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Moxifloxacin-Impregnated Contact Lenses for Treatment of Keratitis in Rabbit Eyes

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ABSTRACT

Moxifloxacin (MOX) was loaded into commercial contact lenses (CLs) via supercritical carbon dioxide ($ScCO_2$) to attain MOX-impregnated CL for keratitis treatment. This study aimed to investigate *Pseudomonas* keratitis treatment with MOX-impregnated CL compared to the traditional eyedrop administration. MOX impregnation was accomplished employing optimum parameters of 2.5 h drug exposure time, 25 MPa pressure, and 313 K for $ScCO_2$ conditions using ethanol co-solvent rendering sustainable delivery, up to 7 days at effective dosage formulation. The MOX-impregnated CL was found to be safe with no significant toxicity on fibroblast cells after 5 days of contact time. Bacterial viability in vivo keratitis treatment in rabbit eyes was significantly decreased to 10^2 from 10^9 CFU/cornea for MOX-impregnated CL treatment, almost similar to exhaustive conventional 0.5% MOX eye drop treatments. The MOX-impregnated CL treatment revealed no conjunctival hyperemia, edema, or secretion for all eyes in the relevant group, and transparent cornea with no keratitis focus was obtained for two of the eyes ($n = 6$). The normal histological structure was seen with MOX-impregnated CL treatment on healthy eyes. Moreover, polymorphonuclear cell infiltration observed in keratitis eyes without any treatment was significantly decreased to a few polymorphonuclear cells in the groups treated with MOX eyedrops and MOX-impregnated CL.

1 | Introduction

Eye diseases are important health problems seriously affecting the quality of life of the human population [1]. More than 2 million patients annually suffer from keratitis caused by microbial infections [2]. Bacterial keratitis requires urgent and appropriate treatment [3]. In particular, *Pseudomonas aeruginosa* keratitis, with widespread and destructive progression, can turn into a suppurative ulcer within 24–48 h and lead to eye loss. The first step in the treatment of microbial keratitis is the administration of antibiotics with different formulations such as eye drops, gels, and ointments. Drops are often the preferred method for treatment due to their ease of preparation and use [4]. As a result, the general approach to *P. aeruginosa* keratitis is to apply drop therapy every hour or every 2 h for long periods of up to a few days [5]. However,

this frequent use of drops can present challenges for patients; significant concerns include non-compliance with daily administration, difficulties in administering the drops particularly for elderly patients, and potential contamination of the bottle after prolonged use [1, 4, 5]. One of the main obstacles in treating eye infections is the unique anatomical structure of the eye. Factors such as tear drainage, limitations in the epithelial transport system, the lack of blood vessels in the cornea, and low corneal barrier permeability can significantly reduce the effectiveness of drugs such as antibiotics, e.g., a reduction of up to 5% in bioavailability [1, 3, 6]. To address this issue, researchers have explored alternative methods such as continuous lavage, which was found to be more effective than traditional topical antibiotic treatment [5]. However, concerns about the use of a pump system, patient immobilization, and potential impact on vision have prompted further investigation and alternatives for eye infection treatments [5]. One potential solution to this issue is the implementation of a mechanism that can increase the bioavailability of active agents. The newly developed system should aim to improve drug efficiency and compliance while also minimizing cytotoxic effects. Additionally, it should have the capability to carry other drugs alongside antibiotics as needed [1]. The use of contact lenses (CLs) in treating keratitis appears to possess the desired features mentioned above [2, 7, 8]. CL are commonly utilized in the treatment of ocular diseases and are generally well tolerated by users [9], affording benefits such as correcting refractive errors and improving corneal deformities [10, 11]. In 2008, the first antibiotic-loaded CL was produced, marking a significant advancement in the field [7]. Subsequent studies have demonstrated that the use of CL results in higher bioavailability of drugs compared to traditional topical drops [1]. This is achieved by minimizing the impact of tear drainage on the fluid at the CL interface, and through the use of various chemical methods to effectively deliver drugs at the appropriate dosage and timing [6, 12, 13]. Thus, bioavailability was increased by 50% [1] while also significantly enhancing the bactericidal effect [6]. However, the majority of studies were conducted in vitro, rather than in living subjects. There is a limited number of studies that have compared the effectiveness of conventional drops and CL treatment in vivo. Kakisu et al. reported gatifloxacin- and moxifloxacin (MOX)-loaded soft CLs employing a soaking process for bacterial inhibition in the eye in vivo. The study showed that hydroxyethyl methacrylate (HEMA)-based CL loaded with MOX released 90% of the loaded drug within 24 h [14]. In a different study, the antibiotic ciprofloxacin was imprinted onto HEMA-based CL, and the in vivo antibacterial activity on *P. aeruginosa* keratitis revealed that drug-imprinted CL could inhibit almost half of the bacteria on the cornea, but cipro drop treatment removed the bacterial colony fully [15]. Polania-Baron et al. [16] designed MOX-filled scleral lenses for keratitis treatment in human eyes. They indicated that the infections in patients can be treated by drug-filled scleral CL successfully; however, these CLs need to be replaced every 24 h. In addition, Maulvi et al. synthesized a novel type of lenses consisting of MOX- and hyaluronic acid-loaded semicircular rings, called implant lenses. The treatment effect of one CL administration was equal to several eye drop treatments for *Staphylococcus aureus*-induced conjunctivitis treatment in vivo [17]. These different types of drug-loaded CL are generally effective materials for in vivo keratitis treatment in animal or human models. However, short-duration drug delivery platforms from the CLs could limit their use in severe infection treatment in clinical use. Therefore, different drug loading techniques should be explored to provide long-term and effective therapeutic dosage formulation as drug delivery devices for keratitis treatment in the animal and human eyes.

Various drug-loading techniques such as adsorption, conjugation, imprinting, or impregnation could be used to design drug-loaded CL, but drug impregnation via supercritical carbon dioxide ($ScCO_2$) provides significant advantages for CL carriers [18–20]. $ScCO_2$ is a phase of CO_2 gas that exists at and above the critical pressure of 72.8 atm and a temperature of 31.0°C [21]. Low viscosity, high diffusivity, zero surface tension, and high solvation power for hydrophobic and/or lipophilic active agents make $ScCO_2$ fluid [22] a great solvent for the drug-loading process [23]. $ScCO_2$ fluid is inert, safe, and improves the amount of drug by easy homogeneous penetration into the carrier material [24]. Furthermore, it can readily be loaded and removed from the CL at the end of the loading process without requiring tedious cleaning procedures and allows sterilization [25]. To overcome lower drug loading with commercial CL using common methods, $ScCO_2$ fluid was chosen to replace toxic organic solvents [26]. Yañez et al. reported the comparison of flurbiprofen loaded into soft CLs by soaking in a water solution of drug and impregnation techniques via $ScCO_2$ to overcome the limitation of common loading processes. The loading of the flurbiprofen anti-inflammatory drug was approximately 10-fold increased with the $ScCO_2$ process in comparison to the soaking process [24]. Utilizing $ScCO_2$ as a drug loading solvent ensures sustainable delivery from CL at effective dosages with longer duration drug release kinetics [27]. Optimization of MOX-impregnated CL with the $ScCO_2$ technique and comparison with the soaking process was also reported in our previous study [28]. It was shown that the MOX loading capacity of silicone-based commercial CLs by the soaking process was significantly enhanced with the $ScCO_2$ system, and the loaded MOX antibiotic could be gradually released at an effective dosage for up to 7 days from the MOX-impregnated CL in a linear profile in vitro. The limitation of short-term MOX delivery from the CL was overcome, and sustainable MOX release from $ScCO_2$ CLs resulted in a similar outcome for the MOX eyedrop treatments achieved for the clinical treatment of severe *Pseudomonas* keratitis.

