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Tese

Infecção experimental de camundongos BALB/c com herpesvírus felino tipo 1 (FHV-1) e avaliação terapêutica de diferentes compostos antivirais

Débora Scopel e Silva

Pelotas, 2017

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Tese apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de concentração: Sanidade Animal).

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Banca examinadora:

Profa. Dra. Silvia de Oliveira Hübner (Orientadora)
Doutora em Ciências Veterinárias pela Universidade Federal do Rio Grande do Sul

Prof. Dr. Gilberto D'Avila Vargas
Doutor em Ciências pela Universidade Federal de Pelotas

Prof. Dr. Éverton Fagonde da Silva
Doutor em Ciências pela Universidade Federal de Pelotas

Dra. Clarissa Caetano de Castro
Doutora em Ciências pela Universidade Federal de Pelotas

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Resumo

SILVA, Débora Scopel e**. Infecção experimental de camundongos BALB/c com herpesvírus felino tipo 1 (FHV-1) e avaliação terapêutica de diferentes compostos antivirais.** 2017. 97f. Tese (Doutorado em Ciências) – Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2017.

O herpesvírus felino tipo 1 (FHV-1) afeta principalmente o trato respiratório superior, provocando rinite, ceratite ulcerativa e conjuntivite, mas é capaz de promover doença sistêmica, provocando pneumonia, necrose hepática, emaciação, abortos e dermatite. O tratamento é sintomático, pois não há um antiviral específico contra o FHV-1. Dessa forma, a pesquisa por compostos antivirais com atividade específica contra o FHV-1 é urgente e necessária. No entanto, pesquisas tendo o gato como modelo experimental são difíceis de serem conduzidas por envolverem questões éticas e humanitárias. O camundongo é um modelo experimental amplamente utilizado na medicina humana e veterinária, mas até o momento não há relatos sobre a infecção experimental de camundongos com o FHV-1. O presente estudo objetivou utilizar camundongos BALB/c como modelos experimentais para reproduzir a infecção pelo FHV-1 e avaliar a eficácia antiviral de diferentes compostos *in vivo*. Três grupos de 14 animais cada foram infectados e tratados com ganciclovir, lactoferrina e o peptídeo P34. Um grupo de 14 animais, grupo controle positivo, foi infectado com FHV-1 e não recebeu tratamento. Outro grupo de 14 animais não foi infectado nem recebeu tratamento (grupo controle negativo). Os animais foram pesados e avaliados diariamente para registrar o desenvolvimento ou não de sinais clínicos. Todos os tratamentos iniciaram 24 h pós-infecção, tiveram duração de 10 dias e foram administrados uma vez ao dia por meio de gavagem (lactoferrina e P34) e injeção intraperitoneal (ganciclovir). Após o término do tratamento foi realizada eutanásia e coleta dos órgãos (fígado, rim, baço e pulmão) para avaliação histopatológica e qPCR. Os resultados foram analisados pelo método analítico Kruskall-Wallis e não foram observadas diferenças estatisticamente significativas no ganho de peso entre os grupos de animais. Os animais infectados e não tratados desenvolveram sinais clínicos típicos de infecção pelo FHV-1, como blefarite, conjuntivite, blefaroespasmo, secreção ocular uni ou bilateral e fotofobia. Os resultados demonstraram que todos os animais foram capazes de se infectar com FHV-1, produzindo doença local e/ou sistêmica em diferentes graus de severidade. Lesões histopatológicas puderam ser observadas no pulmão (pneumonia intersticial, edema, congestão e corpúsculos de inclusão intranucleares), no fígado (congestão, vacuolização de hepatócitos, hepatite, necrose e corpúsculos de inclusão intranucleares), no baço (depleção linfoide) e no rim (nefrite intersticial e nefrose tubular). O órgão que teve maior detecção de DNA viral foi o pulmão, seguido de fígado, baço e rim. Os tratamentos utilizados neste trabalho, na dose, via e frequência de administração não foram capazes de reduzir a carga viral nos animais

tratados. Por esta razão, novas pesquisas deverão ser conduzidas para avaliar essas mesmas drogas, na mesma espécie animal, porém com um diferente regime de tratamento. Os dados gerados neste trabalho sobre a patogênese do FHV-1 em camundongos são inéditos, visto que até o momento só há na literatura relatos de infecção experimental com o FHV-1 no próprio gato, o que torna o camundongo uma opção viável para estudos sobre a patogênese e avaliação de antivirais contra o FHV-1.

Palavras-chave: rinotraqueíte viral felina; modelo experimental; terapia antiviral

Abstract

SILVA, Débora Scopel e**. Experimental infection of BALB/c mice with feline herpesvirus type 1 (FHV-1) and therapeutic evaluation of different antiviral compounds.** 2017. 97f. Thesis (Doctor Degree in Sciences) – Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2017.

Feline herpesvirus type 1 (FHV-1) affects mainly the upper respiratory tract, causing rhinitis, ulcerative keratitis and conjunctivitis, but is able to induce systemic disease, causing pneumonia, hepatic necrosis, emaciation, abortion and dermatitis. Treatment is symptomatic, because there is no specific antiviral against FHV-1. This way, the search for antiviral compounds with activity particularly against FHV-1 is urgent and necessary. Notwithstanding, researches having the cat as the experimental model are difficult to be carried out because of ethical and humanitary concerns. The mouse is an experimental model used widely in human and veterinary medicine, but hitherto there are no reports about experimental infection of mice with FHV-1. The present study aimed to use BALB/c mice as experimental models to reproduce the infection with FHV-1 and evaluat the antiviral efficacy of different compounds *in vivo*. Three groups of 14 animals each were infected and treated with ganciclovir, lactoferrin and the peptide P34. A group of 14 animals, positive control group, was infected with FHV-1 and did not receive treatment.another group of 14 animals was not infecte dor treated (negative control group). Animals were weighed and evaluated daily to record the development of clinical signs or not. All the treatments started 24 h post-infection, had ten days of duration and were administered once a day by gavage (lactoferrin and P34) and intraperitoneal injection (ganciclovir). After the end of the treatment, euthanasia was performed and the organs (liver, kidney, spleen and lung) were collected for histopathology and qPCR. Results were analyzed by the Kruskall-Wallis analytical method and differences statistically significant wer not observed in body weight among the groups. The infected and not treated animals developed typical clinical signs of FHV-1 infection, like blepharitis, conjunctivitis, blepharoespasm, uni or bilateral ocular secretion and photophobia. The results demonstrated that all the animals were able to be infected with FHV-1, producing local and/or systemic disease in different degrees of severity. Histopathological lesions could be observed in lungs (interstitial pneumonia, edema, congestion and intranuclear inclusion bodies), in the liver (congestion, hepatocyte vacuolization, hepatitis, necrosis and e intranuclear inclusion bodies), in the spleen (lymphoid depletion) and in the kidneys (interstitial nephritis and tubular nephrose). The organ with higher viral DNA detection was the lung, followed by the liver, spleen and Kidney. The treatments used in this work, in the dose, via and frequence of administration were not able to reduce viral load in the treated animals. For that reason, new researches might be conducted to evaluate the same drugs, in the same species, however with a different regimen of treatment.

Data generated in this experimental work about the pathogenesis of FHV-1 in mice are extraordinary, since until this moment there are in the literature just reports of experimental infection with FHV-1 using the cat itself, what makes the mouse a viable option to study the pathogenesis of FHV-1 and to evaluate the antiviral activity of drugs against the virus.

Keywords: feline viral rhinotracheitis; experimental model; antiviral therapy

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Lista de Abreviaturas e Siglas

ACV	aciclovir
ATCC	American type culture collection
BoHV-1	bovine herpesvirus type 1
BoHV-5	bovine herpesvirus type 5
CRFK	Crandell-Rees Feline Kidney
C_T	cycle threshold
DNA	ácido desoxirribonucleico
Dpi	dias pós-infecção
EHV-1	equine herpesvirus type 1
E-MEM	Eagle's minimum essential médium
FAVET	Faculdade de Veterinária
FBS	fetal bovine sérum
FHV-1	feline herpesvirus type 1
G1	grupo 1
G2	grupo 2
G3	grupo 3
GCV	ganciclovir
HSV-1	herpes simplex type 1
HSV-2	herpes simplex type 2
MCMV	murine cytomegalovirus
NCG	negative control group
P34	peptídeo P34
PCG	positive control group
PCV	penciclovir

qPCR	quantitative polymerase chain reaction
SRD	sem raça definida
TCID	tissue culture infective doses
TK	thymidine kinase
UFPel	Universidade Federal de Pelotas
URT	upper respiratory tract
URTD	upper respiratory tract disease

Lista de Símbolos

°C	graus Celsius
<	Menor
>	Maior
Kg	Quilograma
µL	Microlitro
µg	Micrograma
Mg	Miligrama
mL	Mililitro
Ng	Nanograma
nM	Nanomolar
%	por cento
®	marca registrada
™	Trademark

Sumário

1 Introdução

O herpesvírus felino tipo 1 (FHV-1) é um membro da família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* que produz uma doença conhecida como rinotraqueíte viral felina (FRANCO et al., 2012). O FHV-1 geralmente está relacionado com doença do trato respiratório superior e ulceração oral em filhotes (MANSELL & REES, 2006). Gatos que sobrevivem à infecção aguda desenvolvem a infecção latente e a reativação da infecção, permitindo a transmissão do vírus a outros animais (FRANCO et al., 2012). O FHV-1 pode provocar rinite e pneumonia intersticial, ceratite ulcerativa, necrose hepática, emaciação, abortos e fetos natimortos (LÓPEZ, 2007), além de dermatite (HARGIS & GINN, 2007). Ocasionalmente lesões de pele podem ser vistas no plano nasal, embora possam ocorrer menos frequentemente na face, patas, orelhas, abdômen (MANSELL & REES, 2006) e região periorbital (GROSS et al., 2009). A mortalidade é maior entre filhotes com menos de seis meses de idade (FRANCO et al., 2012).

O FHV-1 preferencialmente infecta as células mucoepiteliais das tonsilas, conjuntiva e mucosa nasal (GASKELL & POVEY, 1979), mas também há infecção significativa das células do epitélio corneano (NASISSE et al., 1989). Após rápida replicação ocorre dano celular agudo, levando à citólise (GOULD, 2011). A viremia é rara, pois a replicação viral normalmente é restrita a áreas de baixa temperatura corporal, tal como o trato respiratório (GASKELL et al., 2012). Os sinais clínicos se desenvolvem de 3 a 5 dias após a infecção (FRANCO et al., 2012) e perduram por 10 a 14 dias (DAVIDSON, 2009). O vírus pode ser detectado em secreções nasais, conjuntivais ou orofaríngeas 24 h após a infecção e geralmente persiste por 1 a 3 semanas (GASKELL et al., 2012).

