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Refractory bacterial contamination during cell culture

A common challenge in the basic research of skeletal diseases and an effective and convenient solution for this problem

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Abstract

Background: Chronic low back pain, primarily caused by intervertebral disc degeneration (IVDD), significantly impairs quality of life and imposes a substantial economic burden on society. Research on IVDD and tissue repair relies heavily on high-quality primary cell cultures, including nucleus pulposus cells, annulus fibrosus cells, and mesenchymal stem cells. However, refractory bacterial contamination during cell extraction and culture poses a major challenge, hindering experimental progress and compromising the safety of cell-based therapies.

Objective: This study aims to develop a simple and effective method to combat bacterial contamination in rat intervertebral disc and mesenchymal cell cultures, particularly cases resistant to conventional antibiotics like penicillin-streptomycin.

Method: We employed commercially available levofloxacin eye drops as an intervention agent. Through quantitative intervention, we assessed the drug's cytotoxic effects and therapeutic efficacy, establishing an optimized treatment protocol for refractory bacterial contamination.

Result: Our findings demonstrate that levofloxacin eye drops effectively eliminate bacterial contamination without significant cytotoxicity, providing a feasible solution for contaminated cell cultures. A standardized treatment process was developed to ensure reliable outcomes.

Conclusion: This study presents a simple, efficient, and practical approach to managing refractory bacterial contamination in musculoskeletal cell research. The method enhances experimental reliability and supports advancements in IVDD studies and cell-based therapies.

Keywords: annulus fibrosus cell, bacterial contamination, cell culture, intervertebral disc degeneration, levofloxacin, mesenchymal cell, nucleus pulposus cell

1. Introduction

Chronic low back pain, as one of the most common musculoskeletal disorders, affects the quality of life of approximately 568 million people worldwide. Its widespread prevalence, prolonged symptom duration, and status as a leading cause of disability impose a significant economic burden on society.[1,2] Although the causes of low back pain are numerous and complex, it is closely associated with intervertebral disc degeneration (IVDD).[3–5] Current treatments for IVDD primarily involve conservative approaches, such as physical therapy, nonsteroidal anti-inflammatory drugs, and opioids, aimed at alleviating pain symptoms.[6,7] When symptoms become uncontrollable, surgical intervention is often the last resort, despite the risks of complications, including postoperative pain, loss of spinal mobility, and increased incidence of adjacent segment disease.[6] Therefore, investigating the mechanisms underlying IVDD and exploring more effective interventions are crucial for inhibiting IVDD progression and promoting tissue regeneration.

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The factors contributing to IVDD are multifaceted, including mechanical stress, aging, inflammation, and oxidative stress, making it a complex pathological process involving multiple changes.[3–5] Research on the etiology and regenerative repair of IVDD relies on *in vitro* studies using primary cells, including studies based on intervertebral disc cells (primarily nucleus pulposus and annulus fibrosus cells) and stem cell-based regeneration systems (mainly mesenchymal stem cells and intervertebral disc stem/progenitor cells).[2,8–10] A favorable growth environment for primary cells is essential for maintaining cellular specificity, enhancing the accuracy of experimental results, and improving research efficiency.

Primary cells are highly susceptible to microbial contamination during extraction, culture, and processing. Contamination can be introduced through improper aseptic techniques by researchers, inadequately sterilized consumables (e.g., pipettes, culture flasks), or contaminated cell culture reagents (e.g., buffers, media).[11] In most cases, once contamination occurs, it is nearly impossible to effectively eliminate, posing significant challenges and often devastating consequences for time-consuming *in vitro* experiments based on primary cells. Currently, the most common contamination in cell culture is mycoplasma, which is small and difficult to observe under high magnification.[12] However, mycoplasma grows slowly, has a limited impact on the growth state of contaminated cells, and can be eliminated using specific reagents (e.g., tiamulin fumarate and minocycline hydrochloride).[13–15] Another common contamination is bacterial contamination, which is often difficult to detect in its early stages and becomes severe by the time it is noticed. Under bacterial contamination, cell viability drastically declines, with most cells dying quickly, severely disrupting subsequent experiments. Bacterial contamination is particularly challenging to address, especially persistent contamination of unknown origin and refractory contamination resistant to penicillin-streptomycin.[16–18] There is an urgent need for a simple and effective method to resolve refractory bacterial contamination during primary cell culture.

Levofloxacin, with its broad antimicrobial spectrum and high drug utilization, exhibits strong bactericidal effects against Gram-positive bacteria (e.g., Staphylococcus, Streptococcus), Gram-negative bacteria (e.g., Escherichia coli, Pseudomonas aeruginosa, Proteus), and microorganisms such as mycoplasma. It is widely used in the treatment of respiratory infections, urinary tract infections, skin and soft tissue infections, and other diseases.[19–21] Currently, levofloxacin is also selected as a common anti-infective agent for local use in some tissue engineering repair protocols.[22,23] However, the application of levofloxacin as an anti-microbial agent in cell culture to combat bacterial contamination has not been reported. Whether levofloxacin can serve as an effective new antibacterial solution for refractory contamination resistant to penicillin-streptomycin mixtures remains unverified.

This study utilizes commercially available levofloxacin eye drops and adopts a quantitative treatment approach to analyze the cytotoxic effects of levofloxacin on intervertebral disc (IVD) cells and bone marrow mesenchymal stem cells (BMSCs). It confirms that the phenotype of IVD cells and BMSCs remains unaffected after levofloxacin treatment and subsequent cell passage. Finally, by developing a standardized protocol for treating contaminated cells, an effective method for addressing refractory bacterial contamination during cell culture is established, providing a practical intervention for a common challenge in basic research on skeletal diseases.