The purpose of our study is to evaluate and compare the efficacy of MOX-impregnated CL with conventional eye drops in rabbits with induced *P. aeruginosa* keratitis. Therefore, MOX, an antibiotic, was loaded into CL (impregnation technique) by employing a $ScCO_2$ fluid system for the treatment of *P. aeruginosa* keratitis in an animal model. Antibiotic release was determined in balanced salt solution (BSS) and artificial corneal physiological solution at pH 7.5 at 37°C. Furthermore, in vitro cytotoxicity of MOX-impregnated CL was investigated on L929 fibroblast cells. In the animal model, MOX eyedrop treatment, MOX-impregnated CL, and bare CL were used for healthy and *Pseudomonas* keratitis eyes of rabbits to show the potential use of MOX-impregnated CL as an alternative to MOX eyedrops for keratitis treatment. Additionally, we sought to assess the safety of the use of CL and any changes that may occur when using MOX-impregnated CL in eyes without keratitis. In vivo

bacterial inhibition, disease symptoms, grading of corneal infiltration, and histopathological results were determined, with measurements of epithelium and stroma thickness for the MOX-impregnated CL group and compared with the MOX eyedrop treatment that is generally used in clinics and also with the control group.

2 | Materials and Methods

2.1 | Materials

The MOX HCl (Abdi Ibrahim, Turkey) as an antibiotic, dimethyl sulfoxide (DMSO, 99.9%, for analysis, ACS reagent, Carlo Erba, France) and ethanol (99%, BRK, Istanbul, Turkey) as solvents were used as received. CLs (Senofilcon A silicone hydrogel, Acuvue Qasys) were purchased from Johnson & Johnson Vision Care Inc., Turkey. *P. aeruginosa* (ATCC 10145) (Microbiologics, KWIK-STIK, France) was used in the keratitis model. Nutrient agar (NA, Condolab, for cultivation, Madrid, Spain) was used as the growth medium for *P. aeruginosa*. In the cytotoxicity analysis, L929 fibroblast cells (Mouse C3, an connective tissue obtained from the SAP institute, Ankara, Turkey) were used. To prepare the cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM, with 4.5 g/L glucose, 3.7 g/L sodium pyruvate, L-glutamine 0.5 g/mL), fetal bovine serum (FBS, heat inactivated), and penicillin/streptomycin (10,000 U/mL penicillin, 10 mg/mL streptomycin) were used after purchase from Pan Biontech GmbH, Aidenbach, Germany. Furthermore, trypsin (0.25%, EDTA 0.02% in PBS, Pan Biontech GmbH, Aidenbach, Germany), trypan blue (0.5% solution, Biological Industries), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT agent, BioFroxx, Einhausen, Germany), and dimethyl sulfoxide (DMSO, 99.9%, Carlo Erba, France) were used in cytotoxicity tests. Hematoxylin (Bio-Optica, catalog no: 05-M06007), eosin (Bio-Optica, catalog no: 05-M10002), and Erlich Ziehl Neelson (EZN) (ChemBio, catalog no: CB6050.0500) were used for histopathological analysis. Millipore-Direct Q UV3 (Merck Darmstadt, Germany) was used to obtain deionized water at 18.2 MΩ.cm.

2.2 | Preparation of MOX Impregnated Contact Lenses in Supercritical Carbon Dioxide ($ScCO_2$) Fluid

MOX antibiotic was loaded into commercial CL via the drug impregnation technique in a $ScCO_2$ fluid system using the methodology described by Costa et al. with some modification [19]. Drug impregnation was done with a custom-made $ScCO_2$ system (Superex-F-500, Konya, Turkey) contained in a 500 mL stainless-steel reactor consisting of a temperature control system and a high-pressure liquid CO_2 pump. Steel plates with mesh shelves were used to hold CL and carry the drug within the reactor. Six CL and 300 mg MOX were placed on the meshed shelves to provide homogeneous impregnation of drug molecules into CL. The temperature was adjusted to 313 K in the reactor, and 1% ethanol as a co-solvent was introduced into the bottom of the reactor. The steel plate containing CL and the drug was placed in the reactor. The pressure of the reactor was adjusted to 25 MPa, and the impregnation time was 2.5 h. A circulation pump was used to provide homogeneous distribution of the drug throughout the CL. Finally, $ScCO_2$ was slowly removed from the reactor via the pressure valve by releasing the pressure. Then, MOX-impregnated CL were carefully taken out of the reactor and placed in a sterile plastic petri dish for further studies.

2.3 | Determination of the Amount of Drug Loaded Into MOX-Impregnated CL and Release Studies

One MOX-impregnated CL was placed in 3 mL of DMSO for 24 h. Then, the elution solution was measured by UV–Vis spectroscopy (SP-UV300SRB, Spectrum, Quanzhou, China) at 288 nm against a calibration curve for MOX in DMSO to determine the amount of drug released.

The drug release studies from MOX-impregnated CL were done by placing one MOX-impregnated CL in 10 mL of balanced salt solution (BSS) at pH 7.5 at 35°C, similar to physiological eye conditions. Then, the absorbance values of the elution solutions were measured at different times from 1 to 240 h via UV–Vis spectroscopy at 288 nm using a calibration curve for MOX in BSS to estimate the drug release amount over time. The analysis was repeated three times, and the results are expressed as mean ± standard deviation.

2.4 | Cytotoxicity of MOX Antibiotic and MOX-Impregnated CL

The toxicity of MOX antibiotic and MOX-impregnated CL on L929 fibroblast cells was determined through MTT assay. The fibroblast cells were cultured at 37°C under a 5% CO_2 atmosphere, using DMEM supplemented with 10% (v/v) FBS and 1% antibiotics. The cells were seeded into each well in a 48-well plate at 5×10^3 cell/mL concentration. After 24 h, MOX solution in DMEM with concentrations from 10 to 1000 μg/mL and one MOX-impregnated CL were applied to the adhesive cells in 1 mL of culture medium. Cytotoxicity of MOX eye drops and MOX-impregnated CL was investigated for up to 5 days. At the end of the incubation time, the MOX solution or MOX-impregnated CL was removed from the wells and the cells treated with 200 μL of 0.5 mg/mL of MTT reagent in DMEM. After 2 h in the dark, the MTT solution was removed and 400 μL of DMSO was put into each well. A plate reader (Thermo, Multiskan Sky) was used to determine absorbance values at 570 nm. The cell viability% for MOX antibiotic and MOX-impregnated CL was assessed by comparing it with the control group, which was the absorbance of untreated cells. The cytotoxicity tests were repeated three times, and the results are expressed as mean ± standard deviation.

2.5 | Animal Model

The studies with rabbits were carried out at Canakkale Onsekiz Mart University (COMU) Experimental Research Application and Research Center (COMUDAM), with permission from the COMU Animal Experiments Local Ethics Committee (Number of 2022/02-05).

A total of 21 male New Zealand albino rabbits, with an average weight of 2.5–3.0 kg, aged 2.5–3.0 months, were utilized for this study [29, 30]. Upon arrival at Canakkale Onsekiz Mart University, Experimental Research Application and Research Center Animal Laboratory (COMUDAM), the rabbits were quarantined for 7 days before being used in the experiment. They were housed individually in standard rabbit cages in a controlled environment with 12 h of daylight and 12 h of darkness, proper ventilation, and constant temperature. The rabbits were provided with ad libitum access to tap water and feed throughout the duration of the experiment. Rabbits exhibiting signs of infection or congenital eye disease, such as abnormalities in the eyelid, conjunctiva, cornea, lens, or fundus (excluding albinism), were excluded from the study. To prevent any disruption to the experimental model and potential impact on the control eye, the rabbits' movement was restricted during the research. A stabilizer was utilized throughout the experiment for this purpose, as seen in Figure S1.

In the study, 42 eyes of 21 rabbits were used and divided into 4 groups, as listed in Table 1. The bacterial keratitis model was induced using *P. aeruginosa*.

As given in Table 1, *P. aeruginosa* (ATCC 10145) was inoculated into the left eye and the left eye was not treated (Table 1, in group 1). The rabbits' other eyes were left untouched for control purposes in 6 rabbits. In group 2, *P. aeruginosa* was also inoculated into the left eye and standard drop treatment was applied, while the same treatment was applied to the healthy right eyes for control purposes in 6 rabbits. In group 3, *P. aeruginosa* was inoculated into the left eye and an antibiotic-impregnated CL was inserted. The MOX-impregnated CL was also placed in the healthy right eyes for control purposes in 6 rabbits. Group 4 consisted of 3 rabbits and a plain CL without medication was placed in their left and right healthy eyes.