Os gatos portadores da infecção latente são os reservatórios do FHV-1 e constituem a principal fonte de disseminação do agente. A transmissão ocorre principalmente pelo contato direto ou indireto com descargas nasais, aerossóis e, com menor frequência, por fômites contaminados (FRANCO et al., 2012).

O tratamento de animais infectados pelo FHV-1 é um desafio. Os medicamentos antivirais geralmente são caros, exigem um cuidador dedicado, além da aceitação do paciente e de seu tutor. Embora um grande número de antivirais tenha sido desenvolvido para o tratamento das infecções pelos herpesvírus humanos (DE CLERCQ, 2012), a maioria desses compostos foi testada contra o FHV-1 somente *in vitro* e poucos foram avaliados em ensaios clínicos controlados ou em estudos de infecções experimentais. O aciclovir, composto amplamente utilizado no tratamento dos herpesvírus em medicina humana, não possui boa atividade contra o FHV-1 *in vitro* (GASKELL et al., 2007) e ambos, aciclovir e a pró-droga valaciclovir, são muito tóxicos em níveis terapêuticos para administração oral em gatos. Os compostos antivirais ganciclovir (GOULD, 2011) e lactoferrina (BEAUMONT et al., 2003) demonstraram ter grande atividade antiviral contra o FHV-1 *in vitro*, porém eles ainda não foram avaliados *in vivo* (MALIK et al., 2009).

O ganciclovir (GCV) é um análogo acíclico da guanina e, como o aciclovir, sua primeira fase de fosforilação é mediada pela timidina quinase (TK) viral (SAVI, 2004). Pesquisadores demonstraram que o GCV possui eficácia dez vezes superior ao aciclovir contra o FHV-1 (MAGGS & CLARKE, 2004). A lactoferrina é uma glicoproteína produzida pelo epitélio de mucosas de várias espécies de mamíferos e possui propriedades antibacterianas, antifúngicas, antiparasitárias e antivirais (JENSSEN & HANCOCK, 2009). Um estudo demonstrou que a lactoferrina exerce efeito antiviral muito potente sobre a replicação do FHV-1 *in vitro*. Esse efeito parece ser mediado pela inibição da adsorção do FHV-1 à superfície celular e/ou pela inibição da sua penetração na célula (BEAUMONT et al., 2003).

As drogas utilizadas para tratamento sistêmico contra os herpesvírus, por atuarem em nível celular, resultam em variados graus de toxicidade (MAGGS, 2010). Assim, fatores como eficácia, disponibilidade, toxicidade, frequência de aplicação e custo de muitas medicações antivirais estimulam a investigação de novas terapias para tratar as infecções pelo FHV-1. Atualmente, o único composto antiviral disponível no Brasil com eficácia clínica comprovada contra o FHV-1 é o fanciclovir (SILVA et al., 2014a). Estudos realizados recentemente demonstraram ação antiviral contra FHV-1 (SILVA et al., 2014b), herpesvírus bovino tipo 1 (BoHV-1) e vírus da arterite equina (EAV), *in vitro*, exercida por um peptídeo antimicrobiano denominado P34 (CASTRO et al., 2017; SILVA et al., 2017). O peptídeo P34 é produzido por uma espécie de *Bacillus* sp., isolada do conteúdo intestinal do peixe *Leporinus* sp. na

Bacia Amazônica, com atividade antibacteriana já descrita (MOTTA et al., 2007). *In vitro*, o peptídeo P34 apresentou baixa citotoxicidade em células de rim felino (*Crandell Rees Feline Kidney* – CRFK) e alto índice terapêutico contra o FHV-1 (SILVA et al., 2014b), fatores que estimulam a realização de novas pesquisas sobre seu uso *in vivo*.

Embora dados obtidos em ensaios clínicos controlados ou a partir de infecções experimentais em gatos sejam determinantes da real eficácia de drogas contra o FHV-1, esses estudos são extremamente difíceis de serem conduzidos. Pesquisas envolvendo infecção experimental de gatos demandam instalações adequadas e são de difícil manejo, além de apresentarem questões éticas e humanitárias restringentes. Dessa forma, o uso de um modelo experimental de infecção pelo FHV-1 pode permitir abordagens experimentais que não seriam viáveis em gatos, mas que podem ajudar a entender melhor a patogênese da doença e fornecer evidências de novos fármacos antivirais. Apesar dos modelos animais possuírem suas limitações e nunca imitarem completamente a infecção na espécie natural, eles são muito úteis para o rastreio inicial e monitoramento da eficácia antiviral.

A quantidade de informações a respeito das características genéticas e biológicas de linhagens de camundongos os torna modelos animais atrativos para investigar a patogênese e os mecanismos imunes responsáveis pela eliminação dos vírus (AWAN et al., 1990; BAXI et al., 1996; BARTELS et al., 1998; MORI et al., 2012). O perfil clínico nos modelos murinos mimetiza muitos dos sinais observados em algumas doenças humanas e animais (MILLER et al., 1996) e, portanto, a reprodução de infecções virais em animais experimentais demonstrou que esses modelos podem ser utilizados em estudos mais detalhados sobre a patogênese de algumas doenças virais tanto de animais (van WOENSEL et al., 1995; MORI et al., 2012) quanto de seres humanos (CANTELL & PYHÄLÄ, 1973; MAHIET et al., 2012).

Existem vários relatos da reprodução de infecções em modelos experimentais por herpesvirus, outros que não o FHV-1, tais como herpesvírus bovino tipo 1 e 5 (BoHV-1; BoHV-5), herpesvírus equino tipo 1 (EHV-1), além dos herpesvírus simplex tipo 1 e 2 (HSV-1. HSV-2) (van WOENSEL et al., 1995; MALCHIKOV et al., 2009; MAHIET et al., 2012; MORI et al., 2012). Contudo, não há dados até o momento sobre a reprodução da infecção pelo FHV-1 em modelos experimentais, exceto o próprio gato (GASKELL & POVEY, 1979; NASISSE et al., 1989; FONTENELLE et

al., 2008; THOMASY et al., 2011). O presente trabalho objetivou analisar o uso de camundongos como modelos experimentais para reproduzir a infecção pelo FHV-1 e avaliar a atividade do peptídeo antimicrobiano P34, da lactoferrina e do GCV contra o FHV-1 nesse modelo experimental.

2 Artigos

2.1 Artigo 1

Perspectivas terapêuticas no tratamento das infecções pelo herpervírus felino tipo 1

Débora Scopel e Silva; Clarissa Caetano de Castro; Fábio da Silva e Silva; Maureen Hoch Vieira Fernandes; Fátima Lorenzini; João Manuel Chapon Cordeiro; Gilberto D'Avila Vargas; Geferson Fischer; Marcelo de Lima; Silvia de Oliveira Hübner.

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Resumo: O herpesvírus felino tipo 1 é uma causa muito comum de doença do trato respiratório superior entre os felinos. A doença, conhecida como rinotraqueíte viral felina, possui alta morbidade e é autolimitante em animais hígidos, porém causa severos sinais clínicos em felinos debilitados, imunocomprometidos e filhotes. Muitas drogas antivirais comumente utilizadas no tratamento das infecções pelos herpesvírus humanos já foram testadas *in vitro* e *in vivo* contra o herpesvírus felino tipo 1, demonstrando diferentes graus de eficácia e toxicidade. Entretanto, nenhuma droga antiviral foi desenvolvida especificamente contra o herpesvírus felino até o momento, limitando os tratamentos ao uso do aciclovir e do famciclovir. O objetivo do presente artigo foi revisar as terapias convencionais e adjuvantes utilizadas para o tratamento das infecções pelo herpesvírus felino tipo 1, abrangendo os mecanismos de ação e a natureza de alguns compostos antivirais.
Palavras-chave: gatos, rinotraqueíte viral felina, antivirais

Resumen: El herpersvirus felino tipo 1 es una causa frecuente de enfermedades del tracto respiratorio superior de los gatos. La enfermedad, también conocida como rinotraqueítis viral felina, posee alta morbilidad y se autolimita en animales sanos, pero causa signos clínicos graves entre los gatos debilitados, inmuno comprometidos y en animales jóvenes. Muchos fármacos antivirales comumente usados en el tratamiento de las infecciones por herpesvirus humanos ya fueron testados, tanto in vitro como *in vivo*, contra el herpesvirus felino tipo 1 mostrando diferentes grados de eficacia y toxicidad. Hasta el momento no se ha desarrollado ningún fármaco específico contra el herpesvirus felino, y los tratamientos se limitan a la utilización del aciclovir y del famciclovir. El objetivo del presente trabajo ha sido revisar las terapias convencionales y adyuvantes que se utilizan para el tratamiento de las infecciones por herpesvirus felino tipo 1, incluyendo los mecanismos de acción y las características de algunos compuestos antivirales.

Palabras clave: gatos, rinotraqueítis viral felina, antivirales

Abstract: Feline herpesvirus type 1 is a very common cause of upper respiratory tract disease among cats. The disease, known as feline viral rhinotracheitis, has high morbidity and it is self-limiting in healthy cats, but it causes severe clinical signs in kittens and immunocompromised animals. Many antiviral drugs commonly used for the treatment of human herpesvirus were already tested *in vitro* and *in vivo* against

feline herpesvirus type 1, showing different degrees of efficacy and toxicity. Nevertheless, so far no antiviral drug has been developed specifically against feline herpesvirus, limiting therapeutic options to acyclovir and famciclovir. The objective of this work was to review the conventional and adjuvant therapies used for the treatment of feline herpesvirus type 1 infections, including aspects about the mechanisms of action and the nature of some antiviral compounds

Keywords: cats, feline viral rhinotracheitis, antivirals

Introdução

O herpesvírus felino tipo 1 (FHV-1) é um membro da família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Simplexvirus* [1]. Essa família compreende vírus de genoma DNA dupla-fita, envelopados, caracterizados por seu ciclo replicativo curto *in vitro*, rápida disseminação célula a célula e persistência nos gânglios sensoriais de seus hospedeiros, conhecida como latência [2].

