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Research Article

Decreased Collagen Xii Expression and Increased Reactive Oxygen Species Production in Levofloxacin-Treated Tendon

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Abstract

Background: Levofloxacin (LVFX) is widely used for many respiratory, urinary, and oral infections. Although rare, tendinopathy and tendon rupture have been reported in patients treated with LVFX as adverse effect. However, the exact mechanism is not fully elucidated. In this study, we investigated the effects of LVFX on tendon cells and tendon tissue. *Method:* Murine tendon cell line TT-D6 cells were treated with LVFX. Total RNA was extracted from the treated cells and quantitative reverse-transcription polymerase chain reaction (RT-PCR). LVFX-treated TT-D6 cells were subjected to cell proliferation assays and reactive oxygen species production assays. In addition, LVFX was administered to rats, and total RNA was extracted from tendon tissue and quantitatively analyzed for mRNA expression using quantitative RT-PCR. *Results:* Proliferative capacity in TT-D6 cells treated with various concentrations of LVFX showed no significant differences in any of the group comparisons. Quantitative RT-PCR analysis in TT-D6 cells showed that collagen 12a1 (COL12A1) expression was significantly decreased in the LVFX-treated group compared with the control group. The expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 was significantly increased in the LVFX group. On the other hand, there were no significant differences in mRNA expression of decorin, matrix metalloproteinase-9, and Collagen1a1. ROS production was significantly upregulated in LVFX-treated rats, and COL12A1 expression was significantly decreased in LVFX-treated rats compared with controls in tendons collected from LVFX-treated rat models. *Conclusions:* Taken together, COL12A1 reduction may be involved in tendon injury and tendon rupture in LVFX administration, suggesting that increased ROS production may be involved.

Keywords

Levofloxacin, Adverse Effect, Tendon, Type XII Collagen, Reactive Oxygen Species

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1. Introduction

Levofloxacin (LVFX) belongs to the fluoroquinolones (FQs) class of antibiotics that directly inhibits bacterial DNA synthesis. LVFX is widely used for respiratory tract infections, urinary tract infections, and skin infections, and is also frequently used in the oral and maxillofacial region due to its broad antibacterial spectrum, excellent antibacterial activity, and safety [1, 2]. While seizures, arrhythmia, and hypoglycemia have been reported as major adverse effects of FQs, tendon disorders such as tendon rupture have been reported to occur as a rare adverse effect of FQs [3]. FQs-related tendonitis and tendon ruptures tend to occur within one month, whereas LVFX-induced tendon ruptures having an onset time of three days [3].

Tendons, which exist between bone and muscle as anatomically non-contractile elements, are divided into attachment and intermediate parts. The middle part is composed of collagen, proteoglycans, and glycoproteins, with collagen type I making up the majority, but collagen types III, IV, V, VI, XII, and XIV are also included [4]. Expression of type XII collagen found in the search for collagen sequences from chick tendon fibroblasts is unevenly distributed in mesenchymal tissues containing type I collagen during embryonic development, and its distribution is located in tendon, bone, and periodontal tissues in a restricted manner postnatally [5-7]. Type XII collagen forms cross-bridges of collagen I-containing fibers through its collagen domain in complex with tenascin X and decorin [8]. In addition, type XII collagen is involved in the formation of networks between tendon cells and in the development and growth of tendon tissue, and the cross-linking of type XII collagen in tendons plays a vital role in the mechanical properties of tendons [9].

Achilles tendon disorders associated with the administration of FQs have been reported with an Achilles tendon rupture rate of 1 in 5,958 patients treated with FQs and a tendon rupture rate of 1.2 per 10,000 patients treated with FQs [10, 11]. In addition, when Magnetic Resonance Imaging (MRI) was performed on a patient with suspected tendinitis due to FQs, a partial rupture of the Achilles tendon was identified approximately 4 cm above the calcaneal insertion site [12]. On the other hand, tendon damage caused by administration of FQs is not specific to the Achilles tendon, but has also been observed in the long head of the biceps brachii, extensor digitorum longus, and supraspinatus muscles. The Achilles tendon is particularly vulnerable to damage because it plays a weight-bearing role [13].