The administration of *P. aeruginosa* is demonstrated in Figure S2. Before *P. aeruginosa* inoculation into the cornea, rabbits were anesthetized with an intramuscular injection of xylazine hydrochloride (2 mL, 0.2 g). The left eyes of the rabbits were selected for inoculation, and 10 μL of *P. aeruginosa* (10^5 CFU/mL) was injected into the stroma with a 1 mL disposable insulin syringe. The penetration of the fluid into the stroma was observed during the injection. The eyes were examined 24 h later with a hand-held biomicroscope (Reichert) and clinically graded by the ophthalmologist. Rabbit eyes with corneal scores ≥ 3 points and < 5 points were selected as the successful keratitis model [30]. Bare and MOX-impregnated CL were inserted into the infected eye under anesthesia. The eyelid of the rabbits was sutured with 5–0 polypropylene (Prolene, Ethicon) to prevent the CL from coming out of the eye. As indicated in Figure S2, the eyelid was sutured medially and laterally, sufficient to support the edges of the CL. After the animals were placed in their cages, their movements were monitored to evaluate vision. Thus, the aim was to prevent second inflammation by leaving no other tissue inside the eye untouched. MOX is a type of fluoroquinolone antibiotic commonly used to treat bacterial keratitis. To treat the group, MOX drops were prepared at a 0.5% w/v concentration in DI water. For the preparation of MOX drops, 5.45 mg of MOX HCl, equivalent to 5 mg of MOX, was dissolved in 1 mL of DI water. This drug concentration was similar to the MOX drops used for treatment. The drop was then drawn into a sterile insulin syringe and administered. The MOX drop at a 0.5% w/v concentration was initially administered every hour for the first 2 days, and then every 3 h thereafter [31].

During the experiment, the eyes of rabbits were examined and photographed daily for a period of 7 days. However, due to the development of severe symptoms such as suppuration, corneal infiltration, and corneal thinning in the rabbits with *P. aeruginosa* keratitis, they were left untreated after the third day, and the experiments were discontinued. At the end of the 3rd days, the rabbits were sacrificed. In other groups, the experiment could be completed over 7 days. At the end of the 7th days, the rabbits were sacrificed. Disease symptoms were determined by corneal examination by the ophthalmologist based on the discharge, conjunctiva, hypopyon, and corneal infiltration and edema of the rabbit eye at the end of the treatment before sacrificing the animal. The clinical score of the corneal infiltration in *Pseudomonas* keratitis eyes was graded from 0 to 5 according to the severity of opacity and infiltration. Furthermore, the percentage of corneal infiltration area in the infected groups was evaluated by Image J software based on the method proposed by Soleimani et al. [32]. The corneas of all rabbits in different groups were fixed in 10% neutral buffered formalin after sacrifice for histopathological examination by a pathologist.

2.6 | In Vivo Bacterial Inhibition by MOX Drop and MOX-Impregnated CL

Bacterial viability for the eye was determined for keratitis groups to confirm and detect active *P. aeruginosa* infection on the cornea. The corneal swab method, which is commonly used in humans, was applied [33]. Briefly, a bacterial swab was taken from the ulcer and discharged on rabbit eyes using a sterile

TABLE 1 | The groups of *Pseudomonas* keratitis model on rabbit eye.

	Group 1	Group 2	Group 3	Group 4
Right eye	Healthy eye No treatment (G1-H), $n = 6$	Healthy eye MOX drop (G2-H), $n = 6$	Healthy eye MOX impregnated CL (G3-H), $n = 6$	Healthy eye Bare CL (G4-H), $n = 3$
Left eye	Keratitis No treatment (G1-P), $n = 6$	Keratitis MOX drop (G2-P), $n = 6$	Keratitis MOX impregnated CL (G3-P), $n = 6$	Healthy eye Bare CL (G4-H), $n = 3$

cotton stick on day 1st and at the end of the treatment. Then, the bacterial swab was suspended in 1 mL of physiological saline solution (SP), which is 0.9% NaCl. At the end of the treatment, MOX-impregnated CL was removed from the eye before sacrifice and placed into 1 mL of SP. After that, these suspensions were serially diluted with SP, and the living colony was determined by counting after seeding on nutrient agar as a solid bacterial growth medium. In vivo bacterial inhibition % was determined by comparing the number of viable bacteria colonies for the keratitis groups treated with MOX drops and MOX-impregnated CL with the keratitis group that was not treated as a control. All analyses were applied for 6 rabbit eyes, and the results are determined with standard deviation.

2.7 | Histological Examination

Histopathological analysis was performed in a blinded fashion. The corneas were dissected and fixed in 10% neutral buffered formalin for 48 h, and then transferred to increasing concentrations of ethanol (50%, 70%, 80%, 96% and absolute) for dehydration. They were then embedded in paraffin, and 5 μm-thick sections were taken on the transverse plane. The sections were stained with hematoxylin–eosin (H-E) and Erlich Ziehl Neelsen (EZN). The cornea sections were examined using light microscopy (BX51; Olympus; Tokyo, Japan). Quantitative analysis of corneal epithelium and stroma thickness via images was performed with Olympus Cellsens Dimension 1.15 software.

Microscope images of healthy epithelium and stroma thickness were measured and analyzed. After the image analysis program was run (Image J), five different areas were first marked for each tissue section on the micrograph that was given as an example for cornea tissue, corneal epithelium, and stroma thickness. As a result of counting these areas, the values obtained from the program were stored in an EXCEL file. Five animal sections for each group were selected from cornea tissue samples, and then an image analysis method was applied.

2.8 | Statistical Analysis

The cytotoxicity tests were repeated three times ($n = 3$), and the results are expressed as mean ± standard deviation. GraphPad Prism 8 software was used to identify statistical differences in the groups compared to the control group according to the Student's *t*-test with $*p < 0.05$ and $**p < 0.001$ values.

In vivo bacterial inhibition and corneal infiltration area % were identified for 6 rabbit eyes ($n = 6$) and the results are reported with standard deviation. GraphPad Prism 8 software was used to find statistical differences of the groups compared to the control group according to the Student's t-test with $*p < 0.05$ and $**p < 0.001$ values.

Statistical analysis of epithelium and stroma thickness was performed with Sigmaplot 15 by using the Pairwise Multiple Comparison Procedure and Tukey's Test. For each group, 5 rabbit eyes ($n = 5$) were analyzed, and the results are given with standard deviation. Statistical difference was expressed as $*p < 0.05$ compared with the healthy eye as the control group.

3 | Results

3.1 | Preparation of MOX-Impregnated CL

The antibiotic, MOX, was impregnated into soft CLs by using $ScCO_2$ to improve the drug-loading capacity of commercial CL. Drug impregnation was accomplished in a $ScCO_2$ reactor, which had a temperature and pressure controller to provide supercritical conditions. As illustrated in Figure 1, MOX drug powder and silicone hydrogel-based CLs were separately put on the steel plate containing mesh shelves, and this plate was placed into the reactor before adding 1% volume of ethanol as a co-solvent in the bottom of the reactor.

