Toxic Effects of Levofloxacin on Rat Annulus Fibrosus Cells: An *In-vitro* Study

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Statistical Analysis **C**
Data Interpretation **D**
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Background:
Fluoroquinolones are in wide clinical use as safe and effective antibiotics. Articular cartilage, tendons, and epiphyseal growth plates have been recognized as targets of fluoroquinolone-induced connective tissue toxicity. However, the effects of fluoroquinolones on annulus fibrosus (AF) cells are still unknown.

Material/Methods:
The main objective of this study was to investigate the effects of levofloxacin, a typical fluoroquinolone antibiotic drug, on rat AF cells *in vitro*. Rat annulus fibrosus (RAF) cells were treated with levofloxacin at different concentrations (0, 10, 20, 30, 40, 60, 80, and 90 μg/ml) and were assessed to determine the possible cytotoxic effects of levofloxacin. Inverted phase-contrast microscopy was used to accomplish the morphological observation of apoptosis of treated cells. Western blot and real-time quantitative RT-PCR (qPCR) was used to explore the expression of active caspase-3 and MMP-3. Flow cytometry was used to measure the apoptotic incidences.

Results:
Our study showed that levofloxacin, with concentrations at 30, 60, and 90 μg/ml, induced dose-dependent RAF cell apoptosis and higher expression of caspase-3 and MMP-3. More apoptotic cells were observed by inverted phase-contrast microscopy. Moreover, levofloxacin increased the activity of caspase-3, and it also reduced cell viability with different concentrations ranging from 10 to 80 μg/ml.

Conclusions:
Our study results suggest that levofloxacin has cytotoxic effects on RAF cells, characterized by enhancing apoptosis and reducing cell viability, and indicate a potential toxic effect of fluoroquinolones on RAF cells.

MeSH Keywords:
Apoptosis • Caspase 3 • Cell Survival • Levofloxacin

Abbreviations:
RAF – rat annulus fibrosus; **DMEM** – Dulbecco's modified Eagle's medium; **FITC** – fluorescein isothiocyanate; **PI** – propidium iodide; **PBS** – phosphate-buffered saline; **IVD** – intervertebral disc; **ECM** – extracellular matrix; **ACL** – anterior cruciate ligament

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Background

The fluoroquinolone class of antibiotics (e.g., levofloxacin, ciprofloxacin, and moxifloxacin) is becoming popular in the treatment of a wide range of infections due to its good tissue penetration, as reflected by a high volume of distribution [1]. However, recent studies reported that quinolones had adverse effects on the musculoskeletal system, such as myalgia, Achilles tendon rupture, and arthralgia [2,3]. Furthermore, fluoroquinolone-induced connective tissue toxicities were not rare in basic and clinical studies. Articular cartilages, epiphyseal growth plates, and tendons are the major targets for injury [1,4–6]. Sode's research indicated that quinolone use might triple the risk of Achilles tendon rupture [7]. Recent studies showed that levofloxacin could trigger cytotoxic effects on anterior cruciate ligament and fibroblast-like synoviocytes of rabbits [2,8,9]. The mechanism of toxic effects of fluoroquinolones on tendon cells *in vitro* has been well defined [1]. The specific alterations include: enhancing matrix metalloproteinase (MMPs) expression [1,3,10], decreasing expression of some extracellular matrix (ECM) proteins [1,3,11], inhibiting tendon cell proliferation [12,13], and degenerating the ultrastructure [14]. Several studies demonstrated that the alteration of synthesis or the degradation of ECM components plays a prominent role in fluoroquinolone-induced tendon damage. Changes in the expression of tendon matrix components, such as collagen and proteoglycans, have also been previously reported in response to various fluoroquinolones [11,13]. In cultured human tenocytes, the fluoroquinolone ciprofloxacin induces the mRNA of MMP-1, 3, and 13 to higher expression [10,15]. Furthermore, levofloxacin and ciprofloxacin cause a time- and concentration-dependent increase in the apoptosis marker caspase-3 in cultured human tenocytes [1,16]. Previous studies indicated that apoptosis of tenocytes is involved in the pathogenesis of tendon destruction [17]. Ofloxacin was found to reduce the collagen type level by 40% in murine cartilage organoid culture in a dose-dependent manner, while the collagen type remains constant [18]. Another study indicated that the combined administration of fluoroquinolones and glucocorticoids can result in the occurrence of tendon rupture [19]. Programmed cell death has to be considered as a crucial event in the pathogenesis of fluoroquinolone-induced tendinopathies [1].

