

Effects of physiologic concentrations of L-lysine on in vitro replication of feline herpesvirus 1

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Objective—To evaluate the effects of various concentrations of L-lysine on in vitro replication of feline herpesvirus 1 (FHV-1).

Sample—Cultures of Crandell-Rees feline kidney (CRFK) cells.

Procedures—CRFK cells were inoculated with FHV-1 and maintained in media with 20 combinations of L-arginine and L-lysine concentrations. Changes in cell viability were monitored by continuous measurement of electrical impedance of cultured cells and by observation of viral cytopathic effects. Viral load was determined by use of quantitative PCR assay in supernatants obtained from infected cultures at specified time points.

Results—Increases in L-lysine concentration had no effect on the kinetics of cell death in FHV-1-infected cultures. There was also no significant effect ($r^2 < 0.1$) on viral DNA load for L-arginine concentrations $\geq 12 \mu\text{g/mL}$. There was a significant effect of increases in L-lysine concentration on viral DNA load in media supplemented with $6 \mu\text{g}$ of L-arginine/mL (mean \pm SD slope, $-4,641 \pm 1,626$ units; adjusted $r^2 = 0.45$). However, the difference between the lowest ($1 \times 10^{6.28}$ copies/ μL) and highest ($1 \times 10^{6.86}$ copies/ μL) FHV-1 DNA load in these media was < 1 logarithm.

Conclusions and Clinical Relevance—The difference in FHV-1 DNA load was unlikely to be biologically important. Various L-lysine concentrations did not inhibit in vitro replication of FHV-1 at L-arginine concentrations sufficient to maintain cell growth. This conclusion was consistent with results of other studies in which investigators have not detected a consistently beneficial effect when L-lysine is administered to FHV-1-infected cats. (*Am J Vet Res* 2014;75:572–580)

Feline herpesvirus 1 is classified in the family Herpesviridae, subfamily Alphaherpesvirinae, and genus *Varicellovirus* within the newly established order Herpesvirales.¹ Infections with FHV-1 are common among cats throughout the world.^{2,3} Primary FHV-1 infections may not cause clinical signs or can result in clinical disease of differing severity, particularly in young animals.^{4–6} The most common clinical manifestations of FHV-1 infection are rhinitis, conjunctivitis, and keratitis. Clinical signs in affected kittens include fever, lethargy, anorexia, rhinotracheitis with serous to mucopurulent ocular and nasal discharges, sneezing, and sometimes coughing. The virus is shed in oropharyngeal and ocular secretions for up to 1 to 3 weeks.^{4,7} Uncomplicated FHV-1 disease typically resolves within 1 to 2 weeks after infection. However, the virus establishes latency in neuronal tissues, and FHV-1-infected

Received October 28, 2013.

Accepted January 14, 2014.

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Presented in abstract form at the Science Week Conference of the College of Australian and New Zealand Veterinary Scientists, Surfers Paradise, QLD, Australia, July 2013.

The authors thank Janis Bridges for assistance with the statistical analysis.

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ABBREVIATIONS

CI	Cell index
CPE	Cytopathic effect
CRFK	Crandell-Rees feline kidney
DEF GM	L-lysine- and L-arginine-deficient growth medium
DMEM	Dulbecco modified Eagle medium
FHV	Feline herpesvirus
HSV	Herpes simplex virus
NLFK	Northern line feline kidney
qPCR	Quantitative PCR

cats presumably remain infected for life.^{8–10} Such cats may periodically have recrudescence of latent FHV-1 infection, which may or may not be accompanied by clinical disease. The most serious consequence of FHV-1 recrudescence is development of ocular lesions.^{6,11}

The idea of treating herpesvirus infections with orally administered L-lysine originated in the mid-1970s when it was suggested that L-lysine might be a treatment for HSV infections in humans.¹² The premise for treatment with L-lysine was the finding that the titer for HSV grown in esophageal epithelial cells was > 5 -fold as high as when the virus was cultivated in Eagle medium supplemented with $70 \mu\text{g}$ of L-lysine/mL versus $180 \mu\text{g}$ of L-lysine/mL.¹³ In that same study,¹³ the authors suggested that L-lysine was not essential for the

growth of human HSV in vitro. In fact, the lysine-free medium supported growth of the virus better than did complete Eagle medium when either was added to a monolayer of Minn EE cells 48 to 72 hours after inoculation. In contrast, omission of L-arginine or histidine from the culture media profoundly inhibited viral replication in the same experiment.

Because of the similarities between pathogenesis of FHV-1 infections in cats and HSV infections in humans, the data obtained with HSV were anecdotally extrapolated to FHV-1.¹⁴ Conclusions reached for a subsequent in vitro study¹⁵ appeared to support the notion that a high concentration of L-lysine might inhibit FHV-1 replication. In the experience of the authors, this has resulted in the subsequent use of L-lysine for treatment of FHV-1-associated diseases by a number of veterinarians. However, in vivo studies^{7,16–19} conducted to examine the effects of L-lysine supplementation on the severity of rhinitis and conjunctivitis in cats or FHV-1 shedding have provided inconsistent results, without a strong support for the use of L-lysine treatment. Similar studies on the efficacy of L-lysine supplementation on HSV lesions in human populations have yielded conflicting results, with some authors finding a beneficial effect^{20,21} and others failing to detect a beneficial effect.^{22,23}

The main drawback of 1 in vitro study¹³ is that the viability of cells grown under various culture conditions was not reported. Hence, it is difficult to differentiate between the direct effects of various amino acid deficiencies on the growth of the virus and the indirect effects mediated by the influence of the media on the metabolism and viability of cells in which the virus is grown. Because viruses use the cellular machinery for their replication, it is difficult to separate these direct and indirect effects.