O FHV-1 é uma causa comum de doença do trato respiratório superior, conjuntivite (Figura 1) e ceratite em gatos [3]. Além de rinite e pneumonia intersticial, o FHV-1 pode provocar necrose hepática, emaciação, abortos, fetos natimortos [4] e dermatite [5]. O vírus geralmente fica restrito ao trato respiratório superior (Figura 2) e aos olhos, porém pode ocasionalmente invadir os pulmões, levando à pneumonia [6]. A doença, conhecida como rinotraqueíte viral felina [7], também provoca lesões epiteliais que geralmente são vistas no plano nasal, embora possam ocorrer menos frequentemente na face, nas orelhas, nas patas, no abdômen [8,9] e na região periorbital [10]. Os sinais clínicos se desenvolvem de 3 a 5 dias após a infecção [1] e perduram por 10 a 14 dias [11]. Tipicamente, os animais se recuperam em 10 a 21 dias, embora possa ocorrer infecção crônica ou até mesmo óbito [6].

Os sítios primários da replicação viral são os tecidos epiteliais, incluindo a conjuntiva e o epitélio nasal, corneano e faringeano [6]. Após rápida replicação ocorre dano celular agudo, levando à citólise [2] e a lesões necrotizantes [9], promovendo erosão da superfície epitelial (Figura 3) e inflamação [6]. A viremia é um fenômeno raro, pois a replicação viral normalmente fica restrita a áreas de baixa temperatura corporal [12]. O vírus pode ser detectado em suabes nasais, conjuntivais ou orofaríngeos a partir de 24 horas após a infecção, e geralmente persiste por uma a três semanas [2].

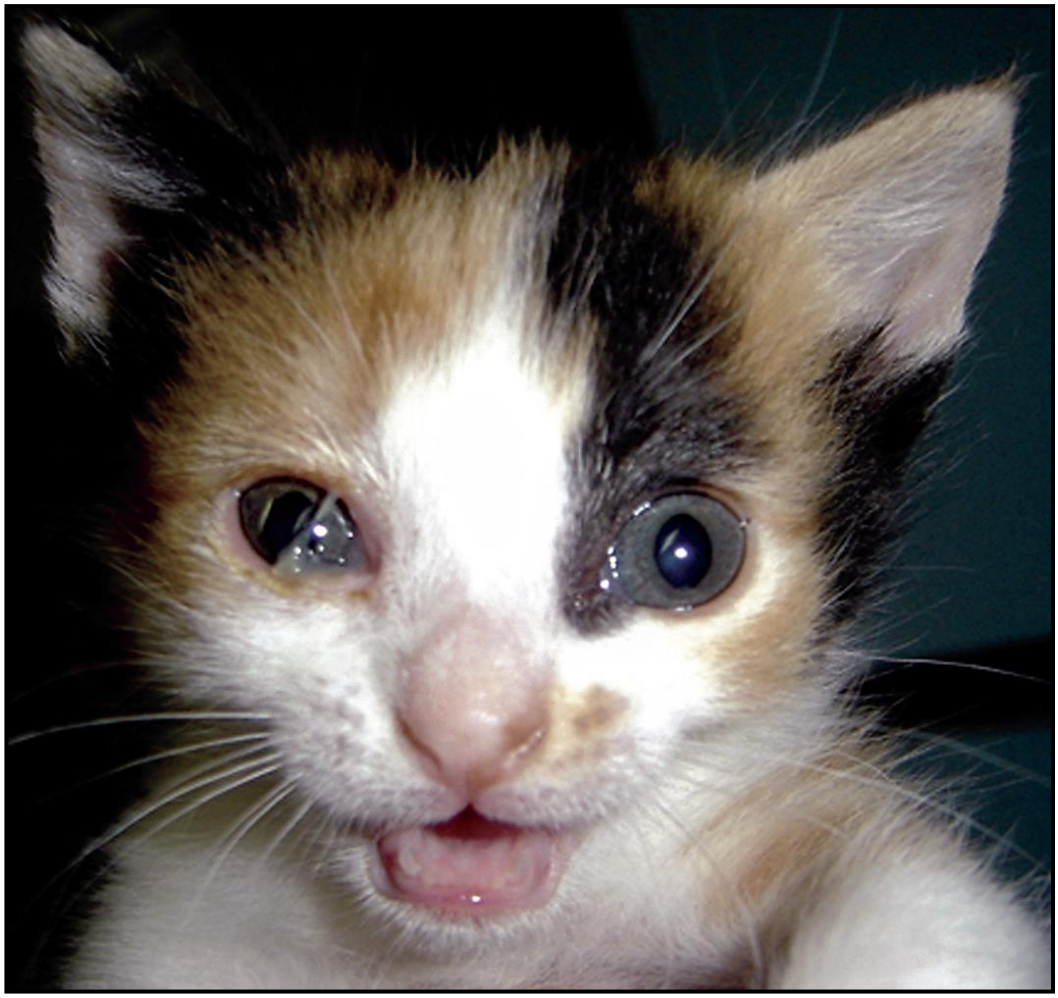

Figura 1 - Filhote sem raça definida (SRD), com aproximadamente quatro semanas de idade, apresentando conjuntivite e secreção ocular purulenta unilateral.

Estudos sorológicos demonstram que o FHV-1 está disseminado na população felina em todo o mundo [2,13], com elevada morbidade e baixa mortalidade [6]. A faixa etária dos gatos acometidos varia de 4 meses a 16 anos, e não foi observada predileção por sexo [14]. Embora os gatos sejam frequentemente infectados em idade jovem, o vírus pode causar problemas por toda a vida do animal [6], devido à latência e à reativação da infecção [15]. A reativação do vírus latente está associada à excreção viral, que ocorre espontaneamente ou devido a fatores estressantes, incluindo administração sistêmica de corticosteroides, coinfecção com outros agentes, troca de ambiente, cirurgias, parto e lactação [6,15].

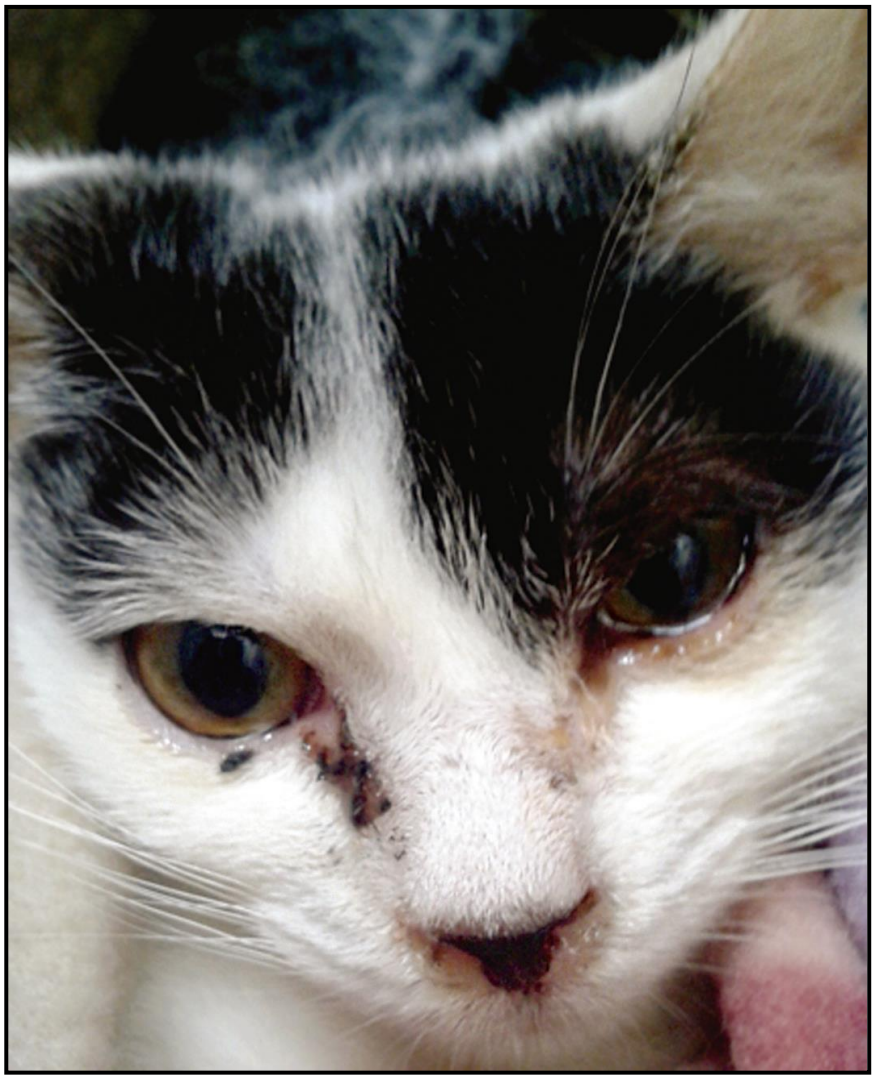

Figura 2 - Felino SRD apresentando secreção serosa nasal e ocular bilaterais.

No ambiente, o FHV-1 é relativamente instável, persistindo por mais de 18 horas em condições úmidas [2] e perdendo cerca de 90% de sua infectividade após 6 horas a 37°C [7]. A principal via de transmissão é o contato direto com fluidos corporais, especialmente secreções respiratórias, por meio de espirros, fômites contaminados ou práticas de manejo não higiênicas [16]. As partículas virais infectantes podem alcançar vários metros após um espirro, disseminando o vírus no ambiente e permitindo que outros gatos expostos se infectem [6].

O diagnóstico pode ser realizado por meio de testes com anticorpo fluorescente [2], reação em cadeia da polimerase (PCR) [17,18], histopatologia e imuno-histoquímica [5,8,19], Elisa (*enzyme-linked immunosorbent assay*), soroneutralização, além de isolamento viral, que é o padrão ouro para diagnóstico definitivo [1] (Figura 4). Entretanto, os testes sorológicos são pouco úteis clinicamente, devido à alta

prevalência de anticorpos [13]. Exames complementares, como a citologia conjuntival, podem ser realizados na infecção aguda pelo FHV-1, na qual se podem observar corpúsculos de inclusão intranucleares [1].