Disorders associated with intervertebral disc (IVD) degeneration are the leading causes of morbidity or life quality deterioration in the elderly [20]. Therefore, the toxic effect of levofloxacin on AF should not been ignored. The AF is a ligament-like tissue. AF and ligament are composed of fibroblasts that produce large amounts of ECM, resulting in a dense cellular structure. However, the toxic effect of levofloxacin on AF has rarely been documented.

As a kind of fluoroquinolone, levofloxacin was ranked as being one of the 3 most frequently used fluoroquinolones in the United States in 2008 [21]. Nevertheless, levofloxacin can induce tendonitis and tendon rupture [22,23]. In this study, we investigated the effects of various doses of levofloxacin on AF cells derived from rat lumbar intervertebral discs. We hope our results contribute to a new assessment of clinical use of levofloxacin in light of its adverse effect on IVD.

Material and Methods

Isolation and culture of RAF cells

SD rats (about 200 g) were obtained from the Laboratory Animal Center of Hebei Medical University (Shijiazhuang, Hebei, China). All procedures were in accordance with the guidelines of the Ethics and Research Committee of Hebei Medical University. Rats were put to death by over-dose anesthesia. L1 to L6 lumber spine was removed en bloc under aseptic conditions. Five lumbar discs (L1–L2, L2–L3, L3–L4, L4–L5, and L5–L6) were dissected from their upper and lower vertebras and were put in a petri dish containing Hank's liquid. Attachments such as the nucleus pulposus (NP) and the surrounding ligament and muscle were also removed to ensure the identity of the RAF tissue. RAF tissues obtained from the same animal were pooled together and washed with Hank's balanced salt solution (HBSS, Gibco), then cut into small pieces (<1 mm^3). Minced RAF tissues were agitated at 37°C for 60 min in an enzyme solution of 0.25% collagenase type II (Sigma Chemical Co., St. Louis, MD). The supernatant liquid was discarded after centrifugation for 5 min and then digested with 0.2% trypsin (Sigma Chemical Co., St. Louis, MD) at 37°C for 10 min. Then it was centrifuged again and supernatant was discarded. Recovered cells were cultured (10^6 cells/flask) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in an incubator under 5% CO_2. The medium was changed every 3 days. After cells reached confluence, they were trypsinized (0.2% trypsin and 0.02% EDTA). RAF cells of the first passage and the second passage were used to perform this experiment. The second-passage cells were seeded at 10^5/well into 6-well plates and then incubated for 2 days. They were washed with PBS and then cultured in 2 ml of medium containing levofloxacin 10, 20, 40, and 80 μg/ml for cell viability and caspase-3 activity assay and 30, 60, and 90 μg/ml for flow cytometry. The controls underwent an identical treatment except for the addition of levofloxacin. RAF cells were collected after 24 h for flow cytometry, and after 48 h for cell viability and caspase-3 activity testing.

Morphological observation

With various concentrations of levofloxacin (30, 60, and 90 μg/ml), the cells were incubated for 24 h. The cultured cells were observed by inverted phase-contrast microscopy (Olympus, Japan) and photographed with a digital camera (Nikon, Japan).

Table 1. Sequence of primers used for qPCR analysis.

