Efficacy of a multidose acyclovir protocol against cyprinid herpesvirus 3 infection in koi (Cyprinus carpio)

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OBJECTIVE
To evaluate the effect of a multidose acyclovir protocol on koi herpesvirus (KHV) viral load and mortality in a cohabitation challenge.

ANIMALS
180 koi fish.

PROCEDURES
Forty fish (shedders) were immersed in a 0.5 KHV plaque-forming units/mL static bath for 8 hours. Mock shedders were treated similarly but exposed to cell culture media. KHV shedders were then transferred into 8 tanks (5 shedders per tank) containing 10 naïve fish (cohabitants) each. Fish in the acyclovir group (AT) received a 10 mg/kg acyclovir intracoelomic injection 1, 3, and 6 days after the first confirmed KHV mortality. Positive controls (PC) were treated similarly but received sterile saline injections. Negative controls (NC) were exposed to mock shedders. Morbidity and mortality were evaluated daily for 50 days post-challenge. Quantitative PCR was used to determine viral load in the gill biopsies of shedders and cohabitants collected at days 19 (T1), 22 (T2), 25 (T3), 34 (T4), and 50 (T5) post-challenge.

RESULTS
Survival curves analyzed by the Gehan-Breslow-Wilcoxon method revealed a delayed onset of mortalities and a significantly lower KHV load at T2 and T3 detected in AT cohabitant fish (P = .042) compared to PC group. However, there were no significant differences in overall mortality or viral loads at T5.

CLINICAL RELEVANCE
The acyclovir protocol used in this study did not control viral infection or mortality at the end of the 50-day trial. Shorter intervals between injections could improve outcomes, but the additional stress inflicted by handling should be considered. Exploring other therapeutic alternatives and doses is warranted.

Ornamental fish aquaculture and trade represent a multimillion-dollar industry. As of 2016, the pet fish population in the United States was approximately 76 million in 8% of US households, and as of 2022, it remains the third most popular pet in the country. Koi fish (Cyprinus carpio) are a popular pet fish worldwide with an increased demand in commercial production of koi to meet the trade demands internationally. 

Cyprinid herpesvirus 3, colloquially known as koi herpesvirus (KHV), is a World Organisation for Animal Health-reportable double-stranded DNA virus in the family Alloherpesviridae. KHV is recognized as one of the most virulent emerging pathogens affecting common carp and koi aquaculture, as well as koi kept by hobbyists. Since its initial emergence in Israel and Germany, naturally occurring KHV infections throughout the ornamental fish industry have now been reported in over 28 countries. KHV infections can result in high mortality (80% to 100%) in naturally occurring outbreaks. The clinical signs and gross changes associated with KHV disease (KHVD) are typically nonspecific and include lethargy, hyporexia, pale and necrotic gills, enophthalmos, reduced skin mucous production sometimes leading to sandpaper texture, and ulcerative hemorrhagic skin lesions. KHVD is temperature dependent, and the disease occurs in the range between 16 °C and 28 °C. Fish that survive KHVD outbreaks can become latent carriers of the virus. In latently infected fish that are stressed, virus replication can occur and shedding of the virus into new populations often results in significant outbreaks.

Currently, strict biosecurity is the only effective method of preventing the introduction of KHV.
In the United States, there are no approved vaccines available against KHV, and although temperature manipulation and prolonged salting have been used to disrupt active viral replication and provide palliative treatment to infected fish, no efficacious targeted treatment against the virus has been reported. Previous research\textsuperscript{10,11} has investigated the antiviral effects of acyclovir against KHV.

Acyclovir is a widely used antiviral drug approved by the US Food and Drug Administration, which represents an option for extralabel use in pet fish under veterinary care. It is a synthetic 20-deoxyguanosine (guanine) nucleoside analog capable of selectively inhibiting the activity of viral DNA polymerase, thus preventing viral replication. Acyclovir has been used against infections caused by herpes simplex virus type 1 and 2 in humans;\textsuperscript{12,13} herpesvirus type 1 in cats;\textsuperscript{14,15} and herpesvirus and papillomavirus in horses.\textsuperscript{16,17} Acyclovir has also been reported to decrease KHV cytopathic effects, viral load, and viral gene expression in vitro using common carp brain cells.\textsuperscript{11} Moreover, a recent pharmacokinetic study\textsuperscript{10} performed in koi fingerlings using a single dose of acyclovir at 10 mg/kg iCe revealed that the plasma concentration remained over the threshold of 3.99 μM for 25.6 hours. This threshold is based on human data; however, it has not been determined for KHV in koi fish. This study\textsuperscript{10} also showed a reduction of cumulative mortality in KHV-infected fish and demonstrated acyclovir can be safely administered intracelomically (iCe) in koi. Thus, acyclovir represents a good option for extralabel use in pet fish under veterinary care.