2. Materials and methods

2.1. Primary cells isolation and culture

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. A total of 10 male Sprague-Dawley (SD) rats were used for the isolation of primary nucleus pulposus (NP) cells, annulus fibrosus (AF) cells, and BMSCs. Rats were euthanized by inhaling an excessive amount of isoflurane. Rat tails, femurs, and humerus were removed under aseptic conditions, and coccygeal IVDs were separated. The gelatinous NP tissue and lamellar AF tissue were separated from the IVD under a microscope. Both ends of the femurs and humerus were removed under aseptic conditions. For rat NP and AF cells isolation, the NP and AF tissue obtained were digested for 30 minutes in a mixture of 0.4% Pronase (Roche Diagnostics, 10165921001) and 0.0125% collagenase. The digested NP and AF tissues were then passed through cell strainers (BD Falcon, 352360) with a pore size of 100 μm and were washed 3 times with phosphate-buffered saline (PBS; Gibco, 20012027). The isolated cells were maincultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, 11320033) containing 20% fetal bovine serum (Gibco 10099141C), supplemented with 1% penicillin-streptomycin combination (Gibco, 15070063) at 37°C in a humidified atmosphere of 5% CO_2. For BMSCs isolation, a syringe with serum-free medium was used, and the bone marrow cavity was slowly flushed. The bone marrow fluid was collected and then filtered through a 40 μm cell strainer to remove impurities and obtain a single-cell suspension. The filtered bone marrow fluid was centrifuged (1200 rpm, 5 minutes), and the supernatant was discarded. The collected cell pellet was resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium containing 10% fetal bovine serum, and then was seeded into a 10 cm culture dish. Different cell treatments were shown in the schematic diagram of the corresponding figures.[24,25]

2.2. RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

Total RNA of NP cells, AF cells, and BMSCs of rats was harvested using MiniBEST Universal RNA Extraction Kit

(TaKaRa, 9767) according to the manufacturer's instructions. Reverse transcription was subsequently performed with PrimeScript RT Master Mix (TaKaRa, RR036A). Synthesized cDNA was then subjected to qPCR analysis using TB Green Premix Ex Taq II (TaKaRa, RR820A). The reactions were performed with CFX96 (Bio-Rad) according to our previous studies.[24] Gene expression levels were reported as relative fold change, with β-actin as an internal control. Primers sequences used in this study are listed in Table 1:

2.3. Apoptosis assay

NP cells were fixed in freshly prepared 4% paraformaldehyde (Solarbio, P1110) for 5 minutes, and subsequently permeabilized with 0.1% Triton X-100 (Beyotime, P0096) for 5 minutes. Then the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using *In Situ* Cell Death Detection Kit (Roche Diagnostics, 11684795910) according to manufacturer's instructions. After the TUNEL staining, these cells were treated with 4',6-Diamidino-2-Phenylindole (DAPI, C1006, Beyotime) at RT for 10 minutes. Finally, the cells were analyzed under a fluorescence microscope (BX53, OLYMPUS).

2.4. Transmission electron microscopy

NP cells were fixed in 2.5% glutaraldehyde overnight and then postfixed in 2% osmium tetroxide for 1 hour. After dehydration in an ascending series of acetone, the samples were embedded in an embedding medium (Epon 812) and cut into ultrathin sections by an LKB-V ultramicrotome. Poststained with uranyl acetate and lead citrate, the samples were visualized using a transmission electron microscope (TEM, H-7650; Hitachi, Tokyo, Japan).

2.5. Statistical analysis

The results were given as means ± SD. Statistical analyses were performed using SPSS 22.0 and GraphPad Prism 9.0 software. Differences between 2 groups were analyzed by Student t test, while differences among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Primary NP cells, AF cells, and BMSCs experienced refractory bacterial contamination

From May 2024 to December 2024, several research groups in our school encountered unexplained cell contamination incidents. Despite various measures such as incubator sterilization, replacement of culture media and serum, cleaning of the biosafety cabinet, and increasing the concentration of penicillin-streptomycin to 5%, the contamination could not be avoided. The specific situation of bacterial contamination is shown in the figure. Under high magnification, the same batch of primary NP cells, AF cells, and BMSCs all exhibited contamination, characterized by the appearance of a large number of rapidly moving rod-shaped objects at the bottom of the dish. These rod-shaped microorganisms appeared translucent under light microscopy and were mainly distributed around the cells, with some closely adhering to the primary cells (Fig. 1A–C). We subsequently performed more than 5 rounds of sterile PBS washing on the contaminated primary NP cells and increased the proportion of penicillin-streptomycin mixture to 2% during subculturing. Unfortunately, these rescue measures had no effect on the bacterial contamination, as the cells exhibited a similar state of contamination as before treatment, with a comparable bacterial load (Fig. 1D). Moreover, we also conducted TEM on the contaminated primary cells. The results of TEM showed that a large number of cells were already dead, while the nuclei of the surviving cells appeared shrunken, with swollen endoplasmic reticulum and mitochondria. The cytoplasm was filled with a large number of phagocytosed bacteria, characterized by an elliptical structure surrounded by a translucent ring-like structure (Fig. 1E).

3.2. Cytotoxicity of levofloxacin for culturing primary cells

We collected the supernatant from contaminated culture dishes of 2 different batches (A1–A4) from 2 research groups for DNA sequencing to analyze the abundance and species of contaminating bacteria. The results (Fig. 2A) showed that the bacterial abundance in these 4 samples was very low, suggesting that the contaminating strains were relatively homogeneous. Further analysis of bacterial species (Fig. 2B,C) revealed that

Table 1

Sequence of qPCR primers used in this study.