No.	Gene	Primer sequence
1	Caspase-3	5'GCTCGCCAATGGTACCGATGT3' (sense)
		5'TTCACGAGTAAGGTCATTTT3' (antisense)
2	MMP-3	5'ACATGGAGACTTTGTCCCTTTTG3' (sense)
		5'TTGGCTGAGTGGTAGAGTCCC3' (antisense)
		3'GCTTACATGTCTCGATCCCACTTAA3' (sense)
		5'CTCGCGCTACTCTCTCTTTCTGG3' (antisense)

Annexin V-FITC/PI staining

The Annexin V-FITC/PI apoptosis detection kit (BD Pharmingen, USA) was used to detect apoptotic cells. The detection process was performed according to the manufacturer's protocol. AF cells were treated with 30, 60, and 90 μg/ml of levofloxacin. After 24 h, the cells were washed with PBS and collected by trypsinization. The cells were washed twice with PBS after centrifugation. From the cell suspension, 1×10^6 cells were re-suspended in 5 ml 1× binding buffer. These cells were incubated for 15 min in the dark along with 5 μl of Annexin V-FITC and 5 μl of PI solution. The samples were analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA). The Annexin V-FITC-/PI- Population was used to reflect the live cells, early apoptotic (FITC+/PI-), and necrotic (FITC+/PI+) cells.

Caspase-3 activity assay

The first-passage AF cells were placed in 6-well plates at 2×10^5 cells per well and controls underwent an identical treatment except for the addition of levofloxacin. Caspase-3 activity was detected using a Caspase-3 activity kit (Beyotime, China), which is based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product, p-nitroaniline (pNA). According to the manufacturer's protocol, treated cells were lysed with lysis buffer (100 μl per 2×10^6 cells) for 15 min on ice and then washed with cold HBSS. After incubating the mixture composed of 10 μl of cell lysate, 80 μl of reaction buffer and 10 μl of 2 mM caspase-3 substrate in 96-well microtiter plates at 37°C for 4 h, caspase-3 activity was quantified in the samples with a microplate spectrophotometer (Biotek) at an absorbance of 405 nm. Caspase-3 activity was expressed as the fold change of enzyme activity compared to that of synchronized cells.

MTS assay

RAF cells (1×10^4) were treated with 0, 10, 20, 40, and 80 μg/ml levofloxacin for 48 h and then seeded into 96-well plates. Cell survival was determined by MTS assay. MTS/PES (Promega, Beijing, China) was added and incubated at 37°C for 4 h. The optical density was measured at 490 nm with a microplate reader (Shimadzu, Kyoto, Japan) and cell viability was normalized as a percentage of control.

Western blot

Active caspase-3 and MMP-3 was detected by Western blot with β-actin as an internal control. Cells were treated with various concentrations (30, 60, and 90 μg/ml) for 24 h, and then we collected the cells. This was accomplished by performing the following steps: (1) Completely lyse each sample by cell lysis buffer, then centrifuge them at 2000 r/min for 10 min. (2) Gather 100 μl of supernatant and add 20 μl lauryl sodium sulfate buffer (6×), then degeneration by boiling water bath for 10 min. (3) Separate protein by electrophoretic with 10% SDS-polyacrylamide gelatin, and then transfer samples to nitrocellulose membrane and confine for 1 h. (4) Add primary antibody of caspase-3 (Bioworld, diluted to 1: 500) and β-actin (Proteintech, diluted to 1:2000) at 37°C for an hour. (5) Second antibody (anti-rabbit IgG) marked with horse radish peroxidase (HRP) was added, at 37°C for 1 h. (6) Scan and analyze the result with digital gel imaging (Chemi Imager 4000, Alpha Innotech US).

Real-time quantitative RT-PCR (q-PCR)