The optimization of loading parameters, such as P, T, and time, was used to attain the highest MOX loading amount in CL based

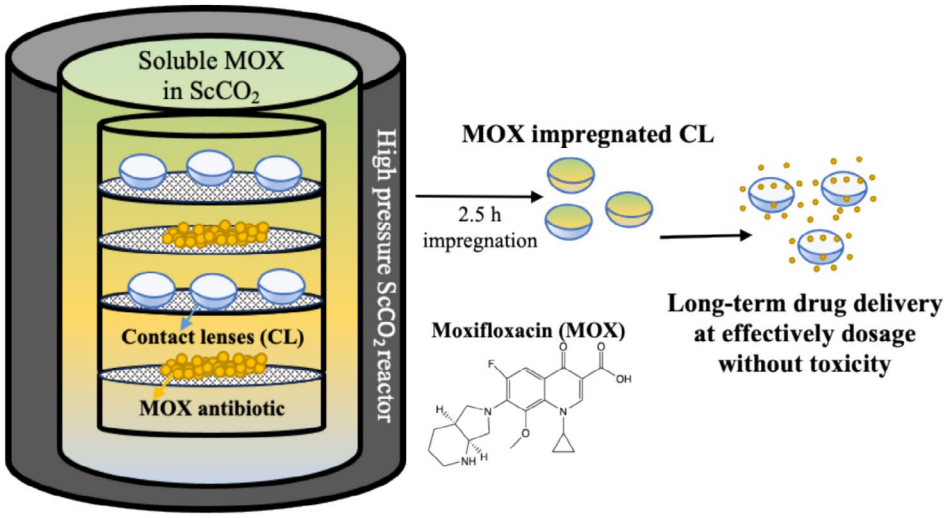

FIGURE 1 | Schematic representation of moxifloxacin (MOX) loading into CL using the impregnation technique at 25 MPa pressure, 313 K temperature for 2.5 h impregnation time with the presence of 1% ethanol as a co-solvent in the high-pressure $ScCO_2$ reactor.

on our previous study [28]. The highest MOX impregnation was obtained at 313 K under 25 MPa pressure for 2.5 h impregnation time, and these conditions were used for the exposure of CL with drug-dissolved $ScCO_2$ fluid via a circulation pump. Under these conditions, the $ScCO_2$ fluid generated in the reactor to dissolve MOX antibiotic was used to transfer the drug into the CL matrix. At the end of the drug loading, MOX-impregnated CL was taken from the reactor and tested as an alternative for the treatment of ocular ulcers. Higher MOX loading capacity was achieved using this procedure, with 102 ± 19 μg MOX impregnation amount for one CL. The MOX delivery from the drug-impregnated CL was investigated at pH 7.5 in BSS at 37°C for up to 10 days, and it was reported that 91 ± 3 μg antibiotic, which is about 90% of the loaded drug, was gradually released from the CL within 7 days [28]. The minimum inhibition concentration (MIC) values for MOX were reported as 1.5 μg/mL MIC_{50} and 32 μg/mL MIC_{90} [34]. Therefore, MOX-impregnated CL delivered an effective dosage of MOX for the treatment of *Pseudomonas* keratitis. The characterization of bare and MOX-impregnated CL and the relevant in vitro release studies were reported in our previous study [28]. As reported in this study, the swollen properties of bare CL did not change, with no foaming and disruption in the CL structure after the $ScCO_2$ process. Furthermore, the transmittance and UV protection ability of bare CL were almost similar to those of MOX-impregnated CL according to UV–Vis spectra.

Toxicity of MOX drops at various concentrations from 10 to 1000 μg/mL and one MOX-impregnated CL for different incubation times up to 5 days was investigated for fibroblast cells. Cell viability of the fibroblasts relative to the control group for each day was determined and summarized in Figure 2a,b respectively.

As seen in Figure 2a, MOX showed no toxicity up to a 50 μg/mL concentration, and the cell viability slightly decreased to 76% ± 3% in the presence of 250 μg/mL MOX at 1 day of

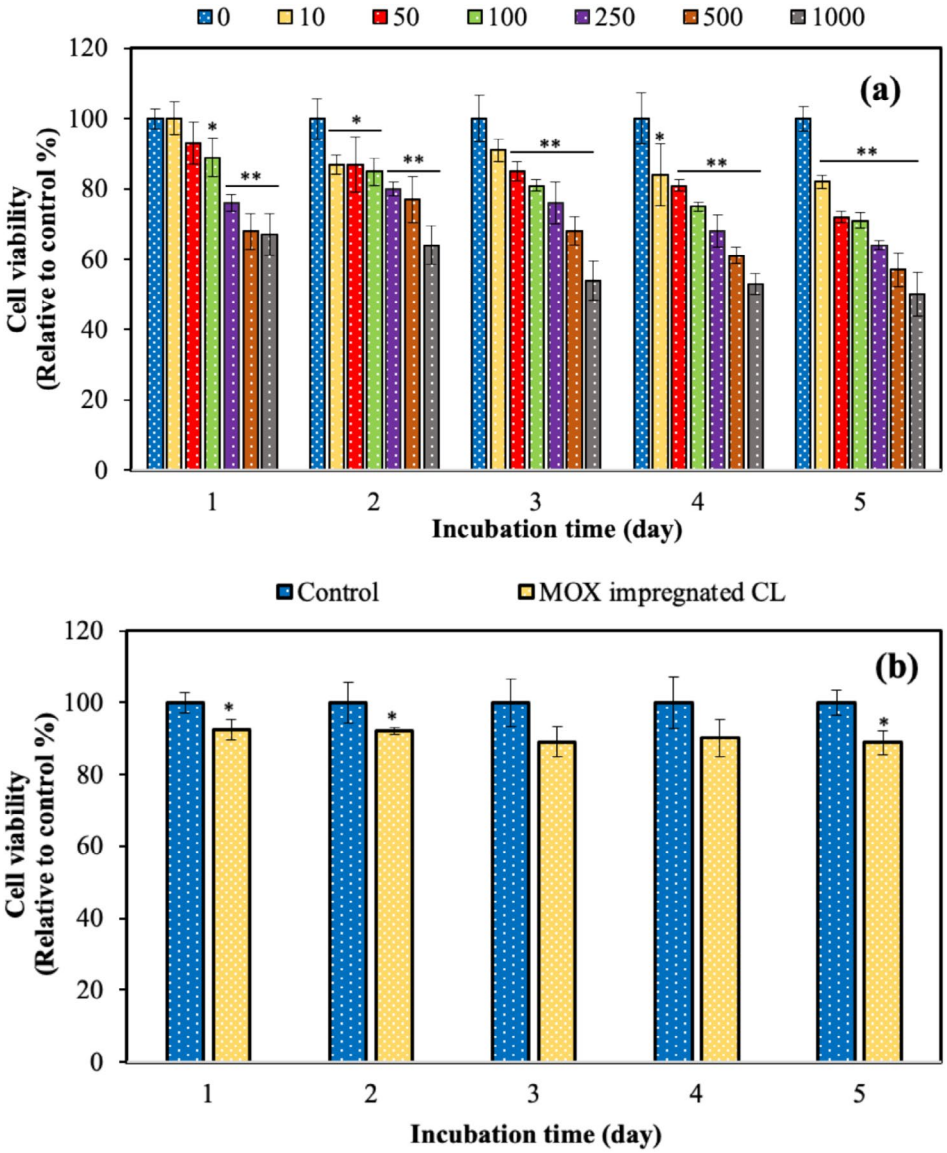

FIGURE 2 | (a) Cytotoxicity of MOX antibiotic at various concentrations from 10 to 1000 μg/mL and (b) MOX-impregnated CL for different incubation times from 1 to 5 days for L929 fibroblast cells using MTT assay for 24 h. Statistical analysis used $*p < 0.05$ and $**p < 0.001$ compared with the control groups for each day ($n = 3$).

incubation. In addition, the cytotoxicity was not significantly changed above a 250 μg/mL concentration up to 1000 μg/mL, with 67% ± 6% viability against fibroblast cells after 1 day. Furthermore, the toxicity of MOX slowly enhanced with the increase in incubation time with the cells. The cell viability % was 81 ± 2 at 50 μg/mL MOX, but 53 ± 6 at a 1000 μg/mL concentration of MOX at 5 days of incubation. Interestingly, one MOX-impregnated CL showed no toxicity on the fibroblast cells, with a maximum of 89% ± 6% cell viability up to 5 days of incubation. To support the cell viability results, OD values of the fibroblasts for control groups and MOX-impregnated CL for up to 5 days of incubation are also demonstrated in Figure S3. According to the MOX release profile, a maximum 90 μg antibiotic was delivered from one CL. In the cell culture study, one MOX-impregnated CL interacted with the cells in 1 mL of cell culture medium. Therefore, it is apparent that the drug delivery from MOX-impregnated CL induces no toxicity for fibroblast cells.

