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Research Article

Pharmacokinetic-Pharmacodynamic Analysis of Cephalexin against *Streptococcus parauberis* in Olive Flounder (*Paralichthys olivaceus*: Temminck and Schlegel)

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Olive flounders are susceptible to annual outbreaks of streptococcosis, which accounts for approximately 10% of all fish farm diseases and is associated with high mortality rates. The development of an antibiotic therapy against streptococcosis is thus necessary. This study determined the therapeutic effects of varying cephalexin concentrations in *Streptococcus parauberis*-infected olive flounders and evaluated its histopathological toxicity and residual concentration in the fish. Compared with the control group, the 200 and 800 mg/kg cephalexin groups showed significant mean survival rates of approximately 10% and 30%, respectively, and the 400 mg/kg group showed the highest survival rate of approximately 40%. The average residual cephalexin concentration in muscle samples on day 1 post-cephalexin administration was 13.21 μg/kg, showing a rapid decrease. At the optimum water temperature (25°C), cephalexin was rapidly metabolized within 24 hr of its administration being terminated, and most of it was excreted from the bodies of the fish. A histopathological analysis showed that the oral administration of cephalexin did not lead to specific inflammatory lesions, and there were no significant differences between the treatment and control groups. Our novel findings suggest that cephalexin is a promising candidate for treating streptococcosis outbreaks in fish farms.

1. Introduction

The olive flounder (*Paralichthys olivaceus*) is an important species of aquacultured fish in South Korea. As the domestic demand for olive flounders has increased, the practice of high-density aquaculture has become popular to enhance productivity; however, this practice has increased the incidence of infectious diseases and associated losses of stock. Several outbreaks of streptococcosis occur in olive flounder farms annually, with the disease representing approximately 10% of all fish farm diseases [1–3]. In particular, *Streptococcus parauberis* strains are the primary causes of streptococcosis, and their incidence has surpassed that of *Streptococcus iniae* [4, 5]. Streptococcosis is caused by gram-positive bacteria, causes hyperpigmentation, exophthalmos, fin hemorrhages, and septicemia, and has a mortality rate of more than 50% in infected fish populations [6]. The number of streptococcosis outbreaks in olive flounder farms is increasing, and there is an urgent need to develop an effective antibiotic therapy.

Cephalexin (CH) is a first-generation cephalosporin antibiotic that was developed in 1967. It was approved by the US Food and Drug Administration for use as an antibiotic in

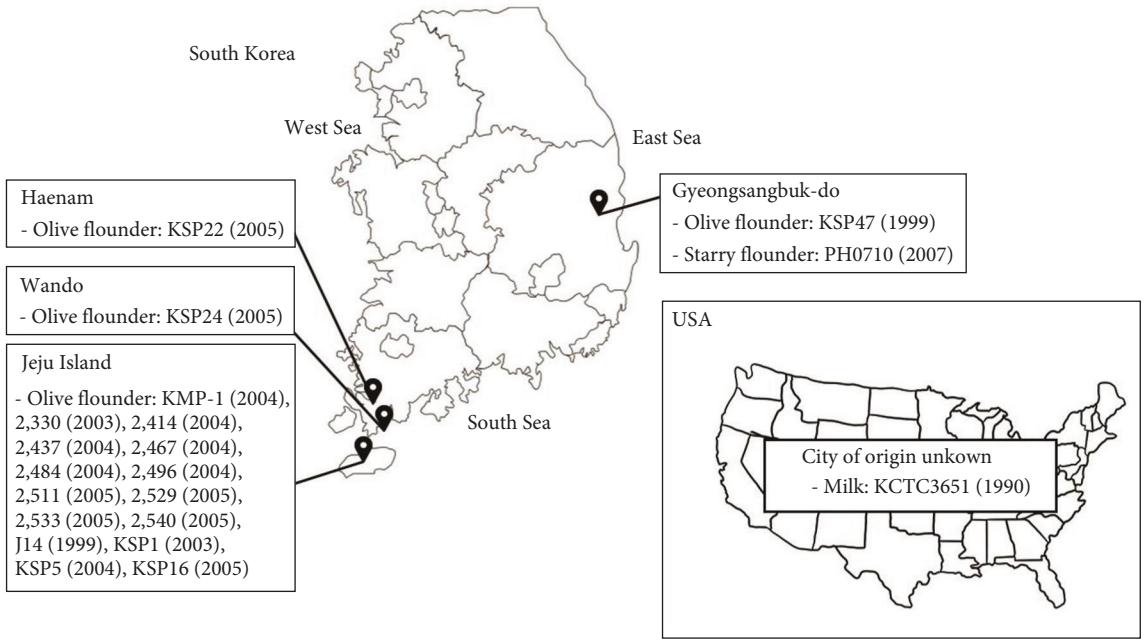

Figure 1: Isolated *Streptococcus parauberis* strains used to evaluate the minimum inhibitory concentration (MIC) of cephalexin *in vitro*. The year of isolation is shown in parentheses. Strain 2,529 was used for the *in vivo* challenge experiment.

humans and animals [7–10]. Its structure contains a β-lactam ring that can inhibit the synthesis of peptidoglycan, which is critical for bacterial cell wall formation. CH, therefore, inhibits the growth of gram-positive and certain Gram-negative bacteria [11]. It is further used as a broad-spectrum antibiotic in livestock and aquaculture facilities to treat infections, such as acute and chronic urinary tract infections, gonorrhea, upper and lower respiratory tract infections, and scarlet fever, caused by staphylococcal and streptococcal species [12–14]. CH also offers the advantages of being inexpensive, relatively safe compared with other antibiotic agents, and can be orally administered in both humans and animals [15].