Tendons are exposed to reactive oxygen species (ROS) generated from surrounding tissues during exercise, which inhibits repair and other functions. Tendon rupture is thought to be caused by the degree of adaptive response to ROS exposure, including intense exercise and genetic factors [14, 15]. Pouzaud et al. demonstrated that FQs showed moderate cytotoxicity on spontaneously immortalized rabbit tendon cell line, suggesting early oxidative stress in the development of fluoroquinolone-induced tendinopathy [16]. Tendon disorders caused by FQs are more likely to occur in patients at high risk, such as the elderly and those on concomitant corticosteroids, because of the low metabolic rate and poor healing response in mature tendon tissue [17]. However, the mechanism of tendon damage caused by LVFX is not fully elucidated.

We hypothesize that the cause of LVFX-induced tendon damage is related to a decrease in type XII collagen. In this study, we examined changes in gene expression such as type XII collagen and the evaluation of ROS production in murine tendon cells treated with LVFX. We also examined changes in type XII collagen gene expression in tendon tissue from rats treated with LVFX.

2. Materials and Methods

2.1. Cell Culture and Reagent

We used murine tendon cells, TT-D6 cell line, which were cultured in α-minimum Eagle's medium (WAKO, Osaka, Japan) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum, at 33 ℃ in a 5% CO_2 atmosphere [18]. We used levofloxacin (LVFX, Tokyo Kasei Kogyo, Tokyo, Japan).

2.2. Cell Proliferation Assay

Cells were seeded onto 96-well plates at 1×10^4 cells per well and incubated for 72 h with LVFX (0, 1.6, 3.1, 6.3, 12.5, 25, 50, 100 μg/mL). To estimate cell proliferation, we used the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Fitchburg, Wisconsin, USA), according to the manufacturer's instructions. Six replicate-measurements were made.

2.3. Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

Cells were seeded onto 6-well plates at 30×10^4 cells per well. LVFX was immediately added to the plates at concentrations of 0, 10, and 100 mg/mL [19], and the cells were collected 72 h later. The harvested cells were rinsed with ice-cold phosphate-buffered saline (PBS). QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) was added to the samples, and the total RNA was extracted. The purity of the RNA was checked using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

The reverse-transcription reaction was performed with a High Capacity cDNA Reverse Transcription kit (Thermo Scientific). qPCR was conducted by TaqMan-based detection

using THUNDERBIRD probe qPCR Mix (Toyobo, Osaka, Japan). TaqMan Gene Expression Assays (Thermo Scientific) used in this study are listed in Table 1. We performed reactions at 95 ℃ for 20 sec, followed by 40 cycles of 95 ℃ for 1 sec, and 60 ℃ for 20 sec with Step One Plus Real-Time PCR System (Thermo Scientific). All experiments were performed in quadruplicate, i.e., each reaction was performed in quadruplicate on four individual samples. Values were normalized to those of glyceraldehyde-3-phosphate dehydrogenase using the 2^{-DD}Ct method. This experiment was repeated at least three times.

2.4. Creation of LVFX-administered Rat Models, Sample Collection and RNA Extraction

Five-week-old male Wistar rats (200 to 300 g in weight) were obtained from Japan SLC (Shizuoka, Japan). LVFX concentrations were based on Bidell et al [20]. Rats were weighed and injected with a dose of 300 mg of LVFX per kg of body weight, 200 mL each in the bilateral anterior and posterior limbs and the abdominal cavity, for a total of 1 mL. This is equivalent to a human concentration of 500 mg per 24 h. The control group received the same amount of Phosphate Buffered Saline (PBS) used as a solvent at the same site. The LVFX-treated model rats served as the treatment group, and the PBS-treated rats served as the control group. Anesthesia was performed by placing a cotton ball containing 2% sevoflurane (Nikko Pharmaceutical, Shizuoka, Japan) as an inhalation anesthetic in the bottom of an anesthesia bottle, placing a wire mesh over the cotton ball, placing a rat on the mesh, and covering the mesh with a lid. Under anesthesia, LVFX was administered into both anterior and posterior limbs and abdominal cavity of rats using a tuberculin syringe (Terumo, Tokyo, Japan). The administration period was 7 days for both the treatment and control groups. On the day following the final administration, the rats were euthanized by inhalation of an excess of sevoflurane. Four rats were used in each experimental group. This experiment has been approved by the University's Animal Experiment Committee (Approval No. 3812). In the euthanized LVFX-treated rat model, tendon specimens were collected separately from the forelimb and hindlimb using a No. 15 shear (Feather Safety Razor, Osaka, Japan). QIAzol Lysis Reagent (Qiagen, Hilden, Germany), 1 mL, was added to the specimens and homogenized using Biomasher II. After homogenization, the supernatant obtained by centrifugation was subjected to RNA extraction according to the protocol of the Fast Gene RNA Basic Kit (Nippon Genetics, Tokyo, Japan).