Gene	Forward	Reverse
β-actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA
Acan	CTGATCCACTGTCCAAGCACCATG	ATCCACGCCAGGCTCCACTC
Col1a1	TGTTGGTCCTGCTGGCAAGAATG	GTCACCTTGTTCGCCTGTCTCAC
Col2a1	ACGCTCAAGTCGCTGAACAACC	ATCCAGTAGTCTCCGCTCTTCCAC
Krt8	ATGTTACCGTCAGCACCACC	GACAAGGGCTTGAGAGGCAC
Runx2	CAGACCAGCAGCACTCCATA	AGACTCATCCATTCTGCCGC
Osx	GGTCCTGGCAACACTCCTAC	AAGAGGTGGGGTGCTGGATA

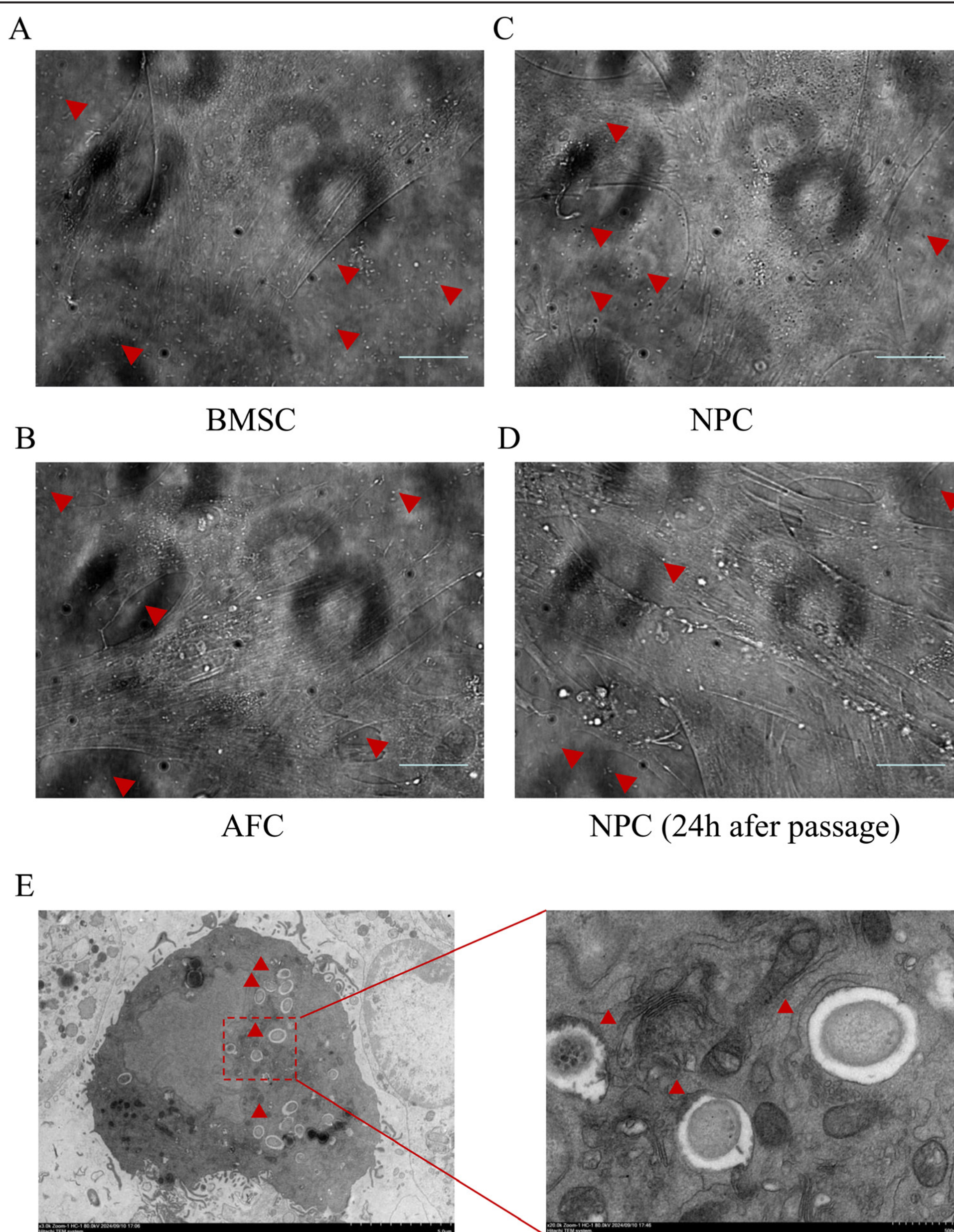

Figure 1. Primary NP cells, AF cells, and BMSCs experienced refractory bacterial contamination: (A–C) Light microscopy images of 3 types of primary cells contaminated with bacteria. The translucent circular objects indicated by the red arrows are bacteria. (D) Light microscopy images of passaged NP cells 24 hours after subculturing. The primary NP cell was contaminated with bacteria. The translucent circular objects indicated by the red arrows are bacteria. (E) Transmission electron microscopy (TEM) images of NP cells. The red arrows indicate bacteria engulfed by the cells. The area enclosed by the red box is the region selected for magnification, and the magnified image is displayed on the right.

