded in paraffin, serially sectioned in a sagittal plane at 8  $\mu$ m and routinely-stained with hematoxylin and eosin. Four tissue sections were used randomly for the histopathological examination for one animal. The thickness of the cartilage of the femoral condyle was measured with Image Analysis System. The thickness of one sample was obtained from the average of those from four sections.

**Microbioassay** CPFX concentration in serum and cartilage was determined by a validated agar diffusion microbioassay using *Escherichia coli* 44102<sup>[8]</sup>. The standard curve was prepared with rat normal serum and saline, and the linear relationship was determined between the concentrations of 0.125 and 16 mg/L. The intra- and inter-day coefficients of variation were less than 5 % in standard curve and the limit of sensitivity of the assay was approximately 0.125 mg/L.

**Isolation and culture of articular chondrocytes** Cartilage fragments were excised from the femuro-tibial joints of the rats and cut into small pieces (1 mm $\times$ 1 mm $\times$ 1 mm). Cartilage pieces were trypsinized for 30 min and treated with 0.1 % collagenase for 3 h at 37 °C. Cells were filtered through nylon mesh (50  $\mu$ m pore size) to remove matrix residues, centrifuged, and seeded  $1\times 10^5$  cells per 50 mm<sup>3</sup> flask in HAM's F-12 culture medium supplemented with 10 % fetal calf serum. After 5-d culture with growth medium in flasks the cells were trypsinized and counted. For the MTT assay, cells were dispensed into 96-well microtiter plates ( $4\times 10^3$  cells per well) and for the DMB assay cells were dispensed into 24-well plates ( $3\times 10^4$  cells per well). After 24 h of pre-incubation with growth medium, CPFX was added at concentrations of 0, 5, 10, 20, 40, 80, and 160 mg/L.

**Determination of proliferation** Cell proliferation was determined by the MTT assay<sup>[9]</sup>. Chondrocytes were incubated with CPFX for 1, 3, and 5 d, respectively. Then MTT (5 g/L, in PBS, pH 7.4) was added and incubated at 37 °C for 4 h. The medium was removed and Me<sub>2</sub>SO was added to dissolve the crystals. The absorbance was measured at 490 nm in a microtiter plate photometer. Data were presented as inhibitory ratio.

**Determination of proteoglycans** After 5-d incubation proteoglycans were determined in culture

media supernatants by the DMB assay<sup>[10]</sup>. Samples in volume of 0.5 mL were mixed with 0.5 mL DMB solution (32 mg/L in 0.05 mol/L formate buffer, pH 3.5) and pipetted into cuvettes. Absorbance was measured using a spectrophotometer at 530 nm. A standard curve was constructed at chondroitin sulfate C 5-100 mg/L. The cells were trypsinized and counted. The content of proteoglycans was presented as  $\mu\text{g}$  per  $1 \times 10^5$  cells.

**Data analysis** Pharmacokinetic analysis was performed using the 3p97 program. Results were expressed as mean $\pm$ SD. Statistical comparisons of the means were performed using multivariate analysis of variance with SPSS software.

**RESULTS**

**Histopathology** No obvious cartilage alterations were observed after treatment with CPFX 400 mg/kg for 7 d. However cartilage was severely lesioned after treatment with CPFX 800 or 1200 mg/kg for 7 d, such as matrix swelling and loss of chondrocytes. The thickness of cartilage in CPFX 800 and 1200 mg/kg groups was significantly decreased compared with the control group, respectively (Fig 1).

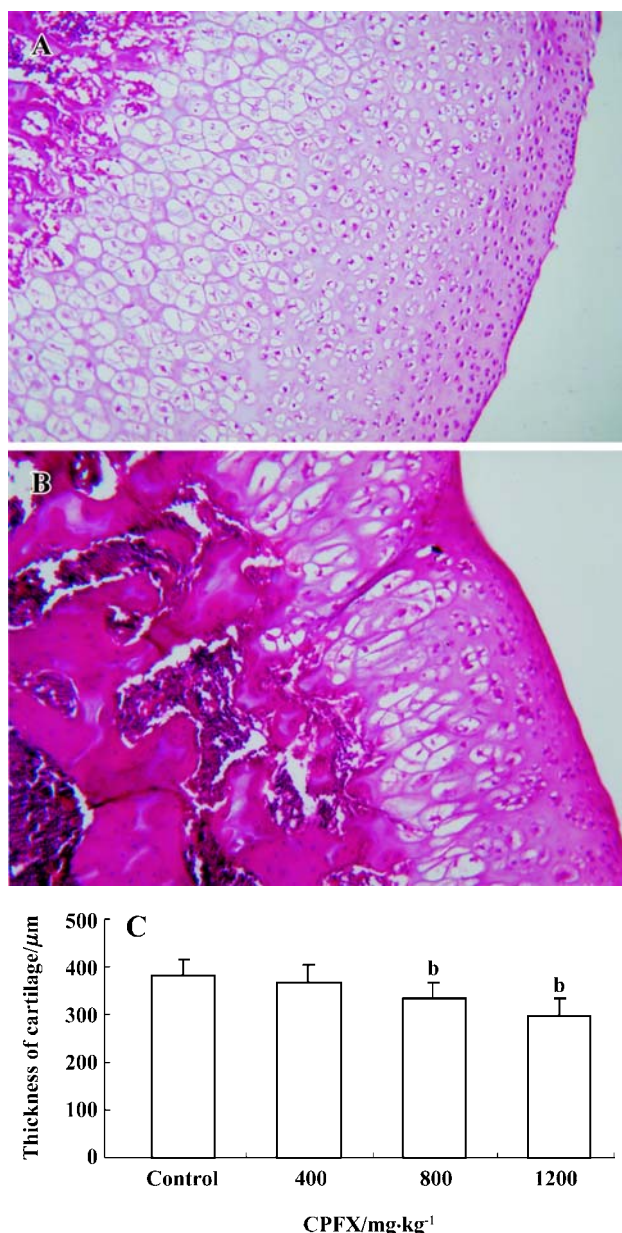
**Toxicokinetics** CPFX was rapidly absorbed after dosing. The concentration of CPFX reached the peak at 1.5 h and then declined gradually (Fig 2). The  $C_{\text{max}}$ ,  $\text{AUC}_{0-\infty}$ , and CPFX concentration in cartilage was increased in a dose-dependent manner. The  $C_{\text{max}}$  and  $\text{AUC}_{0-\infty}$  are similar on d 1 and d 6 (Tab 1).