2.5. Reactive Oxygen Species (ROS) Measurement

Cells were seeded onto 96-well plates at 1×10^4 cells per well and incubated for 72 h with LVFX (0, 10, 100 mg/mL). To measure ROS, the medium was collected and replaced with PBS containing CM-H2DCFDA (C6827, Thermo Fisher Scientific) at a concentration of 5 mM and incubated for 30 min. 2',7'-Dichlorodihydrofluorescin diacetate (DCF) oxidized by ROS was measured using Varioskan flash (Thermo Fisher Scientific) at excitation and fluorescence wavelengths of 485 nm and 535 nm, respectively. Six replicate-measurements were made.

2.6. Statistical Analysis

Comparisons between two groups were analyzed using Student's t-tests, and comparisons among three groups were analyzed using one-way analysis of variance (ANOVA) and Bonferroni–Dunn methods (statistical significance at $p < 0.05$). All values are presented as the mean ± S.E.M. Results are representative examples of more than three independent sets of experiments.

Table 1. *The list of TaqMan Gene Expression Assays.*

Gene name	Assay ID
Mouse Primer	
COL1A1 (Collagen1a1)	Mm00801666_g1
COL12A1 (Collagen12a1)	Mm01148576_m1
DCN (Decorin)	Mm00514535_m1
MMP-2	Mm00439498_m1
MMP-9	Mm00442991_m1
TIMP-2	Mm00441823_m1
ACTB (Beta Actin)	Mm02619580_g1
Rat Primer	
COL12A1 (Collagen12a1)	Rn01521220_m1
GAPDH	Rn01775763_g1

MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix. metalloproteinase-9, TIMP-2: Tissue inhibitor of metalloproteinase-2, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

3. Results

3.1. LVFX Does Not Inhibit Cell Proliferation in TT-D6 Cells

First, we examined whether LVFX treatment causes changes in cell proliferative capacity in TT-D6 cells. No significant differences were observed in the treatment group compared to the control group (Figure 1).

3.2. LVFX Inhibits Gene Expression of Type XII Collagen in TT-D6 Cells

We focused on type XII collagen (COL12A1) because it is involved in the formation of networks between tendon cells and in the development and growth of tendon tissue [9]. Expectdly, mRNA expression of COL12A1 was decreased in LVFX-treated TT-D6 cells (Figure 2).

3.3. LVFX Upregulates Gene Expression of Both MMP-2 and TIMP-2 in TT-D6 Cells

Tsai et al. demonstrated that ciprofloxacin which is an antibiotic agent in the fluoroquinolone class modulates the expression of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase (TIMPs) and type I collagen (COL1A1) in primary Achilles tendon cells [21]. In reference to this report, we investigated whether LVFX affects the expression of COL1A1, MMPs, and TIMPs in TT-D6 cells. Both MMP-2 and TIMP-2 mRNA expression is upregulated in LVFX-treated cells compared to the control (Figure 3A and 3B), whereas no significant differences in gene expression of MMP-9 and COL1A1 were observed between the control and LVFX-treated cells (Figure 3C and 3D). As collagen XII interacts with decorin (DCN) [9], we examined DCN expression in LVFX-treated TT-D6 cells. Unexpectedly, no change in DCN gene expression between the control and LVFX-treated cells (Figure 3E).

3.4. COL12A1 Gene Expression in Tendon Tissue Is Decreased in LVFX-Treated Rat Models

Total RNA was extracted from tendon tissue collected from LVFX-treated rat models, and the expression of COL12A1 was quantitatively evaluated by qRT-PCR. As expected, the expression of COL12A1 was significantly decreased in LVFX-treated rats compared with PBS-treated rats, the control group (Figure 4).