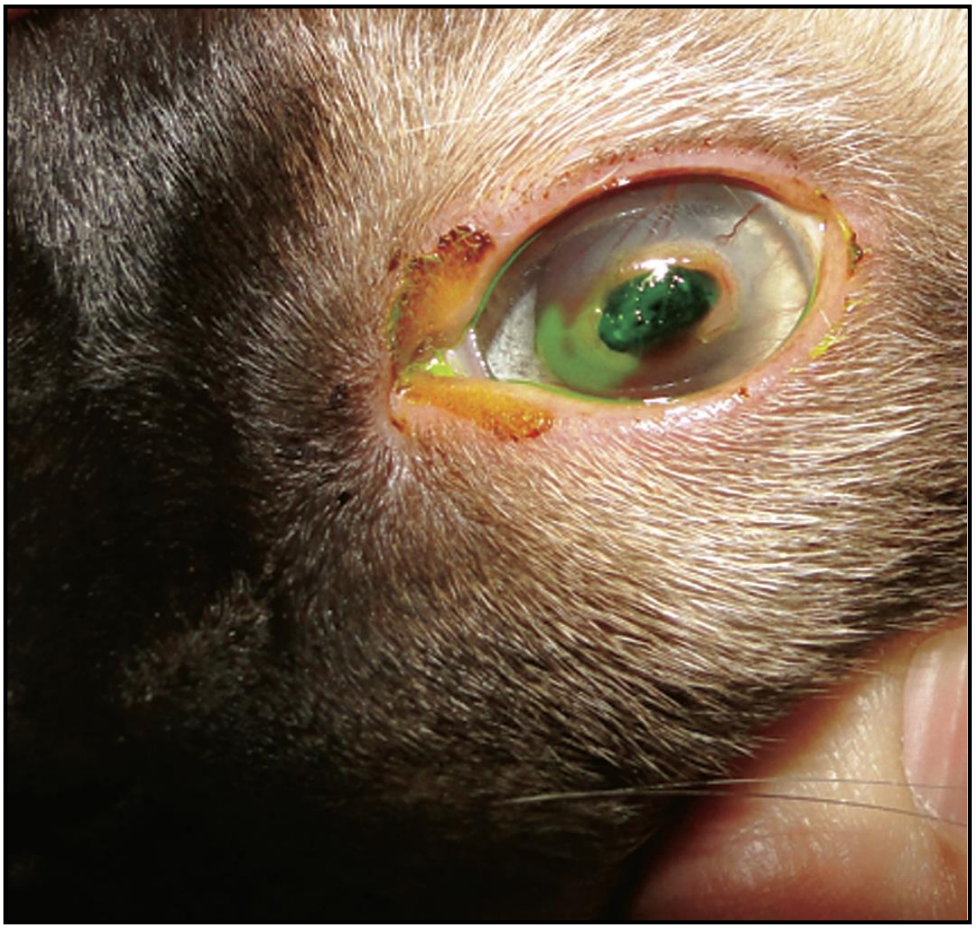

Figura 3 - Felina siamesa com oito anos de idade apresentando sequestro corneano e intensa ceratite devido à infecção pelo FHV-1. Observar a impregnação da córnea pela fluoresceína.

Existem vacinas vivas modificadas e inativadas disponíveis contra o FHV-1, entretanto elas não conferem proteção contra a reativação da latência [6], além de proporcionarem apenas proteção parcial contra os sinais clínicos e nenhuma proteção contra a reativação ou liberação do vírus [9], o que reforça a importância da existência de fármacos antivirais.

Os agentes antivirais eficazes e não tóxicos contra os herpesvírus humanos não são necessariamente efetivos e seguros contra o herpesvírus felino [20]. Embora um grande número de agentes antivirais tenha sido estudado *in vitro* contra o FHV-1, poucos foram avaliados em ensaios clínicos [2], além de nenhum composto antiviral

ter sido desenvolvido especificamente contra o FHV-1 [20]. Dessa forma, um protocolo de tratamento eficaz, específico e seguro contra o FHV-1 ainda não está disponível [6]. O objetivo do presente artigo foi revisar as terapias antivirais convencionais e adjuvantes utilizadas para o tratamento das infecções pelo FHV-1.

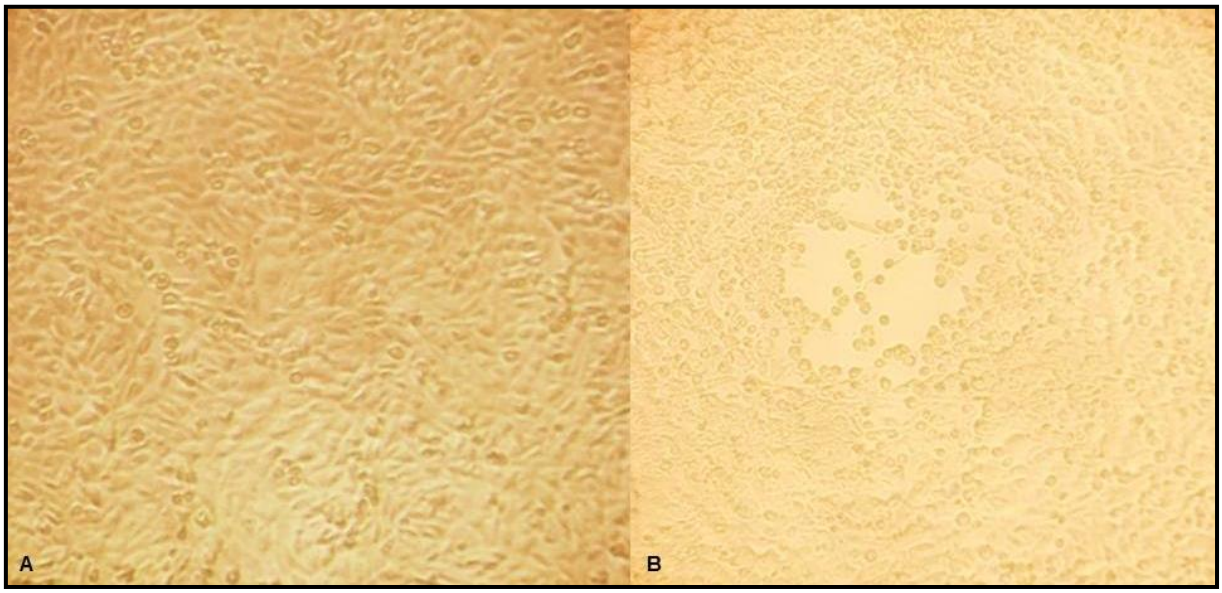

Figura 4 - Microfotografias de cultivo de células de rim felino (*CRFK – Crandell Rees feline kidney*) utilizado para o isolamento viral. Na imagem **A** podem-se visualizar células não infectadas e, na imagem **B**, células apresentando efeito citopático característico de uma infecção pelo FHV-1 (120x)

Os médicos veterinários de pequenos animais frequentemente são desafiados a tomar decisões clínicas e terapêuticas em relação ao manejo de gatos infectados com o FHV-1 [21]. Um grande número de antivirais foi desenvolvido para o tratamento das infecções pelos herpesvírus humanos [22] e muitos deles já foram avaliados contra o FHV-1 [23]. Existe uma variedade de agentes antivirais para tratamento oral ou tópico (oftálmico) de felinos infectados com o FHV-1. Contudo, todos os agentes antivirais atualmente utilizados em gatos infectados com FHV-1 são virostáticos, apenas retardando a replicação do vírus enquanto o sistema imune do hospedeiro o elimina e, por isso, necessitam de administração frequente para serem eficazes [6]. Além disso, devido ao fato de os vírus serem micro-organismos intracelulares obrigatórios, os antivirais tendem a ser mais tóxicos do que os agentes antibacterianos [20].

Os compostos antivirais administrados por via oral ou tópica necessitam ser metabolizados pelo hospedeiro ou pelo vírus antes de atingirem sua forma ativa [24]. Muitos desses agentes antivirais foram avaliados somente em seres humanos e podem não ser processados adequadamente pelos gatos ou pelo FHV-1, sendo necessários mais estudos sobre a eficácia e a farmacocinética dessas drogas em felinos. O conhecimento das propriedades farmacológicas dos compostos antivirais pode guiar a conduta terapêutica do médico veterinário para o tratamento dos felinos acometidos pelo FHV-1 [20].

A seguir serão descritos alguns aspectos relacionados à eficácia, à farmacocinética e à segurança de alguns antivirais propostos para o tratamento de gatos infectados com o FHV-1.

Antivirais análogos de nucleosídeos

O grupo mais eficaz de drogas anti-herpes é o de análogos acíclicos de nucleosídeos [2]. São compostos virostáticos que atuam como substratos alternativos, competindo com os nucleosídeos fisiológicos [6]. A incorporação de análogos de nucleosídeos aborta a síntese de DNA catalisada pela DNA polimerase, visto que não se podem formar ligações que estabilizem a dupla cadeia [23]. Para ser metabolicamente ativa, a maioria dos nucleosídeos acíclicos requer fosforilação pela timidina quinase (TK) de origem viral [2]. Após a fosforilação viral pela TK, ocorrem etapas adicionais de fosforilação que são mediadas pelas quinases celulares do hospedeiro [25].

- *Aciclovir*

O aciclovir é um análogo acíclico da guanosina comumente prescrito para seres humanos com doença herpética [26]. A formulação tópica é bem tolerada em gatos [2], porém o aciclovir sistêmico foi associado à supressão da medula óssea [27]. Além disso, estudos experimentais demonstraram que o aciclovir é ineficaz contra o FHV-1 [28], embora sua atividade antiviral *in vitro* tenha sido aumentada significativamente quando utilizado em conjunto com o interferon alfa recombinante humano [29]. Apesar desses relatos, um teste clínico prospectivo sugeriu que o aciclovir tópico aplicado cinco vezes ao dia pode ser eficaz para o tratamento de gatos com ceratite causada por herpesvírus [30]. A terapia tópica com aciclovir a 3% deve ser realizada a cada 4 ou 6 horas, durante 21 dias [2]. A terapia sistêmica é controversa, pois mesmo

utilizando doses muito altas (50 a 100mg/kg), o fármaco não atinge boas concentrações no plasma [6].

- *Valaciclovir*

É a pró-droga do aciclovir que é absorvida com maior eficácia pelo trato gastrointestinal em seres humanos e felinos [20]. Contudo, não deve jamais ser utilizada em felinos, pois em animais experimentalmente infectados com o FHV-1, o valaciclovir induziu supressão da medula óssea, necrose renal e hepática fatal, além de não reduzir a excreção do vírus ou a severidade da doença clínica [31].

- *Ganciclovir*

O ganciclovir é um análogo acíclico da guanina e, como o aciclovir, sua primeira fase de fosforilação é mediada pela TK viral [32]. Está disponível para administração tópica, intravitreal ou sistêmica (oral ou intravenosa) em seres humanos com infecção pelo herpes simplex tipo 1 (HSV-1) [20]. Estudos *in vitro* indicam boa eficácia contra o FHV-1 e, portanto, essa é uma opção promissora de tratamento, embora ainda não tenham sido feitas triagens clínicas [2]. Pesquisadores demonstraram que o ganciclovir possui eficácia dez vezes superior ao aciclovir contra o FHV-1, sugerindo que existe uma diferença na captação celular da droga, na eficácia da fosforilação pelo hospedeiro ou pelo vírus [25] ou ainda na estabilidade dos metabólitos [28].