Pseudomonadota, primarily Pigmentiphaga, was the dominant contaminating strain. To address the issue of refractory bacterial contamination during primary cell culture, we utilized levofloxacin eye drops, a readily available broad-spectrum antimicrobial agent, to treat the cultured cells. A diagrammatic sketch illustrates the process of cell extraction, treatment, and detection (Fig. 2D). For the ease of use and quantification in cell culture, we used the common unit of "drops" (approximately 0.04 mL per drop, 24.4 mg/5 mL) for measurement. Cell Counting Kit-8 (CCK-8) results showed that lower concentrations of levofloxacin (< 0.2 drop/mL) had no effect on the cell viability of primary NP cells, AF cells, and BMSCs. However, at concentrations exceeding 0.5 drop/mL, levofloxacin exhibited inhibitory effects on cell viability, reducing it to approximately 60% of normal levels at 4 drops/mL. Compared with primary AF cells and BMSCs, primary NP cells showed better tolerance to levofloxacin, although the statistical variability within each group was significant, likely due to the confluent and slow growth characteristics of primary NP cells (Fig. 2E–G). Subsequently, we performed similar

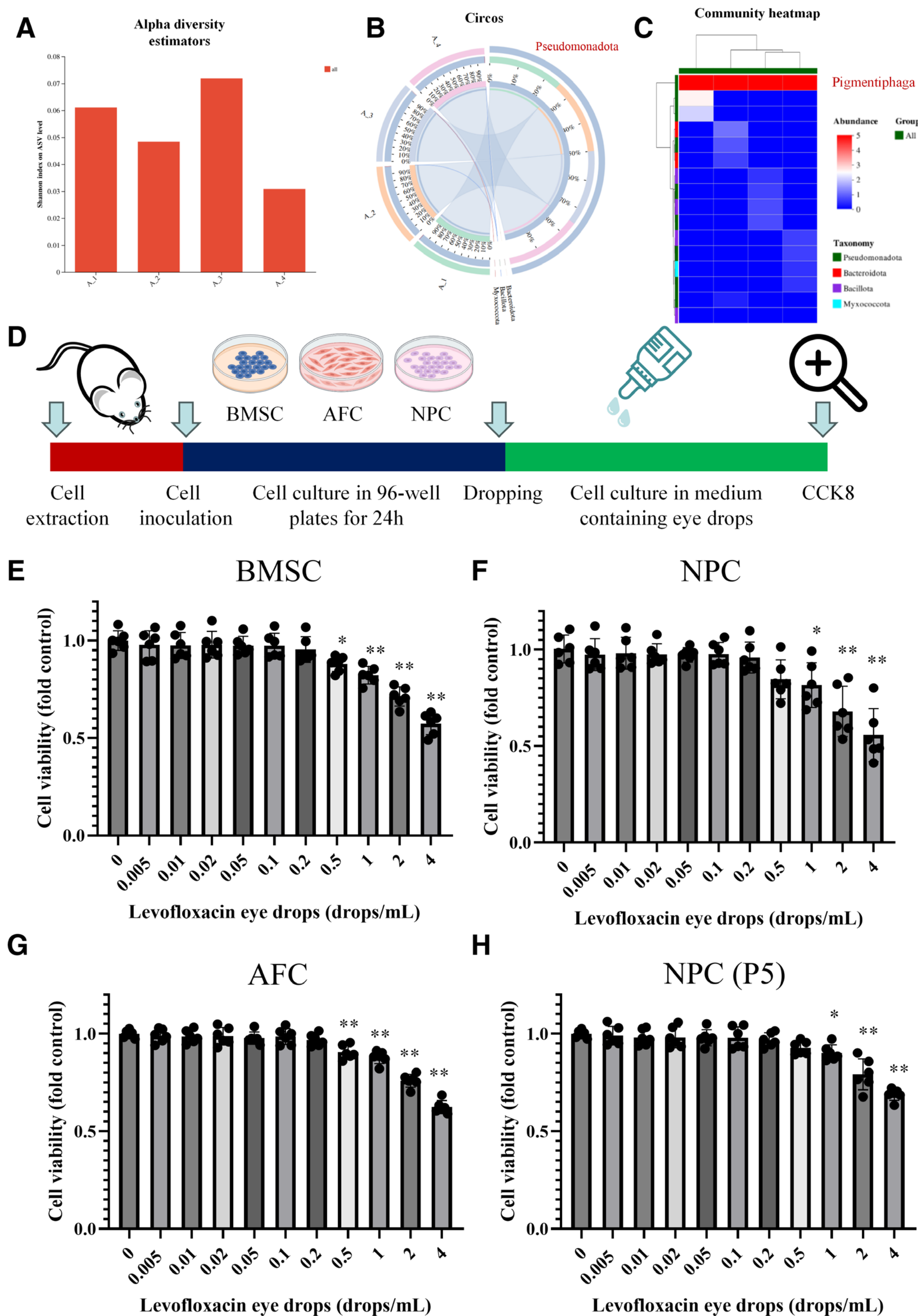

Figure 2. Cytotoxicity of levofloxacin for culturing primary cells. (A) Alpha diversity estimators of the sequencing data. (B) Circos sample-species relationship plot of the sequencing data. (C) Community heatmap of the sequencing data. (D) Schematic diagram of the cell treatment process. (E–H) CCK-8 results of 3 types of primary cells and P5 NP cells treated with different concentrations of levofloxacin.

treatments on P5 NP cells. The results indicated that P5 NP cells exhibited even greater tolerance to levofloxacin, with less impact on cell viability, suggesting that this treatment may have lower toxicity effects on nonprimary cells or even cell lines (Fig. 2H).

