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In Vitro Discrimination of Fluoroquinolones Toxicity on Tendon Cells: Involvement of Oxidative Stress

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ABSTRACT

Tendinopathy are classic side effects observed with fluoroquinolones antibiotics. A previously validated model based on a spontaneously immortalized rabbit tendon cell line (Teno cell line) was used to evaluate cellular responses to the fluoroquinolones pefloxacin (PEF), ofloxacin (OFX), levofloxacin (LVX), and ciprofloxacin (CIP), in various concentrations. Cell viability, redox status changes, reduced glutathione content, and reactive oxygen species production were assessed using neutral red, Alamar blue, monobromobimane and 2,7-dichlorofluorescin-diacetate fluorescent probes, respectively. Living adherent tenocytes were analyzed using a cold light cytofluorometer adapted to 96-well microplates. All fluoroquinolones showed moderate cytotoxicity after 24 h and more severe, significant toxicity after 72 h on tendon cells. Moreover, two groups of fluoroquinolones may be differentiated: intrinsic toxicity for tendon cells was high with ciprofloxacin and pefloxacin [redox status decrease was 80 and 62% ($*p < 0.05$) for PEF and CIP at 1 mM for 72 h, respectively], but moderate with ofloxacin and levofloxacin LVX [redox status decrease was 30 and 22% ($*p < 0.05$) for OFX and LVX at 1 mM during 72 h, respectively]. Our model supports a role for early oxidative stress in the development of fluoroquinolone-induced tendinopathy. Moreover, our study indicates that intrinsic toxicity to tendon cells varies across fluoroquinolones. The Teno cell line may be a useful model for detecting and evaluating tendon toxicity of new fluoroquinolones and other drugs associated with tendinopathy.

Fluoroquinolones antimicrobial agents are widely used in clinical practice as broad-spectrum antimicrobials with excellent bioavailability. However, they have been reported to induce tendinopathies and, less often, arthralgia and myalgia (Jorgensen et al., 1991; Hayem and Carbon, 1995; Stahlmann and Lode, 2000; Stahlmann, 2002). The main target is the Achilles tendon, where complete rupture can occur; other sites of involvement include the shoulders, knees, hand, and plantar fascia. More than 400 cases of FQ-induced tendinopathy have been reported (Ribard et al., 1992; Zabraniecki et al., 1996; Lewis et al., 1999; Van Der Linden et al., 2001).

Risk factors for fluoroquinolones-induced tendinopathy include older age, corticosteroid therapy and renal dysfunction. Achilles cause prolonged functional impairment. This pathology can be observed in patients only a few hours or days after receiving a single oral dose of FQ, and results in a serious source of invalidity (Stahlmann and Lode, 2000).

The mechanisms underlying fluoroquinolone-induced tendinopathy remain incompletely understood, and the absence of an animal model has been an obstacle to their elucidation. Several studies have investigated fluoroquinolone toxicity for joint tissues using either flow cytometry (Hayem et al., 1994, 1996; Ratinaud and Valet, 1994; Thuong-Guyot et al., 1994) or histology (Kato et al., 1995; Kashida and Kato, 1997; Shakibaei and Stahlmann, 2001). However, these methods do not allow for the measurement of labile markers for oxidative stress and are not well suited to systematic toxicological screening. Microtitration cold light cytofluorometry that we developed (Rat, 1994; Rat et al., 1994, 1995, 1996, 1997) can be used to assess drug effects and to screen for toxicity (Rat et al., 1996) on living adherent cells without prior trypsination or dispersion. Therefore, it limits artifacts that might take place during monodispersion or extraction processes, which are often used in flow cytometry and biochemistry.

The aim of this study was to further assess the discrimination of intrinsic tenotoxicity potential of four fluoroquinolones: pefloxacin (PEF), ciprofloxacin (CIP), levofloxacin (LVX), and ofloxacin (OFX) using methods that comply with the recommendations issued by the European Center for the Validation of Alternative Methods (Clothier, 1989; Balls and

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ABBREVIATIONS: FQ, fluoroquinolones; PEF, pefloxacin; CIP, ciprofloxacin; LVX, levofloxacin; OFX, ofloxacin; DCFH-DA, 2,7-dihydrodichlorofluorescein diactetate; PBS, phosphate-buffered saline; FCS, fetal calf serum; kb, kilobase(s); ROS, reactive oxygen species.

Fentem, 1992). We used an immortalized rabbit-tendon cell line that express type I collagen. The cells were exposed for variable period to OFX, PEF, CIP, or LVX. Cell viability, redox status, reactive oxygen species, and intracellular reduced glutathione content were assessed using neutral red, Alamar blue, 2,7-dichlorofluorescin-diacetate (DCFH-DA), and monobromobimane probes, respectively. Such methodology allowed us to investigate the mechanism of fluoroquinolone tenotoxicity.

Materials and Methods

Reagents Supply. PEF, LVX, and OFX were from Aventis (Strasbourg, France) and CIP was from Bayer AG (Wuppertal, Germany). Monobromobimane, Neutral Red, and DCFH-DA were purchased from Molecular Probes (Eugene, OR). Alamar blue was obtained from Interchim (Asnières, France). Dimethyl sulfoxide and ascorbate were obtained from Sigma-Aldrich (St-Quentin-Falavier, France). The culture medium (Ham's F-12), trypsin, and calcium- and magnesium-free phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ulis, France). Fetal calf serum (FCS) came from Dominique Dutscher (Brumath, France).

Fluoroquinolone Stock Solutions. Fluoroquinolones stock solutions (10^{-2} M) were prepared in HCl (0.05 N) and stored at 4°C. These solutions were diluted in Ham's F-12 medium containing 2.5% FCS to obtain concentrations ranging from 0.01 μM to 1 mM. Two cellular controls were used, namely, cells incubated with Ham's F-12 medium containing 2.5% FCS without xenobiotic (cellular control) and cells incubated with the highest HCl concentration used in the tested solution (vehicle control). No significant toxicity had been observed previously when tendon cells were exposed to the vehicle alone (0.005 N HCl).

Cell Culturing. In previous report, we cultured and characterized juvenile rabbit's tenocytes (Bernard-Beaubois et al., 1997, 1998). After this study, a spontaneously immortalized clone of tenocytes that we called Teno cell line was isolated from rabbit Achilles tendon. Cells were cultured in 75-cm^2 flasks containing Ham's F-12 medium supplemented with 10% FCS, 4 μg/ml gentamicin, 2 mM glutamin and incubated at 37°C in a moist atmosphere with 5% CO_2. The culture medium was changed every 2 days. Only cells expressing type I collagen from passages 88 to 92 were used.