- *Idoxuridina*

A idoxuridina é um análogo da timidina originalmente desenvolvido para o tratamento de seres humanos infectados com o HSV-1 [24]. Após a fosforilação intracelular a idoxuridina compete com a timidina para sua incorporação no DNA viral, deixando o vírus resultante incapaz de se replicar [20]. Entretanto, a idoxuridina age com menor eficácia contra o FHV-1 quando comparada ao HSV-1 e, por ser um inibidor inespecífico da síntese de DNA, afeta qualquer processo que necessite de timidina [20]. A idoxuridina deve ser aplicada quatro a seis vezes ao dia no olho afetado (durante até 21 dias) para atingir sua máxima eficácia [6], estando disponível comercialmente na forma de solução oftálmica a 0,1% ou pomada a 0,5% e é bem tolerada pela maioria dos gatos [20].

- *Trifluorotimidina*

Também conhecida como trifluridina, a trifluorotimidina é um análogo da timidina [33]. Este fármaco demonstrou eficácia superior à idoxuridina e similar ao aciclovir e à vidarabina para o tratamento de ceratite por HSV-1 [34]. Teoricamente, ela é a droga antiviral tópica de escolha para o tratamento da conjuntivite pelo FHV-1 [2,20], porém,

deve ser administrada com frequência e não é bem tolerada em gatos, presumivelmente pela irritação ocular relatada em seres humanos [20]. Apresenta-se na forma de solução oftálmica a 1%, devendo ser aplicada quatro a seis vezes ao dia, durante 14 a 21 dias [6].

- *Vidarabina*

A vidarabina é um análogo da adenosina não seletivo e está associada a notável toxicidade quando administrada pela via sistêmica [35]. A droga tópica é bem tolerada em gatos [21] na forma de pomada oftálmica a 3%, devendo ser aplicada quatro a seis vezes por dia no olho afetado [20], durante até 21 dias [6].

- *Cidofovir*

O cidofovir é um análogo da citosina usado para tratar retinite causada por citomegalovírus em seres humanos, possuindo também um grande espectro de ação contra outros vírus [2]. Estudos demonstraram eficácia contra o FHV-1 *in vitro* e *in vivo* [28,36,37], entretanto, ainda há poucos dados que suportem sua segurança a longo prazo como agente tópico em gatos [20]. O metabólito ativo do cidofovir tem longa ação antiviral, possuindo uma meia-vida intracelular de 65 horas [2]. A administração tópica de cidofovir a 0,5%, a cada 12 horas, durante 21 dias, reduziu significativamente a excreção do vírus e a severidade dos sinais clínicos em gatos infectados experimentalmente [37]. No entanto, o uso oftálmico experimental foi associado à estenose do ducto nasolacrimal em seres humanos e em coelhos [38], além de nefrotoxicidade em seres humanos [39]. Infelizmente, essa droga ainda não está disponível comercialmente no Brasil.

- *Penciclovir*

O penciclovir é um análogo da desoxiguanosina, com mecanismo de ação similar ao do aciclovir e do ganciclovir [40], que possui alta eficácia contra o FHV-1 *in vitro* [25] e *in vivo* [41,42], provavelmente devido à grande eficiência da fosforilação pela TK viral. A dose recomendada é de 90mg/kg por via oral uma a três vezes ao dia, durante 3 semanas [2,43].

- *Famciclovir*

O famciclovir é a pró-droga do penciclovir com metabolismo mais complexo, envolvendo oxidação por enzimas hepáticas [2]. Ele demonstrou ter efeito favorável na redução da severidade dos sinais clínicos decorrentes da infecção pelo FHV-1 [42]. Um estudo recente avaliou a utilização de altas doses de famciclovir, administradas oralmente, em gatos experimentalmente infectados com o FHV-1, demonstrando

redução da conjuntivite e da excreção viral [43]. Atualmente, o famciclovir e o cidofovir são os únicos compostos antivirais com comprovada eficácia clínica contra FHV-1, devendo ser considerados drogas preferenciais para o tratamento da infecção pelo FHV-1 [2]. A dosagem recomendada é de 62 a 125mg/gato, uma a três vezes ao dia por via oral, por até 21 dias [42].

- *Ribavirina*

A ribavirina foi o primeiro análogo de nucleosídeos sintético que demonstrou ter atividade contra vírus RNA e DNA e seu mecanismo de ação principal é a inibição da enzima celular inosina monofosfato desidrogenase [44]. Essa droga demonstrou ter atividade promissora contra o FHV-1 *in vitro* [45], contudo, sua administração *in vivo* provocou sérios efeitos colaterais, como, por exemplo: supressão de medula óssea, degeneração hepática [46], trombocitopenia, perda de peso, icterícia e distúrbios de coagulação [47]. Sendo assim, não deve ser utilizada nesta espécie.

Antivirais análogos do pirofosfato

- *Foscarnet*

Foscarnet é um análogo do pirofosfato que inibe a DNA polimerase e é eficaz no tratamento das infecções por herpesvírus resistentes ao aciclovir em seres humanos [39]. Um estudo comparativo constatou que a eficácia do foscarnet contra o FHV-1 *in vitro* era similar à do aciclovir [28].

Compostos antivirais adjuvantes

Fatores como eficácia, disponibilidade, toxicidade, frequência de aplicação e custo de muitas medicações antivirais levaram a um grande interesse por terapias adjuvantes no tratamento das infecções pelo FHV-1 [21], inclusive compostos provenientes da fauna e da flora brasileiras [48].

- *Interferons*

Interferons (IFNs) são moléculas proteicas produzidas e secretadas pelas células em resposta à infecção viral e são conhecidas por terem amplas atividades imunológicas e antivirais [49]. Os INFs são classificados em dois grupos amplos: o tipo I inclui os INFs alfa, beta e ômega (IFN-α, IFN-β, IFN-ω), que podem ser produzidos pela maioria dos tipos celulares após a infecção viral; o tipo II é representado pelo INF gama (IFN-γ) que é produzido somente por algumas células do sistema imune [2].

Os IFNs se ligam a receptores celulares que ativam enzimas inibindo a síntese, a morfogênese e a liberação do vírus, resultando em uma ação virostática [6]. Os IFNs α-humano e ω-felino podem resultar na diminuição dos efeitos citopáticos produzidos pelo vírus *in vitro* [50,51], e na remissão dos sinais clínicos e da excreção viral [52]. Contudo, a administração do INF-ω previamente à infecção pelo FHV-1 não resultou em diminuição da excreção viral ou dos sinais clínicos dos animais [53]. O IFN-α humano e o INF-ω felino estão disponíveis comercialmente na forma de produtos liofilizados [6,53], entretanto, o INF-ω felino ainda não está disponível no Brasil. Em um estudo *in vitro*, o IFN-α humano demonstrou ser eficaz quando utilizado em associação com o aciclovir [29]. A dose do INF-α humano é de 30 UI (unidades internacionais)/gato por via oral uma vez ao dia, semana sim, semana não, até a resolução dos sinais clínicos [6]; já a dose do INF-ω felino é 1 MU/kg (1 milhão de unidades por quilo) a cada 24 horas, por 5 dias, por via subcutânea [54].

- *Lisina*

A lisina é um aminoácido antagônico à arginina capaz de reduzir a replicação *in vitro* de vírus que possuem proteínas ricas em arginina, como o HSV [55] e o FHV-1 [20,56]. Alguns estudos demonstraram que gatos infectados experimentalmente com o FHV-1 e que receberam lisina tiveram redução significativa da conjuntivite quando comparados aos animais do grupo controle [57]. Os dados desses estudos sugerem que a administração oral de lisina é segura em gatos [58], podendo reduzir tanto a reativação do vírus em animais latentemente infectados quanto os sinais clínicos decorrentes da infecção [57]. Todavia, a redução da suplementação com arginina não é recomendada em gatos [6], pois, na falta de arginina, a conversão da amônia em ureia é suprimida, resultando em níveis aumentados de amônia no sangue, o que leva à hipersalivação, hiperestesia, êmese, tremores musculares, ataxia, espasmos tetânicos, coma e morte [59]. A dosagem recomendada de lisina é 250 a 500mg/gato por via oral, a cada 12 ou 24 horas, durante até 3 semanas [6,20].

- *Lactoferrina*

A lactoferrina é uma glicoproteína produzida pelo epitélio de mucosas de várias espécies de mamíferos e possui propriedades antibacterianas, antifúngicas, antiparasitárias e antivirais [60]. Um estudo demonstrou que a lactoferrina exerce efeito antiviral muito potente sobre a replicação do FHV-1 *in vitro*. Esse efeito parece ser mediado pela inibição da adsorção do FHV-1 à superfície celular e/ou pela inibição da sua penetração na célula [61].

- *P34*

O peptídeo antimicrobiano P34 é uma substância produzida por uma espécie de *Bacillus* sp. que foi isolada do conteúdo intestinal do peixe *Leporinus sp.* na bacia Amazônica [62]. Estudos realizados por nosso grupo de pesquisa demonstraram que o P34 possui atividade antiviral contra o FHV-1 *in vitro* [48] e, portanto, pesquisas científicas adicionais *in vivo* deverão ser conduzidas.

Os dados gerados em estudos *in vitro* e *in vivo* proporcionaram grande base para o uso de drogas antivirais, bem como das terapias adjuvantes direcionadas aos felinos [20], como demonstra a figura 5. Além de reduzir o número de placas provocadas pelos herpesvírus, os agentes antivirais também devem exercer efeito redutor sobre o tamanho das placas por eles induzidas e, a redução do tamanho da placa pode ser um parâmetro para avaliar a habilidade do agente antiviral de restringir o tamanho das lesões macroscópicas *in vivo* [63]. Aciclovir, penciclovir e suas respectivas pró-drogas valaciclovir e famciclovir são as drogas de escolha para o tratamento das infecções herpéticas em seres humanos [44]. Os análogos dos nucleosídeos tais como: cidofovir [28], idoxuridina [28,64], trifluridina [30] e vidarabina [64] demonstraram ser eficazes na redução do número de placas do FHV-1 em ensaios *in vitro* [65]. Embora o aciclovir e o foscarnet tenham eficácia comprovada contra os herpesvírus humanos, eles possuem baixa eficácia na redução da formação de placas pelo FHV-1 [28].