To provide support for the in vivo use of L-lysine supplementation for treatment of FHV-1 infections, information on the in vitro effects of L-lysine on FHV-1 replication in healthy, viable cells would be most relevant. Inhibition of viral replication would be expected if the cell culture environment does not adequately support cell growth. To illustrate this point, viability of CRFK cells grown in various combinations of L-lysine (0, 200, and 300 µg/mL) and L-arginine (0, 2.5, and 5 µg/mL) concentrations was < 50%, compared with viability for cells grown in the control medium, and no cells were viable in medium without L-lysine and L-arginine supplementation.¹⁵ The titer of FHV-1 grown in all of the L-lysine- and L-arginine-supplemented media that supported cell growth was reduced, compared with the titer of FHV-1 grown in control medium (DMEM containing 84 µg of L-arginine/mL and 146 µg of L-lysine/mL). However, although virus replication in media containing 2.5 µg of L-arginine/mL was reduced with increasing concentrations of L-lysine, this relationship was reversed in media containing 5 µg of L-arginine/mL.¹⁵

In clinically normal cats, mean plasma concentrations of L-lysine are between 11.7 and 16.8 µg/mL, whereas those of L-arginine are between 17.1 and 25.1 µg/mL.^{16,19,24} Dietary supplementation of L-lysine results in mean plasma concentrations between 42 and 97 µg/mL, depending on the supplementation regimen

used.^{7,19,24} Although these values are extracellular rather than intracellular concentrations of L-lysine, they provide guidance as to the physiologic range within which in vitro amino acid concentrations have relevance for potential in vivo effects. Hence, the ranges of L-lysine and L-arginine concentrations reported in 1 study¹⁵ were outside the physiologic ranges expected to be achievable in vivo.

Therefore, the objective of the study reported here was to examine the effects of L-lysine and L-arginine on in vitro replication of FHV-1. Specifically, we intended to determine whether there was significant inhibition of FHV-1 replication when the concentrations of L-lysine and L-arginine were within the range required to support cell growth in vitro and within the range achievable in vivo.

Materials and Methods

Cell culture—Crandell-Rees feline kidney cells or NLFK cells were used for propagation of FHV-1. Cells initially were maintained in growth medium that consisted of advanced minimal essential medium^a supplemented with 4% (vol/vol) fetal bovine serum,^b 1% (vol/vol) L-alanyl-L-glutamine dipeptide,^c and 1% (vol/vol) penicillin-streptomycin solution^c (final concentration, 100 U of penicillin/mL and 100 µg of streptomycin/mL). Cells were cultured in accordance with standard cell culture protocols.²⁵

Preparation of virus stock—A New Zealand field isolate (V060996) of FHV-1 was purified by use of the limited dilution method in NLFK cells. Briefly, supernatants from the highest dilution of the virus that caused viral CPE in NLFK cells was collected and used to inoculate tissue culture flasks seeded with fresh NLFK cells. Flasks were maintained at 37°C in a 5% CO₂ humidified atmosphere and observed daily for viral CPE. When 100% of the cells had viral CPE, the flasks were subjected to 2 freeze-thaw cycles; supernatants then were collected, clarified by means of low-speed centrifugation (300 × g for 10 minutes), divided into aliquots, and stored at –80°C for further use. One aliquot of the virus was thawed and titrated in accordance with standard methods.²⁶

Propagation of FHV-1 in media with various concentrations of L-arginine and L-lysine—The DEF GM consisted of DMEM^d supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) L-alanyl-L-glutamine dipeptide, and 1% (vol/vol) penicillin-streptomycin solution. To assess the effect of various L-lysine and L-arginine concentrations on viral growth, DEF GM was supplemented with defined concentrations of L-lysine^e and L-arginine^e (Appendix). Routine maintenance of cells was achieved with DEF GM supplemented with 126 µg of L-arginine/mL and 320 µg of L-lysine/mL. All experiments were performed with CRFK cells; the cells were maintained in accordance with standard cell culture protocols.²⁵

Experiment 1—Seven 96-well tissue culture plates were used in the experiment; 6 plates were used for collection of samples and determination of DNA load, and the other plate was used for real-time cell monitoring.^f

On each plate, all wells in 6 columns received an aliquot (0.1 mL) of medium with a low concentration of L-arginine (36 µg/mL), and the other 6 columns received an aliquot of medium with a high concentration of L-arginine (252 µg/mL). The concentration of L-lysine in the medium differed across rows. The media added to columns 1 through 6 of each of the low- and high-L-arginine wells also contained serial concentrations of L-lysine (20, 40, 80, 160, 320, and 640 µg/mL). Crandell-Rees feline kidney cells grown in maintenance media were passaged, collected, and resuspended at a concentration of 4×10^5 cells/mL of DEF GM and added (50 µL) to each well of the plates. Finally, 50 µL of virus suspension containing 2×10^3 TCID₅₀ of FHV-1 was added to each well in rows 1 through 4 of each plate, and 50 µL of DEF GM (cell control samples) was added to each well in rows 4 through 8 of each plate. Thus, each well contained 2×10^4 cells mixed with 2×10^3 TCID₅₀ of FHV-1 (moiety of infection, 0.1) in DEF GM supplemented with L-arginine to achieve a final concentration of 18 µg/mL (low arginine) or 126 µg/mL (high arginine) and final L-lysine concentrations of 10, 20, 40, 80, 160, and 320 µg/mL for columns 1 through 6, respectively, for each of the low- and high-L-arginine media. Plates were incubated at 37°C in a 5% CO₂ humidified atmosphere for a predetermined amount of time. The plate used for real-time cell monitoring was incubated under the same conditions as for the tissue culture plates but in a separate incubator.

Experiment 2—Experiment 2 was performed as described for experiment 1, with 2 modifications. For the first modification, each well in 6 columns of the plates received an aliquot of DEF GM supplemented with 12 µg of L-arginine/mL (low arginine; final concentration, 6 µg/mL), whereas each well in the other 6 columns of the plates received an aliquot of DEF GM supplemented with 24 µg of L-arginine/mL (high arginine; final concentration, 12 µg of L-arginine/mL). For the second modification, 2×10^4 TCID₅₀ of FHV-1 (moiety of infection, 1) was added to each well in rows 1 through 4 of each plate.