Injections and oral administration are the two most common methods used to introduce antimicrobial agents in fish farms. The injection route is less frequently used in aquaculture, owing to the high labor costs involved and the possibility of damaging the skin of the fish or causing infection. The oral route is preferred as it overcomes these limitations [16, 17] and can even be applied to small-sized fish. The oral route of administration is thus considered to be the optimal method for safely and homogenously delivering antimicrobial agents to farmed fish.

The therapeutic efficacy of CH in fish has been reported against pasteurellosis in cultured yellowtail fish (*Seriola quinqueradiata*) [18]; however, the efficacy, absorption, decomposition, and metabolism of antimicrobial agents differ among various fish species. It is thus necessary to determine the optimal dose, mode of administration, and residual concentration of each antibiotic and synthetic antibacterial agents for each fish species [19, 20].

To the best of our knowledge, the efficacy and safety of CH in olive flounders have not yet been investigated. Therefore, in this study, we aimed to determine the therapeutic effects of orally administered CH (at varying concentrations) in olive flounders infected with *S. parauberis* and to evaluate the histopathologic toxicity and residual concentrations of CH within the bodies of the fish.

2. Materials and Methods

2.1. Sensitivity and Efficacy of Cephalexin against S. parauberis Strains In Vitro

2.1.1. Bacteria. The *in vitro* efficacy of CH was tested against 20 strains of *S. parauberis* isolated from Jeju, Gyeongbukdo, and Wando in South Korea and the United States (Figure 1). The *S. parauberis* strains were cultured and maintained at 28°C in Luria-Bertani (LB) broth to determine their sensitivity to CH.

2.2. Measurement of the Minimum Inhibitory Concentration (MIC). Each *S. parauberis* strain was cultured by being shaken in LB broth until an absorbance of 1.0 at 280 nm, equivalent to 1×10^5 colony forming units (CFUs)/mL, was reached. Next, 10 μL of 10-fold serially-diluted CH (TCI, C2248, Japan, stock solution 50 μg/mL) was used for inhibitory efficacy testing. The bacteria were cultured for 16 hr in a shaking incubator set at 28°C. The MIC was defined as the CH concentration at which bacterial growth (corresponding to colony formation on an agar LB plate) was not observed for each strain. *S. parauberis* suspensions were prepared according to the Clinical and Laboratory Standards Institute guideline VET04-A2 for MIC tests (CLSI, 2014).

2.3. Effects of Cephalexin in Olive Flounder

2.3.1. Experimental Fish. Olive flounders ($n = 330$; average length: 15 ± 0.98 cm) obtained from a fish farm in Taean City, Chungcheongnam-do, South Korea, were acclimatized

Table 1: Primer sequences and polymerase chain reaction conditions were used to detect *Streptococcus parauberis*.

Pathogen	Primer name	Sequence	Product size (bp)	Reference
S. parauberis	Spa 2,152 Fw	5′-TTT CGT CTG AGG CAA TGT TG-3′	718 bp	[21]
	Spa 2,870 Re	5′-GCT TCA TAT ATC GCT ATA CT-3′		
PCR step	Temperature	Reaction time	Cycles	
Predenaturation	95°C	5 min	1	
Denaturation	95°C	30 s		
Annealing	48°C	30 s	30	
Extension	72°C	1 min		
Final extension	72°C	5 min	1	

for 1 week in an aqua tank system maintained at 18°C. During the acclimation period, 10 fish were randomly sampled and tested using polymerase chain reaction (PCR) to determine whether they were free of *S. parauberis* infection. Table 1 lists the PCR primer sequences and conditions used for detecting *S. parauberis* [21]. Each treatment group was divided into eight subgroups, including the control group, with 10 fish per group and four replicates. Each group was housed in a separate 100-L capacity recirculation-type aquarium (20 L of seawater changed per day) set at 18°C with the salinity levels adjusted to 30% (adjusted using Reef salt Mix; KENT MARINE; sea salt, USA).

2.3.2. Efficacy of Cephalexin in S. parauberis-Infected Fish. Of the *S. parauberis* strains, strain 2,529 (Jeju Island, South Korea, 2015) was selected for the MIC experiment as it showed moderate CH sensitivity. For virulence recovery, the *S. parauberis* strain 2,529 was cultured at 28°C for 24 hr on an LB plate containing 5% horse blood lysates. The fish in all the groups (except the control group) were equally infected with 4.2×10^7 CFUs/100 μL of *S. parauberis* (lethal dose 50) via intraperitoneal injections. The CH agent (Aquacefa, Chamshin Holdings Co. Ltd., Korea) was formulated in eight doses (0, 50, 100, 200, 400, 800, 1,600, and 3,200 mg/kg of fish body weight) and orally administered once daily for 7 days using the intubation method. The control group was orally administered an equal amount of distilled water (DW) instead of the CH agent (Figure 2). The cumulative mortality rates were measured for 19 days, starting from the first day of the oral administration of the CH agent. The presence of *S. parauberis* was subsequently confirmed in the kidneys, spleens, and livers of the dead fish using PCR analyses.