We performed q-PCR to detect expression level of mRNA encoding caspase-3 and MMP-3. Trizol method (GibcoBRL) was used to isolate total RNA according to the manufacturer's instructions. Total RNA was measured fluorometrically using the CyQuant-Cell Proliferation Assay Kit (Molecular Probes). cDNA synthesis was performed using the ThermoScript@RT-PCR System (Invitrogen, Carlsbad, CA, USA). For semi-quantification of the genes of interest, we utilized the DyNAmo SYBR Gren 2-step qRT-PCR Kit (Finnzymes) in a total volume of 20 μL, performing real-time PCR reaction in an Mx300P cycler. Amplicons of caspase-3 and MMP-3 were amplified with primers listed in Table 1. Standard curves were run in each optimized assay, which produced a linear plot of threshold cycle (Ct) against log
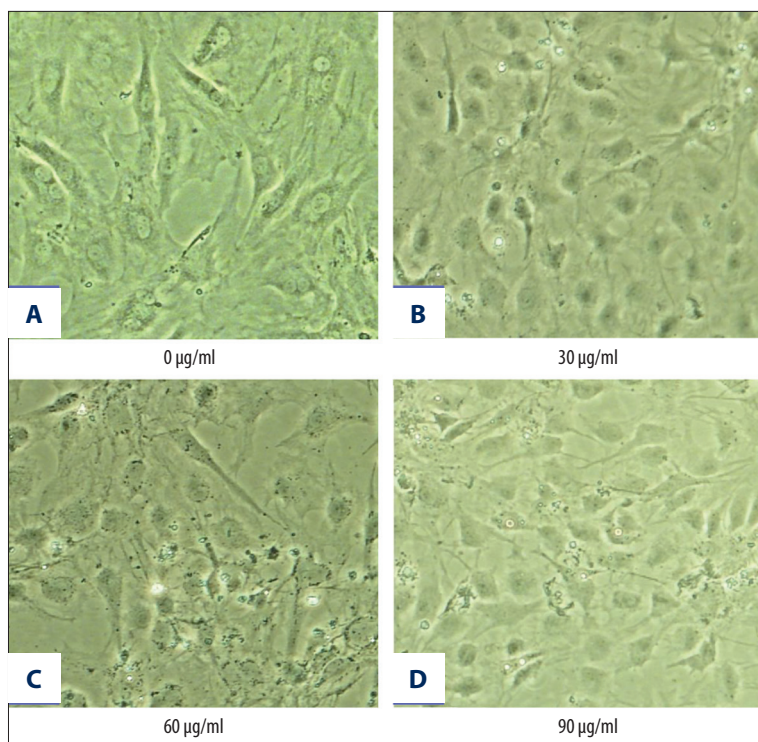
Figure 1. Morphological observation. Cells in control group grew well, with fusiform or polygonal shapes. When treated with levofloxacin at different concentrations (30, 60, and 90 µg/ml), cells presented shrinkage phenomenon and more vacuoles at different degrees were found in a concentration-depended manner.
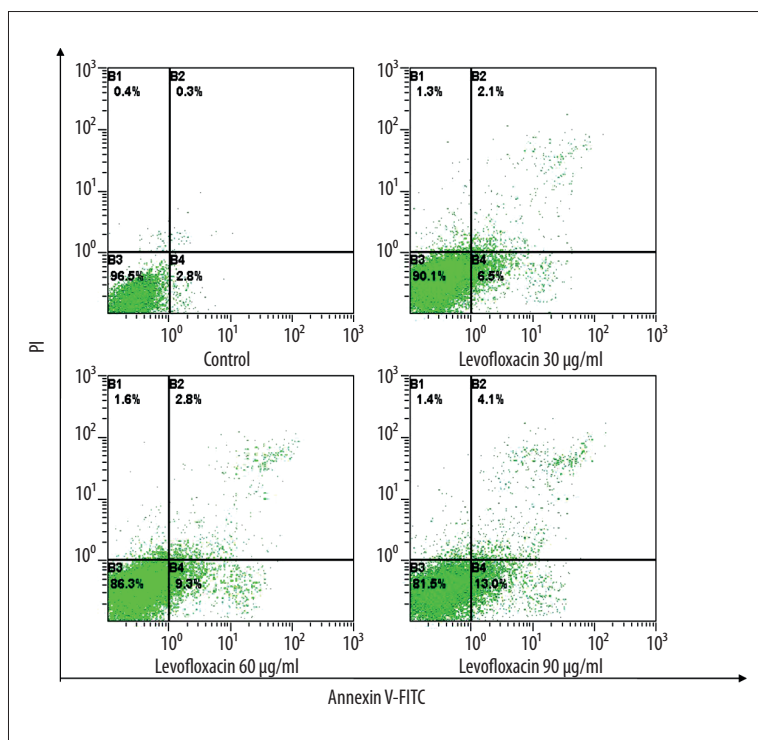