Verification of free amino acid concentrations—To determine the accuracy of the amino acid dilutions, concentrations of L-lysine and L-arginine were measured in selected aliquots of media collected 6 hours after the start of incubation and stored at -20°C. Eight media were selected, with concentrations ranging from 10 to 320 µg of L-lysine/mL and 6 to 126 µg of L-arginine/mL. Concentrations of free amino acids were assayed in thawed aliquots by use of high-performance liquid chromatography at a commercial laboratory.⁵

Assessment of viral growth and cell viability—At predetermined time points (6, 12, 24, 48, and 72 hours after inoculation with FHV-1), one of the tissue culture plates was removed from the incubator and used for processing. The plate was examined for evidence of viral CPE by visual observation of cells with an inverted microscope ocular.

Cell viability in FHV-1-infected cells and uninfected control cells grown under the same conditions in the designated plate was monitored by use of a real-time

system^f and analyzed with proprietary software.^h The system measured the electrical impedance created by a monolayer of cells grown on the base of the gold-coated culture well. The higher the number of cells present on the base of the well, the larger the impedance between electrodes in the base of the culture well and the culture media. Changes in cell number, morphology, adhesion, or viability can result in changes in impedance. A unitless variable termed the CI was derived to measure the relative change in electrical impedance over time. Measurements were collected for the cell-monitoring plate at 15-minute intervals for 72 hours.

Collection of samples—At each predetermined time point (6, 12, 24, 48, and 72 hours after inoculation with FHV-1), cell culture supernatants, which included detached cells, were collected from 1 of the 6 replicate 96-well plates. Collected supernatants were stored at -20°C until further use. Extractions of DNA were performed with a commercial kit,ⁱ which was used in accordance with the manufacturer's instructions; DNA extractions were used for determination of viral load.

qPCR assay—A qPCR assay was used to enumerate the FHV-1 DNA load over time, which was used as a marker for virus replication. A subset of uninfected control cells was included as a negative control sample. The DNA extracted from cell culture supernatants was used as a template in the qPCR assay at a dilution of 1:10. The qPCR assay was performed with primers and a probe as described elsewhere.¹¹ The reaction mixture (final volume, 10 µL) contained 0.4 µM of each primer (FHV forward: 3'-AGAGGCTAACGGACCATCGA-5' and FHV reverse: 5'-GCCCGTGGTGGCTCTAAA C-3'), 80nM of FHV TaqMan probe (FAM-TATATGTGTC-CACCACCTTCAGGATCTACTGTCGT-TAMRA) in a reaction mix,^j and 2 µL of template DNA. Samples were subjected to an incubation step with uracil N-glycosylase (2 minutes at 42°C), followed by an initial denaturation-enzyme activation step (5 minutes at 95°C) and then by 45 cycles (denaturation for 3 seconds at 95°C and annealing-extension for 30 seconds at 60°C, with acquisition of fluorescence at the end of each annealing-extension step). Quantitative FHV-1 standards were prepared by cloning gel-purified FHV-1 PCR assay product (81 bp) into a plasmid vector^k in accordance with the manufacturer's instructions. The plasmid-containing FHV-1 DNA was linearized by digestion with a restriction endonuclease^l and serially diluted (range, 10⁷ to 10⁰ copies of FHV-1 DNA/µL). Negative (uninfected CRFK cells) and nontemplate (water or elution buffer) control samples were included in each run of the assay. All assays were performed in triplicate. The concentration of FHV-1 DNA in each sample was calculated on the basis of the standard curve computed by use of software supplied with the real-time instrument^m; results were expressed as the number of FHV-1 copies/µL of template.

Statistical analysis—All data were tested for normality with the Anderson-Darling test. Data that were not normally distributed were logarithmically transformed, which normalized the data. The association between CI and L-lysine concentration was tested for each

L-arginine concentration by use of linear regression. The CI for each L-lysine concentration was compared between L-arginine concentrations with a 1-way ANOVA followed by a Tukey multiple comparison test. To evaluate the effect of increases in L-lysine concentration on viral replication, the association between viral DNA load and L-lysine concentration was tested for each concentration of L-arginine by use of linear regression. Linear regression also was used to compare planned amino acid concentrations with measured concentrations. Significance was defined as values of $P \leq 0.05$.

Results

Effect of various L-lysine and L-arginine concentrations on cell growth—The CI curves of uninfected cells cultured in media with various L-arginine and L-lysine concentrations were determined throughout the 72-hour culture period. Increasing the L-lysine concentration did not significantly affect cell growth (adjusted $r^2 < 0.3$) for cells grown in media with 12 and 18 μg of L-arginine/mL (Figure 1). Increasing the L-lysine concentration significantly decreased the CI of cells grown in media with 6 μg of L-arginine/mL (mean \pm SD slope, -0.003152 ± 0.000639 ; adjusted $r^2 = 0.53$; $P < 0.001$) and, to a lesser degree, the CI of cells grown in media with 126 μg of L-arginine/mL (mean slope, -0.001949 ± 0.000538 ; adjusted $r^2 = 0.37$; $P = 0.002$). The CI of cells grown in media with 6 μg of L-arginine/mL was significantly less than the CI of cells