3.3. Levofloxacin treatment on primary cells showed no effect of cell viability on subcultured cells

To determine whether treating primary cells with levofloxacin affects the viability of subsequent generations, we used a high concentration of levofloxacin (higher than

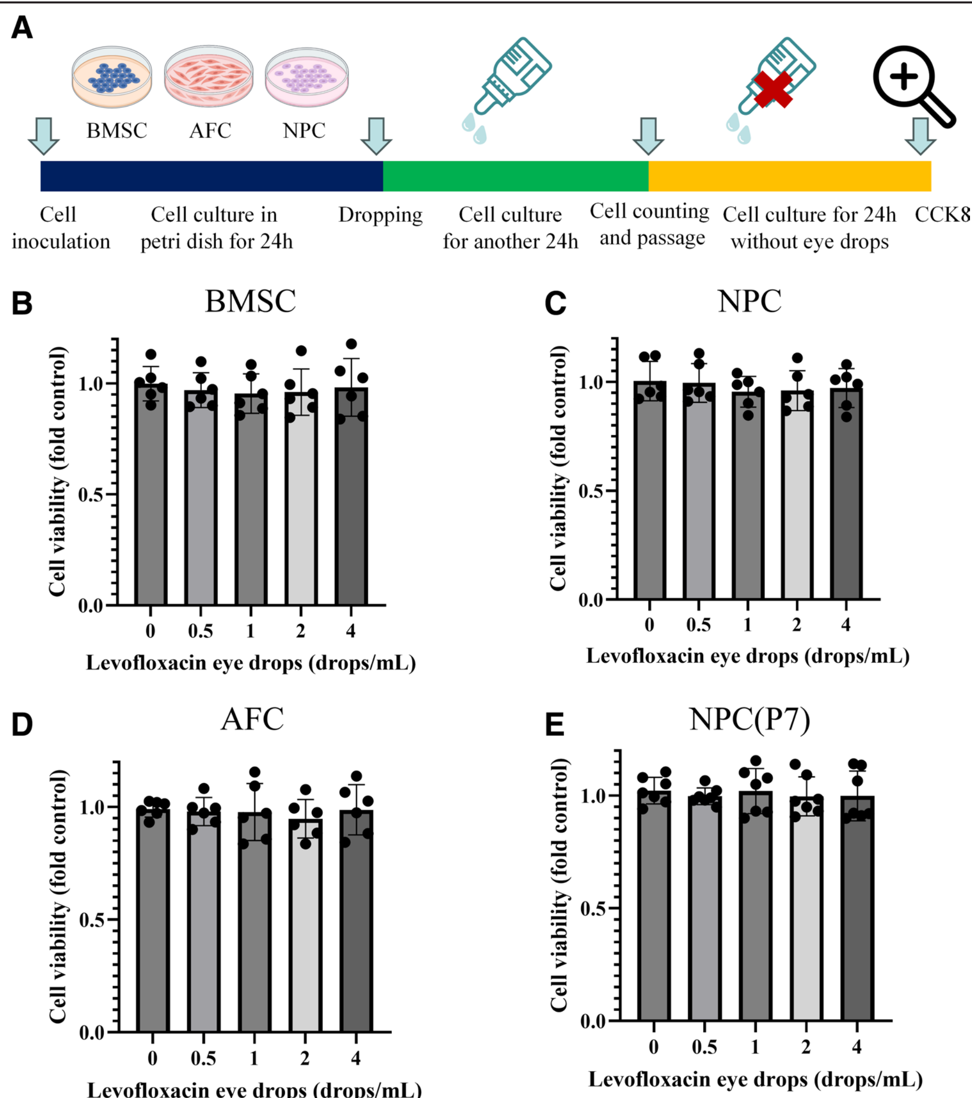

Figure 3. Levofloxacin treatment on primary cells showed no effect of cell viability on subcultured cells. (A) Schematic diagram of the cell treatment process. (B–E) CCK-8 results of 4 types of passaged cells. Three types of primary cells and P7 NP cells were treated with different concentrations of levofloxacin.

0.5 drop/mL), known to impact cell viability, to treat primary NP cells, AF cells, and BMSCs. The treatment and detection methods are illustrated in the schematic diagram (Fig. 3A). After 1 day of treatment with a high concentration of levofloxacin, the morphology of the primary cells showed no significant changes. The treated cells were then passaged and cultured in medium without levofloxacin, while the control group consisted of normally passaged cells that were not treated with levofloxacin. CCK-8 results showed that although the high concentration of levofloxacin inhibited the viability of primary NP cells, AF cells, and BMSCs during the primary cell culture process, this treatment did not exhibit any inhibitory aftereffects on the viability of the passaged cells (Fig. 3B–D). Additionally, we applied the same treatment protocol to P7 NP cells, and the CCK-8 results demonstrated a similar effect (Fig. 3E). These findings suggest that primary cells treated with levofloxacin may exhibit a cell state similar to that of untreated passaged cells after subculturing.