Figure 2. Annexin V-FITC/PI staining assay. RAF cells were treated with levofloxacin at concentrations of 30, 60, and 90 µg/ml for 24 h (n=5 samples per group). The percentage of the cell forms were calculated using Cell Quest Pro (mean values were given; the experiment was performed 3 times). In each plot, the indication for each quadrant is shown as follows: lower left: viable cells; lower right: early apoptotic cells; upper right: necrotic cells. FITC: fluorescein isothiocyanate, PI: propidium iodide.

(dilution). The amount of target was quantified based on the concentration of the standard curve and is presented as relative Ct value. The quantity of target was normalized against the quantity of β-actin.

Statistic al analysis

All statistic analyses were completed using SPSS 13.0 for windows. Each assay was repeated at least 3 times independently to acquire reliable quantitative data. Mean ± standard
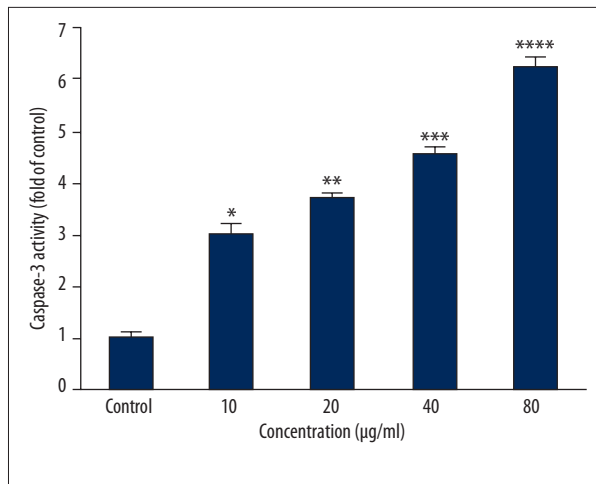
Figure 3. Active caspase-3 activity assay. RAF cells were treated with 10–80 μg/ml levofloxacin for 48 h. The results are expressed as fold of control. Bars present the Mean ±S.D. (n=6 samples per group). * P<0.05, compared to the control values.
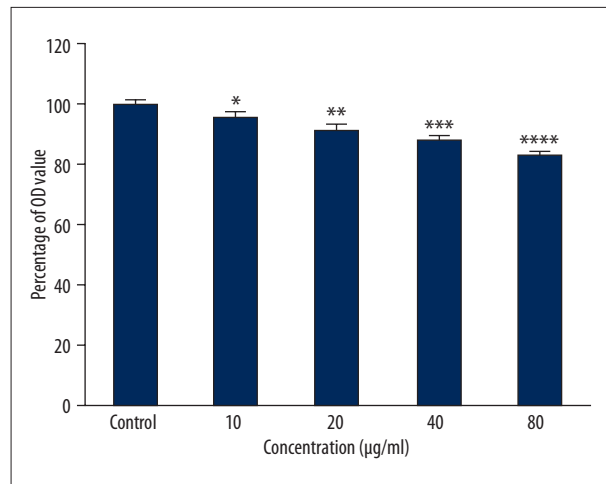

Figure 4. Cell viability assay. RAF cells were treated with 10–80 μg/ml levofloxacin for 48 h, and their viability was determined by MTS assay. The results are expressed as a percentage of OD value of control. Bars show the Mean ±S.D. (n=6 samples per group). * P<0.05, compared to the control values.

deviation (SD) is used to express the result. One-way ANOVA method was used to compare different groups, accompanied by Dunnett t test as a post hoc test. P<0.05 was considered statistically significant.

Results

Morphological observation

As is shown in Figure 1, the cultured cells without levofloxacin grew well, and their shapes were fusiform or polygonal. However, the cells treated with levofloxacin at different concentrations (30, 60, and 90 μg/ml) presented shrinkage phenomenon and typical degenerative changes, such as multiple vacuoles in the cytoplasm.