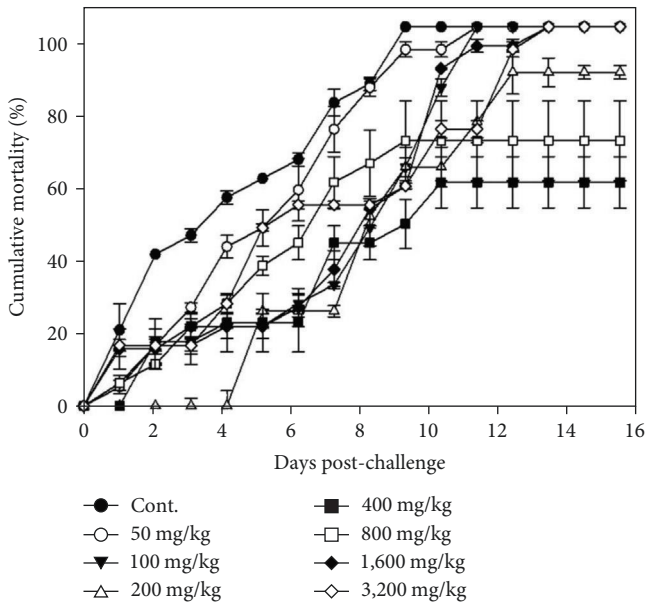

Figure 2: Cumulative mortality rate of olive flounders following the oral administration of different cephalexin agent concentrations (0, 50, 100, 200, 400, 800, 1,600, and 3,200 mg/kg of fish body weight) for 7 days post-*S. parauberis* infection.

2.4. Measurement of Residual Cephalexin Concentrations in the Muscle of Olive Flounder

2.4.1. Standard Curve and Recovery Rate Measurements of Cephalexin. CH was extracted from the muscles of the fish in accordance with the Korean Food Standards Codex developed for animal tissues. A standard CH stock solution was diluted to seven concentrations (0, 10, 25, 50, 100, 200, and 400 ppb) using 50% methanol (SAMCHUN, Korea).

As the analytical method used in this study [22] was developed for other animal food products rather than fish, the technique was validated in olive flounder tissues according to the criteria of the method validation procedure [23]. In addition, the CH levels were measured three times consecutively at the start and end of the analysis to determine whether there were any significant time-dependent or instrument-related changes in the standard curve linearities of the spiked CH standards.

2.4.2. Cephalexin Oral Administration and Sampling of Olive Flounder Muscle. Olive flounders ($n = 64$; average length: 15 ± 0.22 cm) were obtained from a fish farm in Taean City, Chungcheongnam-do, South Korea, and acclimatized for 1 week in an aqua tank system maintained at 18°C. The CH administration (test) and CH nonadministration (control) groups were divided into separate water tanks in duplicate. To evaluate the effects of water temperature on the residual CH concentration in the fish muscle, analyses were conducted at an optimum water temperature (22°C; Experiment I) and low water temperature (15°C; Experiment II).

The test and control groups were orally administered 400 and 0 mg/kg of CH, respectively, by intubation for 7 days. Subsequently, 16 fish per group (two fish poolings; total,

eight samples) were sampled on days 1, 3, 6, 12, 19, 26, and 31 following the completion of the CH administration to measure the residual CH concentrations in the fish muscles, which are edible parts of the fish.

2.5. Sample Extraction and Liquid Chromatography–Tandem Mass Spectrometry Analysis of Cephalexin. Tissue samples (2 g) obtained from the fish in each group were accurately weighed and homogenized with a 10-mL solution containing 1 mL of ammonium acetate buffer (Sigma–Aldrich, USA) and 9 mL of 2 mM ammonium formate (Junsei, Japan). The samples were cultured by being shaken for 5 min and then centrifuged at 10,000 × *g* for 10 min. The supernatant was separated, and 250 mg of C18 (SUPELCO, USA) and 250 mg of PSA (SUPELCO, USA) were added to it. The samples were then incubated (with shaking) for 1 min, followed by 10 min of centrifugation at 10,000 × *g* and 4°C. The samples were then concentrated from 5 to 1 mL using a nitrogen-blowing concentration evaporator (Goojung EvaT-0200, South Korea) at 40°C. The concentrated samples were filtered using a 0.2-μm polyvinylidene fluoride syringe filter (Whatman). Finally, a CH residual analysis was performed using a high-performance liquid chromatography-tandem mass spectrometry system that included a residual concentration analyzer, liquid chromatograph (ACQUITY H Class UPLC, WATERS, USA) functioning as a sample injector, and mass spectrometer detection system (Xevo TQ-S micro, WATERS) equipped with an Acquity UPLC BEH C18 reversed-phase column (2.1 × 50 mm, 1.7 μm, WATERS).