3.2 | Comparison of MOX-Impregnated CL and MOX Drops for In Vivo Keratitis Treatment in Rabbits

In the animal model, 24 rabbits were used for the experiment and 42 eyes of 21 rabbits were included in the protocol. Table 1 reveals the distribution of eyes according to group. Although the groups were planned to consist of 6 eyes for the experiment, the two rabbits did not regain consciousness after anesthesia. One rabbit injured its eye while trying to escape from the cage. These rabbits were excluded from the study.

Rabbits were examined until an appropriate keratitis model was established after bacterial inoculation. By the end of the second day, all rabbits that were inoculated with bacteria had developed an appropriate keratitis model. Treatment was initiated for the groups that were to receive it. During treatment, all rabbits that received eye drops showed resistance to the treatment. After the drops were administered, they exhibited a strong blinking reflex and shook their heads.

Digital camera images of the rabbit eyes for all groups are given in Figure 3a. The images were taken before infection and 1st and 7th days after bacterial injection.

In keratitis groups, discharge, conjunctiva, hypopyon, and cornea infiltration and edema were evaluated by the ophthalmologist, and the number of rabbits with these disease symptoms is given in Table 2.

In Group 1, no secretions, conjunctival redness, edema, keratitis, or hypopyon were observed in any of the right healthy eyes for G1-H group. However, at the 48th hour, all left keratitis eyes in G1-P group showed grade 4 secretion and hyperemia. The corneal thickness increased in all eyes with keratitis. On the 3rd day, hypopyon could not be identified due to intense corneal opacity. Clinical score for corneal infiltration in *Pseudomonas* keratitis eyes at the end of the treatment with number of affected rabbit eyes ($n = 6$) are given in Table 3. In keratitis without any treatment group (G1-P), only one eye had grade 3 corneal focus, the other five eyes had grade 4 corneal focus. The percentage of corneal infiltration area of the infected eyes was examined by Image J analysis and the results were given in Figure 3b. Accordingly, approximately 78% ± 15% infiltration area was measured for G1-P keratitis control group. Bacterial viability as colony forming unit (CFU)/cornea was determined by bacterial swab culture taken from the cornea at the end of treatment, as seen in Figure 3c. The keratitis group without any treatment had approximately 10^9 CFU bacteria on the cornea.

In Group 2, no secretions, conjunctival redness, edema, keratitis, or hypopyon were observed in any of the right healthy eyes with MOX drop treatment in the G2-H group. However, three of the left keratitis eyes administered MOX drops in G2-P did exhibit grade 2 conjunctival redness. One cornea was completely healed, while another had a grade 1 keratitis focus. Three corneas had a grade 3 keratitis focus, and one had a grade 4 keratitis focus. Additionally, all eyes with keratitis showed an increase in corneal thickness. Two eyes also had hypopyon, which was only present in eyes with grade 3 keratitis. However, after starting treatment, secretions disappeared in all eyes. As indicated in Figure 3b, bacterial viability on the cornea revealed 73% ± 9% inhibition with nearly 10^3 *Pseudomonas* viability on the cornea after treatment with MOX drops.

In Group 3, none of the right healthy eyes with MOX-impregnated CL inserted in the G3-H group showed any signs of secretions, conjunctival redness, edema, keratitis, or hypopyon. The removed CLs did not have any debris formation. Similarly, none of the left keratitis eyes treated with MOX-impregnated CL in the G3-P group showed conjunctival hyperemia or edema, and there were no secretions present in any of the eyes. In two of the eyes, the keratitis focus disappeared, while one cornea had grade 2 keratitis focus, two had grade 3, and one had grade 4. The corneal thickness increased in all eyes with keratitis. Only one eye had hypopyon, and it was the same eye that had grade 3 corneal keratitis. As shown in Figure 3b, the corneal infiltration was significantly decreased after treatment with MOX drop and MOX-impregnated CL, e.g., 47% ± 27% and 23% ± 22% infiltration areas in the eyes of the G2-P and G3-P groups, respectively. Furthermore, bacterial viability on the cornea treated with MOX-impregnated CL was significantly decreased to approximately 10^2 from 10^9 CFU/cornea, with 80% ± 14% bacterial inhibition, as shown in Figure 3b.

In Group 4, no secretions, conjunctival redness, edema, keratitis, or hypopyon were present in both eyes, including the healthy eye treated with soft CL in the G4-H group, and no debris was found.

3.3 | Histological Analysis

Corneal examination of the healthy eye for the control group (G1-H), MOX drops (G2-H), MOX-impregnated CL (G3-H), and bare CL (G4-H) treatments found a normal histological structure formed of many layers, including the outer stratified squamous nonkeratinized corneal epithelium, the stroma, Descemet's membrane, and endothelial cells, as seen in Figure 4.

In H&E and EZN stained sections, the superficial stroma layer displayed great aggregation of polymorphonuclear cell

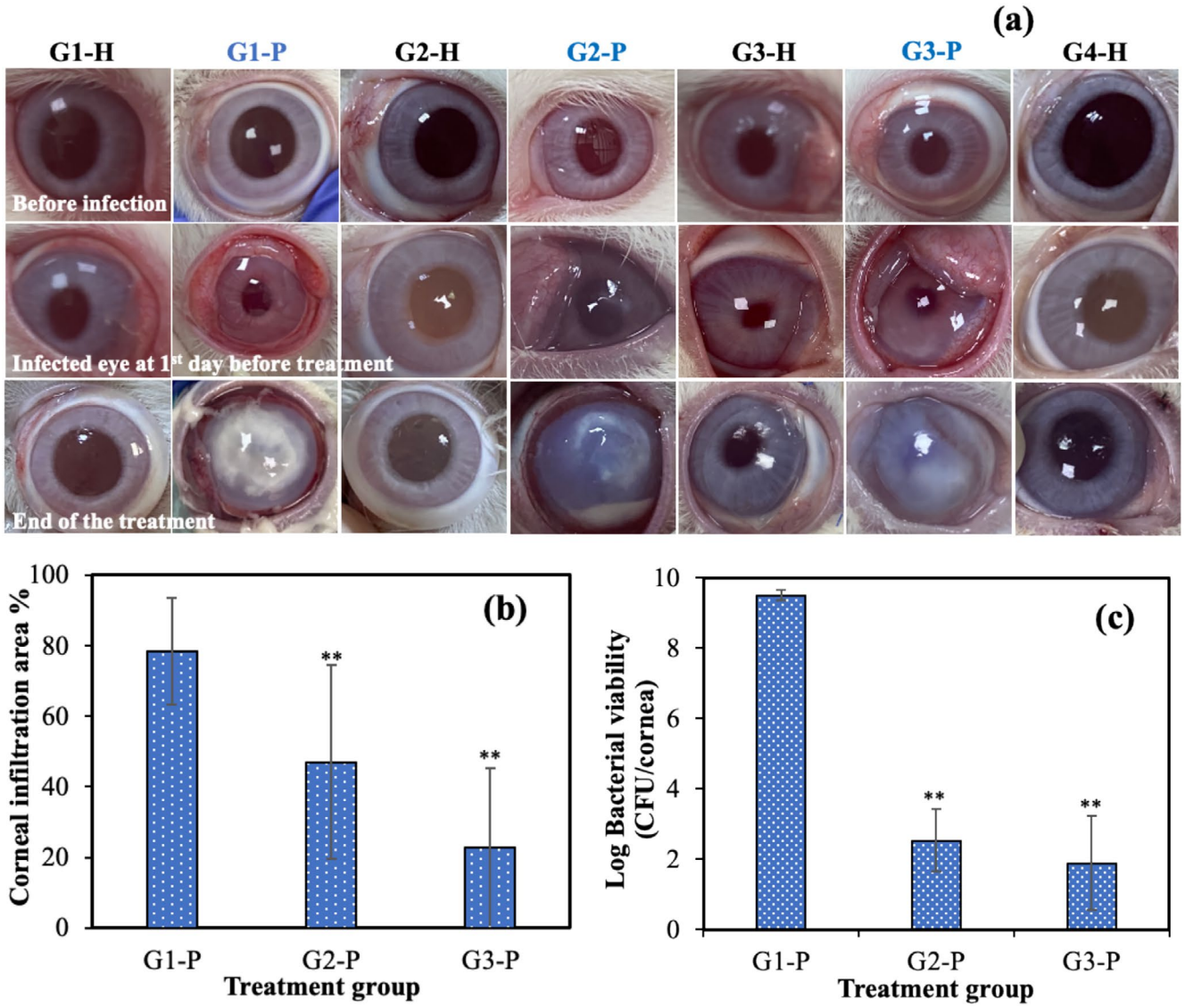

FIGURE 3 | (a) Photographs of the rabbit eyes in all groups. The images were taken before infection, 1st day before treatment, and at the end of the treatment. (b) Percentage of corneal infiltration area, and (c) bacterial viability of *Pseudomonas* keratitis (CFU/cornea) for keratitis groups with G1-P control, G2-P MOX drop treatment, and G3-P MOX-impregnated CL treatment at the end of treatment. Statistical analyses are expressed as $*p < 0.05$ and $**p < 0.001$ compared with the control group G1-P ($n = 6$).