Once a confluent cell layer was obtained, the medium was aspirated without disturbing the cell layer. The cell layer was washed once with warm sterile PBS, which was aspirated. Then, 1 ml of 0.25% trypsin was added to each 75-cm^2 flask, and the flasks were rocked gently to ensure that the cell layer was covered and that trypsin had penetrated between the cells. The flasks were replaced for a few minutes at 37°C in a 5% CO_2 atmosphere. The cells were viewed under a light microscope. The culture medium containing trypsin-inactivating factors was added to the flask. After centrifugation at room temperature, 700*g* for 3 min, the culture medium was removed and the cell pellets were resuspended in the medium, and then transferred into culture flasks. Finally, the cells were incubated at 37°C in a 5% CO_2 atmosphere. With the same cells culture medium, rabbit primary tenocytes have been obtained according to Bernard-Beaubois et al. (1997, 1998) protocols, rabbit fibroblast SIRC were obtained from American Type Culture Collection (Manassas, VA) (ATCC CCL-60).

Before microtitration analysis, cells were seeded into 96-well microplates (Costar-Dominique Dutscher, Brumath, France) with 5500 cells/well and kept at 37°C for 72 h. Finally, the cells were then exposed to one of the studied fluoroquinolones.

Light Microscopic Analysis. Rabbit primary tenocytes and Teno cell s were cultured in petri dishes containing Ham's F-12 medium supplemented with 10% FCS, 4 μg/ml gentamicin, and 2 mM glutamin. The dishes were incubated at 37°C in a moist atmosphere with 5% CO_2. The cultures were examined under a Leica inverted microscope (DMIR-B; Leica, Wetzlar, Germany).

RNA Extraction and Northern Blot Analysis of Rabbit Teno Cell Line, Primary Rabbit Tenocytes, and Rabbit Fibroblast (SIRC) Cells. Collagen I expression with and without ascorbate (50 and 100 μg/ml) was evaluate by Northern blotting. The steady-state level of mRNA encoding collagen I was measured by hybridizing the membranes with appropriate cDNA probes (Chomczynski and Sacchi, 1987; Mäkelä et al., 1988).

Total RNA was extracted from cells grown to confluence in standard medium according to the method of Chomczynski and Sacchi (1987) using RNAzolTM (Bioprobe, Montreuil sous Bois, France). Purified total RNA (12 μg) was electrophoresed overnight at 50 V in 1% agarose gel under denaturing conditions and transferred onto a 'positive' nylon membrane (Appligène, Graffenstaten, France). The probes used for hybridization were the carboxy-propeptide domain of human α1(I) procollagen mRNAs (pH Cal 1U, 670 base pairs) (Mäkelä et al., 1988) and a mouse cDNA of 28S rRNA (4.7 kb) (Arnheim, 1979).

Inserts were prepared by restriction enzyme digestion of plasmids followed by electrophoresis and by purification from the agarose using Spin-X centrifuge filter units (Costar, Brunath, France) according to the manufacturer's instructions. DNA probes were labeled with [^{32}P]α-dCTP by random priming (Amersham, Saclay, France). Prehybridization (2 h) and hybridization (24 h) were performed at 65°C in 0.5 M sodium phosphate buffer (pH 7.2), containing 7% SDS, 2 mM EDTA, and 0.5 mg/ml herring sperm DNA.

After hybridization, the membranes were washed twice for 30 min in 40 mM phosphate buffer containing 1% SDS. The first washing was performed at 20°C and the second at 65°C. The filters were dried then exposed at −80°C to X-ray films (Kodak-X-OMAT AR or Hyperfilm; Amersham) with intensifying screens. Dehybridization was achieved by washing the filters at 80°C in 1% SDS for 1 to 2 h and was controlled by autoradiography of the stripped filters.

Cold Light Cytofluorometry. All fluorescence measures were performed using a highly sensitive (femtograms to picograms per milliliter) and specific cold light cytofluorometer (Fluorolite 1000-Dynex) (Rat et al., 1994). This apparatus used in combination with an extended photomultiplicator (280–900 nm) and a 100% photonic transmission in air enables highly sensitive detection with UV probes (5 pg/ml), green probes (femtograms to picograms per milliliter) and red probes (30 pg/ml). Moreover, cold light cytofluorometer is adapted to intracellular fluorescence analysis on living adherent cells in microplates that permit labile markers assessment.

Cell Incubation with Fluoroquinolones. After removal of the culture medium, the fluoroquinolones were added in various concentrations (0.01 μM, 0.1 μM, 1.0 μM, 10 μM, 0.1 mM, and 1.0 mM; 200 μl/well). The microplates with the fluoroquinolones were incubated at 37°C in a moist atmosphere with 5% CO_2 for 24 h or 72 h.

Cell Viability Assay: Neutral Red Test. The neutral red assay is based on incorporation of the supravital dye neutral red into living cells. Neutral red stock solution (0.4%) was prepared in distilled water and stored at room temperature. Before staining, a fresh 1:80 dilution of the dye (50 μg/ml final concentration) in the medium was prepared. In accordance with the validated test of Borenfreund and Puerner (1984), 200 μl/well of medium containing neutral red were added to living cells, and the microplates were incubated at 37°C in moist atmosphere with 5% CO_2 for 3 h. Ethanol-acetic acid solution (200 μl/well) was added to reveal the fluorescent neutral red signal (Rat et al., 1994, 1995, 1996). The microplates were shaken for 15 min, and fluorescence was measured (excitation 535 nm/emission 600 nm) (Essig-Marcello and Van Burkirk, 1990; Rat et al., 1994).

Redox Status Assay: Alamar Blue Test. Alamar blue assay (De Fries and Mitsuhashi, 1995; Larson et al., 1997) uses a visible blue fluorogen probe resazurin, which is reduced to a red fluorescent compound (resorufin) by cellular redox enzymes. The Alamar blue solution, which is devoid of cytotoxicity, was added undiluted (20 μl) to the wells containing the fluoroquinolone solutions (200 μl), and

the microplates were incubated at 37°C for 12 h. Intracellular fluorometric detection (excitation 535 nm/emission 600 nm) was performed using cold light cytofluorometry.

Reactive Oxygen Species Production Assay: DCFH-DA Test. ROS production was detected using the fluorogen DCFH-DA, which is activated by cellular esterases and reacts with cellular peroxidase to become a fluorescent product (Bass et al., 1983). Before fluoroquinolone treatment, DCFH-DA (20 μM/20 min) was added to each well containing the living adherent cells (Osseni et al., 1999). The fluorescence signal (excitation 485 nm/emission 535 nm), which is proportional to ROS production (Osseni et al., 1999), was measured using cold light cytofluorometry.