Based on pharmacokinetic studies in humans, acyclovir and other nucleoside analogues have been shown to have a short elimination half-life and variable bioavailability.\textsuperscript{18–22} Therefore, multidose protocols have been suggested. A pharmacokinetic study\textsuperscript{16} of valacyclovir, the prodrug of acyclovir, showed that a multidose valacyclovir protocol is required to maintain effective serum concentrations that decrease viral replication of herpes virus type 1 in horses. In mice, a multidose protocol at 3, 6, 8, and 18 hours post hyperthermic stress was efficacious against reactivation of induced herpes simplex virus type 1.\textsuperscript{23}

The objective of this study was to evaluate a multidose acyclovir protocol and its effect on viral load and mortality in a KHV cohabitation challenge. We hypothesize that multiple acyclovir injections at 10 mg/kg iCe every 72 hours will decrease viral load in targeted tissues and decrease KHV-associated mortality.

**Materials and Methods**

**Virus**

Challenges were performed with isolate KHV-U recovered from an ornamental fish during a previous KHV outbreak. The isolate was propagated in koi fin cells (KF-1) for 21 days at 20°C as described by Hedrick et al.\textsuperscript{8} Virus concentration was determined by plaque method using 0.75% methylcellulose overlay and staining with 0.6% (wt/vol) crystal violet in 60% formalin solution after 7 days.

**Animals**

All animal procedures were approved by the University of California-Davis Institutional Animal Care and Use Committee. Nine hundred koi fingerlings weighing between 7 and 113 g, were obtained from a commercial ornamental fish producer in Florida with no history of KHV infection. Animals were housed at the University of California-Davis Center for Aquatic Biology and Aquaculture in 900 L of dechlorinated well water (18 ± 1°C) under flow-through conditions with constant aeration for over 1 year prior to study commencement. Upon arrival, a random sample of 30 koi fingerlings was selected for general health assessment and gill clip KHV testing using quantitative PCR (qPCR) and serum ELISA as described in Soto et al.\textsuperscript{24} Additionally, approximately 6 weeks prior to the commencement of this study, a random sample of 6 koi fingerlings (same population) were euthanized via immersion in at least 500 mg/L buffered tricaine methanesulfonate (MS222; 1:1 sodium bicarbonate; Aqualife TMS Fish Anesthetic; Syndel) for 30 min and then pithed with an 18-gauge needle, according to the American Veterinary Medical Association guidelines for euthanasia. Following euthanasia, blood was collected from the caudal vein, and gill and kidney samples were collected for molecular diagnosis and viral cell culture in KF-1 as above. Animals tested negative for KHV via cell culture, molecular, and serological methods. A subpopulation of 180 animals was randomly collected and used in the current study. Animals were fed at 1% body weight per day using a commercially available koi feed (Classic Fry and Koi color; Skretting). Approximately 1 week prior to the challenge, 120 naïve fish (cohabitants) were transferred into 132-L freshwater flow-through tanks with similar flow rates and supplemental aeration and maintained at a density of 10 fish per tank. The other 60 animals were exposed to KHV or sterile cell culture media (controls) via immersion as described below and considered “shedders” in the cohabitation challenge.

**Cohabitation challenge**

Cohabitation challenges were performed as described by Quijano Cardé et al.\textsuperscript{10} A subgroup of 40 koi fingerlings was exposed to a 0.5 KHV plaque-forming units/mL bath for 8 hours and deemed “KHV shedders.” Another subgroup of 20 koi fingerlings was treated similarly but exposed to sterile cell culture media and was deemed “mock shedders.” Fish were divided into three experimental groups with 4 replicate tanks per group: (1) each negative control (NC) tank consisted of 10 cohabitant fish and 5 mock shedders; (2) each positive control (PC) tank had 10 cohabitant fish and 5 KHV shedders; and (3) each acyclovir treatment group (AT) tank had 10 cohabitant fish and 5 KHV shedders.