Annexin V-FITC/PI staining assay

Levofloxacin increased the percentage of early apoptotic cells in a concentration-dependent manner, as is shown in Figure 2. After cells were treated with levofloxacin at a concentration of 30, 60, or 90 μg/ml for 24 h, the apoptotic rate of RAF cells was 6.38%, 9.20%, and 13.06%. The data indicated that levofloxacin induced apoptosis of RAF cells in a dose-dependent manner.

Caspase-3 activity assay

As is shown in Figure 3, caspase-3 activity, a marker of apoptosis, was significantly increased after RAF cells were treated with levofloxacin (10 to 80 μg/ml) for 48 h in a concentration-dependent manner. This result provides further evidence
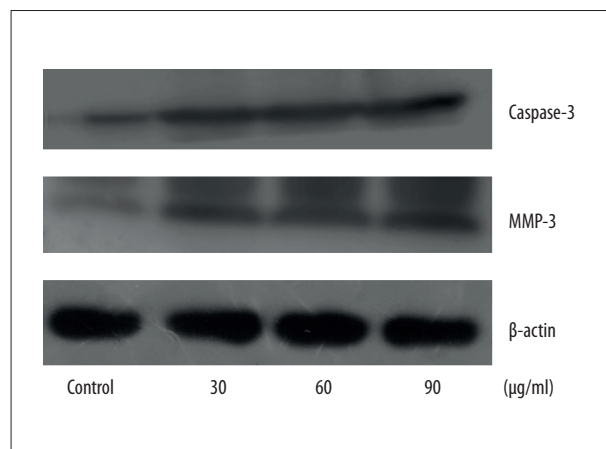

Figure 5. Protein level of caspase-3 and MMP-3 by Western blot. The result shows the expression of caspase-3 and MMP-3 at different concentrations of levofloxacin (30, 60, and 90 μg/ml). The indicated proteins were detected by Western blot with β-actin as an internal control.

that levofloxacin could induce the apoptosis of RAF cells by enhancing the activity of active caspase-3.