3.4.Levofloxacin treatment on primary cells showed no effect of cell specificity on subcultured cells

To determine whether treating primary cells with levofloxacin affects the cell specificity of subsequent generations, we used a high concentration of levofloxacin (1 drop/mL) to treat primary NP cells, AF cells, and BMSCs. The treatment and detection methods are illustrated in the schematic diagram (Fig. 4A). After 1 day of treatment of levofloxacin, these cells were harvested and experienced a qPCR analysis. qPCR results showed that some marker genes or downstream effector genes of these cells were sharply inhibited after the treatment of levofloxacin, including Krt8, Acan, Col2a1 in NP cells, Acan, Col1a1, Col2a1 in AF cells, and Col1a1, Runx2, Osx in BMSCs (Figs. 4B–4D). These results indicated a negative-immediacy effect of levofloxacin on IVD cells and BMSCs. However, the effect of levofloxacin on subcultured cells showed some attractive results (Fig. 4E). Compared with the untreated passaged cells, these treated passaged cells

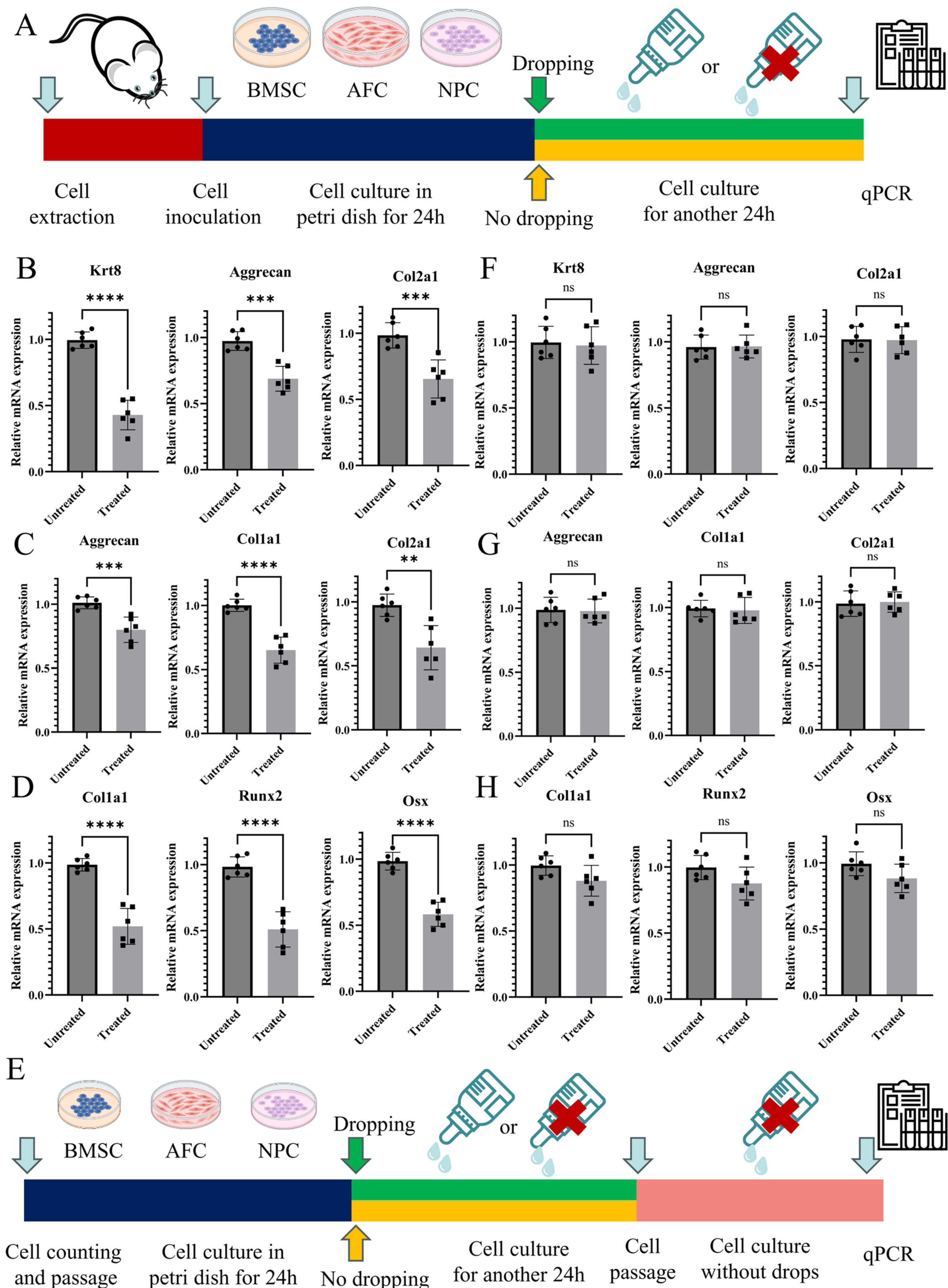

Figure 4. Levofloxacin treatment on primary cells showed no effect of cell specificity on subcultured cells. (A) Schematic diagram of the cell treatment process. (B–D) qPCR analysis of some marker genes or downstream effector genes of NP cell, AF cell and BMSC. (E) Schematic diagram of the cell treatment process. (F-H) qPCR analysis of some marker genes or downstream effector genes of NP cell, AF cell, and BMSC.

showed no significant difference in the expression of these marker genes or downstream effector genes, indicating a potential use of levofloxacin on antibacterial treatment during cell culture in skeletal disease study (Fig. 4F–H).

3.5.Levofloxacin treatment on primary cells showed no effect on apoptosis

To determine whether the effect of levofloxacin on cell viability is mediated through the intervention of apoptosis, we