Considerações finais

Estudos recentes têm demonstrado uma nova perspectiva para o uso de drogas antivirais e terapias adjuvantes direcionadas aos felinos. Entretanto, é importante salientar que os resultados obtidos *in vitro* ou em infecções experimentais nem sempre representam o que ocorre em animais naturalmente infectados. Ainda que a doença seja autolimitante em animais imunocompetentes, a latência neural e a doença recrudescente são uma realidade para os animais imunocomprometidos. Alguns dos agentes antivirais desenvolvidos para o tratamento da doença herpética em seres humanos atuam contra o FHV-1 e a maioria deles tem sido segura quando aplicada em gatos domésticos. No entanto, cabe ressaltar a necessidade de pesquisas científicas no que se refere ao desenvolvimento, à avaliação da eficácia e ao mecanismo de ação de drogas antivirais seguras contra o FHV-1, visto que ainda

não há nenhuma droga antiviral específica contra o agente e o que temos disponível de maneira eficaz no Brasil se resume ao aciclovir e ao fanciclovir.

Figura 5 - Relação da eficácia e da toxicidade dos antivirais avaliados para o tratamento das infecções pelo FHV-1.

Antiviral	**Eficácia *in vitro***	**Eficácia *in vivo***	**Toxicidade**
Aciclovir [21,27,28]	Moderada	Moderada	Moderada
Valaciclovir [31]	Ausente	Ausente	Alta
Ganciclovir [2,25,28]	Presente	NT	NT
Idoxuridina [6,20]	Moderada	Moderada	Ausente
Trifluorotimidina [2,20]	Presente	Presente	Presente
Vidarabina [20,21]	Presente	Presente	Ausente
Cidofovir [28,36,37]	Presente	Presente	Moderada
Penciclovir [25,41,42]	Presente	Presente	Ausente
Famciclovir [42,43]	Presente	Presente	Ausente
Ribavirina [45,46,47]	Presente	Presente	Alta
Foscarnet [28]	Presente	NT	NT
Interferon [50,51,53]	Presente	Presente	Ausente
Lisina [57,58]	Presente	Presente	Ausente
Lactoferrina [60,61]	Presente	NT	NT
P34 [48]	Presente	NT	NT

NT: não testado

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2.2 Artigo 2

Mice as the Experimental Model of Infection With Feline Herpesvirus Type 1

Débora Scopel e Silva; Clarissa Caetano de Castro; Fábio da Silva e Silva; Marcelo de Lima; Gilberto D'Avila Vargas; Geferson Fischer; Fabiane Borelli Grecco; Silvia de Oliveira Hübner.

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Débora Scopel e Silva[1]; Clarissa Caetano de Castro[1]; Fábio da Silva e Silva[1], Marcelo de Lima[1]; Gilberto D'Avila Vargas[1]; Geferson Fischer[1]; Fabiane Borelli Grecco[2]; Silvia de Oliveira Hübner[1]

[1] Laboratory of Animal Virology and Immunology, Universidade Federal de Pelotas, Campus Capão do Leão s/n°, Zip Code: 96010-900; Capão do Leão, Rio Grande do Sul, Brazil – scopeldebora@yahoo.com.br; clarissac.decastro@gmail.com; silvamedvet@hotmail.com; mdelima.ufpel@gmail.com; gdavilavargas@gmail.com; geferson.fischer@gmail.com; sohubner@yahoo.com.br

[2] Professor of the Veterinary College, Pathology Department, Universidade Federal de Pelotas, Campus Capão do Leão s/n°, Zip Code: 96010-900; Capão do Leão, Rio Grande do Sul, Brazil - fabigrecco@ig.com.br

Abstract

Feline herpesvirus type 1 (FHV-1) is an important causative of respiratory and oular disease worldwide. A specific treatment against this agent is not available yet. For that reason research about antiviral drugs is encouraged. Mice have been largely used to study the pathogenesis of several human and animal viruses, specially herpesviruses. The objective of this study was to verify if BALB/c mice would be suitable experimental models for the infection with FHV-1. Animals were infected intranasally with FHV-1 and produced typical clinical signs of disease starting three days post-infection and lasting until the day of euthanasia. The mice were euthanized ten days post-infection and the organs were collected to perform histopathology and real-time PCR. The results showed that mice were infected with FHV-1 and reproduced the disease, since lesions and viral DNA could be found in all the organs sampled, being the lung the most affected organ. This paper is the first reporting

experimental infection of BALB/c mice with FHV-1 and demonstrated that this species can be a useful tool to understand pathogenesis of FHV-1 and also to perform antiviral trials against this agent.

Key-words: BALB/c mouse, feline rhinotracheitis, experimental model.

Introduction

Feline herpesvirus 1 (FHV-1) is classified in the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus* within the order *Herpesvirales* (Davison et al., 2009). FHV-1 is a common and important cause of respiratory and ocular disease, dermatitis, and potentially intraocular disease in cats (Maggs, 2005). Infections with FHV-1 are common among cats throughout the world (DiGangi et al., 2012; Zicola et al., 2009). FHV-1 primarilly infects domestic cats, but lions and cheetahs are also susceptible (Povey, 1979; Gaskell et al., 2007). 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 (Cave et al., 2014).

Cats affected by both primary infection and viral recrudescence are likely to have ocular disease (Townsend et al., 2004). Conjunctivitis is the most common ocular condition, followed by corneal epithelial ulceration and keratitis, with or without ulceration (Stiles & Pogranichniy, 2008). Conjunctivitis is manifested by conjunctival hyperemia, with or without chemosis, tearing and discomfort (Stiles, 2014). Uncomplicated FHV-1 disease typically resolves within 1 to 2 weeks after infection (Cave et al., 2014). However, the virus establishes latency in neuronal tissues, and FHV-1–infected cats presumably remain infected for life (Parzefall et al., 2010). Such cats may periodically have recrudescence of latent FHV-1 infection, which may or may not be accompanied by clinical disease (Cave et al., 2014).

Currently, there is no specific drug available for the treatment of FHV-1 infection. Based on the literature, veterinary clinicians mostly use the antiviral drugs availabe for human herpesviruses to treat herpesviral infections in cats (Silva et al., 2014). Whenever a drug developed for the treatment of humans infected with a herpesvirus is used to treat a cat infected with FHV-1, two major assumptions must be made: that the drug is efficacious against FHV-1 and that it is safe in cats (Thomasy & Maggs, 2016). For this reason research in the field of veterinary antiviral therapy is encouraged.

Several points must be considered when determining the antiviral efficacy of a drug. Methodical investigation of *in vitro* efficacy against FHV-1, followed by pharmacokinetic and safety trials in normal cats, subsequent placebo-controlled efficacy studies in experimentally inoculated animals, and, finally, carefully designed and monitored clinical trials in client-owned animals, is critical (Thomasy & Maggs, 2016). Although data obtained in controlled clinical trials or experimental infections in cats reflect the real efficacy of drugs against FHV-1, these studies are extremely difficult to be carried out. Research involving experimental infection of cats demands suitable accomodations, besides humanitary and ethical restrictive concerns. In that way, the use of a carefully projected experimental model of infection with FHV-1 could allow experimental approaches that would not be viable in cats, but could help to understand better the pathogenesis of the disease and provide evidences of new antiviral drugs.

Although experimental models have their limitations and never mimic completely the infection in their natural host, they are very useful in the initial trial and monitoring of the antiviral efficacy. The amount of informations about the genetic and biologic characteristics of mice lineages make them attractive animal models to investigate the pathogenesis and the immune mechanisms responsible for virus clearance (Awan et al., 1990; Baxi et al., 1996; Bartels et al., 1998; Mori et al., 2012).

There are several reports of the reproduction of herpesviral infections (except for FHV-1) in animal models, like bovine herpesvirus types 1 and 5 (BoHV-1; BoHV-5), equine herpesvirus type 1 (EHV-1), herpes simplex type 1 and 2 (van Woensel et al., 1995; Malchikov et al., 2009; Mahiet et al., 2012; Mori et al., 2012). However, until now, there is no data about the reproduction of infection with FHV- 1 in animal models, except for the cat itself (Gaskell & Povey, 1979; Nasisse et al., 1989; Fontenelle et al., 2008; Thomasy et al., 2011). The present study aimed to evaluate BALB/c mice as experimental models for the infection with FHV-1.

Material and Methods

Virus and Cells

The strain B927 of FHV-1, provided by Desidério Finamor Veterinary Research Institute (IPVDF), was propagated in Crandell-Rees Feline Kidney (CRFK - ATCC® Number: CCL-94™, USA) cells cultivated in Eagle's minimum essential medium (E-MEM – Sigma Aldrich, USA) supplemented with 10% of bovine fetal serum (BFS, Gibco, USA), penicillin (Sigma Aldrich, USA), streptomycin (Vetec, Brasil), amphotericin B (Cristália, Brasil) and enrofloxacin (Bayer, Brasil), in an incubator at 37°C. The virus was titrated in monolayers of CRFK cells before use and the titer was considered the highest dilution of the culture that showed cytopathic effect.

Animals

Thirty three female BALB/c mice (4 weeks old) were obtained from the Central Bioterium of the Federal University of Pelotas (UFPel). All mice were acclimatized for one week before experimental manipulation and were housed at a temperature of 22-24°C, humidity of 40-60% and a light/dark cycle of 12/12 h in the animal facility of the university. Experimental and control mice were housed separately in mini-isolators. Autoclaved water and comercial pellets

of food were given *ad libitum*. All the animal handling was carried out in a bio-safety cabinet and the procedures were approved by the the Ethical Committee for Animal Experimentation (protocol number 23110.009152/2013-59).

Experimental Infection

Twenty three BALB/c mice were inoculated intranasally with a viral suspension of FHV-1 (10 µL/nostril/animal), with a titer of 10^5 tissue culture infective doses ($TCID_{50}$/100 µL). The control group (ten animals) received autoclaved distilled water in the same way. Animals were observed daily for clinical signs of disease, body weight changes and mortality. The animals were euthanized ten days post-infection (10 dpi) by an anaesthetic overdose and the organs were collected for histopathology and quantitative PCR (qPCR). Kidney, liver, lung and spleen fragments were stored at -70°C for qPCR and in formalin 10% for the histopathological analysis.

DNA Extraction and Quantification

DNA extraction was performed from the collected organs with the kit Wizard SV Genomic DNA Purification System (PROMEGA, USA) according to the manufacturer's protocol.

DNA concentrations of each sample were assessed by fluorometry in a QUBIT 3.0 device, with the kit QUBIT ssDNA Assay (Thermo Fisher Scientific, USA), according to the manufacturer's reccomendations. Briefly, an aliquot of 1 to 20 µL of each sample was diluted into a buffer solution with dye supplied by the kit. The reading was performed in ng/µL.

Histopathology

Samples of each tissue were embedded in parafin wax, sectioned and stained with hematoxylin and eosin (HE) in the Department of Pathology of the Veterinary College (FAVET/UFPel). A histopathologist who was not aware of sample assignment of the

experimental groups analyzed the samples. The sections were analyzed with a light microscope.