MTS assay

Levofloxacin treatment decreased RAF cell viability in a concentration-dependent manner, as displayed in Figure 4 (P<0.05). Compared with the control group, the cell viability of different groups decreased to 95.8%, 91.5%, 88.2%, and 83.2% at concentration of 10, 20, 40, and 80 μg/ml respectively.
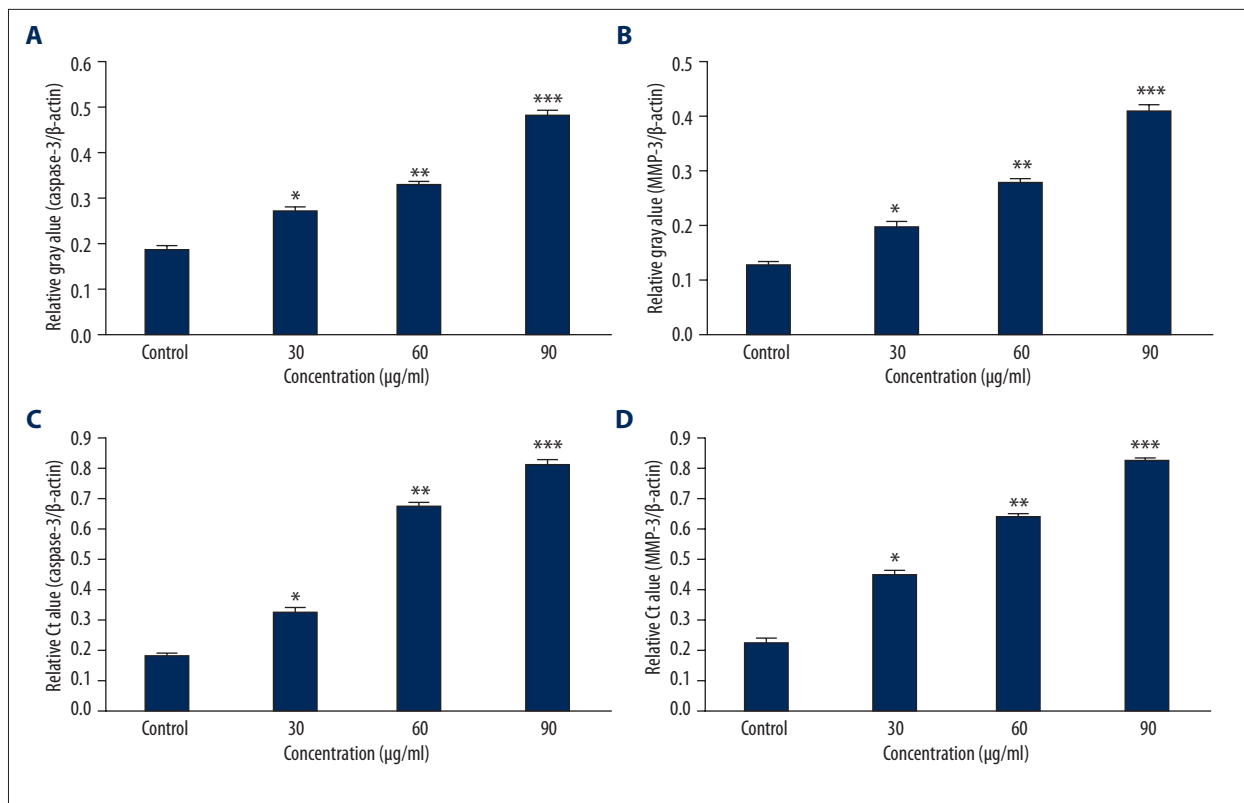
Figure 6. (**A, B**). Protein level of caspase-3 and MMP-3 by Western blot. The results show that the expression of caspase-3 and MMP-3 of the control group was low, but after treatment with levofloxacin, the expression of caspase-3 and MMP-3 was higher. A positive correlation was found between the expression of caspase-3, MMP-3, and the concentration of levofloxacin (Mean ±SD; n=5, p<0.05 compared to control). (**C, D**) The expression level of caspase-3 and MMP-3 detected by qPCR. The mRNA level was determined by real-time quantitative R-T RNA. Four group of RAF cells were stimulated by different concentrations of levofloxacin, 0 μg/ml (as control group), 30 μg/ml, 60 μg/ml, and 90 μg/ml, cultured for 24 h. Levofloxacin significantly increased the mRNA expression of caspase-3 and MMP-3 in a concentration-dependent manner (Mean ±SD; n=5, p<0.05 compared to control).

Western blot and qPCR

To investigate active caspase-3 and MMP-3, we performed Western blot testing and qPCR on RAF cells treated with levofloxacin 30, 60, and 90 μg/ml for 24 h. As shown in Figures 5 and 6, levofloxacin significantly increased the expression of active caspase-3 and MMP-3 in a concentration-dependent manner (p<0.05).

Discussion

In previous studies research on the adverse effects of quinolones were mainly focused on tendon, cartilage, and ligament, and they all underwent mechanical stress force directly. In this study, we investigated the effect of levofloxacin, one of the quinolones, on RAF cells. Annulus fibrosus is a ligament-like tissue, connecting 2 adjacent vertebrae. IVD degeneration is an age-associated and multi-factorial process. The degenerative process can be asymptomatic, but sometimes it also may

lead to acute or chronic back pain, which often ultimately is treated surgically, with varying results in terms of effectiveness and patient satisfaction [24–26]. During human IVD degeneration, a continuous increase of cell death has been recognized for many years [27–29].