TABLE 2 | Observed disease symptoms depend on the cornea for all groups in the keratitis model with number of rabbit eye ($n = 6$).

	Number of rabbits ($n = 6$)						
Disease finding	**G1-H**	**G1-P**	**G2-H**	**G2-P**	**G3-H**	**G3-P**	**G4-H**
Discharge	0	6	0	0	0	0	0
Conjunctiva (hyperemia/edema)	0	6	0	3	0	0	0
Hypopyon	0	N.I.[a]	0	2	0	1	0
Cornea (infiltration/edema)	0	6	0	5	0	4	0

[a]Hypopyon could not be identified.

infiltrations in the *Pseudomonas* keratitis group (G1-P) and keratitis group treated with MOX drops (G2-P), as shown in Figure 5a,c.

Lack of epithelial organization was detected in the same groups. Polymorphonuclear cell infiltrations were few in the keratitis group treated with MOX-impregnated CL (G3-P) compared to G1-P and G2-P groups, as indicated in Figure 5d. Larger areas of edema were observed in the stroma of the keratitis group compared to the MOX treatment and MOX-impregnated CL groups. Furthermore, it was determined that the Descemet membrane and endothelial cell layer disappeared in the keratitis group (G1-P). As seen in Figure 5e,f, the Descemet membrane and endothelial cell layer were detected in the MOX treatment and MOX-impregnated CL treatment groups. Foreign body giant cell formation was not observed in any group.

TABLE 3 | Clinical score for corneal infiltration in *Pseudomonas* keratitis eyes at the end of the treatment with the number of affected rabbit eyes ($n = 6$).

Grading	Number of rabbits ($n = 6$)		
	Keratitis control (G1-P)	Keratitis MOX drop (G2-P)	Keratitis MOX impregnated CL (G3-P)
0: No infiltration	0	1	2
1: Transparent cornea, rare spot infiltration, clearly visible iris	0	1	0
2: Mild corneal opacity, infiltration 1/4–1/2 of the entire cornea surface, visible iris	0	0	1
3: Severe corneal opacity, infiltration 1/2–3/4 of the entire cornea surface, faint iris	1	3	2
4: Infiltration 3/4 of the entire cornea surface, white opacity, invisible iris and pupil	5	1	1
5: Corneal perforated	0	0	0

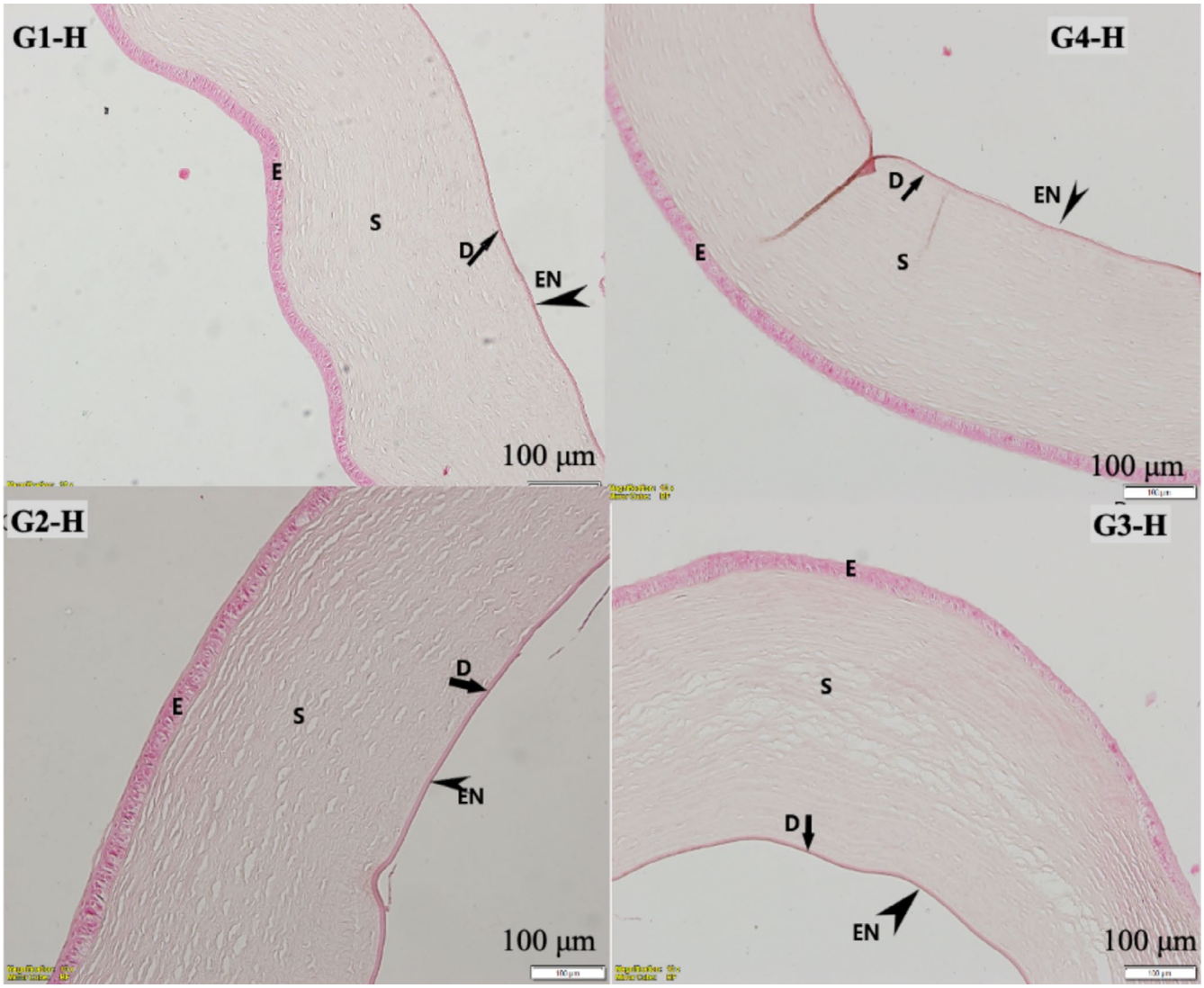

FIGURE 4 | Histopathological images of the cornea for the healthy eyes in control (G1-H), MOX drop treatment (G2-H), MOX impregnated CL (G3-H), and bare CL (G4-H) groups. The outer stratified squamous nonkeratinized corneal epithelium (E), stroma (S), descemet's membrane (D) and endothelial cells (EN) can be seen (H-E).