Reduced Glutathione Assay: Monobromobimane Test. Monobromobimane, which is an UV fluorogen bimane probe, reacts specifically with glutathione to form a fluorescent derivative (Kosower and Kosower, 1987; Han et al., 1997). The stock solution (16.6 mg/ml in 100% dimethyl sulfoxide), stored at 4°C, was diluted in Ca^{2+}- and Mg^{2+}-free PBS to obtain 100 μM monobromobimane. The diluted solution was added directly on the living cells in the microplates. Fifteen minutes later, fluorometric detection (excitation 390 nm/emission 480 nm) was performed using cold light cytofluorometry (Rat, 1994).

Expression of Results and Statistical Analysis. Each drug concentration was tested in different independent experiments to obtain minimum 12 single values for each concentration. All results were obtained in fluorescence arbitrary units and were then converted into percentages of the control value. Single-factor analysis of variance was performed with single values, followed by a bilateral Dunnett's test (Dunnett, 1955, 1964) at a level of significance of 0.05 (SigmaStat 2.03; SPSS Sciences, Chicago, IL).

Results

Setting Up of the Teno Cell Line Model

The Teno cell line is one of the first tendon cell lines spontaneously immortalized from rabbit tendon cells that is usable for pharmacotoxicology study. Specific differentiation parameters of primary tenocytes such as morphology and collagen I expression have been assessed.

Morphology Control with Light Microscope Analysis. Rabbit primary tenocytes and Teno cell line exhibited a similar morphology. The cells were elongated cells and arranged in long chains (Fig. 1).

Collagen I Expression. Type I collagen transcripts were detected in primary tenocytes culture and in Teno cell line but not in rabbit fibroblast cell line (SIRC). Moreover, the amount of mRNA encoding collagen I protein increased in primary tenocytes and Teno cell line culture after treatment with 50 and 100 μg/ml ascorbate but not in rabbit fibroblast cell cultures (SIRC). Type I collagen mRNA was detected at passage 80, 90, and 92 of Teno cell cultures (Fig. 2).

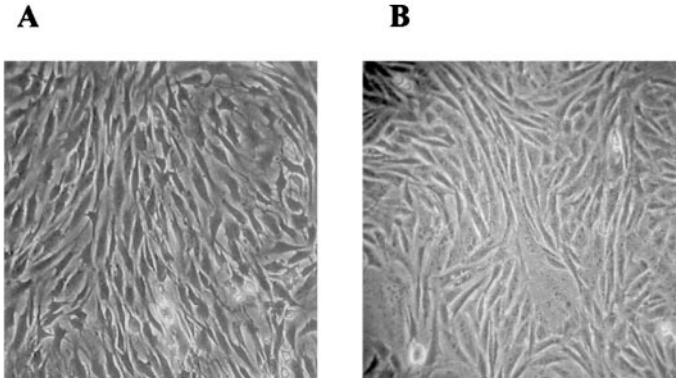

Fig. 1. The rabbit Teno cell line and primary rabbit tenocytes show similar morphological characteristics when viewed under an inverted light microscope. Primary rabbit tenocytes (X200) (A) and rabbit Teno cell line (200×) (B).

Fluoroquinolones Cytotoxicity

Cell Viability-Membrane Integrity Assessment: Neutral Red Test. Figure 3 shows the rate of cytotoxicity induced by fluoroquinolones. Cell viability was assessed with fluoroquinolones concentrations from 0.01 μM to 1.0 mM, a range that includes the therapeutic level in humans. After fluoroquinolones exposure of 24 h, a significant ($^*p < 0.05$) decrease in cell viability ($^*p < 0.05$) was seen only with the highest concentrations (1.0 mM) of PEF (58% versus control) and CIP (32.3% versus control). At this incubation time, the IC_{50} (concentration associated with a 50% reduction of survival versus control) was approximately 1.0 mM for PEF and >1.0 mM for the other fluoroquinolones. Moreover, significant ($^*p < 0.05$) delayed tendon toxicity was detected after

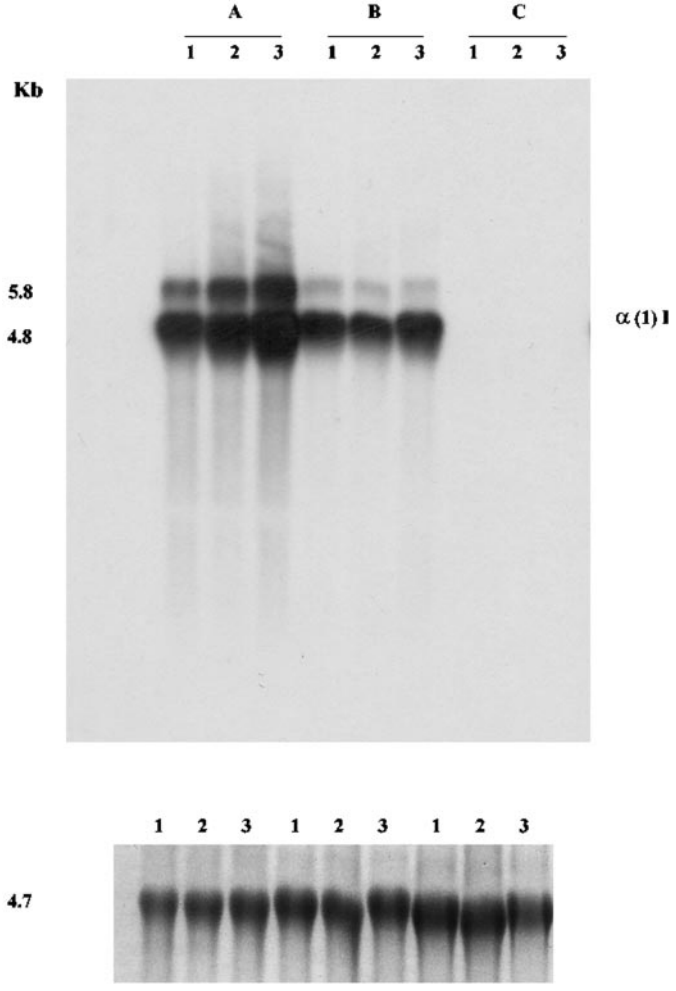

Fig. 2. Top, Northern blot analysis (three experiments) of total RNA extracted from primary rabbit tenocyte, rabbit Teno cell line, and rabbit fibroblast (SIRC) incubated with or without ascorbate at different concentrations during 48 h. Equal amounts of total RNA (12 μg) were loaded in each lane and hybridized with a type I collagen cDNA probe. Sizes of type I collagen mRNA transcripts were 5.8 and 4.8 kb. Bottom, mouse cDNA of 28S rRNA (4.7 kb). A, primary rabbit tenocyte. B, rabbit Teno cell line P90. C, rabbit fibroblast cell line (SIRC). 1, cellular control 0 μg/ml ascorbate; 2, 50 μg/ml ascorbate; 3, 100 μg/ml ascorbate; α (1)I, Alpha 1 (type I) chain.