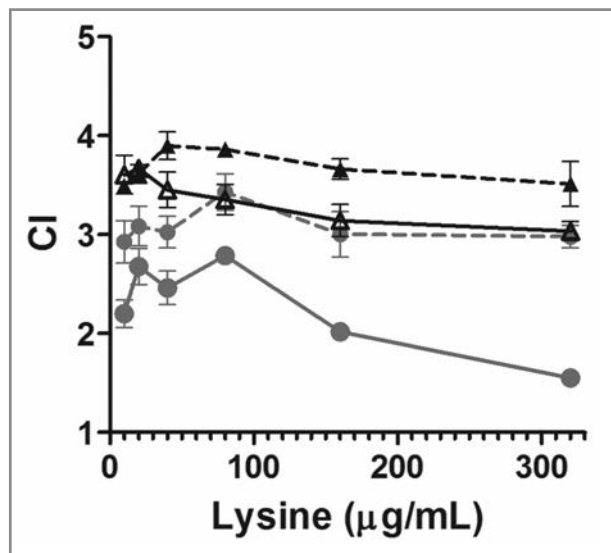


Figure 1—Viability for uninfected CRFK cells seeded at 2×10^4 cells/well and cultured for 72 hours in media with various concentrations of L-lysine and L-arginine in experiment 1 (final L-arginine concentrations, 18 $\mu\text{g}/\text{mL}$ [black triangles and dashed black line] and 126 $\mu\text{g}/\text{mL}$ [white triangles and solid black line]) and experiment 2 (final L-arginine concentrations, 6 $\mu\text{g}/\text{mL}$ [gray circles and solid gray line] and 12 $\mu\text{g}/\text{mL}$ [gray circles and dashed gray line]). The CI is proportional to the confluence of the cells. Increasing the L-lysine concentration significantly decreased the CI of cells grown in media with 6 μg of L-arginine/mL (mean \pm SD slope, -0.003152 ± 0.000639 ; adjusted $r^2 = 0.53$; $P < 0.001$) and 126 μg of L-arginine/mL (mean slope, -0.001949 ± 0.000538 ; adjusted $r^2 = 0.37$; $P = 0.002$). The CI of cells grown in media with 6 μg of L-arginine/mL was significantly ($P < 0.05$) lower than the CI of cells grown under all other conditions, except for L-lysine concentrations of 20 and 40 $\mu\text{g}/\text{mL}$.

grown under all other conditions, although this difference was not significant for media with 20 and 40 μg of L-lysine/mL. The CI of cells in those wells was not significantly different from the CI of cells grown with the corresponding L-lysine concentrations in media supplemented with 12 μg of L-arginine/mL. Thus, increasing the L-lysine concentration had some inhibitory effect on cell growth, which was most evident for cells grown in media with an L-arginine concentration of 6 $\mu\text{g}/\text{mL}$ and an L-lysine concentration $> 80 \mu\text{g}/\text{mL}$.

Effect of changes in L-lysine and L-arginine concentrations on viability of FHV-1-infected cells—In contrast to the CI curves of uninfected cells, the CI curves of cells infected with FHV-1 reached a peak at approximately 15 and 19 hours after initiation of culture in experiments 1 and 2, respectively. This was followed by a rapid decrease of the CI because of death of infected cells, which corresponded with the first appearance of typical herpesvirus CPE in replicate cell cultures. There was no significant difference between the CIs of FHV-1-infected cells grown in media with various concentrations of L-arginine and L-lysine. The medium that yielded the highest CI curve over time was 6 μg of L-arginine/mL and 320 μg of L-lysine/mL, whereas the medium that yielded the lowest CI curve over time was 6 μg of L-arginine/mL and 10 μg of L-lysine/mL (Figure 2). All other CI curves from both experiments were between those 2 CI curves.

Effect of various L-lysine and L-arginine concentrations on FHV-1 DNA load—The number of viral DNA copies increased rapidly during culture. The kinetics of FHV-1 DNA load in cells grown in media supplemented with the lowest (6 $\mu\text{g}/\text{mL}$) and highest (126 $\mu\text{g}/\text{mL}$) concentrations of L-arginine and various concentrations of L-lysine were determined (Figures 3 and 4). The maximum viral DNA load was detected at 48 to 72 hours after inoculation; values ranged from 5.3

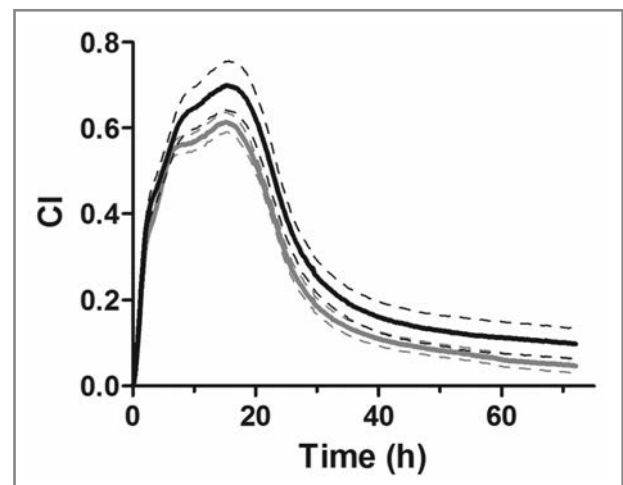


Figure 2—Representative CI curves for cells inoculated with FHV-1 and cultured for 72 hours in experiment 2. The CRFK cells were seeded (2×10^4 cells/well) and inoculated with FHV-1 (time 0; moiety of infection, 1). The CI was determined at 15-minute intervals for each of quadruplicate wells, and the mean CI was calculated. Data represent the mean (solid line) and SEM (dashed lines) for wells that yielded the highest CI curve (6 μg of L-arginine/mL and 320 μg of L-lysine/mL [black lines]) and lowest CI curve (6 μg of L-arginine/mL and 10 μg of L-lysine/mL [gray lines]).