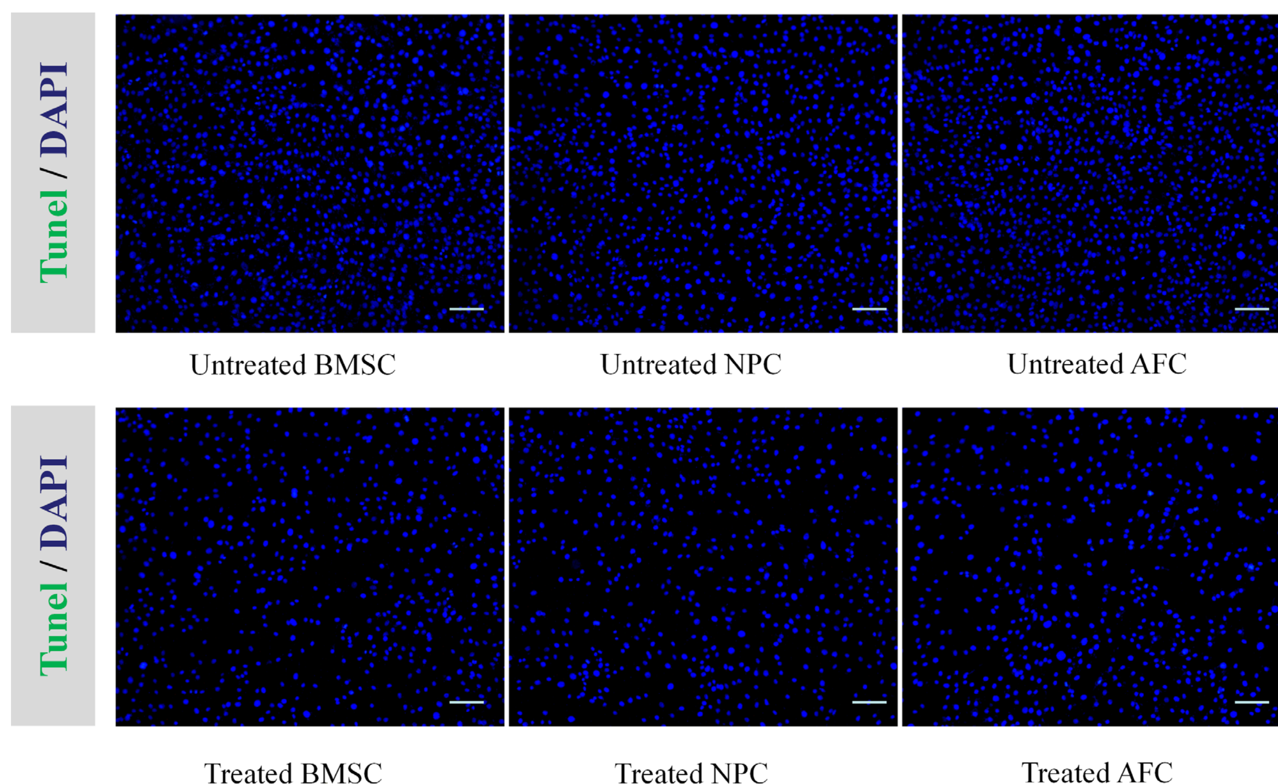

Figure 5. Levofloxacin treatment on primary cells showed no effect on apoptosis. TUNEL staining images of NP cells treated with levofloxacin or not. Nucleus was stained by DAPI dye, which showed as round blue dots.

conducted a TUNEL staining assay. The TUNEL staining results showed that, compared with the untreated group, the number of cells in the levofloxacin-treated group was significantly reduced, as evidenced by fewer DAPI-stained cells being observed. However, there was no difference in the number of TUNEL-positive cells between the 2 groups, and no TUNEL-positive cells were observed in the treated group (Fig. 5). This suggests that the reduction in cell number caused by levofloxacin may be due to its impact on cell proliferation rate rather than through the induction of apoptosis.

3.6. Levofloxacin treatment protocol for refractory bacterial contamination

After several months of exploration, we developed a comprehensive protocol for addressing refractory bacterial contamination in cell culture, as illustrated in Figure 6A. When early-stage bacterial contamination occurs, we thoroughly wash the contaminated cells with sterile PBS to significantly reduce the bacterial load. After cleaning, the cells are passaged, and once they have fully adhered, they are treated with levofloxacin at a concentration of 1 drop/mL for 24 hours. The cells are then washed 3 more times with sterile PBS. Following the washing step, the cells are treated again with the same concentration of levofloxacin. After treatment, the cells can be seeded onto the appropriate culture plates, subjected to the required experimental procedures, and subsequently analyzed. Figure 6B demonstrates the state of cells after the full treatment process. Cells that were only washed with sterile PBS but not treated with levofloxacin still exhibited a large number of motile bacteria 24 hours after seeding. In contrast, cells treated with levofloxacin showed no live bacteria 24 hours after seeding, indicating the effectiveness of this antibacterial strategy.

4. Discussion

The issue of contamination during cell culture, particularly refractory bacterial contamination with unknown causes, represents a significant challenge impacting fundamental research in orthopedic degenerative diseases. Although traditional methods to combat contamination—such as training laboratory personnel in aseptic techniques, replacing cell culture consumables, and thoroughly cleaning incubators or workstations—may alleviate the problem to some extent, the recurrence of bacterial contamination is a common occurrence due to the inability to accurately pinpoint the source of contamination. Presently, reagents targeting mycoplasma contamination have been developed and widely applied[13–15]; however, measures to address bacterial contamination in the context of basic orthopedic research have not been extensively studied or discussed. Our research has, for the first time, developed a simple and feasible antibacterial method that successfully resolves the issue of early-stage bacterial contamination in primary cell cultures.