Prior to the challenge, fish were anesthetized using 100 mg/L tricaine methanesulfonate (MS-222; Aqualife TMS Fish Anesthetic; Syndel) buffered (1:1 wt/wt) with sodium bicarbonate via immersion, and a fin clip of the dorsal aspect of the caudal fin was performed in shedders as a method of identification to monitor shedder and cohabitant fish in the
cohabitation challenge. Fish were then divided among the experimentally infected groups (4 tanks per treatment) for a cohabitation model (1:2 ratio, shedders: cohabitant fish). Fish were monitored twice daily postchallenge during which time any fish exhibiting moderate to severe signs of disease was euthanized as previously described and gills clips were collected for KHV quantification via qPCR.

After the first mortality from cohabitation exposure was confirmed KHV positive via qPCR, the treatment protocol was initiated. For each day of treatment, fish were anesthetized using buffered MS-222 as described above. Weight was obtained for each fish for dosing calculations, and identification as “cohabitant” or “shedder” was recorded. The AT group received 10 mg/kg acyclovir (AuroMedics Pharma LLC), and the PC and NC groups received sterile phosphate-buffered saline (PBS) administered via an intracoelomic injection cranial to the pelvic fins. A gill biopsy was performed to quantify viral load per fish and estimate the prevalence of infection in the group. The koi fingerlings were then returned to their tanks and recovered. All acyclovir injections were performed by the same individual (MSH). Positive controls were exposed to KHV but received PBS injections instead of acyclovir. Negative controls were exposed only to sterile cell culture media and injected with PBS. The same procedures were repeated every 72 hours for a total of 3 treatments, which were considered the time points T1, T2, and T3. Additionally, to monitor the KHV load in treated and control fish, gill clips were collected from all animals 9 days after the third treatment (T4).

Morbidity and mortality were recorded for 50 days, and gills were collected from mortalities and euthanized moribund animals for KHV infection confirmation and viral load assessment via qPCR (n = 10, 10, and 2 for the positive control, acyclovir, and negative control, respectively). Fifteen days postchallenge (T5), surviving fish were euthanized as previously described and the gills of 12 koi fingerlings per group were sampled to assess viral load and final prevalence of KHV.

Viral load quantification
The DNeasy Blood & Tissues kit (Qiagen) was used to extract DNA from samples kept at −80°C following the manufacturer’s recommendation. DNA was eluted in 100 μL of elution buffer. qPCR using the QuantStudio3 qPCR System (Thermo Fisher Scientific) was performed to evaluate viral load throughout T1-5. qPCR was performed in all samples for T1, T2, T3, T4, and T5 from PC and AT. Negative control samples were pooled (n = 3 fish samples per pool) and processed similarly.

Statistical analysis
Statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad Software). Multiple unpaired t tests were used to compare viral load at each time point among shedders and cohabitant fish in each group. Survival curves were determined for each group using the Kaplan-Meier method at different time points. Gehan-Breslow-Wilcoxon test was used to compare survivability among PC and AT fish. All comparisons were considered significant at P ≤ .05.

Results
Morbidity and mortality
Clinical signs and gross changes in KHV-infected fish were evident in shedders and cohabitants and included anorexia, lethargy, hemorrhagic and ulcerative skin lesions, hyphema, exophthalmia, and pale gills (Figure 1). The first mortality was confirmed as KHV positive via qPCR at day 15 post-exposure from a shedder in the PC group, and the first mortality in the AT group was registered 19 days post-exposure (Figure 2). Treatments started on day 19 post-exposure (T1) and were repeated on day 22 (T2), and day 25 (T3).