Real Time PCR

The samples were amplified by real time quantitative PCR, using the methodology of absolute quantification (Lefever et al., 2009).

Primers *forward* 5' GAC GTG GTG AAT TAT CAG CTG AAG 3', *reverse* 5' AAG GTA TGG TGC GGC AAA TC 3' and the probe 5' FAM-TGC TGC CTA TAT CAC CGC CCA CTA TCA A 3'TAMRA, were designed to amplify a conserved fragment of 77 base pairs of the thymidine kinase gene (TK) of FHV-1 (GenBank sequence KR296657.1).

PCR reaction was performed using 10 µl of 2x QuantiNova Probe PCR Master Mix (QIAGEN® USA), 1 µl (450 nM) of each primer, 1 µl (250 nM) of the probe, 3 µl of RNAse-free water and 4 µl of the extracted DNA. Each cycle of amplification was composed of an initial denaturation at 95ºC during 2 minutes, followed by 50 cycles at 95ºC during 5 seconds and 30 seconds at 60ºC.

The cycle threshold (C_T) for each sample was determined with the number of the cycle in which the fluorescence produced surpassed the limit of detection. The analysis were performed with the device BIOER LineGene 9600 (Hangzhou Bioer Technology, China).

For the quantification, a standard curve was performed with serial 10 fold dilutions of the positive control, which was the vaccine Feligen® CR/P (Virbac, Brazil) .

Statistical analysis

The statistical analysis was performed with the program Prism (GraphPad Software, USA). The Kruskall-Wallis analythical method was used to compare viral load among the organs of each animal. Values were considered significant when $p < 0.05$.

Results

Experimental Infection

Daily evaluations of the experimentally infected mice revealed some clinical signs related to upper respiratory tract disease (URTD). Uni or bilateral mild serous ocular discharge and conjunctivitis were observed in 9 animals (39,13%), all the mice presented blepharitis (Fig. 1) and blepharospasm (100%), ruffled fur was seen in 8 animals (34,78%) and photophobia was recorded in 6 animals (26,08%). The clinical signs appeared on the 3rd day post-infection and lasted until the day of euthanasia. Mice who received distilled water instead of FHV-1 did not present any type of clinical sign. There was no difference in body weight between the infected and control animals ($p>0.05$). Mortality was not observed.

Histopathology

Notable macroscopic lesions were not observed at the time of necropsy. However, multiple histopathological lesions could be verified in the same infected animal. The main histopathological lesions found in the infected animals were: interstitial pneumonia (10/23), pulmonary edema (1/23) and congestion (2/23); hepatitis (5/23), hepatic congestion (11/23), hepatocyte vacuolization (12/23) and necrosis (3/23), lymphoid depletion in the spleen (5/23), interstitial nephritis (5/23) and tubular nephrosis (5/23); apart from inclusion bodies in the lungs and liver (7/23) (Fig. 2).

Figure 1: FHV-1 experimentally infected BALB/c mice showing (**A**) blepharitis and (**B**) photophoby.

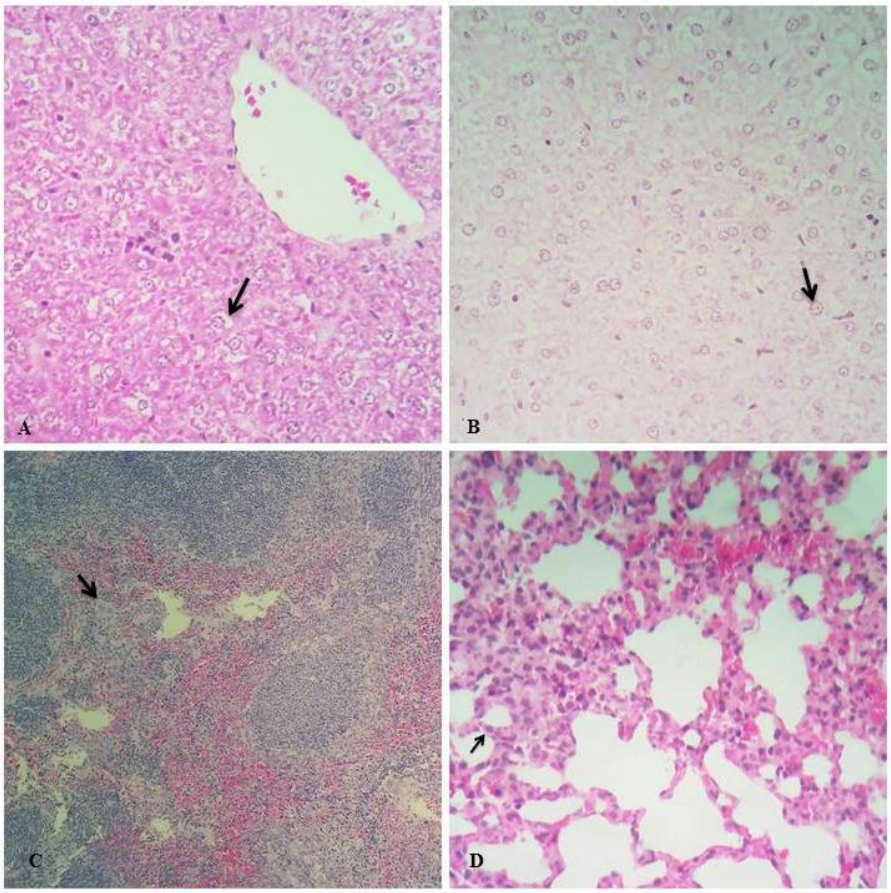

Figure 2. **A**: Liver presenting hepatocyte vacuolization (arrow). **B**: Liver, in a higher magnification presenting intranuclear inclusion bodies in hepatocytes (arrow). **C**: Spleen with lymphoid depletion (arrow) and **D**: interstitial pneumonia with mononuclear infiltrate and presence of pneumocytes type II (arrow). Microphotographs stained with HE.

qPCR

It was possible to detect viral DNA at least in one organ from each animal experimentally infected. The most affected organ was the lung (18/23), followed by the liver (14/23), spleen (10/23) and kidney (9/23) as can be seen in Fig. 3. It is important to say that viral DNA could be detected in more than one organ in some animals and the amount of viral load was statistically significant among the organs ($p<0.05$).

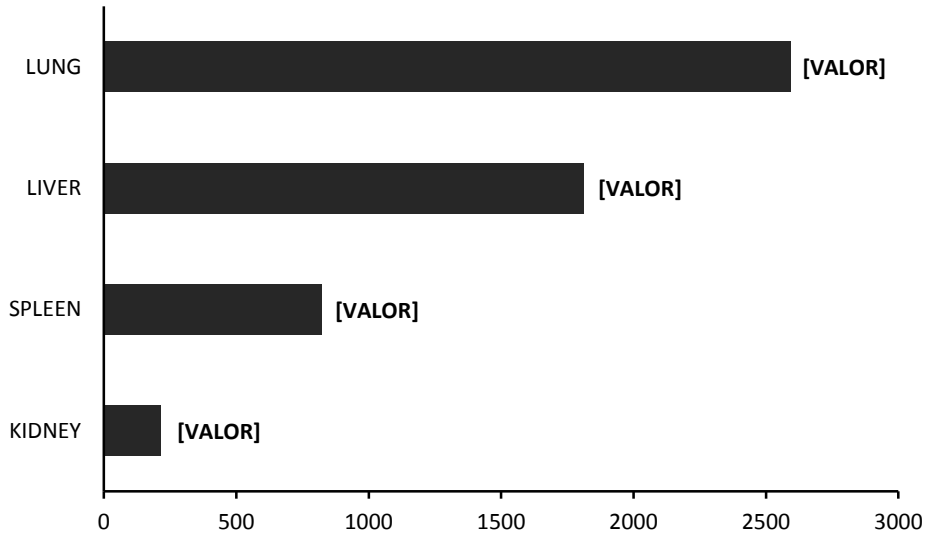

Figure 3: FHV-1 DNA copies/ mL in each organ sampled from the infected mice.

Discussion

Pathogenesis studies of FHV-1 infection have almost exclusively been done on live animals (Maes, 2012), *in vitro* organ cultures like the tracheal mucosa (Leeming et al., 2006) or *ex vivo* explants (Li et al., 2015). Mucosal explants have recently been shown for several herpesviruses to be an excelent system to study kinetics of viral invasion (Maes, 2012). This system has been used to compare the invasiveness of different herpesviruses (Vandekerckhove et al., 2009; Li et al., 2015). It can also be used for strain comparison and to study the role of individual or combinations of viral genes as determinants of viral virulence

(Glorieux et al., 2007; Steukers et al., 2012). However, this system has the same problem of using FHV-1 natural host: the source of the tissue explaint would still remain the cat. So, the murine model could replace the cat and allow the infection to occur in the context of an immune host.

In this work, evidence that mice can be infected by FHV-1 is provided. According to our histopathological and molecular analysis all the animals were infected by FHV-1, except for the controls. Viral DNA could be found in several organs of all of the inoculated mice, preferentially in the lungs (78,26%), followed by the liver (60,86%), spleen (43,47%) and kidneys (39,13%). This indicates that FHV-1 had been able to infect all of these animals and to spread within the organism in a productive manner.

The incubation period described for FHV-1 infected cats varies from 2 to 6 days (Maes, 2012), which is in accordance with the presentation of clinical signs in the experimentally infected animals in our study. Clinical findings in the experimentally infected mice appeared at 3 dpi and even if mild, they were very similar to the symptoms observed in naturally (Gaskell et al., 2007; Lara, 2012) and experimentally FHV-1 infected cats (Nasisse et al., 1989; Richter et al, 2009). All the infcected mice presented al least one type of clinical sign and the symptoms perdured until the day of euthanasia (10 dpi).

The virus rapidly replicates in epithelial cells of, basically, the upper respiratoty tract (URT) and the eyes, and then ascends via axons of sensory neurons to establish lifelong latency within the trigeminal ganglia (Maggs, 2005). FHV-1 is primarily an upper respiratory and ocular pathogen, with only sporadic involvement of the lungs, as viremia levels are low because of the temperature sensitivity of this virus (Maes, 2012). This would favor the virus to replicate in the URT instead of a systemic infection, but the animals of the present study presented both URT and systemic infection, as lesions like interstitial pneumonia, hepatitis,

interstitial nephritis and lymphoid depletion in the spleen could be found. Similar features were described in naturally FHV-1 infected cats (Hoover et al., 1970; Chvala- Mannsberger et al., 2009) and in EHV-1 experimentally infected mice (van Woensel et al., 1995; Mori et al., 2012).