Cell specificity is an issue that cannot be overlooked in orthopedic research. Cells constitute complex tissues, such

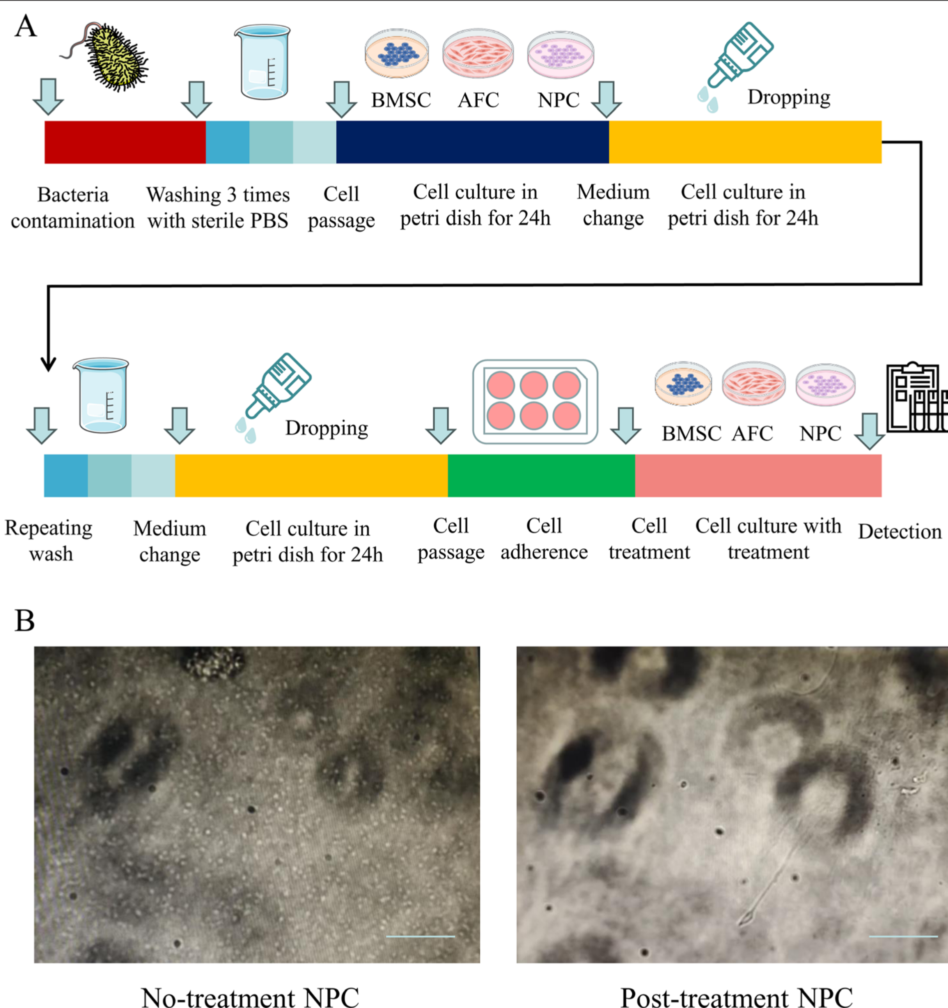

Figure 6. Levofloxacin treatment protocol for refractory bacterial contamination. (A) Schematic diagram of levofloxacin treatment protocol. (B) Light microscopy images of treated and untreated primary NP cells contaminated with bacteria.

as intervertebral discs, which exhibit significant cellular heterogeneity.[26,27] In recent years, single-cell sequencing data have also revealed that even within the nucleus pulposus tissue alone, there exist distinct cell subpopulations performing different cellular functions.[28–31] An effective antibacterial measure must not compromise the specific functions of these cells. Our study demonstrates that treating primary NP cells, AF cells, and BMSCs with levofloxacin does not affect the cell-specific functions of their progeny, thereby enhancing the feasibility of our approach.

Levofloxacin eye drops, a commonly used clinical antibacterial topical medication, are readily available in almost every pharmacy and hospital dispensary, which increases the accessibility of the drug. The main component of levofloxacin eye drops is levofloxacin, with excipients including sodium chloride and sterile water. The inherent antibacterial and sterile properties of these eye drops make their application in cell culture possible.[32] The standardized outlet design facilitates convenient and controlled quantitative use of the antibacterial drug. The use of levofloxacin eye drops reduces potential contamination during drug preparation, simplifies experimental procedures, and reduces workload, making the antibacterial process for refractory bacterial contamination during cell culture straightforward.

However, our study does have certain limitations. The best approach to any microbial contamination remains identifying and completely eliminating the source of contamination. For instance, if bacteria are observed under a microscope in the culture medium or serum used, replacing them with sterile alternatives is the most direct solution. Placing sterile culture medium in a petri dish for cell culture in an incubator and observing motile bacteria after a period of culture may suggest that incubator sterilization is a viable option. These troubleshooting methods can reduce the use of external antibacterial drugs and may be more beneficial for proper cell culture. Our antibacterial solution is more suited to situations where contamination recurs without an identifiable source. Additionally, cells treated with our antibacterial protocol should be used promptly to minimize potential adverse effects from repeated treatments.

5. Conclusion

Our research provides an effective solution to the problem of refractory cell contamination encountered during cell

culture. This solution has no significant adverse effects on several cell types commonly used in the study of orthopedic degenerative diseases and can, to some extent, resolve the issue of cells being unusable for subsequent research due to early-stage contamination.

Ethical statement

Ethics approval was obtained from the Institutional Review Board of Xijing Hospital of Fourth Military Medical University (KY20203146-1), and informed consent was obtained from each donor. The work presented in this paper was performed according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Conflicts of interest

The authors have no conflicts of interest to disclose.

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Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors on request.

Author contributions

Writing original draft, Conceptualization, Methodology, Project administration, Supervision, Review & editing: Dong Wang, Zhuojing Luo.Writing original draft, Conceptualization, Methodology, Project administration, Formal analysis, Review & editing: Chu Gao, Chuxin Zhou.Investigation, Software, Validation, Resources: Xin He, Di Wang, Jianxin Mao.

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