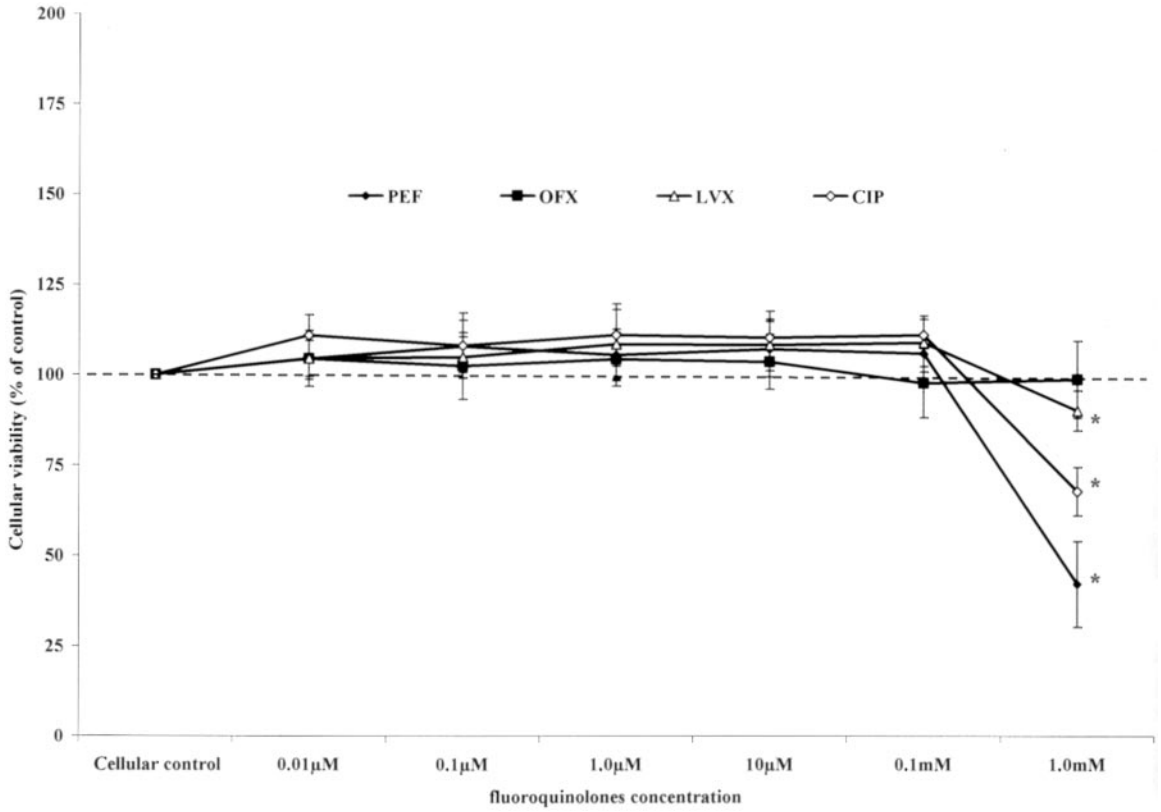

Fig. 3. Cell viability evaluated after 24 h of continuous exposure of cells to fluoroquinolones. Cell viability was evaluated with neutral red probe directly on living tendon cells. Specific fluorometric detection of neutral red was performed with cold light fluorometry (excitation 535 nm/emission 600 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate ($n = 12$) (*, $p < 0.05$).

72 h (Fig. 4), being the most marked with the highest concentration (1.0 mM) of fluoroquinolones. At 72 h, the IC_{50} values were about 1.0 mM for PEF and CIP, and >1.0 mM for OFX, and viability showed significant ($*p < 0.05$) decreases of 64.7, 43, and 16.5% with these drugs, respectively, compared with the cellular control; no significant difference in cell viability was found between LVX and the control.

Fluoroquinolones Induced Oxidative Stress

Redox Status Assay: Alamar Blue Test. At 24 h (Fig. 5), a significant ($*p < 0.05$) decrease in redox status was found with the four fluoroquinolones only at high concentrations (0.1–1.0 mM). With PEF, a significant ($*p < 0.05$) fall in redox status occurred with all concentrations. With CIP at concentrations of 10 μM, 0.1 mM, and 1.0 mM, redox status decreased significantly ($*p < 0.05$), by 6.1, 10, and 31.7%, respectively. With OFX, significant ($*p < 0.05$) redox status decrease of 3.4, 4.7, 9.5m and 26.2% occurred with concentrations of 1.0 μM, 10 μM, 0.1 mM, and 1.0 mM, respectively. With LVX in concentrations of 0.1 mM and 1.0 mM, redox status decreased significantly ($*p < 0.05$), by 5.2 and 19%, respectively.

After 72 h of incubation (Fig. 6), with PEF in concentrations of 10 μM, 0.1 mM, and 1.0 mM, redox status fell significantly ($*p < 0.05$), by 6.4, 6.4, and 80.1%, respectively. With CIP, redox status decreased significantly ($*p < 0.05$) with all concentrations studied. With OFX, significant ($*p < 0.05$) decrease in redox status of 7.8, 6.6, 12.2, and 30.1% was seen with concentrations of 1.0 μM, 10 μM, 0.1 mM, and 1.0 mM, respectively. With LVX in concentrations of 10 μM, 0.1 mM, and 1.0 mM, redox status fell significantly ($*p < 0.05$) by 6.7, 6.7, and 21.9%, respectively.

Reactive Oxygen Species Production. As shown in Fig. 7, the tenocytes were incubated with a range of fluoroquinolones concentrations (0.01 μM–1.0 mM) for 45 min. At low concentrations (<10 μM), ROS production was not significantly different from the control with LVX and CIP, whereas significant ($*p < 0.05$) overproduction occurred with PEF (1.0 μM) and OFX (all concentrations). With high concentrations (10 μM–1.0 mM), a concentration-dependent increase in ROS production occurred with the four fluoroquinolones. With PEF, the increase was 33.9 and 207.4% at concentrations of 10 μM and 0.1 mM, respectively. With CIP, ROS production increased significantly ($*p < 0.05$), by 18.6 and 192.4% at 0.1 mM. At 1.0 mM, ROS production was lower with PEF and CIP than with OFX and LVX.

Moreover, ROS production increased significantly ($*p < 0.05$) with OFX and LVX in therapeutic (10 μM) and supratherapeutic concentrations (0.1 mM and 1.0 mM). With OFX, significant ($*p < 0.05$) ROS production occurred with all concentrations (13.7, 6.7, 8.4, 26.8, 155.6, and 173.5% with 0.01 μM, 0.1 μM, 1.0 μM, 10 μM, 0.1 mM, and 1.0 mM, respectively). With LVX, the increase in ROS production was 23, 161, and 164.1% with concentrations of 10 μM, 0.1 mM, and 1.0 mM, respectively.