2.6. Histotoxicity of Cephalexin in Olive Flounder

2.6.1. Cephalexin Administration in Fish. Olive flounders ($n = 36$; average length: 15 ± 0.51 cm) were obtained from a fish farm in Taean City, Chungcheongnam-do, South Korea, and acclimatized for 1 week in an aqua tank system maintained at 18°C. Each treatment group was divided into two groups (including the control group), with 18 fish per group.

The optimal therapeutic effect was observed when 400 mg/kg of CH was administered to the *S. parauberis*-infected fish for 7 days. To evaluate the toxicity in the fish organs at this dose, the histopathological toxicity of CH in the livers, spleens, and kidneys was examined after feeding 400 mg/kg of CH to the fish for 10 days; the feeding period was extended from 7 to 10 days to increase the reliability of the results. The CH administration (test group) and control groups were divided into separate water tanks. The test group was orally administered 400 mg/kg of CH for 10 days, and the control group was orally administered an equal volume of DW. On days 1, 5, and 10 from the start of the oral administration of CH and days 3, 6, and 12 following the completion of oral CH administration, organs were resected from three fish in each group to conduct a histopathological analysis.

2.6.2. Histopathological Toxicity Analysis. As mentioned in the section above ("*Cephalexin administration in fish*"), three fish in each group were anesthetized in an MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma–Aldrich) anesthetic bath on each of the sampling dates. Organ tissues from the livers, spleens, kidneys, and stomachs were then cut and fixed in a 10% formalin solution (formaldehyde solution, 35.0%, SAMCHUN) for 48 hr. The tissues were trimmed using an automated tissue processing system (TP 1020, Leica, Germany), dehydrated in a series of graded ethanol solutions (70%–100%), cleaned in xylene (SAMCHUN), and embedded in paraffin. The embedded tissues were serially sectioned to a thickness of approximately 5–6 μm using a microtome (Leica, RM 2135, Germany), subjected to hematoxylin and eosin (H&E) staining, and analyzed using an optical microscope (Leica, DM500, Germany).

Table 2: *In vitro* minimum inhibitory concentrations (MICs) of CH against *S. parauberis*.

No.	Strain code	MIC (μg/mL)
1	PH0710	4.64
2	KSP47	4.89
3, 4	KMP-1, J14	4.92
5	2529	4.93
6	KSP22	4.97
7	KSP16	4.99
8	KSP24	5
9, 10	2,437, 2,540	49.62–49.64
11	KCTC3651,	49.72
12–16	2,533, 2,414, 2,330, 2,511, 2,496	49.80–49.87
17–19	2,484, 2,467, KSP5	49.91–49.92
20	KSP1	>50

2.7. Statistical Analyses. The data are expressed as the mean ± standard deviation. The results were statistically analyzed using log-rank tests and Kaplan–Meier analyses (GraphPad Software, USA). Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. In Vitro Sensitivity and Efficacy of Cephalexin against S. parauberis Strains. The MICs of CH were measured for the 20 *S. parauberis* strains. The results revealed that eight strains (*S. parauberis* KMP-1, 2529, J14, KSP16, KSP22, KSP24, KSP47, and PH0710) were inhibited at ≤5 μg/mL of CH, while 11 strains (*S. parauberis* KCTC3651, 2330, 2414, 2437, 2467, 2484, 2496, 2511, 2533, 2540, and KSP5) were inhibited at 49–50 μg/mL of CH. The remaining strain (*S. parauberis* KSP1) exhibited high resistance, with a MIC of 50 μg/mL or more (Table 2). To test the therapeutic effects of CH in bacteria-infected olive flounders, the *S. parauberis* strain 2,529, which is highly sensitive to CH, was selected.

3.2. Effects of Cephalexin in S. parauberis-Infected Fish. After infecting the olive flounders with *S. parauberis*, the CH agent was orally administered at different doses for 7 days to evaluate its therapeutic effects. Compared with the control group, the group administered 400 mg/kg of fish weight showed a significantly higher survival rate of approximately 40%. Until day 13 post-oral administration, 100% mortality

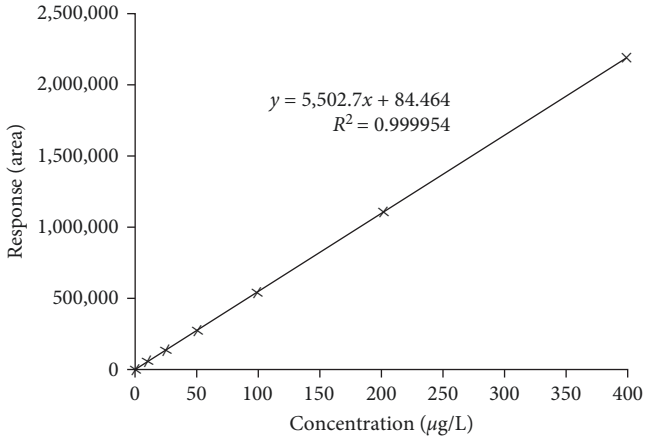

Figure 3: Standard curve of cephalexin was generated using standard solutions containing seven cephalexin concentrations (0, 10, 25, 50, 100, 200, and 400 ppb) obtained using liquid chromatography–tandem mass spectrometry.