Previous studies showed that when combined with IL-1β, ciprofloxacin could increase the expression of MMPs in human tendon cells after being incubated with ciprofloxacin at a concentration of 150 μM (50 μg/ml) for 48 h, but in the absence of IL-1β, ciprofloxacin caused no significant effects on the expression of certain MMPs, even at a high concentration [10,15]. In another study, levofloxacin was found to significantly increase the mRNA expression of MMP-3 and MMP-13 in a concentration-dependent manner, even at the low concentration of 14 μM without IL-1β [8], which was in line with the result in our study that levofloxacin showed similar cytotoxicity on RAF cells. These above-mentioned results seem to indicate that ACL (anterior cruciate ligament) cells and AF cells are more susceptible to cytotoxicity by fluoroquinolones than

tendon cells or that levofloxacin is more cytotoxic than ciprofloxacin. Several other studies have also shown that ofloxacin can induce apoptosis of chondrocytes [30]. Recently Yang et al. showed that levofloxacin could induce apoptosis of rat nucleus pulposus cells via Bax/Bcl-2/caspase-3 pathway, which was reversed by 17β-estradiol through upregulating integrin α2β1 [31,32]. In addition, (lev)ofloxacin-induced apoptosis was observed in osteoclast precursor cells and hepatocarcinoma cells [33,34]. These findings indicate that apoptosis may be a common adverse effect of levofloxacin on tissues and cells.

Apoptosis is a common event in many physiological processes, like histogenesis during prenatal development, and it also protects the body against many diseases such as cancer [35]. A study by Jia-xuan Qiu et al. showed that plumbagin could induce the apoptosis of human tongue carcinoma cells through the mitochondria pathway [36]. Some kinds of apoptosis could be inhibited. Recent research demonstrated that glucagon-like peptide-1 (GLP-1) could protect vascular endothelial cells against advanced glycation end-products (AGEs)-induced apoptosis [37]. However, uncontrolled apoptosis may be pathogenic and lead to tissue structure damage. In this study we investigate the effect of levofloxacin on RAF cells. When the concentration rang was 30 to 90 μg/ml and the cells treated for 24 h, the levofloxacin increased the apoptosis, and at concentrations of 10 to 80 μg/ml for 48 h significantly increase the caspase-3 activity in a concentration-dependent manner and decrease cell viability. Moreover, in our study levofloxacin was used on cell monolayers at concentrations of 10–90 μg/ml, which were approximated to those reached in patient plasma during therapy with levofloxacin [38]. AF is a ligament-like tissue outside intervertebral disc, and it has many functions, such as maintaining nucleus pulposus colloid composition and keeping the position and shape of nucleus pulposus to ensure the entire disc load and its role in bearing. The biological performance of the AF is weak. Intervertebral disc degeneration may cause many clinical diseases, such as lumbar intervertebral disc herniation (LDH), instability of the lumber spine, and lumbar scoliosis.

Conclusions

In summary, we investigated levofloxacin-induced apoptosis in RAF cells for the first time in this study. Programmed cell death possibly plays an essential role in the pathogenesis of levofloxacin-induced cytotoxicity on RAF cells. The cytotoxicity promotes apoptosis, reduction of cell viability, and increased activity and expression of caspase-3 and MMP-3. These findings suggest a potential adverse effect of levofloxacin on AF, and the possibility that levofloxacin causes lumber diseases should not be ignored. In addition, some problems should be highlighted, for instance, whether other fluoroquinolones have similar toxic effects on RAF cells and whether levofloxacin has similar toxic effects on other tissues, such as posterior longitudinal ligament. The mechanism of the toxic effect of fluoroquinolones on cells derived from dense connective tissues of the locomotor system, such as tendons or ligaments, are targets deserving further study. The effect of fluoroquinolones on cells and tissues *in vivo* is also a problem that remains to be investigated. More explorations of its clinical significance are needed in the future.

Because it was limited by the available technology and equipment, this work inevitably has some deficiencies. Further studies should be done in the future. For instance, we can continue to explore the relationship between apoptosis and time. We can also explore the mechanism of the toxic effect of fluoroquinolones and the mechanism of signal transduction. Electron microscopy can be used to observe the changes of ultrastructure of the treated cells. The effect of fluoroquinolones on cells and tissues *in vivo* is worth exploring as well. We hope our study can contribute to further research on the cytotoxicity of fluoroquinolones.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. The authors indicated no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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