Reduced Glutathione Assessment

This oxidative stress was correlated with glutathione depletion after 72 h (Fig. 8) of fluoroquinolone exposure. Sig-

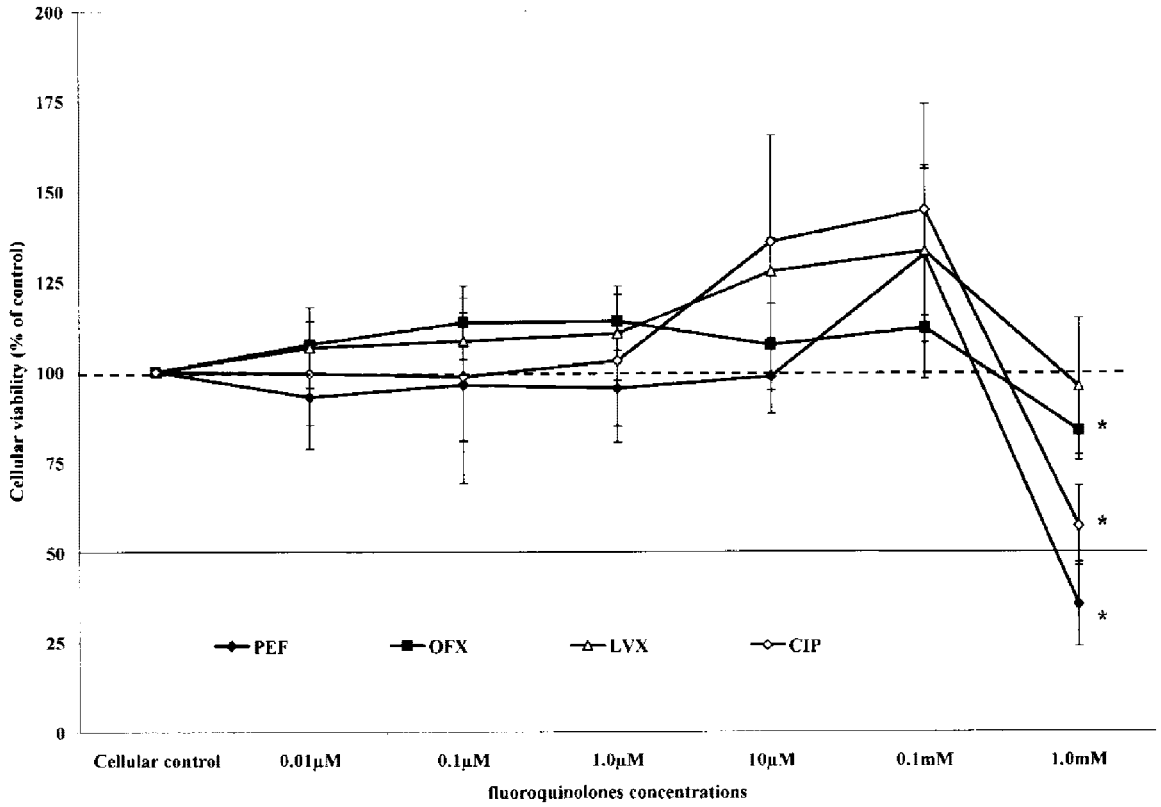

Fig. 4. Cell viability evaluated after 72 h of continuous exposure of cells to fluoroquinolones. Cell viability was evaluated with neutral red probe directly on living tendon cells. The specific fluorometric detection of neutral red was performed with cold light fluorometry (excitation 535 nm/emission 600 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate ($n = 12$) (*, $p < 0.05$).

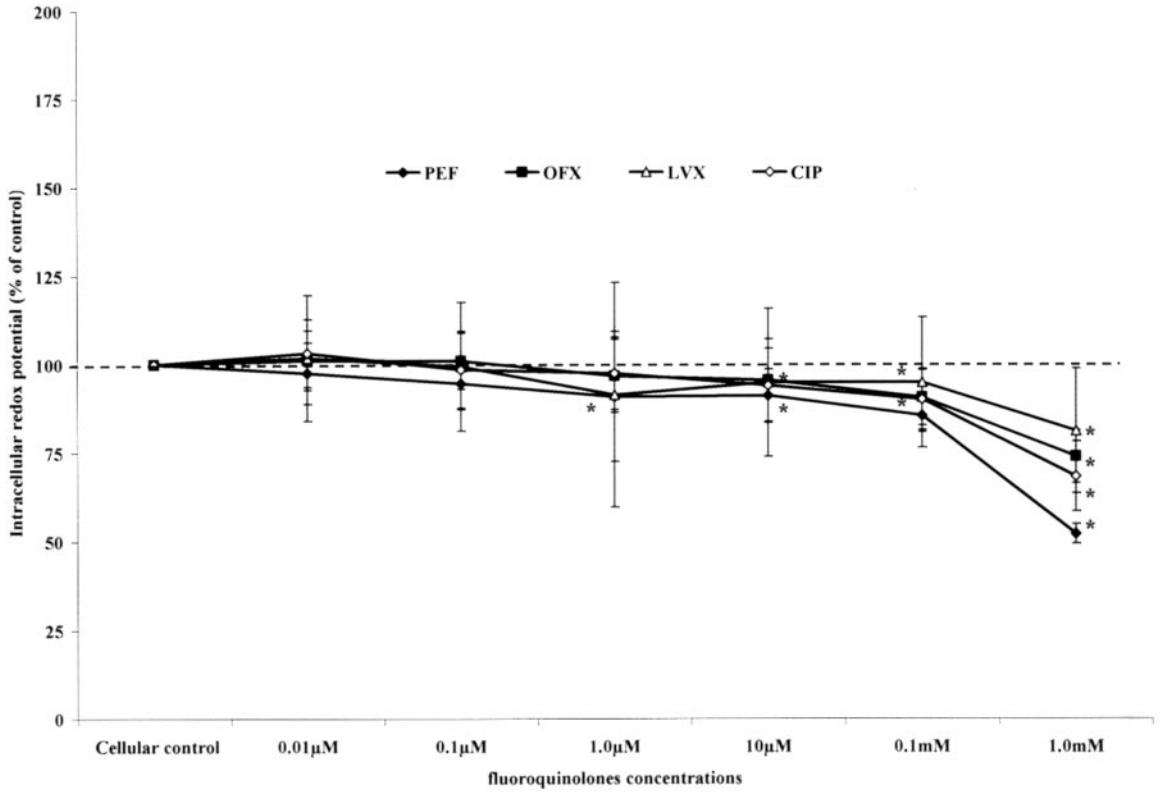

Fig. 5. Intracellular redox potential evaluated after 24 h of continuous exposure of cells to fluoroquinolones. Intracellular redox potential was evaluated with Alamar blue probe directly on living tendon cells. The specific fluorometric detection of Alamar blue was performed with cold light fluorometry (excitation 535 nm/emission 600 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate ($n = 12$) (*, $p < 0.05$).