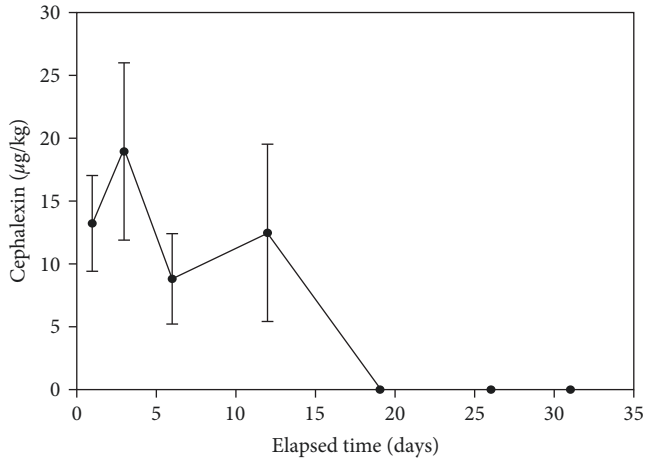

Figure 4: Residual cephalexin concentration in olive flounder muscles 31 days following the oral administration of cephalexin at 400 mg/kg of fish body weight at the optimum water temperature (22°C) for 7 days. Data are expressed as the mean ± standard deviation (SD) from eight fish at each time point.

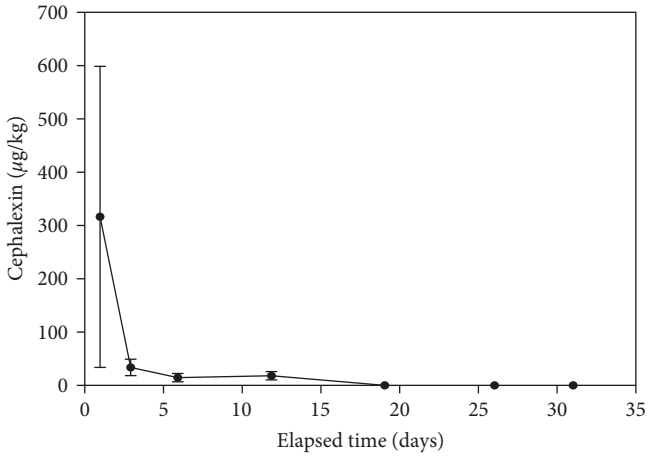

Figure 5: Residual cephalexin concentration in olive flounder muscles 31 days following the oral administration of cephalexin at 400 mg/kg of fish body weight at a comparatively low water temperature (15°C) for 7 days. Data are expressed as the mean ± SD from eight fish at each time point.

was observed in the groups administered with 0 (control group), 50, 100, 1,600, and 3,200 mg/kg of the CH agent; however, the groups administered with 200 and 800 mg/kg of the CH agent showed significant survival (mean survival rates: approximately 10% and 30%, respectively), which was maintained for up to 15 days (Figure 2). In conclusion, the oral administration of the CH agent (400 mg/kg of fish weight) showed the optimal therapeutic effects against *S. parauberis* infection.

3.3. Cephalexin Pharmacokinetics in Olive Flounder Muscle (Residual Concentration)

3.3.1. Standard Curve and Recovery Rate Measurements of Cephalexin. A calibration curve (Figure 3) was obtained by plotting the peak area ratios of seven standard CH concentrations (0, 10, 25, 50, 100, 200, and 400 ppb). The calculated regression line rendered a perfect fit ($R^2 > 0.9994$) for the standard solutions. The CH recovery from the olive flounder muscles ranged from 80% to 90%, as determined by extrapolating these values from known standard concentrations.

3.3.2. Experiment I: *Residual Cephalexin Concentration in the Muscle of Olive Flounders Grown at the Optimal Water Temperature (22°C).* The residual CH concentration in the muscles of the fish grown at a water temperature of 22°C was measured on days 1, 3, 6, 12, 19, 26, and 31 following the oral administration of 400 mg/kg of CH. On day 1 post-CH administration, the residual CH concentration in the fish muscle samples was considerably low, with an average of 13.21 μg/kg. On day 3, the average intramuscular CH concentration was 18.94 μg/kg. From day 12 onward, CH was only detected in two out of the eight samples but could not be detected in any samples from days 19 to 26 and 31. In the control groups, CH was not detected in any of the fish muscle samples at any of the time points (Figure 4).

3.3.3. Experiment II: *Residual Cephalexin Concentrations in Olive Flounder Muscles at a Low Water Temperature (15°C).* The residual CH concentrations in the muscles of the fish grown at a water temperature of 15°C were measured on days 1, 3, 6, 12, 19, 26, and 31 following the oral administration of 400 mg/kg of CH. Similar to the findings at a water temperature of 22°C, the average residual CH concentration in the fish muscle samples on day 1 was 313.9 μg/kg of fish body weight. On days 3 and 6, the CH concentrations in the fish muscle samples declined to 30.8 μg/kg and 11.2 μg/kg, respectively, and gradually decreased thereafter until day 19. On days 19, 26, and 31, CH was not detected in any of the fish muscle samples. CH was not detected in any of the fish muscle samples from the control group at any of the time points (Figure 5).