3.5. LVFX Promotes ROS Production in TT-D6 Cells

Considering the possibility that overproduction of ROS may be toxic to the extracellular matrix, we examined changes in ROS production upon LVFX treatment in TT-D6 cells. In LVFX-treated cells, DCF oxidized and generated by ROS was significantly increased compared to the control (Figure 5).

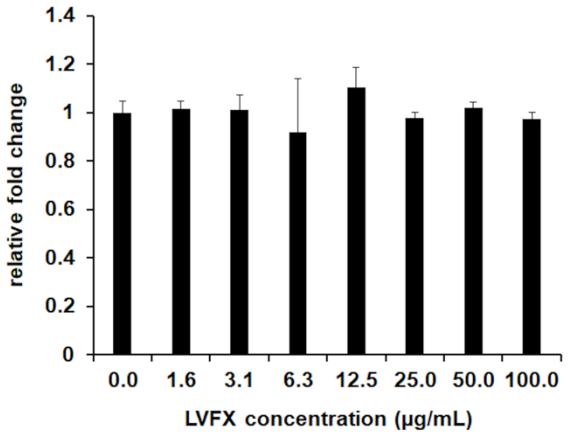


Figure 1. *No effect of levofloxacin on cell proliferation.*

Effect of LVFX (0, 1.6, 3.1, 6.3, 12.5, 25, 50, 100 μg/mL) on. proliferation of TT-D6 cells incubated for 3 days. Data are expressed as mean ± S.E.M. *p < 0.05.

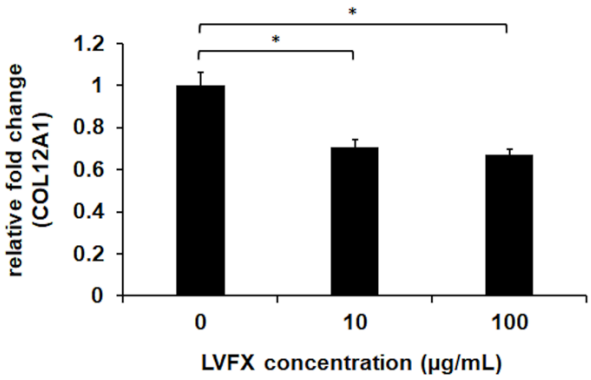
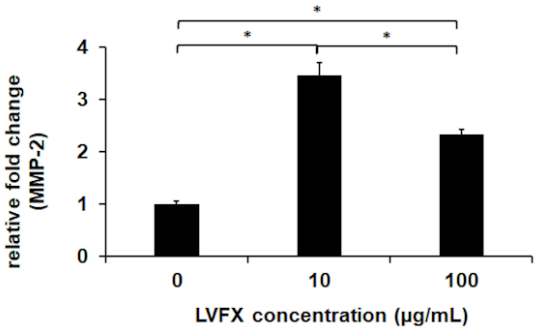


Figure 2. *Inhibition of COL12A1 expression in LVFX-treated tendon cell line.*

Expression of COL12A1 mRNA in TT-D6 cells treated with. LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. Data are expressed as mean ± S.E.M. *p < 0.05.



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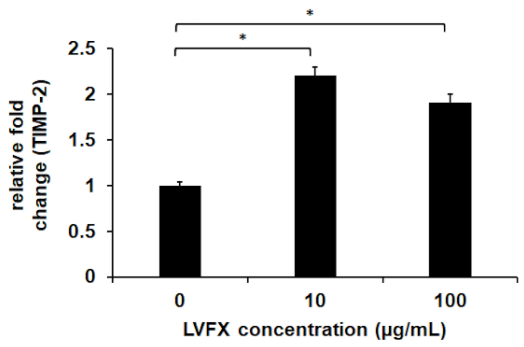

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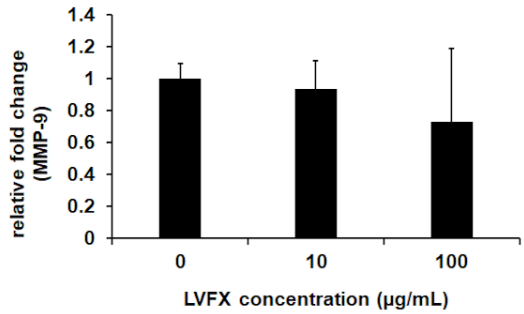