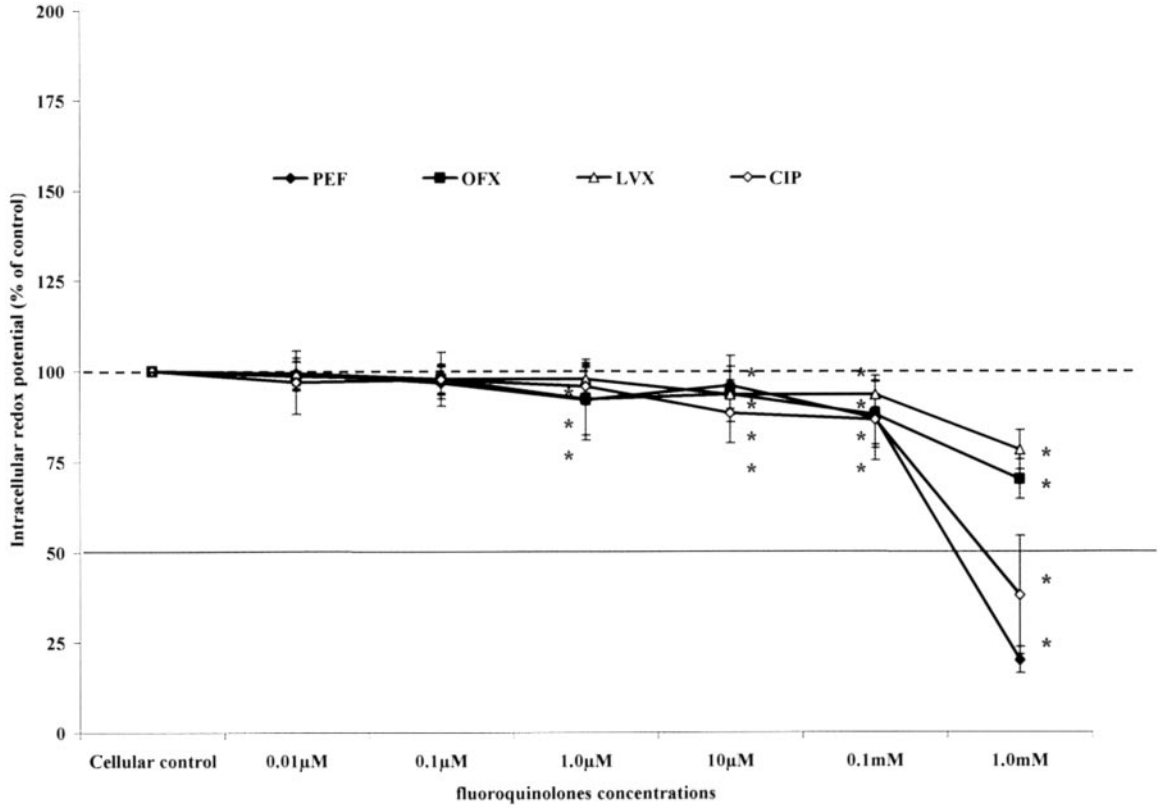

Fig. 6. Intracellular redox potential evaluated after 72 h of continuous exposure of cells to fluoroquinolones. Intracellular redox potential was evaluated with Alamar blue probe directly on living tendon cells. The specific fluorometric detection of Alamar blue was performed with cold light fluorometry (excitation 535 nm/emission 600 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate (n = 12) (*, $p < 0.05$).

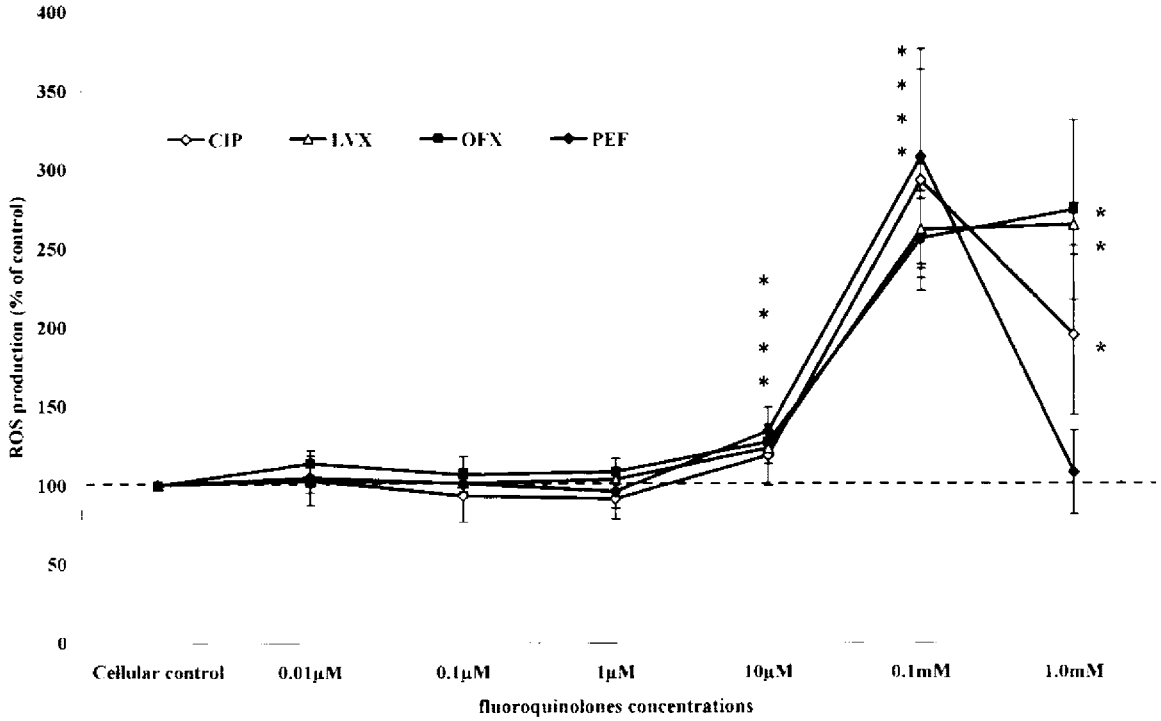

Fig. 7. Evaluation of the reactive oxygen species after continuous exposure of cells to pefloxacin, ciprofloxacin, ofloxacin, and levofloxacin (45 min). Reactive oxygen species were detected with fluorogen DCFH-DA probe. DCF fluorescence is proportional to reactive oxygen species production. The fluorometric detection of DCF was performed directly on living tendon cells with cold light fluorometry technology (excitation 485 nm/emission 535 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate (n = 12) (*, $p < 0.05$).