3.3.4. Histotoxicity of Cephalexin in Olive Flounder. A comparative analysis was performed for the CH contents of the

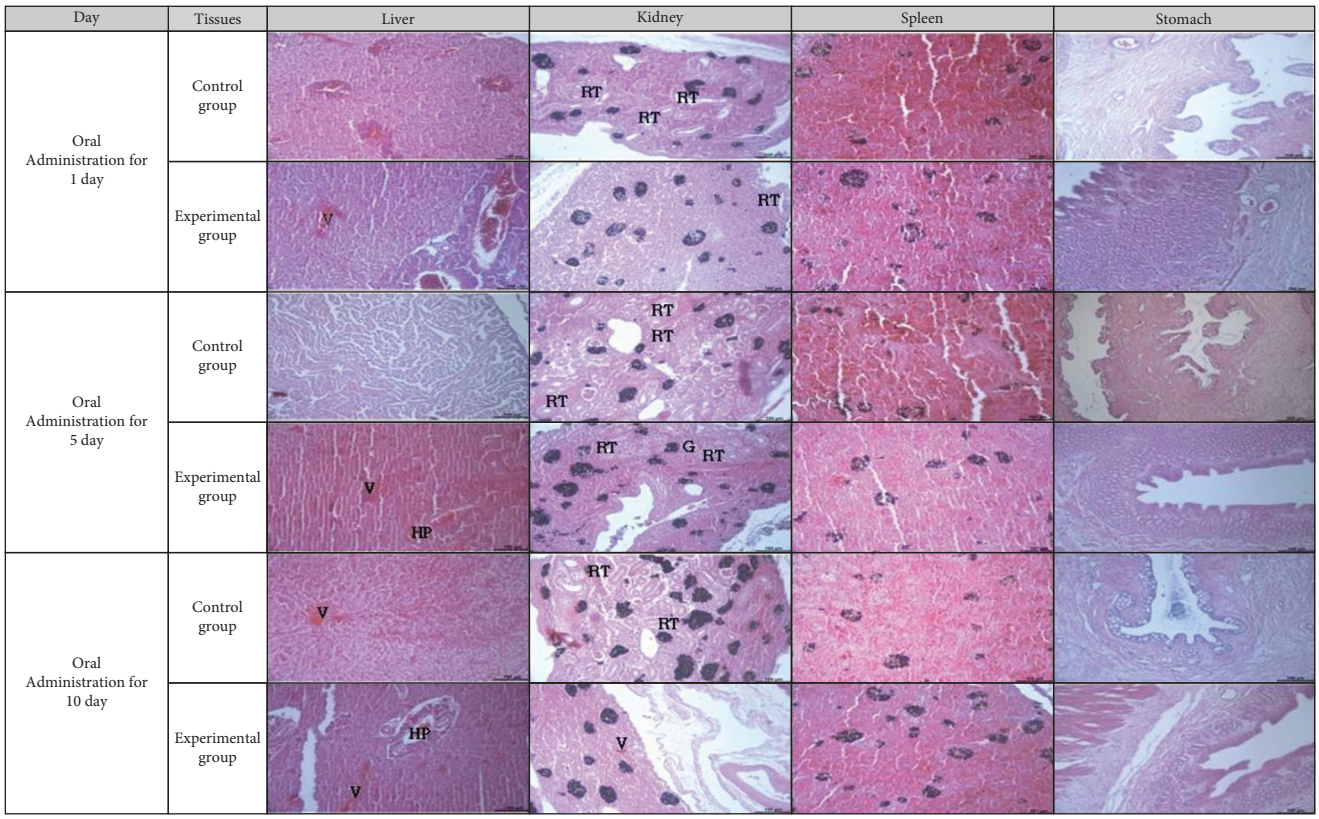

FIGURE 6: Images following the hematoxylin and eosin (H&E) staining (scale bar = 100 μm) of the liver, kidney, spleen, and stomach tissues collected on days 1, 5, and 10 following the initiation of oral CH administration (400 mg/kg) in the experimental group and distilled water in the control group. HP, hepatopancreatic tissue; IT, interstitial tissue; RT, renal tubule; G, glomerulus; V, venous.