$\times 10^6$ copies/ μL to 9.3×10^6 copies/ μL for experiment 1 and from 3.0×10^6 copies/ μL to 7.3×10^6 copies/ μL for experiment 2. The numbers of viral DNA copies per microliter of supernatant after culture for 72 hours in all 24 media were determined (Figure 5). At that time point, there was no significant effect of increasing the media concentration of L-lysine on the viral DNA load

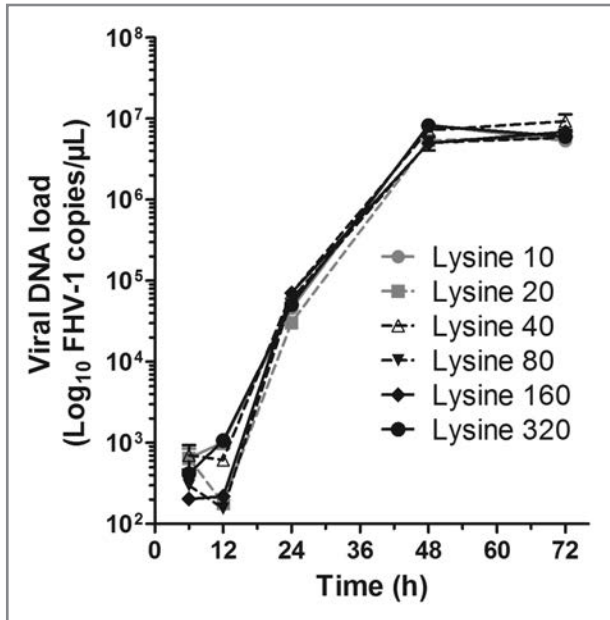


Figure 3—Change in FHV-1 DNA load over time in experiment 1. The CRFK cells were seeded (2×10^4 cells/well) and inoculated with FHV-1 (time 0; moiety of infection, 0.1). Cells were grown in media supplemented with L-arginine at 126 $\mu\text{g}/\text{mL}$ and various concentrations of L-lysine (10 to 320 $\mu\text{g}/\text{mL}$). Data represent the mean \pm SEM for triplicate wells.

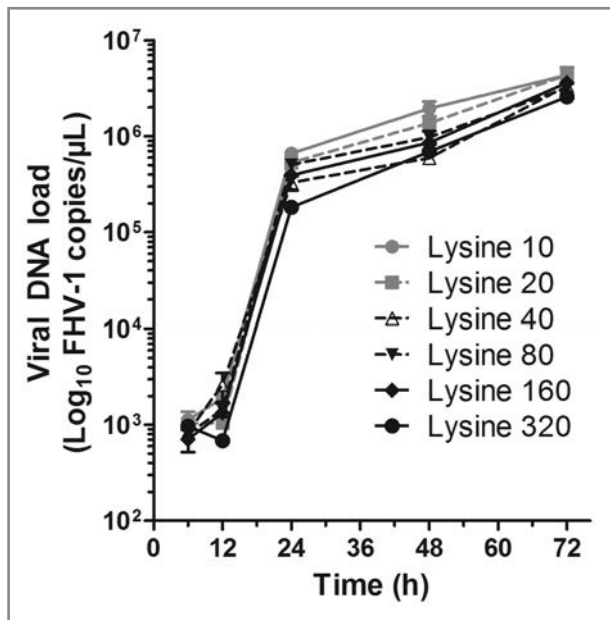


Figure 4—Change in FHV-1 DNA load over time in experiment 2. The CRFK cells were seeded (2×10^4 cells/well) and inoculated with FHV-1 (time 0; moiety of infection, 1). Cells were grown in media supplemented with L-arginine at 6 $\mu\text{g}/\text{mL}$ and various concentrations of L-lysine (10 to 320 $\mu\text{g}/\text{mL}$). Data represent the mean \pm SEM for triplicate wells.

when L-arginine was supplemented at 12, 18, and 126 $\mu\text{g}/\text{mL}$ ($r^2 < 0.1$; all $P > 0.3$). There was a significant effect of increasing the concentration of L-lysine on the viral DNA load in media supplemented with 6 μg of L-arginine/ mL (mean \pm SD slope, $-4,641 \pm 1,626$; adjusted $r^2 = 0.45$; $P = 0.017$). However, the difference between the lowest ($10^{6.28}$ copies/ μL) and highest ($10^{6.86}$ copies/ μL) FHV-1 DNA load in media supplemented with 320 or 10 μg of L-lysine/ mL , respectively, was only 0.58 log. In addition, at each concentration of L-lysine, the number of viral DNA copies at the 72-hour time point was similar (within 1 log) in media supplemented with all 4 L-arginine concentrations. At the 48-hour time point, there was no significant effect of increasing the media concentration of L-lysine on viral DNA load when L-arginine was supplemented at 6 $\mu\text{g}/\text{mL}$ and 12 $\mu\text{g}/\text{mL}$ ($r^2 < 0.2$; all $P > 0.2$). There was a small but significant effect of increasing the concentration of L-lysine on viral DNA load in media supplemented with 18 μg of L-arginine/ mL (mean slope, $-1.599 \times 10^6 \pm 0.645 \times 10^6$; $r^2 = 0.28$; $P = 0.02$). In addition, a slightly higher viral DNA load was detected in wells with higher L-lysine concentrations than in wells with lower L-lysine concentrations in media supplemented with 126 μg of L-arginine/ mL (mean slope, $2.173 \times 10^6 \pm 0.537 \times 10^6$; $r^2 = 0.51$; $P < 0.001$). However, the difference in viral load detected at various L-lysine concentrations for these 2 L-arginine concentrations was < 0.5 log and thus not likely to be biologically important. All uninfected control cells had negative results when tested for FHV-1 DNA.

Measurement of amino acid concentrations—Free amino acid (L-lysine and L-arginine) concentrations