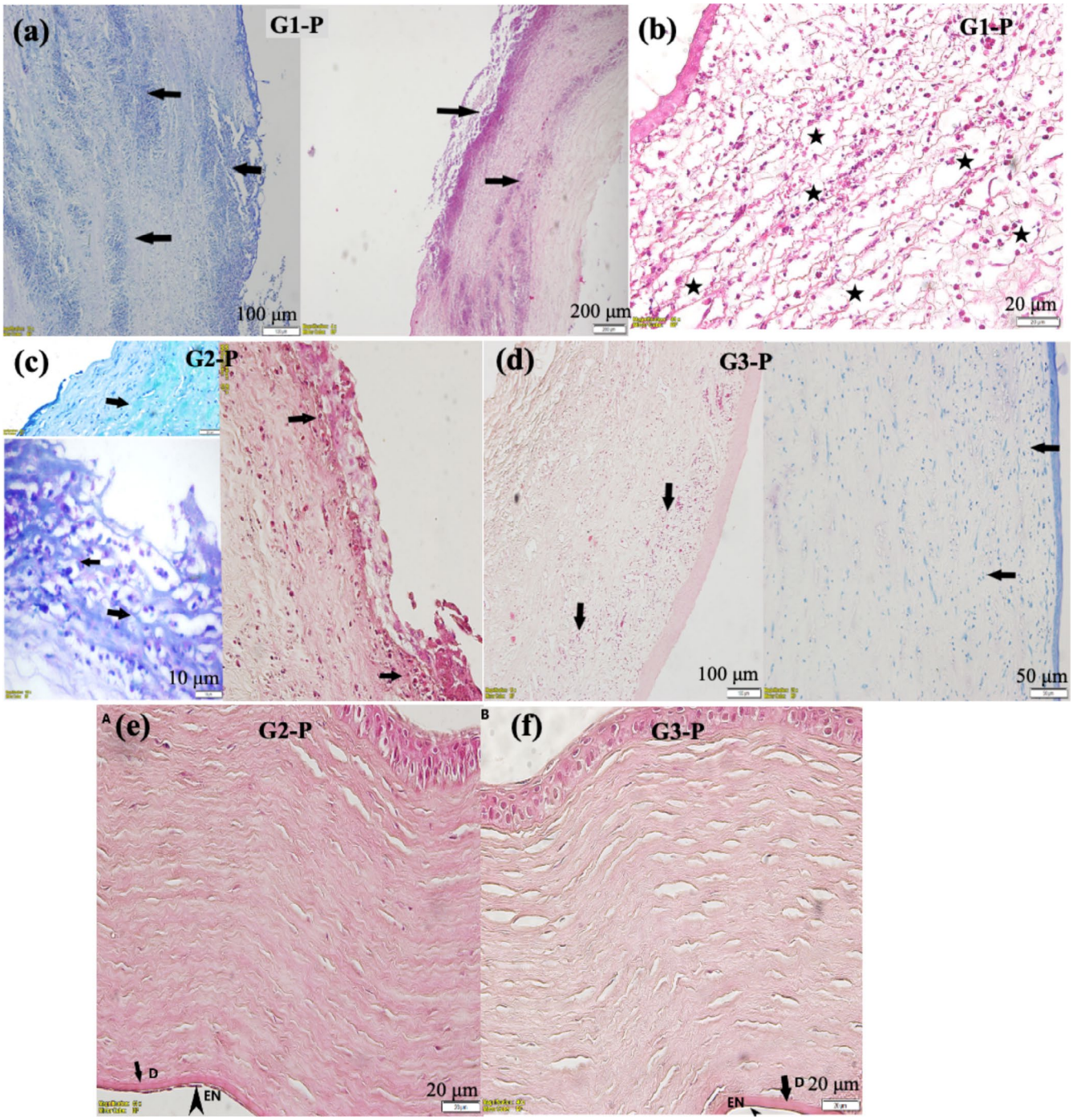

FIGURE 5 | Histopathological images of cornea for the keratitis eyes including (a, b) control (G1-P), (c) MOX drop treatment (G2-P), (d) MOX-impregnated CL (G3-P). There are many polymorphonuclear cells in the areas indicated by the arrows (EZN and H-E) and intense edema in the areas indicated by the stars for the control group (H-E). Polymorphonuclear cells are indicated by the arrows for MOX-impregnated CL treatment, and few polymorphonuclear cells are present in the areas indicated by the arrows for MOX-impregnated CL treatment. Descemet membrane and endothelial cell layer (e) MOX drop treatment and (f) MOX-impregnated CL (H-E).

Corneal epithelium and stroma thickness for each group were investigated by image analysis, and the results are summarized in Figure 6a,b, respectively.

According to the results, epithelium and stroma were approximately 20.1 ± 1.6 and $270 \pm 45\,\mu m$ thick in healthy corneal tissue, whereas the keratitis eye had 6.8 ± 2.4 and $1088 \pm 99\,\mu m$ epithelium and stroma thickness, respectively. As seen in Figure 6a, epithelium thickness was not significantly changed with MOX drop treatment. For MOX-impregnated CL treatment, epithelium thickness increased to $29.1 \pm 8.5\,\mu m$ in healthy eyes, but slightly decreased to $14.9 \pm 1.6\,\mu m$ in keratitis eyes. Similar to MOX-impregnated CL, bare CL slightly enhanced the thickness of the epithelium to $23.3 \pm 5.2\,\mu m$. As indicated in Figure 6b, stroma thickness was slightly decreased to $141 \pm 11\,\mu m$ for the G2-H group, which used MOX drop treatment on healthy eyes.

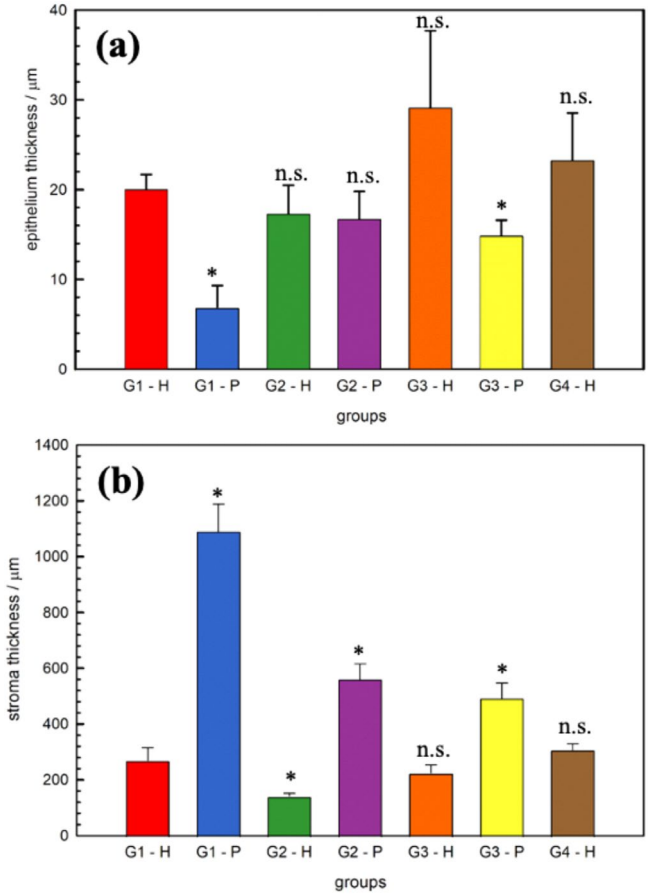

FIGURE 6 | (a) Epithelium and (b) stroma thickness for each group. Statistical analysis is expressed as $^*p < 0.05$ in comparison with the control group G1-H ($n = 5$, n.s. = no significance).

No statistical differences were present for MOX-impregnated or bare CL treatment in healthy eyes of the G3-H and G4-H groups, with 255 ± 28 and $307 \pm 22\,\mu m$ stroma thickness values, respectively. With keratitis treatment, the $1088 \pm 99\,\mu m$ stroma thickness for keratitis cornea decreased to $561 \pm 54\,\mu m$ in the MOX drop treatment G2-P group and to $493 \pm 53\,\mu m$ in the MOX-impregnated CL G3-P group.