Cumulative mortalities in the PC and AT were 31/60 (51.6%) and 33/60 (55%), respectively (Supplementary Table 1). Survival curves for all treatments are presented in Figure 2. There were 2 mortalities in the NC group which had evidence of trauma and were confirmed to be unrelated to KHV via qPCR testing of the gills. Of the total mortalities, 4/33 in the AT and 3/31 in the PC group were euthanized due to the onset of clinical signs and moribund appearance. The highest number of mortalities per treatment (n = 4) occurred at days 25 and 36 post-exposure in treatments PC and AT, respectively. Gehan-Breslow-Wilcoxon test comparisons between AT and PC were significantly different at day 25 (P = .046) and 34 (P = .047). All other comparisons revealed no significant difference (P > .05). In all time points, PC shedders and AT shedders presented greater mortality when compared to PC and AT cohabitants. At the end of the trial (T5) there was no significant difference in survival between the PC and AT groups (P > .05).
Koi Herpesvirus loads determined by qPCR

All NC fish were qPCR negative for KHV. At T1 and T2, the viral loads were similar between cohabitant AT and PC fish and between shedders in AT and PC groups (P > .05), but significantly lower viral loads were present in cohabitant fish in comparison to shedders in exposed groups (P < .05; Figure 3). Both shedders and cohabitant fish presented significantly lower viral loads when compared to mortalities (P < .05). A similar trend in viral loads among groups was observed at T3; however, by this time point, similar viral loads were detected between mortalities and shedders in PC and AT treatments (P > .05). By T4, cohabitants and shedders in the AT treatments presented similar viral loads as those in mortalities. Significantly higher viral loads were detected in cohabitant AT fish when compared to cohabitant PC fish (P = .0032). The viral loads at end of the trial (T5) were statistically significantly lower in the survivors than in mortalities (P < .05) but were similar in AT and PC survivors (P > .05).

Discussion

The 3-dose acyclovir protocol used in this study was not effective in controlling KHVD long-term as similar viral loads and mortality were observed.
between treated and positive control groups at the end of the trial. There was however a significant increase in survival in the AT group at T3 (after 2 doses of acyclovir) and at T4 (after fish had received 3 doses of acyclovir; Figure 2). A similar response was noted by Quijano Cardé et al.\textsuperscript{10} after a single dose of acyclovir significantly reduced mortality in KHV-infected fish approximately 25 to 30 days post-challenge when compared with the positive control group. These results suggest that there is a positive effect on survivability while receiving acyclovir, and this effect decreases with time after the last dose. Based on these results it could be speculated that longer periods of treatment and/or increased dose frequency could be more effective at improving survivability.

The acyclovir dose used in this study was obtained from a pharmacokinetic study\textsuperscript{10} that established that a dose of 10 mg/kg was safe for koi and resulted in a mean peak plasma concentration ($C_{\text{max}}$) of 141 μM, a time to maximum concentration ($T_{\text{max}}$) of 45 minutes post-injection, an elimination rate constant of 0.05 per hour, and a half-life of 14 hours in fish kept in similar conditions as in the present study. Moreover, concentrations > 66.67 μM inhibited the cytopathic effect induced by KHV in vitro,\textsuperscript{11} further supporting the potential use of acyclovir against KHV infections. The 72-hour interval in this study was selected to minimize handling and reduce animal stress. However, the highest number of mortalities registered in a day from all treatment groups occurred at T1 and T3, immediately after handling and injection. The increased mortality following treatment days could be associated with debilitated fish experiencing an anesthetic complication. In fish, repeated and frequent dosing of intracoelomic injections is not an ideal protocol for therapeutics, given that the stress involved in the procedure itself can induce immunosuppression and viral reactivation. This reactivation has been previously reported in fish experiencing an anesthetic complication. In addition, the need for an anesthetic for the injection administration carries an increased risk of complications given the extent of gill pathology typically seen in koi affected with KHV. Given the challenges of repeatedly administering intracoelomic injections, and in most cases the potentially large number of individuals to be treated, other routes of administration should be explored. In fish medicine, oral and immersion routes are generally preferred as they reduce handling stress and improve the ability to treat a population in a time- and cost-effective manner. In humans, the oral formulation of acyclovir has incomplete absorption, has fairly low bioavailability (15% to 30%),\textsuperscript{26} has other pharmacokinetic parameters that can be variable, and has been reported to have a short half-life of 2.5 to 3 hours.\textsuperscript{27} While the current oral dosing recommendation for acyclovir in humans is 3 to 5 times per day, an extended-release version of this medication for use as an antitherpetic has been proposed. Alternatives to increase the bioavailability and decrease the dosing frequency of acyclovir have also been explored by using different routes of administration. For example, a study\textsuperscript{28} performed in beagles described the use of a gastroretentive system designed to prolong gastric transit time and allow continued absorption, which resulted in improved oral bioavailability of acyclovir with a significantly prolonged $T_{\text{max}}$. Other different formulations such as microemulsions have been investigated. A study\textsuperscript{12} that tested a model of herpes simplex virus type 1 infection reported that the emulsion could inhibit the development of cutaneous herpetic lesions. In addition to other previously discussed variables, the timing and duration of medication administration have been shown to impact the efficacy of acyclovir in controlling or limiting viral replication. Studies\textsuperscript{13,29} performed in mice investigating the efficacy of acyclovir in induced herpetic simplex virus type 1 showed that efficacy was dependent on the timing of the first dose and the length of exposure to acyclovir. Therefore, based on these studies, frequently repeated administration is required to achieve adequate therapeutic effects.