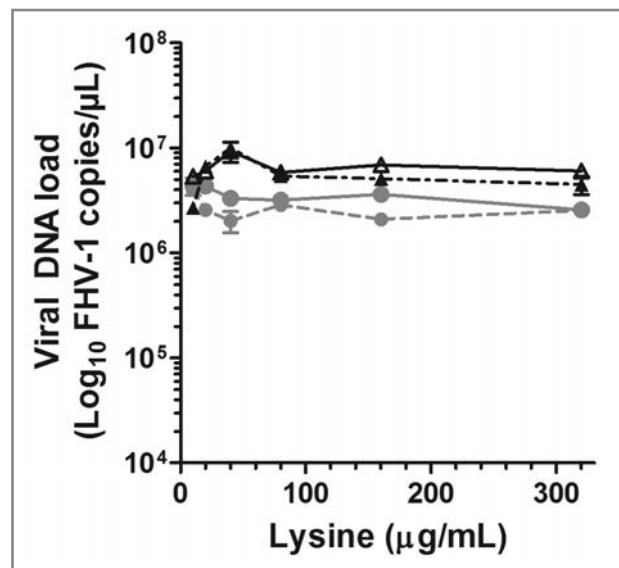


Figure 5—The FHV-1 DNA load in supernatants collected after CRFK cells were cultured for 72 hours in 24 media with various concentrations of L-lysine and L-arginine in experiment 1 (final L-arginine concentrations, 18 and 126 $\mu\text{g}/\text{mL}$) and experiment 2 (final L-arginine concentrations, 6 and 12 $\mu\text{g}/\text{mL}$). Increasing the concentration of L-lysine had a significant effect on the viral DNA load in media supplemented with 6 μg of L-arginine/ mL (mean \pm SD slope, $-4,641 \pm 1,626$; adjusted $r^2 = 0.45$; $P = 0.017$, although this effect was most likely not biologically important. Data represent the mean \pm SEM for triplicate wells. See Figure 1 for remainder of key.

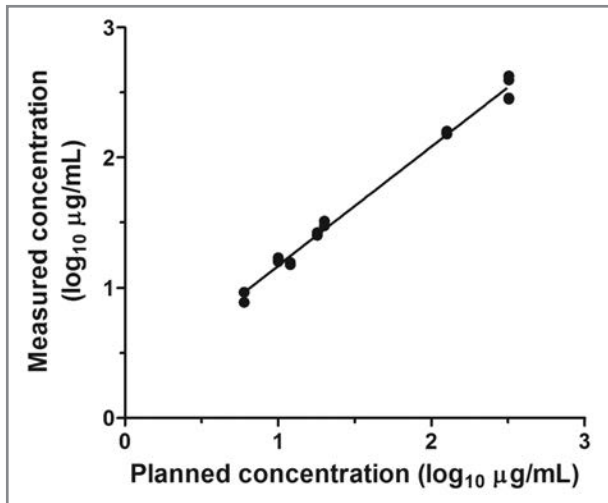


Figure 6—Correlation between planned concentrations of L-lysine and L-arginine in the cell culture media and measured concentrations of these 2 amino acids after CRFK cells were cultured for 6 hours with or without FHV-1. Data were not normally distributed; thus, the data are reported as the log₁₀ of the concentration. The regression line ($r^2 = 0.99$; $P < 0.001$; $n = 11$ amino acid concentrations) was defined by the equation $y = 0.9139x + 0.2565$.

were assayed in supernatant obtained from selected wells representing 8 cell culture conditions, which included 4 wells infected with FHV-1 and the corresponding 4 control (uninfected) wells. There was a strong correlation between measured and planned amino acid concentrations (Figure 6).

Discussion

Since the early 2000s, L-lysine has been recommended as an orally administered treatment to reduce respiratory tract or ocular disease and shedding of FHV-1 in cats infected with the virus. The underlying premise for that recommendation is the 2000 study¹⁵ in which investigators determined that replication of FHV-1 was inhibited *in vitro* when cell culture media were supplemented with extra L-lysine. However, viral growth in that study¹⁵ was suppressed in media supplemented with 200 or 300 µg of L-lysine/mL only in the presence of 2.5 µg of L-arginine/mL but not in the presence of 5 µg of L-arginine/mL. In addition, viability of cells in all tested media was poor. This highlighted the finding that alterations in culture conditions could have a direct effect on cells, independent of any effect on the inoculated virus. In fact, only approximately 10% of cells grown under conditions that were most suppressive to FHV-1 replication were viable. Similar to results described in that 2000 study,¹⁵ growth of cells in media supplemented with the lowest amount of L-arginine (6 µg/mL) in the present study was reduced in comparison with that in media with a higher L-arginine concentration. Thus, the reduction in viral titers in cells grown in media suboptimal for supporting cell growth could simply have reflected lower numbers of cells available for virus infection and replication, rather than direct inhibition of viral growth.

The intent of the present study was to reevaluate the *in vitro* effects of various concentrations of L-lysine

and L-arginine but with concentrations that were within the range necessary to support cell growth. Under the experimental conditions described for the present study, various concentrations of L-lysine (10 to 320 µg/mL) and L-arginine (6 to 126 µg/mL) had no effect on kinetics of FHV-1–induced cell death in FHV-1–infected cells. When the L-arginine concentration was between 18 and 126 µg/mL, there was no effect of L-lysine on the viral DNA load. Although there was a significant reduction in the viral DNA load in wells containing media supplemented with 6 µg of L-arginine/mL, the difference in viral DNA copy number was only 0.58 log over the entire range of L-lysine concentrations tested, and thus this difference was unlikely to be biologically important. Additionally, it is worth mentioning that the reduction in FHV-1 load paralleled the reduction in cell viability, as assessed by the shape of CI curves. Therefore, as mentioned previously, the observed effects may simply be explained by poorer support of FHV-1 replication by cells with poorer viability.

A novel method for measuring cytotoxic effects was used in the present study. We used a real-time analysis system developed to improve testing of cytotoxic effects; details of the system have been described elsewhere.²⁷ The system allows continuous monitoring of cell adhesion, proliferation, spreading, death, and detachment, thus creating a kinetic description of the cellular effects of viral infection. The system is sensitive enough to detect small differences in cellular responses to noxious stimuli that may not be detected by conventional endpoint assays.²⁸ We chose to use this system for the experiments to maximize the chance of detecting differences in cellular changes induced by viral replication throughout the entire 72 hours of the present study, rather than detecting differences only at pre-selected time points. However, we did not detect effects of changes in concentrations of L-lysine or L-arginine on the kinetics of FHV-1–infected cell death as determined by serial measurements of the CI.