**Proliferation of cultured chondrocytes** CPFX inhibited chondrocytes proliferation in a dose-dependent and time-dependent manner. The threshold concentration of CPFX to inhibit chondrocytes proliferation was 40 mg/L after 1-d culture, 20 mg/L after 3-d culture, and 10 mg/L after 5-d culture (Fig 3).

**Soluble proteoglycans** CPFX 10-80 mg/L decreased the secretion of soluble proteoglycans after incubation with chondrocytes for 5 d (Fig 4).

**DISCUSSION**

For most xenobiotic compounds, kinetics in animals and humans differ considerably. Therefore, data from toxicological studies can be interpreted only on the basis of the kinetics of the compound in the species studied. In the present study, the toxicity of CPFX on knee joint cartilage in juvenile rats was investigated after oral administration once daily for seven consecutive days, resembling the length of a clinical treatment period.

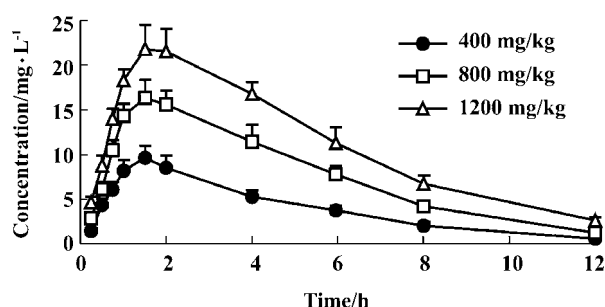
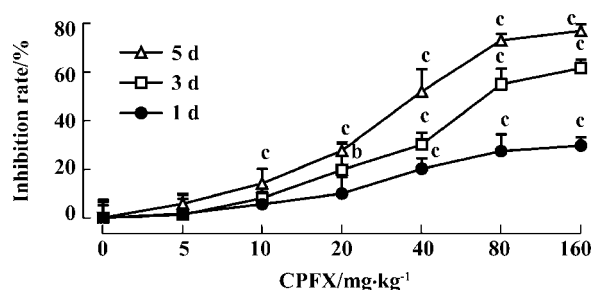
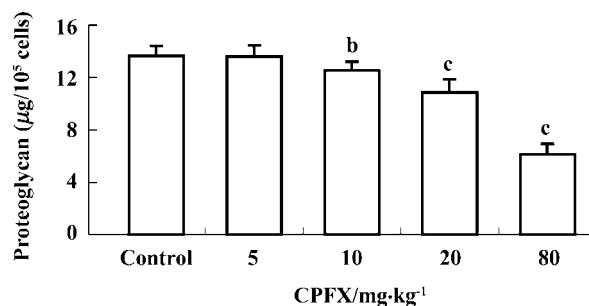


**Fig 1. Articular cartilage of femoral condyle from 4-week old rats. (A) Control; (B) CPFX 1200 mg/kg.  $\times 200$ . (C) Effect of CPFX 400-1200 mg/kg on thickness of the cartilage.  $n=5$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs Control.**

CPFX decreased thickness of the articular cartilage of the femoral condyle at 800 and 1200 mg/kg but not at 400 mg/kg. Although the doses we used was 50, 100, and 150 fold than the therapeutic doses in humans, the mean peak concentrations in the serum of juvenile rats were only approximately 4, 6, and 8 times than those in human volunteers after administration of a single oral dose of CPFX 500 mg<sup>[11]</sup>. Thus, it is very important to include toxicokinetic investigations to provide a reasonable basis for risk assessment. Our study showed that

**Tab 1. Main pharmacokinetic parameters on d 1 and d 6 and the concentration of CPFX in cartilage 4 h after dosing on d 7 in 4-week old rats after treatment with CPFX 400, 800, and 1200 mg/kg,  $n=5$ . Mean $\pm$ SD.**