While both oral administration and immersion therapy are commonly used methods for treating infectious diseases in groups of fish, limited information is available for these routes using acyclovir. In one study,\textsuperscript{30} where chum salmon (Oncorhynchus keta) were experimentally infected with an Oncorhynchus masou virus (a virus within the Herpesviridae family), orally administered acyclovir did not affect survival, while fish in the immersion therapy group had reduced mortality. Further studies need to be performed to ascertain the viability of acyclovir as either an oral medication or immersion therapy in different species of fish facing diverse viral infections.

Viral loads were measured from gill tissue, as it has been shown that gills are one of the main target tissues for KHV. The ease of access and high vascularity of this tissue makes it the ideal tissue for diagnosis during KHV outbreaks.\textsuperscript{24,31} However, future studies should also evaluate viral loads in other target organs during an active infection, such as the spleen and kidney.\textsuperscript{31} In the present study, while the viral loads were consistently higher in the PC cohabitant fish when compared to the AT cohabitant fish for most of the study, there was no statistically significant difference in viral loads among groups in all time points except for T4, which interestingly revealed a higher viral load in the AT cohabitant group when compared to PC cohabitants. Another related interesting finding was that while acyclovir did prolong survival at the early time points (during acyclovir therapy) (Figure 2), increased mortalities were observed in the AT cohabitant group after approximately 7 days post-acyclovir treatment (approx 32 days post-challenge). It could be hypothesized that while heavily infected fish in the PC treatments had already succumbed to mortality in earlier days, acyclovir treatment kept viral loads under the threshold of KHV-associated mortality. However, once the acyclovir in the system got metabolized, KHV was able to replicate and cause mortalities resulting in the greater mortality that occurred in AT treatments between days 30 to 40 post-challenge (Figure 2). A piece of supportive evidence for this
hypothesis is that the previously mentioned increase in viral load in this group at T4 would be consistent with an outbreak in a group previously relatively controlled. While all fish groups are kept in identical systems and treated the same throughout the study, it is not uncommon to see outbreaks of KHV in just one group, or even just one replicate tank.

Early diagnosis and initiation of treatment seem critical for timely acyclovir therapy in koi. Our cohabitation model and study were designed to replicate a natural outbreak and clinical intervention; however, early monitoring via qPCR of gill biopsies and initiation of treatment prior to first mortality in the pond could have resulted in greater therapeutic success. In mammals, it has been suggested that acyclovir therapeutic efficacy is temporal and dose-dependent. In a mice model of herpes simplex virus type I infection, viral reactivation was evaluated after hyperthermic stress. Acyclovir administered 6 hours after hyperthermic stress resulted in a 90% reduction in viral reactivation. However, when the first dose of acyclovir was delayed by an additional 12 hours (hour 18), there was no appreciable reduction in viral activation. Efficacy increased when a multidose 50 mg/kg intraperitoneal acyclovir protocol was administered 3, 6, 12, and 18 hours after a heat stress event. The efficacy decreased with protocols that delayed the initiation of treatment. This could suggest that an earlier intervention after KHV exposure may have prevented viral replication.

In the case of a devastating disease such as KHV, the development of new alternatives for prevention and treatment is crucial for aquaculture. Further studies looking into acyclovir pharmacodynamics and an ideal route and interval of administration are needed to make an informed recommendation when applying acyclovir as an extralabel drug to the treatment of pet koi fish infected with KHV.

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**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org