4 | Discussion

Bacterial keratitis symptoms include pain, sensitivity to light, redness of the eye, discharge, corneal infiltration, and reduced vision. *P. aeruginosa* keratitis is a fast-progressing corneal disease. This type of keratitis, like other microbial keratitis, is primarily treated using eye drops. If keratitis is not promptly and appropriately treated, it can lead to blindness within a matter of days [3]. The most commonly used eye drops contain antibiotics from the quinolone group [2, 13]. The ease of removal of eye drops from the surface of the eye and their limited ability to penetrate the cornea may reduce their effectiveness. In cases of rapid keratitis progression caused by *P. aeruginosa*, it is essential to increase the frequency of drop administration due to low bioavailability [3]. This challenge can significantly impact patient compliance and disrupt the efficacy of treatment. Moreover, the reluctance to administer eye drops, particularly in children, and the tendency to blink and remove the drops may decrease the likelihood of achieving the therapeutic dose [35]. The same reflex was observed in rabbits in this study. Patient compliance is often cited as the primary challenge in treating keratitis. This is due to the fact that in order for the medication to effectively treat the condition, it must be applied frequently to the fornix conjunctiva. This can be particularly challenging for elderly patients, children, and those with medical conditions that make it difficult for them to administer the drops themselves. Properly administering the drops at specific intervals is crucial in maintaining a therapeutic dose on the ocular surface. Alternatively, a carrier system can be used to release a consistent amount of medication into the eye. This delivery system should not release the drug immediately but should be able to maintain adequate drug concentration on the eye surface daily, weekly, or even monthly, as if effective drops were being instilled at least 6 times a day. Therefore, it seems appropriate to attempt the use of intraocular carrier systems to facilitate treatment [3].

CLs are a safe and versatile tool that can be effectively used for various ocular diseases. In addition to their primary function, carrier systems have proven to be extremely advantageous in the treatment of numerous diseases [3]. There is a belief that they can be utilized with great effectiveness in the treatment of keratitis [36, 37]. They can be placed close to the focus of infection. They prevent the drug from being cleared by tears and may increase bioavailability by increasing turnover up to 30 min. CL can ensure timely and effective distribution of the drug, which is crucial as it aids in the treatment of infections and promotes local healing. The most important problem in CL carrier systems is sudden drug release, and this lack of continuity in drug release depends on the loading technique [1, 31]. In addition, drug-loaded CL could lose their properties such as transparency, wettability, oxygen-carrying ability, hardness, etc., during the loading process [12, 35, 36]. These issues include unhygienic use of CL, unsuitability for all patients, uncertainty about shelf life stability, difficulty in determining the most effective dose and release rate for each disease being treated, as well as the potential of CL to further exacerbate inflammation [1, 3, 38, 39]. In the current study, the MOX antibiotic was impregnated into CL using $ScCO_2$ fluid technology to provide high antibiotic loading, sustainable and long-term delivery at effective dosages for the treatment of *Pseudomonas* keratitis. This work is a continuation of our previous study about the optimization of MOX impregnation into soft CL via a $ScCO_2$ system for sustainable and long-term release. During the in vitro testing, the drug was released with a controlled release profile in a controlled manner without any initial burst release. The structural properties of the lens were well-preserved after the impregnation process. Also, $ScCO_2$ fluid significantly improved the dissolution and access of MOX into the CL matrix because of its well-known high mass transfer ability and almost zero surface tension [40]. Furthermore, homogeneous drug impregnation was accomplished, and sterile MOX-impregnated CL were obtained through the sterilization capability of $ScCO_2$ as a green solvent without leaving any residue.

Over the years, different types of drug-loaded CLs have been developed and frequently used in ocular applications. The most well-known diseases treated in this way are keratitis, glaucoma, keratoconus, and dry eye [41]. Most of the CL studies about the

treatment of infections in the literature were conducted in vitro. It was mentioned that bactericidal effects are observed by coating CLs with various molecules. Among the coatings of CL reported in the literature are epigallocatechin gallate, polyethyleneimine-graft-polyethylene glycol methacrylate, glycidyl methacrylate, Zn-CuO, MEL4, and PVA-anionic collagen to produce bacteria-specific coatings [29, 42–45]. The use of zwitterionic coating effectively prevented the adhesion of multiple types of bacteria and fungi [46]. These coatings were prepared with the aim of protecting microbial adhesion, rather than being therapeutic. In a study testing the release of ofloxacin in vitro from CL loaded with drugs by the soaking technique, a bactericidal effect was demonstrated, although this effect diminished significantly after 48 h [13]. In another study, maximum release from CLs loaded with ciprofloxacin by molecular imprinting could only be observed in the first 24 h, and most of the antibiotic was released within only 3 h [47]. In a study where attempts were made to treat *P. aeruginosa* in vivo, although the treatment was continued for a short time, improvement was observed. It was also reported that an intense release was observed only in the first 8 h from the MOX-imprinted CL [15]. It showed the same effect on in vitro *S. aureus* and *P. aeruginosa* [31], which is very similar to the present study. In another study, MOX-imprinted silicone hydrogel CL material rendered extended drug release and was found to be effective against the *S. aureus* strain, but the maximum effect was observed in the first days, and no cytotoxicity was detected [18]. In a Polymyxin B study where release was examined, the imprinting technique released the drug regularly and was not toxic to the chorioallantois membrane [48]. Also, an improvement in the keratitis model was detected after 7 days using the Morgan lens as a drug delivery device, but the toxic effect could not be evaluated in vivo [6]. In the current study, the possibility of a toxic effect was examined on corneal cells in vivo in rabbits, and no toxic effect was observed by comparing with the control group. MOX-impregnated CL prepared employing $ScCO_2$ technology had a gradual release profile up to 200 h without any significant toxicity, which is an excellent result compared to CL loaded with drugs by using common methods such as soaking (from the corresponding drug solution) in the literature.

In in vivo studies inducing a bacterial keratitis model in rabbit eyes where bacterial inhibition was achieved especially in the first 48 h, using a hydrogel carrier as therapeutic CL for delivery of gatifloxacin as a slow release and long-term treatment was not fully optimized [49]. The present study involved the monitoring of rabbits using CL for up to 7 days. A significant decrease in eye secretions was observed after the first 24 h. At the end of 7 days, 2 corneas were completely healed, while keratitis focus progression and corneal perforation were not observed in 4 others in group 3. There were no side effects of the CL observed, such as redness, corneal epithelium defect, or corneal thinning, in eyes that did not exhibit keratitis but were exposed to CLs. One of the reasons why the CL group had more successful results may be due to the resistance to treatment in the eye drop group. A better response may have been obtained in rabbits where more drops remained in the eye. Here, microbial load from tears and aqueous matter could not be studied, and this is one of the limiting factors of the current study. Furthermore, an additional factor contributing to the success of CL is their potential as drug-eluting bandage CLs, effectively alleviating pain, promoting epithelialization, and mitigating the effects of disease-induced corneal defects [6, 50]. The rabbits' eyelids were stitched to prevent the CLs coming out of the eyes, so not all areas of the rabbits' corneas could be evaluated every day. This may be considered another limiting factor in the current study. Another limitation of the study is that the number of subjects increased before the study, making it impossible to conduct a Draize test. However, groups 2, 3, and 4 were used as both control group and test group for the Draize test. No side effects were observed in the healthy eyes of subjects that received the antibiotic drops or wore the CLs.

5 | Conclusions

MOX antibiotic was impregnated into CL via $ScCO_2$, providing a promising alternative to eye drops for the treatment of *Pseudomonas* keratitis in vivo. The use of $ScCO_2$ provided many advantages without affecting the CL, e.g., no structural features, including transparency, wettability, and hardness of CL, which could hinder the vision were detected after the drug-loading process. The prepared MOX-impregnated CL allowed prolonged and gradual antibiotic release from the matrix for up to 7 days with no cytotoxicity. Similarly, MOX-impregnated CL provided long-term therapeutic treatment comparable to the conventional treatment of MOX eye drops for in vivo keratitis treatment. MOX-impregnated CL were safe and biocompatible in animal models. The results of this study highlight the promising potential of new treatments in eye-related diseases using CL for long-lasting therapeutic benefits, while also prioritizing safety and biocompatibility for patients. MOX-impregnated CL are convenient, safe, and afford independent patient usability. As research in this area continues to advance, these innovative approaches have great promise to significantly enhance the efficacy and convenience of corneal treatments for various ocular conditions. Overall, MOX-impregnated CL prepared via the $ScCO_2$ impregnation technique is considered an excellent system that can replace eye drops to facilitate keratitis treatment without any toxicity according to the in vivo model.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data generated is contained within this manuscript and in Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.