CPF <sub>X</sub> /mg·kg <sup>-1</sup>	$C_{max}$ /mg·L <sup>-1</sup> d 1	AUC/mg·h·L <sup>-1</sup> d 1	$C_{max}$ /mg·L <sup>-1</sup> d 6	AUC/mg·h·L <sup>-1</sup> d 6	Concentration in cartilage/ $\mu$ g·g <sup>-1</sup>
400	9.6 $\pm$ 1.3	48.1 $\pm$ 7.2	9.4 $\pm$ 1.6	51.1 $\pm$ 6.0	7.4 $\pm$ 2.1
800	16.3 $\pm$ 2.1	97.2 $\pm$ 12.3	15.2 $\pm$ 2.2	95.4 $\pm$ 10.6	13.4 $\pm$ 2.8
1200	21.8 $\pm$ 2.5	143.1 $\pm$ 22.3	23.7 $\pm$ 3.0	148.8 $\pm$ 19.8	20.3 $\pm$ 3.5

**Fig 2. Serum concentration-time profile after a single oral dose of CPF<sub>X</sub> 400, 800, and 1200 mg/kg in 4-week old rats.  $n=5$ . Mean $\pm$ SD.****Fig 3. Inhibitory effects of CPF<sub>X</sub> on proliferation of cultured chondrocytes from 4-week old rats.  $n=4$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control.****Fig 4. Effect of CPF<sub>X</sub> on secretion of soluble proteoglycans from cultured chondrocytes of 4-week old rat.  $n=5$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control.**

concentration of CPF<sub>X</sub> in serum reached the peak at 1.5 h after administration of an oral dose of 400, 800, and 1200 mg/kg, respectively. The  $C_{max}$  and AUC<sub>0-∞</sub> were similar on d 1 and d 6 indicating no accumulation after multiple doses. The cartilage lesions become severe with an increase of  $C_{max}$ , AUC<sub>0-∞</sub>, and concentration in cartilage.

Stahlmann *et al* reported that lesions in knee joint cartilage from juvenile rats were detectable only after oral treatment with sparfloxacin 1800 mg/kg but not 600 mg/kg. The corresponding  $C_{max}$  was 12.4 mg/L and the AUC<sub>0-∞</sub> was 228 mg·h·L<sup>-1</sup> in serum. At all time points the concentrations of sparfloxacin in joint cartilage were significantly higher than those in serum<sup>[5]</sup>. Kato *et al* discovered that repeated oral administration of levofloxacin 100 mg/kg in rats for 7 d induced lesions in the articular cartilage of the femoral condyle, while at dose of 30 mg/kg did not. The corresponding data for levofloxacin concentration in serum and articular cartilage were 6.24 g/L and 4.93  $\mu$ g/g in 30 mg/kg group, and 21.98 g/L and 12.20  $\mu$ g/g in 100 mg/kg group, respectively<sup>[6]</sup>. The concentration of levofloxacin in plasma was higher than that in cartilage. In our study no significant differences between concentration of CPF<sub>X</sub> in serum and cartilage were observed. Nagai *et al* demonstrated that although garenoxacin concentrations in plasma and joint tissue in beagle dogs were higher than those for ciprofloxacin and norfloxacin, the articular toxicity of garenoxacin was much less than that of the other two antimicrobials<sup>[12]</sup>.

To confirm our results obtained *in vivo*, we investigated the effects of CPF<sub>X</sub> on proliferation of cultured chondrocytes and secretion of soluble proteoglycans. CPF<sub>X</sub> inhibited chondrocytes proliferation and secretion of soluble proteoglycans in a concentration- and time-dependent manner. The minimal toxic concentration of CPF<sub>X</sub> was 10 mg/L similar with that in *in*

*vivo* experiments. So the cultured cells would be appropriate to evaluate the toxicity of QNs on chondrocytes *in vitro*. CPFX caused cartilage lesion by inhibiting proliferation of chondrocytes and secretion of proteoglycans.

Hildebrand *et al* reported that QNs decreased soluble proteoglycan levels in the culture medium and the incorporation of <sup>35</sup>S-sulfate into high-molecular-weight molecules in cultures of dog chondrocytes<sup>[13]</sup>. Egerbacher *et al* also observed that in cultivated horse and dog chondrocytes, cell proliferation decreased in CPFX- and enrofloxacin-treated groups<sup>[14]</sup>. Our results were consistent with them.

The specific mechanism responsible for the initiation of quinolone-induced arthropathy has not been determined. The most plausible explanation for the mechanism involved the chelation of magnesium ions by QNs, resulting in changed function of chondrocyte surface integrin receptors<sup>[15,16]</sup>. QNs treatment plus Mg<sup>2+</sup>-free medium increased the negative effects on cell morphology and adhesion seen in the QNs or Mg<sup>2+</sup>-free groups. Mg<sup>2+</sup> supplement reduced the effects of QNs *in vitro*<sup>[14]</sup>.

In conclusion, concentrations of CPFX in serum and cartilage could provide a better basis for a reasonable risk assessment.

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