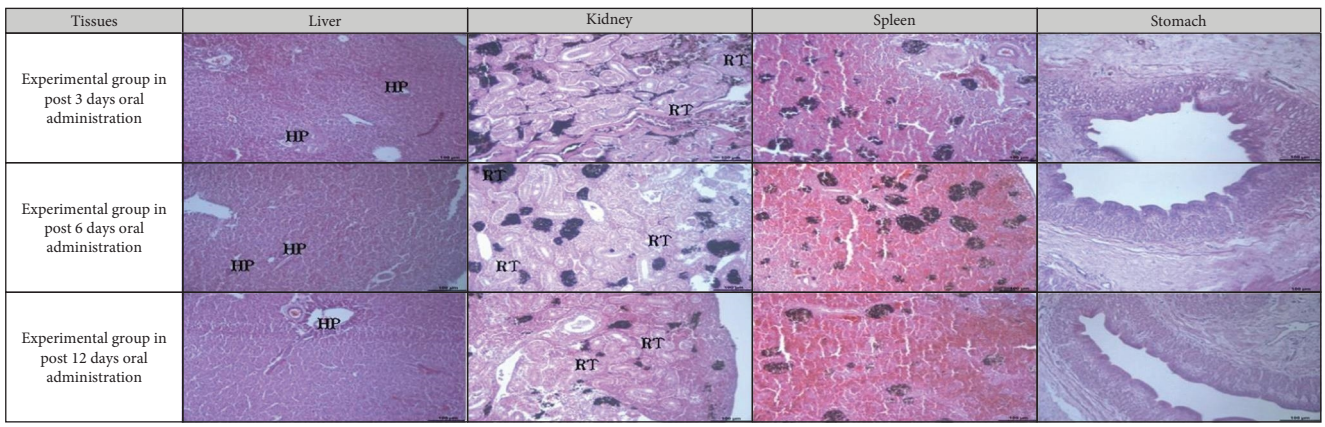

FIGURE 7: Images following the H&E staining (scale bar = 100 μm) of the liver, kidney, spleen, and stomach tissues collected on days 3, 6, and 12 following the completion of oral CH administration (400 mg/kg) until day 10 in olive flounders. HP, hepatopancreatic tissue; IT, interstitial tissue; RT, renal tubule; G, glomerulus; V, venous.

organ tissues (livers, kidneys, spleens, and stomachs) of the test and control groups on days 1, 5, and 10 following the initiation of CH administration and on days 3, 6, and 12 following the completion of the CH (400 mg/kg) treatment for 10 days. There were no notable differences between the two groups (Figures 6 and 7). A histopathological toxicity analysis revealed no specific inflammatory lesions and no significant differences were observed between the groups. These results show that the oral administration of CH (400 mg/kg of fish body weight) for 7 days did not induce organ toxicity in the olive flounders.

4. Discussion

Administering antibiotics is currently the method of choice for treating most bacterial diseases in fish farms. Of the

antibiotics that may be used, CH, which is a derivative of cephalosporin, is advantageous because it is easily absorbed from the intestinal tract and can be administered orally [11, 24, 25].

CH is useful for treating various nonspecific infections caused by staphylococci, streptococci, *Enterobacteriaceae*, and some anaerobic bacteria [26]. The MIC of CH against *S. iniae* is known, but to the best of our knowledge, there are no available data reporting the MIC of CH against *S. parauberis*, which causes streptococcosis. Lim et al. [27] and Park et al. [28] previously reported that the MICs of CH against *S. iniae* range from 0.5 to 20 μg/mL and 0.125 to 256 μg/mL, respectively. In our study, the MIC analysis showed that nine of the 20 *S. parauberis* isolates were inhibited by CH at concentrations of $\leq$5 μg/mL. When the CH concentration was $<$50 μg/mL, all but one of the *S. parauberis* isolates were inhibited, which suggests that resistant strains can emerge at various concentrations of CH.

Several reports have highlighted the presence of antibiotic-resistant bacteria in finfish within aquaculture settings [29–33]. This resistance emerged within a few years of treating infections with antibacterial drugs [34, 35]; it has since limited the applicability of using these antibiotics to control bacterial diseases in fish [36] and has become a public health concern. Thus, antibiotic susceptibility testing for pathogenic bacteria is crucial for identifying bacterial characteristics and determining the resistance of each strain [37]. Here, we determined the CH tolerance ranges of 20 *S. parauberis* isolates derived from South Korea because these strains can acquire CH resistance more easily than they can acquire resistance to other antibiotics [13]. *S. parauberis* generally shows cross-resistance to CH, and when a heavy inoculum is used, such cross-resistance can also be observed with other cephalosporins and ampicillin [38–41]. Antibiotics, such as cephalosporins, inhibit cell wall synthesis and are only effective against actively growing bacteria; thus, they are more effective when administered at prolonged dose intervals as this allows for a short bacterial binary fission period between doses [42].

It is critical to evaluate the efficacy of an antibiotic against a bacterial disease to determine its ability to treat infections and prevent antibiotic resistance. CH has high systemic bioavailability in fish following oral administration, but the appropriate concentration, dosage, and administration duration must be determined before treating susceptible gram-positive infections, particularly those caused by *Streptococcus* and *Staphylococcus* spp. in fish farms. This is important as CH is primarily used in fish farms in South Korea during streptococcosis outbreaks. To the best of our knowledge, the present study is the first to report the therapeutic effects of CH in olive flounders.

The present study analyzed the effects of varying CH doses on the *S. parauberis* strain 2,589, which causes streptococcosis in olive flounders. Our results were obtained by conducting *in vitro* and *in vivo* experiments to determine the optimal CH dose regimen for *S. parauberis*-infected olive flounders and showed that the optimal oral CH dose and duration for treating *S. parauberis* infection was 400 mg/kg of fish body weight for 7 days. The results suggest that oral CH administration can effectively treat gram-positive bacteria-induced diseases in olive flounders. Therefore, we determined the therapeutic efficacy of CH, its residual profiles, and the sensitivity to orally administered CH for treating streptococcosis in cultured olive flounders.