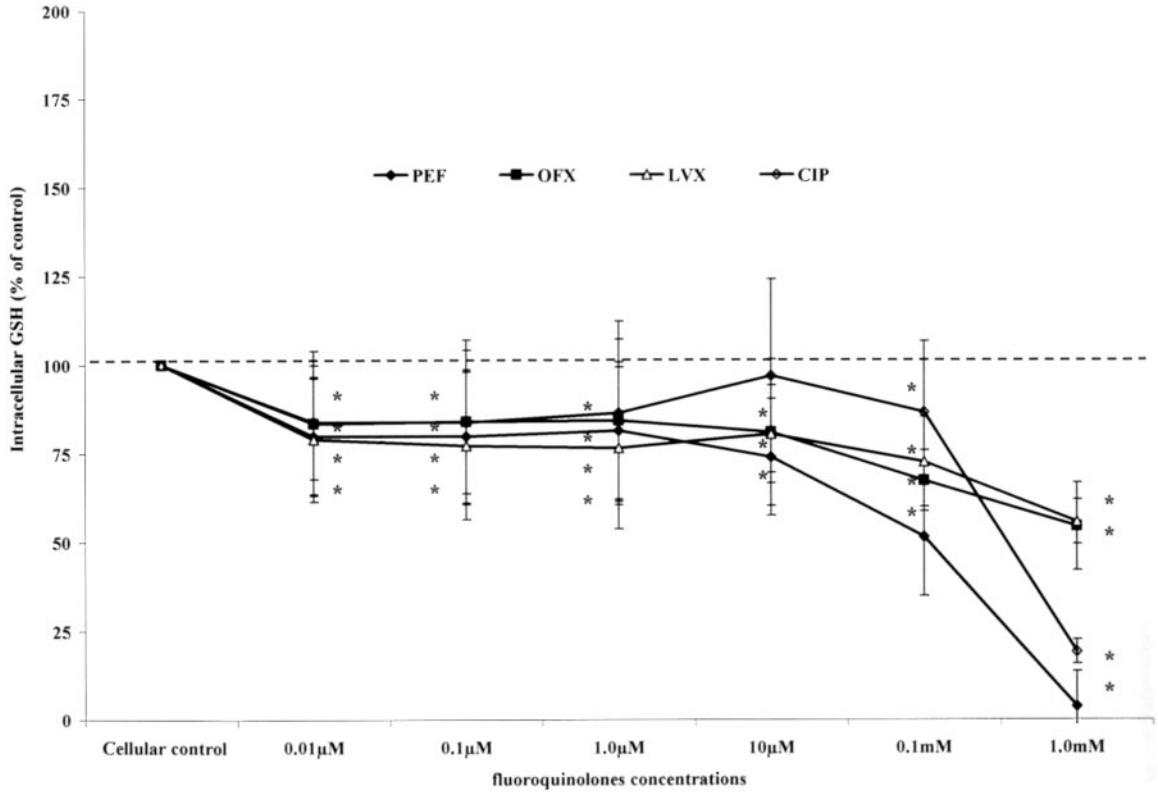

Fig. 8. Intracellular glutathione content evaluation after continuous exposure to fluoroquinolones (72 h). Glutathione content was evaluated with monobromobimane fluorogen probe without extraction or fixation. The fluorometric detection of the probe was performed directly on living tendon cells with cold light fluorometry (excitation 390 nm/emissiom 480 nm). Values are mean percentage of control ± S.D. from two independent experiments in sixplicate ($n = 12$) (*, $p < 0.05$).

nificant (*$p < 0.05$) decrease in glutathione content fell by 20.2, 20.2, 18.7, 26.1, 48.5, and 96.3% at 0.01 μM, 0.1 μM, 1 μM, 10 μM, 0.1 mM, and 1.0 mM, respectively. With CIP, glutathione content decreased by 16.3, 16.3, 13.7, 13.4, and 80.8% at concentrations of 0.01 μM, 0.1 μM, 1.0 μM, 0.1 mM, and 1.0 mM, respectively.

With OFX, the decrease in glutathione content was 16.6, 16.2, 15.8, 19.1, 32.7, and 45.5% with 0.01 μM, 0.1 μM, 1.0 μM, 10 μM, 0.1 mM, and 1.0 mM, respectively. With the same concentrations of LVX, glutathione content fell by 21.1, 22.9, 23.5, 19.6, 27.4, and 44.3%, respectively.

Discussion

Although in vivo (Kato et al., 1995; Kashida and Kato, 1997; Shakibaei and Stahlmann, 2001) and in vitro (Kempka et al., 1996; Bernard-Beaubois et al., 1998; Williams et al., 2000) studies have investigated the potential mechanisms underlying fluoroquinolone-induced tendinopathy, no reproducible model has been proposed to detect and predict intrinsic fluoroquinolone tendon toxicity. Furthermore, most studies were performed directly on animals using biochemical and histological methods that do not allow for an accurate evaluation of oxidative stress, a factor that has been implicated in the development of fluoroquinolone tendinopathy (Kato et al., 1995; Kashida and Kato, 1997; Simonin et al., 2000). One of the main objectives of our study was to avoid the artifacts commonly generated by monodispersion or extraction procedures. Microtitration cold light cytofluorometry (Rat, 1994; Rat et al., 1994, 1995) detects labile markers of oxidative pathways and subjects the cells to only minimal mechanical or enzymatic stress.

Cellular Model Adapted to Tenotoxicity Discrimination. The morphology of Teno cell line was very similar to that of the primary tenocytes. The elongated cells were arranged in long chains, a pattern commonly reported for normal tenocytes (Ippolito et al., 1980; Bernard-Beaubois et al., 1997). Moreover, the rabbit Teno cell line but not rabbit fibroblast cell line expressed collagen I mRNA and clearly responded to ascorbate, which is known to enhance collagen I production (Kao et al., 1976; Schwarz and Bissell, 1977).

In our study, we used the Teno cell line to compare the intrinsic tendon toxicity of four fluoroquinolones. We found marked differences, with PEF and CIP exhibiting the highest toxicity at the concentrations seen in articular tissues, compared with OFX and LVX.