In a 1964 study,¹³ HSV replication was inhibited when the L-lysine concentration was increased from 70 to 180 µg/mL in Eagle medium (which contains 126 µg of L-arginine/mL), although the difference in viral titer between the 2 media was only 0.75 log. We did not find this to be the case for FHV-1 replication in the present study, despite the fact we evaluated higher L-lysine concentrations at the same concentration of L-arginine. Possible explanations for the difference in these findings include differences in sensitivity to L-lysine among herpesviruses, cell culture systems, or viral detection methods used (DNA quantification vs determining the number of plaque-forming units).

Genomes of the alphaherpesviruses, including FHV-1, are similar, and several viral proteins have been described.²⁹ All 20 common amino acids, including L-lysine and L-arginine, are used in FHV-1 proteins.³⁰ Plasma concentrations of L-lysine and L-arginine in healthy cats are between 12 and 16.2 µg/mL for L-lysine and between 12.2 and 17.4 µg/mL for L-arginine.²⁴ Dietary supplementation can increase mean plasma concentrations of L-lysine to 42 µg/mL, but higher L-lysine concentrations in the diet cause a reduction in food intake.²⁴ It is difficult to compare *in vitro* cell culture

media concentrations to in vivo plasma concentrations because plasma concentrations are physiologically regulated, whereas media concentrations gradually decrease unless the media are replenished. For this reason, concentrations of amino acids in media used for in vitro cell culture are typically much greater than plasma concentrations of the same amino acids. For example, DMEM contains 146 µg of L-lysine/mL and 84 µg of L-arginine/mL, respectively.

It is even more difficult to relate extracellular concentrations of L-lysine and L-arginine to intracellular concentrations of these amino acids, although it is the latter that are used by the replicating virus. The same pathway is used for transport of L-lysine and L-arginine into mammalian cells, which creates a potential for competition between the 2 amino acids.³¹ Thus, it has been suggested that the ratio of L-lysine to L-arginine, rather than the concentration of each amino acid, is critical in achieving an inhibitory effect on viral replication.³² Investigators in 1 study¹⁵ evaluated ratios of L-lysine to L-arginine as high as 120 (300 µg of L-lysine/mL to 2.5 µg of L-arginine/mL). At that ratio, there was a nonsignificant reduction in the titer for FHV-1, compared with the titer for FHV-1 in the control medium. Although the authors of that study¹⁵ stated that the reduction in FHV-1 titer was > 50%, actual titers were not reported. Therefore, the biological importance of this reduction is uncertain. By comparison, the reduction in viral titers observed in the present study for FHV-1 grown in media supplemented with 6 µg of L-arginine/mL was also > 50% between wells with 10 µg of L-lysine/mL (7.3×10^6 TCID₅₀/mL) and wells with 320 µg of L-lysine/mL (1.9×10^6 TCID₅₀/mL). However, this corresponded to a difference of only 0.58 log, which is unlikely to be biologically important. The maximum ratio of L-lysine to L-arginine tested in the present experiments was 53.3 (320 µg of L-lysine/mL to 6 µg of L-arginine/mL). If a higher ratio is needed before there is inhibition of FHV-1, it is difficult to imagine how that could be achieved in vivo. Plasma L-arginine concentrations may decrease to < 6 µg/mL in cats from which food is withheld after being fed a diet devoid of L-arginine; this is associated with a severe risk of hyperammonemia and encephalopathy.^{33,34} Similarly, dietary supplementation of L-lysine that resulted in plasma concentrations > 42 µg/mL also reduced food intake.²⁴ Thus, it would be difficult to obtain a plasma concentration ratio of L-lysine to L-arginine > 50 in healthy cats in vivo.

In the present study, a qPCR assay was used to quantify viral replication. It is possible that not all viral DNA was derived from infectious virions. Consequently, we cannot fully exclude the possibility that altering the ratio of L-lysine to L-arginine had an effect on replication of the infectious virus without altering the amount of viral DNA production. Titration of the infectious virus in cell culture supernatants could have been used to test this hypothesis, but this was not performed as part of the present study. However, real-time impedance data did not reveal obvious differences in the kinetics of cell death among the various media formulations, which suggested that production of defective viruses was not a likely explanation for the results.

A lack of in vitro effects does not exclude the possibility of an in vivo effect. In an evaluation of cutaneous HSV-1 infection, guinea pigs were inoculated cutaneously with HSV-1 at the same time as direct application of L-lysine or placebo solutions.³⁵ Placebo-treated guinea pigs developed typical cutaneous lesions, but the L-lysine-treated group did not. In addition, HSV-1 was recovered from the skin of all guinea pigs, regardless of clinical signs, but HSV-1 was recovered only from the dorsal root ganglia of the L-lysine-treated group but not from the dorsal root ganglia of the placebo-treated group. The authors of that study³⁵ suggested that the presence of L-lysine at the site of infection altered the tropism for the dorsal root ganglia and prevented the development of local lesions through an immunomodulatory effect. Macrophage function is affected by the availability of L-arginine, which is required for nitric oxide production and for lipopolysaccharide-induced intracellular signaling.^{36,37} However, a direct role for L-lysine in affecting immune responses has not been reported to our knowledge. In poultry, a dietary excess of L-lysine increases renal arginase activity and can lead to sufficient loss of L-arginine as to cause arginine deficiency.³⁸ Suppression of growth has been detected in dogs fed high concentrations of L-lysine.³⁹ However, at the doses achievable for healthy cats, increasing the L-lysine intake did not significantly suppress plasma L-arginine concentrations.²⁴ We cannot exclude that these relationships are altered in ill cats. For example, human patients with sepsis have reduced plasma concentrations of L-arginine.^{40,41} By extrapolation, the relative ratio of plasma concentrations of L-lysine to L-arginine may be higher in diseased cats than in healthy cats, and dietary supplementation with the same amount of L-lysine may yield different biological effects for different physiologic conditions.