Although only a limited number of studies have investigated the use of CH in fish, the effects of CH and its pharmacokinetics have been well-studied in humans. CH remains stable in gastric acid and is almost completely absorbed in the upper gastrointestinal tract. Following its oral administration in normal fasting participants, CH is rapidly absorbed with peak blood levels achieved at 1 hr, although the time taken to reach peak levels varies considerably [43]. CH is excreted almost exclusively by the kidneys via both glomerular filtration and tubular excretion [44]. Like most other cephalosporins, CH is not metabolized or inactivated in the body [45, 46], and its half-life is approximately 30–60 min in individuals with normal renal function [45, 47]. Therefore, upon oral administration, the therapeutic levels of CH are maintained in the body for 6–8 hr, after which more than 90% of it is excreted or unchanged in the urine within 16 hr, and CH is consequently administered once every 6–12 hr. According to Katharios et al. [48], CH administered intraperitoneally at 200 mg/kg in sea bream rapidly reached its maximum serum concentration (5.4 mg/kg) 1 hr post-treatment and was quickly eliminated with a half-life of less than 1.83 hr. We determined the residual CH concentrations in the muscles of olive flounders grown at optimal (25°C) and low (15°C) water temperatures after they were orally administered with 400 mg/kg of CH for 7 days. The average residual CH concentration in the olive flounder muscle samples on day 1 post-CH administration was 13.21 μg/kg, suggesting a rapid decrease in the CH concentrations in the fish bodies compared to those of the orally administered doses. In the olive flounders grown at 25°C, CH was rapidly metabolized within 24 hr of administration, and most of it was excreted from the bodies of the fish. In contrast, in the olive flounders that were grown at 15°C, the metabolic function of the fish was lowered, which delayed CH degradation or excretion. Therefore, CH is more likely to be retained at a higher concentration when the water temperature is lower. Notably, however, in the fish grown at both of the water temperatures, CH was equally eliminated within 19 days.

Data reporting the efficacy and safety of CH in teleost species are currently insufficient, but CH has been used to control a wide range of bacterial infections in ornamental and aquaculture fish [48]. In this study, we administered the highest effective CH dose (400 mg/kg of fish body weight) to olive flounders for 10 days and then extended this to 12 days. We then conducted histopathological tests on tissue samples collected from the first day of CH administration to day 12 after the termination of the treatment. No significant differences were observed between the experimental and control groups, and no specific inflammatory lesions were observed in the tissues. Thus, the histopathological analysis revealed no abnormal findings, and these results suggest that CH does

not induce organ toxicity in olive flounders because it is rapidly excreted from the body. Due to its extremely short half-life, there is limited evidence that CH accumulates in the sera of humans with normal renal function, and it is thus deemed to exert low levels of toxicity [49]. In addition, no toxicity has been reported in seabream-administered CH at doses of 50–400 mg/kg, as determined by monitoring their physiological status, hematology, blood biochemistry, and histology for 30 days [48]. Animal studies have also shown that CH exerts low levels of toxicity following oral and parenteral administration [13, 50].

The present study analyzed the effects of treating olive flounders with various concentrations of CH and confirmed the optimal dose for treating streptococcosis-causing *S. parauberis* in the fish. The safety of using CH was then confirmed through histopathological observations in different tissue organs obtained from the start of CH administration to day 12 after terminating the treatment.

5. Conclusions

The development of an antibiotic therapy against streptococcosis in olive flounders is necessary. This study determined the therapeutic effects of various CH concentrations in *S. parauberis*-infected olive flounders and evaluated its histopathological toxicity and residual concentration in the fish. The survival rate of the olive flounders that had been infected with *S. parauberis* was highest when the fish were treated with CH at a concentration of 400 mg/kg of fish weight. CH metabolized into the fish muscle more rapidly at 22°C than at 15°C. The histopathological analysis further revealed that CH did not induce organ toxicity. These results may be used as index data to determine the safety and effectiveness of using CH in medicated food in fish farms. In conclusion, we propose that CH is a promising candidate for treating streptococcosis outbreaks in fish farms.

Data Availability

The data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Ethical Approval

All experiments on the fish used in this study were conducted in accordance with the institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee of Sun Moon University (approval number: SM-2021-02-01).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yue Jai Kang, Hee Jae Choi, and Ji-Hoon Lee were involved in conceptualization and methodology. Kyu Seok Cho, Hee Jae Choi, and Ji-Hoon Lee were involved in investigation. Da Yeon Choi, Saran Hori, and Ji-Eun Lee were involved in formal analysis. Seong Hee Choi, Jun-Hwan Kim, and Yue Jai Kang were involved in writing—original draft, resources, validation, and data curation. Seong Hee Choi and Yue Jai Kang were involved in conceptualization and project administration and reviewed and edited the article. All authors have read and agreed to the published version of the manuscript. Hee-Jae Choi and Ji-Hoon Lee contributed equally to this work.

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