Tenotoxicity and Tissue Diffusion of Fluoroquinolones

FQ Cytotoxicity on Living Tendon Cell. Viability and redox status assays (neutral red and Alamar blue tests) showed alterations in membrane integrity and redox status, respectively, with the four fluoroquinolones after 24 h of exposure. Moreover, a significant delayed toxicity (72 h) occurred with PEF and CIP, compared with OFX and LVX. To provide a basis for extrapolating these results to clinical practice, we took into account the pharmacokinetic properties of the four fluoroquinolones tested in our study (Montay et al., 1984; Gonzalez and Hendwood, 1989; Stahlmann et al., 1995; Chien et al., 1997; Zhanel and Noreddin, 2001) (Table 1).

The range of concentrations tested (0.01 μM–1.0 mM) in-

TABLE 1
Pharmacokinetic properties of the four fluoroquinolones tested in our study

Fluoroquinolones Single Oral Dose	Plasma C_{max}	Bioavailability
	μM	%
Pefloxacin, 500 mg	8.2–9.2	100
Levofloxacin, 500 mg	14	≥99
Ofloxacin, 400 mg to 800 mg	14–30	95
Ciprofloxacin, 500 mg	5.6–6.9	56–77

cluded the peak plasma concentrations seen in humans with the four fluoroquinolones tested (5.6–30 μM after a single oral dose of 500 mg) and the highest concentrations observed in articular tissue (Meissner et al., 1990; Yabe et al., 2001).

Recently, Yabe et al. (2001) reported that intraarticular OFX concentrations in juvenile dogs were approximately 1.8 to 2.0 times higher than the serum concentration 2 h after dosing. Similarly, Meissner et al. (1990) reported that half-life of OFX was longer in synovial tissues than in serum. OFX, PEF, and LVX diffuse more easily in tissues than does CIP, a fact that may contribute to the differences in tendon toxicity seen in clinical practice (Van Der Linden et al., 2001). Thus, comparison of these studies permits to conclude that FQ seems to penetrate well in articular tissues to an extent concentration higher than in serum. From the pharmacokinetics data, it can be deduced that OFX, PEF, and LVX are more diffusible than CIP and may explain that OFX, PEF, and LVX are more tenotoxic than CIP, as reported in clinic (Van Der Linden et al., 2001). The high intrinsic tenotoxicity of CIP observed in our study is limited in clinic due to low tissue diffusion. Moreover, up to now, it was difficult to classify LVX due to the lack of clinical observations. Our in vitro model permits to hypothesize that LVX has the same tenotoxicity in clinic as OFX. This hypothesis was suggested because of similar intrinsic tenotoxicity and similar tissular diffusion of OFX and LVX.

Fluoroquinolone-Induced Oxidative Stress and Glutathione Depletion. Because we were able to evaluate ROS production induced by direct fluoroquinolone exposure of living adherent tendon cells, our study provides the first strong evidence implicating ROS in fluoroquinolone tendon toxicity. Our methodological approach provides reliable and rapid detection of transient and labile markers such as ROS. In our study, all tested fluoroquinolones stimulated the ROS production, either at all tested concentrations or at the higher concentrations. After cellular exposition to high concentrations of PEF and CIP, a decrease in oxidative stress expression was observed probably due to highly mitochondrial alteration. A likely hypothesis is that the glutathione content falls rapidly so that intracellular antioxidant enzymes lose their ability to modulate the overproduction of ROS. Although intrinsic tendon toxicity is a class effect of fluoroquinolones, cell viability and glutathione content depletion distinguished two levels of toxicity: PEF and CIP were the most toxic, showing the most toxic effect on tendon cells, whereas OFX and LVX were moderately cytotoxic.

The present study strongly supports a role for oxidative stress among the mechanisms involved in fluoroquinolones-induced tendon toxicity, in keeping with earlier findings by Simonin et al. (2000). This hypothesis is in agreement with putative mechanisms of chondrotoxicity (Hayem et al., 1994, 1996; Ratinaud and Valet, 1994; Thuong-Guyot et al., 1994; Simonin et al., 2000). Hayem et al. (1994) documented mitochondrial alterations due to ROS overproduction in rabbit articular chondrocytes exposed to fluoroquinolones. In addition, both neutral red testing to evaluate cell viability/membrane integrity and Alamar blue assays to assess redox status provided evidence of cell membrane damage associate to necrosis. Cell death mechanism is directly dependent of fluoroquinolone concentrations. At high concentrations, we observed necrosis phenomenon. With low concentrations, an apoptosis mechanism could be suggested (chromatin condensation with DNA probe; data not shown). Of course, additional study should be realized to confirmed cell death mechanism.

ROS play multiple roles in health and disease. They have a direct toxic effect on cells and cellular components, serve as important messenger molecules in the induction of several genes, and act as second messengers in inflammatory processes. A Causal relationship has been demonstrated between intracellular ROS and induction of matrix metalloproteinases (Brenneissen and Puerner, 1997).

These data support the possibility that fluoroquinolone-induced ROS overproduction may activate matrix metalloproteinases, thereby contributing to damage the tendon microstructure. As suggested by Corps et al. (2002), matrix metalloproteinases expression can be selectively activated by fluoroquinolones in tendon cells. Another study (Williams et al., 2000) showed that fluoroquinolones increased proteolytic activity. This indirect enhancement of matrix metalloproteinases by fluoroquinolones might be ascribable to increased ROS production. The direct toxic effect of ROS on tendon cells and the indirect toxic effect mediated by enhancement of matrix metalloproteinases might act in synergy to cause fluoroquinolone-related tendinopathy.

It is the first time that oxidative stress mechanism was demonstrated in tenotoxicity induced by fluoroquinolones. Of course, additional in vitro study will be required to confirm the role played by ROS in the tenotoxicity of fluoroquinolones and to define whether ROS production was a limiting factor or just a component of the tenotoxicity.

Discrimination of Fluoroquinolone Intrinsic Tenotoxicity. Intrinsic toxicity of fluoroquinolones may not be the only mechanism underlying tendon rupture in patients treated with fluoroquinolones. The tissular diffusion of each fluoroquinolones, individual susceptibility, and other predisposing factors such as age, diet, end-stage renal failure, and corticotherapy should also be taken into account (Hayem and Carbon, 1995; Zabraniecki et al., 1996; Der Linden et al., 2001). This study demonstrates that intrinsic tendon toxicity varies across fluoroquinolones. After 72 h of exposure, we found evidence of marked tendon cytotoxicity with PEF and CIP and moderate tendon cytotoxicity with OFX and LVX. Our tendon cell model permit a reliable discrimination of fluoroquinolones intrinsic tenotoxicity using cellular markers such as membrane integrity, redox status, and reduce glutathione content.

In conclusion, the present study indicates involvement of oxidative stress in fluoroquinolone tendon toxicity and provides new insights into the underlying mechanisms. Our model using a tendon cell line and a microtitration technology based on specific fluorescent probes seems reliable for the

toxicological screening of new fluoroquinolones, or other drugs believed to exhibit tendon toxicity.

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