The efficacy of oral administration of supplementary L-lysine to cats with various stages of FHV-1 infection has been evaluated.^{7,16-19} In 1 study,¹⁹ cats in a treatment group received 600 mg of L-lysine twice daily as a bolus beginning 6 hours prior to inoculation with FHV-1; this resulted in a mean plasma L-lysine concentration of 97 µg/mL. All cats, regardless of whether they received supplementary L-lysine, developed clinical signs of ocular or respiratory tract disease after inoculation with FHV-1. Although clinical scores were reduced in cats receiving L-lysine, compared with clinical scores for the control group, there was no difference in viral shedding between the 2 groups.¹⁹ In another study,⁷ administration of a once-daily bolus of 400 mg of L-lysine to cats previously infected with FHV-1 resulted in peak plasma L-lysine concentrations of 65 µg/mL and reduced viral shedding but had no effect on clinical signs. In that study,⁷ most cats remained healthy regardless of L-lysine administration despite an attempt to recrudesce latent FHV-1 infection by the administration of corticosteroids. Thus, these results of controlled studies provide little support for the use of supplementary L-lysine as an aid to reduce clinical disease or virus shedding following FHV-1 infection.

Effects of supplemental L-lysine on ocular and respiratory tract disease in naturally infected cats has been evaluated.¹⁶⁻¹⁹ Results of these studies should be interpreted

ed with caution because FHV-1 is not the only pathogen involved in infectious rhinitis and conjunctivitis in cats under natural conditions. Nonetheless, incorporation of L-lysine into a basal diet of cats from a research colony affected by endemic rhinitis and conjunctivitis did not reduce clinical signs in the treated group.¹⁶ Indeed, signs of ocular and respiratory tract disease were detected in 22 of 25 (88%) cats fed a lysine-supplemented diet, compared with only 15 of 25 (60%) cats in the control group. Of 7 cats that seroconverted to FHV-1 during the study, 6 were in the L-lysine-supplemented group. The mean serum concentration of L-lysine was 43 µg/mL for the supplemented group, compared with 14 µg/mL for the control group.¹⁶

Similarly, we are not aware of beneficial effects of supplemental L-lysine on the prevalence of respiratory tract and ocular disease in shelter populations of cats. In 1 study,¹⁷ there was no difference in the rates of development of mild rhinitis or conjunctivitis between cats receiving an L-lysine-supplemented diet and control cats for a period of 4 weeks. However, moderate to severe disease was more common in the L-lysine-supplemented group during week 4 than in the control group, and FHV-1 DNA was detected more commonly in ocular swab specimens obtained from L-lysine-treated cats than from control cats. In that study,¹⁷ L-lysine was added (1.7% [wt/wt]) to a basal diet of the supplemented group, which resulted in a mean plasma concentration of 36 µg/mL at the end of the 4-week study. In another study,¹⁸ healthy cats and kittens were assigned to receive no treatment or a daily bolus of L-lysine (500 or 250 mg) over a period of 5 months. Plasma L-lysine concentrations were not measured, but the treatment did not reduce the frequency or severity of clinical signs in the L-lysine-treated animals (n = 144 cats or kittens), compared with results for the untreated control cats or kittens (147). The conclusions of the authors of that study¹⁸ were that lysine supplementation may not be effective in preventing upper respiratory tract infections in cats in a stressful shelter situation and that time, staffing, and money may be better spent developing infection control practices, limiting fomite transmission, and generally reducing stress. The results of the in vitro investigation reported here supported the conclusions from in vivo studies.

The data reported here provided an extension of a previous study.¹⁵ Although in some aspects, the data for the present study were similar to those reported in that other study,¹⁵ our interpretation of these data and our conclusions differed from those reported previously. We tested the hypothesis that increasing the concentrations of L-lysine in media supplemented with L-arginine at concentrations compatible with supporting cell growth would inhibit FHV-1 replication in vitro. We rejected that hypothesis and therefore find that the premise for administering supplemental L-lysine is false. The lack of evidence for benefits of supplemental L-lysine on the outcome of FHV-1 infection in vivo is consistent with data for the present study. Thus, data from the present study were in agreement with the notion that, contrary to popular belief, there is currently little scientific merit in recommending L-lysine treatment to cats for control of upper respiratory tract disease attributable to infection with FHV-1.

- a. Advanced MEM, Invitrogen NZ Ltd, Auckland, New Zealand.
- b. HyClone fetal bovine serum, Thermo Scientific, Albany, New Zealand.
- c. Glutamax, Life Technologies Inc, Carlsbad, Calif.
- d. Thermo Fischer Scientific, Albany, New Zealand.
- e. Sigma-Aldrich NZ, Auckland, New Zealand.
- f. Provided by Roche Products NZ Ltd, Auckland, New Zealand.
- g. Massey University Nutrition Laboratory, Palmerston North, New Zealand.
- h. RTCA software, version 1.2.0909, ACEA Biosciences Inc, San Diego, Calif.
- i. High Pure PCR Template Preparation Kit, Roche Applied Sciences, Nonnenwald, Penzberg, Germany.
- j. Perfecta Taq qPCR FastMix UNG, Quanta BioSciences Inc, Gaithersburg, Md.
- k. pCR 4Blunt-TOPO vector, Invitrogen NZ Ltd, Auckland, New Zealand.
- l. Pst 1, Fermentas, Thermo Scientific, Albany, New Zealand.
- m. Qiagen, Limburg, The Netherlands.

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Appendix

Ratios of L-lysine to L-arginine in amino acid-deficient media used to propagate CRFK cells and FHV-1.

L-lysine (µg/mL)	L-arginine (µg/mL)			
	6	12	18	126
10	1.7	0.8	0.6	0.1
20	3.3	1.7	1.1	0.2
40	6.7	3.3	2.2	0.3
60	10.0	5.0	3.3	0.5
80	13.3	6.7	4.4	0.6
160	26.7	13.3	8.9	1.3
320	53.3	26.7	17.8	2.5

Various concentrations of L-lysine and L-arginine were added to DMEM supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) L-alanyl-L-glutamine dipeptide, and 1% (vol/vol) penicillin-streptomycin solution.