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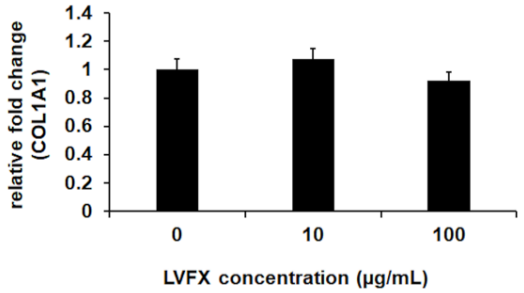

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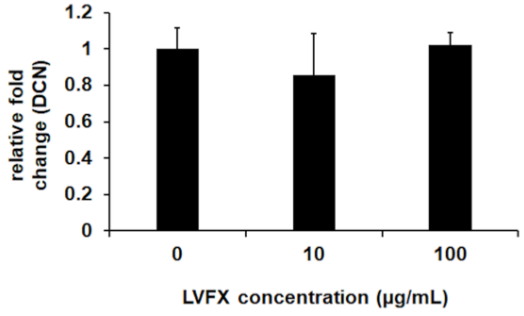

E

Figure 3. *Upregulation of MMP-2 and TIMP-2 in LVFX-treated. tendon cell line.*

A) Expression of MMP-2 mRNA in TT-D6 cells treated with LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. B) Expression of TIMP-2 mRNA in TT-D6 cells treated with LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. C) Expression of MMP-9 mRNA in TT-D6 cells treated with LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. D) Expression of COL1A1 mRNA in TT-D6 cells treated with LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. E) Expression of DCN mRNA in TT-D6 cells treated with LVFX (10, 100 μg/mL) for 72 h estimated by quantitative RT-PCR analysis. Data are expressed as mean ± S.E.M. *p < 0.05.

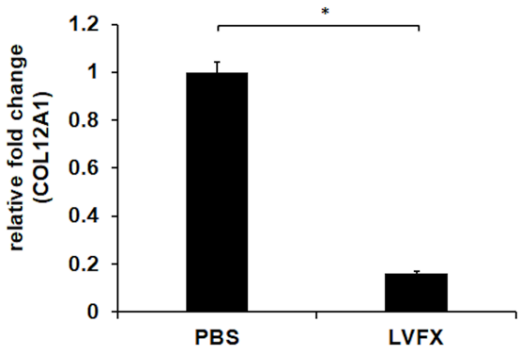

Figure 4. *Inhibition of COL12A1 expression in LVFX-treated. tendon tissue.*

Expression of COL12A1 mRNA in bilateral anterior and posterior. limbs and the abdominal cavity of rats (N=4) which are administered to LVFX estimated by quantitative RT-PCR analysis. Data are expressed as mean ± S.E.M. *p < 0.05.

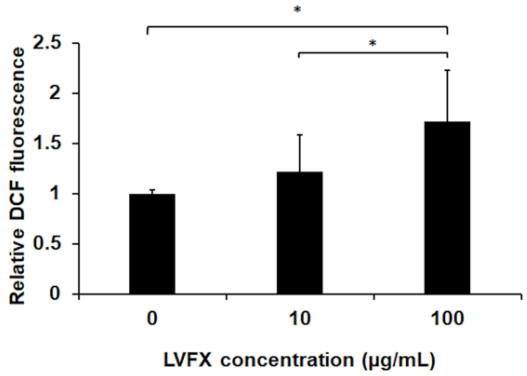

Figure 5. *Promotion of ROS production in LVFX-treated tendon. cell line.*

Effect of LVFX (10, 100 μg/mL) on ROS production of TT-D6 cells incubated for 3 days. Data are expressed as mean ± S.E.M. *p < 0.05.

4. Discussion

Type XII collagen is involved in intercellular communica-

tion and mechanical properties by forming collagen cross-links between adjacent cells during tissue development and growth [8, 22]. The study by Izu et al. showed that fibrous collagen I was reduced in XII-deficient tendon cells compared to controls, suggesting that collagen XII signaling specifically alters tendon cell biogenesis [23]. In COL12A1-deficient mice, changes in the columnar structure of fibers in the long axis direction, disruption of tendon cell-to-cell communication and cell adhesion are observed, with gait abnormalities and rupture of the anterior cruciate ligament as the phenotype [23, 24]. The expression of type XII collagen in Achilles tendon and anterior cruciate ligament was decreased in 17-week-old mice, although there was no significant difference between 4-week-old and 17-week-old mice [23]. Thus, type XII collagen is important for the acquisition of tendon function and mechanical properties, and suppression of type XII collagen expression may cause tendon disorders. Our study showed that LVFX had no effect on the proliferative potential of tendon cells, COL12A1 was decreased, and COL12A1 expression was also decreased in tendon tissue from LVFX-treated rats. In other words, our results suggest that LVFX may cause tendinopathy by inducing a decrease in COL12A1 in tendons. Type XII collagen binds to type I collagen via DCN [9], but our results showed no significant difference in DCN expression after LVFX administration. Tendon damage caused by LVFX may be related to a decrease in type XII collagen independently of DCN.

Overproduction of ROS directly affects the extracellular matrix [16]. In the present study, our results showed no significant difference between the control group and the low concentration group (LVFX10), but the high concentration group (LVFX100) significantly increased ROS production suggesting that the production of ROS may have decreased the expression of COL12A1. On the other hand, Zhang et al. reported that ROS generated via mitochondria may cause tendon dysfunction [24], and further studies on the pathway of ROS generation and the mechanism of action of ROS on type XII collagen are needed.

Tsai et al. treated primary cultures of rat tendon tissue with Ciprofloxacin and reported that MMP-2 was upregulated, COL1A1 was decreased, and TIMP-2 expression was unchanged [21]. Our results agreed that MMP-2 was elevated and MMP-9 was unchanged, but TIMP-2 was elevated and COL1A1 was unchanged, which was not consistent with the results of Tsai et al. In the experiments of Tsai et al. used heterogeneous primary cultured cells from rat tendon tissue, whereas we used a homogeneous mouse tendon cell line, TT-D6 cells, which may have caused the inclusion of cells derived from peritendinous tissue in the experiments of Tsai et al. The difference in results may be due to the inclusion of cells derived from peritendinous tissues in their experiments. Pouzaud et al. reported that the tendon toxicity of FQs varied depending on their type [16], and it is possible that differences in results may have been caused by differences in the drugs used. Karousou et al. speculated that the elevated gene expression of TIMPs in ruptured tendon tissue may be a tissue response to overproduction of MMPs [25]. The local balance between MMPs and TIMPs is important for the maintenance of the extracellular matrix of tendons, and the resulting changes in collagen synthesis and degradation may affect tendons. Our experiments suggest that increased TIMP-2 expression may be a response to increased MMP-2 expression.

This study has the following limitations. LVFX effects on cells other than tendon cells have not been studied. The effects of non-new quinolone antimicrobial agents on tendon cells have not been studied. The influence of ROS on the development of tendon damage in LVFX was suggested, but inhibition experiments with antioxidants are needed.

5. Conclusions

Through our experiments, we have found that LVFX increased ROS production and decreased COL12A1 expression in tendon cells. In addition, a decrease in COL12A1 expression was also observed in the LVFX-treated rat model. These new findings indicate that LVFX-induced type XII collagen reduction may cause tendinopathy, an adverse effect of LVFX treatment. This study showed the decrease of COL12A and the increase of ROS before Achilles tendon rupture treated with LVFX occurs. Monitoring COL12A and ROS levels in the blood of patients treated with LVFX may serve as a potential biomarker for assessing the risk of Achilles tendon rupture.

Abbreviations

LVFX	Levofloxacin
FQs	Fluoroquinolones
ROS	Reactive Oxygen Species
PBS	Phosphate-Buffered Saline
DCF	Dichlorodihydrofluorescin
MMPs	Matrix Metalloproteinases
TIMPs	Tissue Inhibitors of Metalloproteinase
COL	Collagen
DCN	Decorin

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Seiji Asoda: Conceptualization, Data curation, Formal Analysis, Investigation, Project administration, Validation, Writing – original draft, Writing – review & editing

Toshinori Iwai: Conceptualization, Data curation, Formal Analysis, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing

Shinnosuke Nogami: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing

Ko Ito: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing

Data Availability Statement

The data supporting the outcome of this research work has been reported in this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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