Since until this moment there is no data regarding the use of mice as animal models for the experimental infection with FHV-1, comparisons had to be made with other herpesviruses in the same genus. Studies using a mouse model showed neuroinvasion after intranasal inoculation, thereby indicating the ability of EHV-1 to penetrate the brain following replication in tissues outside the central nervous system (Awan et al., 1990; Gosztonyi et al., 2009; Yu et al., 2010). The study conducted by Mori et al. (2012) described that the mice inculated intranasally with EHV-1 produced a mild respiratory infection of short duration, which was confirmed by the development of lesions and viral isolation. For example, BALB/c mice inoculated intranasally with EHV-1 exhibited many features similar to the infection in the horse (Mori et al., 2012). This included respiratory signs, local viral replication in the respiratory mucosa and cell-associated viremia (Walker et al., 1999), as demonstrated in this work.

Our findings suggest that viral migration occurred over the course of infection, as histopathological lesions and viral DNA could be detected in all the organs analyzedadjacent organs as demonstrated in cats (Gaskell & Povey, 1979; Chvala- Mannsberger et al., 2009) and in mice infected with EHV-1 (Mori et al., 2012). Such systemic infection in mice also could be detected by Abril et al. (2004) for BoHV-1 and 5, but the inoculation was performed intraperitoneally and with higher virus titers. Systemic disease is more likely to be caused by intraperitoneal or intravenous injections (Barr et al. 2007). In contrast, peripheral infections have been initiated by ocular, vaginal, skin, and nasal/oral tissue inoculation (Kollias et al., 2015).

Because the objective of this study was to verify if BALB/c mice would be potential models for the reproducibility of FHV-1 infection and generalized spread of the virus, internal organs were selected to perform the histologic and molecular analysis. Nevertheless, the animals presented clinical signs consistent with acute URTD, then it would be advisable to perform another study with similar conditions, but sampling the upper airways (nasal planum/nares, nasal turbinates, trachea and mandibular/rectropharyngeal lymph nodes) and the eyes (palpebral conjunctiva, third eyelid and corneas), as the primary replication sites of FHV-1 in the cat include mucosae of the nasal septum, turbinate, nasopharynx, conjuntivae, tonsils, mandibular lymph nodes and upper trachea (Maes, 2012).

A hallmark of alphaherpesvirus biology is that acute infection is followed by lifelong persistence of the viral genome in latent form in nervous and lymphoid tissues (Maes, 2012). So, another structure that would be a very important sampling site for the study of FHV-1 pathogenesis in mice is the trigeminal ganglia to verify latency. This is already well-established for human herpesviruses in the mouse model (Cook et al., 1991; Neumann et al., 2007; Stuart & Keadle, 2012).

Experimental infection models allow studies that are close to the field situation, but raise many ethical issues especially in companion animals such as cats (Li et al., 2015). For this reason, and in benefit of the health of domestic cats, the mouse model described here could be an interesting tool for the investigation of FHV-1 infection and future antiviral therapy research, making possible the evaluation of new drugs against the virus and contributing to the understanding of shared aspects of herpesviruses biology.

Conclusions

From our study it is possible to conclude that BALB/c mice are able to be infected by FHV-1 and to produce local and systemic disease. To our knowledge this is the first report hitherto of

FHV-1 infecting mice experimentally, thence data are extraordinary. Although a lot still has to be researched and discovered about the pathogenesis of FHV-1 in these animal models, the amount of informations produced here is primordial to elucidate initial trials in experimental infection with feline viruses in this species.

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2.3 Artigo 3

Activity of ganciclovir, lactoferrin and peptide P34 in mice experimentally infected with feline herpesvirus type 1

Débora Scopel e Silva; Clarissa Caetano de Castro; Fábio da Silva e Silva; Marcelo de Lima; Gilberto D'Avila Vargas; Geferson Fischer; Amanda de Souza da Motta; Silvia de Oliveira Hübner.

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Activity of ganciclovir, lactoferrin and peptide P34 in mice experimentally infected with feline herpesvirus type 1

Débora Scopel e Silva[1]; Clarissa Caetano de Castro[1]; Fábio da Silva e Silva[1], Marcelo de Lima[1]; Gilberto D'Avila Vargas[1]; Geferson Fischer[1]; Amanda de Souza da Motta[2]; Silvia de Oliveira Hübner[1]

[1] Universidade Federal de Pelotas, Laboratory of Animal Virology and Immunology, Campus Capão do Leão, Rio Grande do Sul, Brazil – scopeldebora@yahoo.com.br; clarissac.decastro@gmail.com; silvamedvet@hotmail.com; mdelima.ufpel@gmail.com; gdavilavargas@gmail.com; geferson.fischer@gmail.com; sohubner@yahoo.com.br

[2] Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Av. Sarmento Leite, n° 500, Prédio 12101, Zip Code: 90050-170, Campus Centro, Porto Alegre, Rio Grande do Sul, Brazil - amanda.motta@ufrgs.br

Abstract

Feline herpesvirus type 1 (FHV-1) is the major pathogen of feline upper respiratory tract disease and ocular disease among cats of all ages worldwide. Some antiviral drugs commonly used for the treatment of human herpesvirus were already tested *in vitro* and *in vivo* against feline herpesvirus type 1, but so far no antiviral drug has been developed specifically against FHV-1. Ganciclovir, a drug commonly used to treat human herpesviruses, was effective against FHV-1 *in vitro*, as well as lactoferrin and the peptide P34. The objective of this work was to evaluate for the first time the antiviral activity of ganciclovir, lactoferrin and the peptide P34 against FHV-1 *in vivo*, using BALB/c mice as experimental models. Mice were infected intranasally with FHV-1 and treated with ganciclovir, lactoferrin or the peptide P34

24 h after infection. One group was infected and not treated as the positive control group and another group was not infected nor treated, as the negative control group. After ten days of treatment, the animals were euthanized and the organs were collected to perform real-time PCR. The animals of the treated groups had markedly less clinical signs than the animals of the positive control group. qPCR analysis did not reveal a decrease of the viral load in the organs of the treated animals, suggesting that these antiviral compounds, in this dosage and frequence had no antiviral effect against FHV-1 *in vivo*, in this proposed experimental model.
Key-words: feline rhinotracheitis, antiviral therapy, BALB/c mouse

Introduction

Respiratory infections in cats are frequent and of great importance in feline internal medicine.[1] Feline herpesvirus type 1 (FHV-1), an enveloped double-stranded DNA virus, belongs to the *Varicellovirus* genus of the subfamily *Alphaherpesvirinae* [2] is the major pathogen of feline upper respiratory tract disease (URTD) and ocular disease[3] in cats of all ages, but preferentially in newborn and young cats.[2] Although there are comercially available vaccines against the main pathogens involved in the URTD, including FHV-1, for up to thirty years, the prevalence of these microrganisms in the feline population remains high.[1,4,5]

Clinical signs observed in both natural and experimental infections in cats are sneezing, loss of appetite, pyrexia, depression, nasal and ocular discharge, conjunctivitis dyspnea and cough.[2] The involvement of the eye includes corneal ulcers, stromal keratitis, corneal sequestration and blindness.[6,7] Corneal sequestration is more common in recurrent cases of keratitis due to FHV-1 latency and recrudescence.[8] Animals with chronic respiratory disease can present severe complications that frequently result in euthanasia.[9]

Many antiviral drugs commonly used for the treatment of human herpesviruses were already tested *in vitro* and *in vivo* against feline herpesvirus type 1, showing different degrees of

efficacy and toxicity. Nevertheless, so far no antiviral drug has been developed specifically against FHV-1.[10]

One drug commonly used for the treatment of herpes simplex virus types 1 and 2 and human herpesvirus type 6 is ganciclovir (GCV).[11,12] Ganciclovir (9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl] guanine), like acyclovir, is a synthetic purine nucleoside, and an analog of guanosine.[13] This drug is phosphorylated by viral thymidine kinases (TK) of the herpesvirus family and other viruses, into GCV monophosphate.[14] Within virus-infected cells, both viral and cellular TKs catalyze further phosphorylation of GCV monophosphate into GCV triphosphate, the active metabolite.[15] GCV triphosphate accumulates in virus-infected host cells, interfering with viral replication in two ways: 1) it is directly incorporated into viral strand primer DNA, inducing DNA single- and double-strand breaks, and resulting in viral DNA chain termination[16]; and 2) being a derivative of 2′-deoxyguanosine, it competes with deoxyguanosine triphosphate for binding to viral DNA-polymerase, interrupting new viral DNA synthesis.[17] GCV has demonstrated antiviral activity against FHV-1 *in vitro,*[18] but so far it has not been tested *in vivo*.

A natural antiviral compound with reported antiviral activity against FHV-1 *in vitro* is lactoferrin.[19] Lactoferrin is one of the most abundant secretion proteins in man and it is found in external secretions, especially milk, tears and saliva, but is also present in seminal plasma and vaginal mucus.[20] Lactoferrin derived from human or bovine milk has been shown to exhibit potent *in vitro* activity against cytomegalovirus[21] poliovirus[22], respiratory syncytial virus, [23] human immunodeficiency virus,[24] human herpesviruses type 1 and 2 (HSV-1 and HSV-2).[25] Lactoferrin appears to be an ideal antiviral agent since it interrupts viral replication at the level of viral attachment, a specific and essential step of the replicative cycle, without affecting normal host-cell metabolism.[24] Other advantages include easy availability, very low cytotoxicity and low cost.[19]

Our research group has recently reported the antiviral activity of an antimicrobial peptide against FHV-1 *in vitro*[26] as well as bovine herpesvirys type 1 [27] and equine arteritis virus [28]. This peptide, named P34, is produced by a species of *Bacillus* isolated from the intestinal contents of a fish (*Leporinus* sp.) found in the Amazon basin.[29] This peptide is already well characterized [30], studied [31,32] and applied experimentally in food safety[33,34], as the peptide P34 had antibacterial activity detected against Gram-positive and Gram-negative bacteria, like *Escherichia coli* (*E. coli*) and *Lysteria monocytogenes*, respectively.[35]

Ideally, to be the most trustworthy, the cat would be the best model to evaluate the antiviral activity of drugs against FHV-1 *in vivo*. However, studies with cats are difficult to be conducted because of ethical concerns about the use of animals for experimental trials in our country. Besides, such kind of research would require a relatively high number of animals and then it would become costly and laborious. The amount of information regarding the genetic and biological characteristics of inbred mouse strains make them attractive animal models for investigating the pathogenesis and immune mechanisms responsible for viral clearance.[36] As other studies already have been performed using BALB/c mice as animal models for the infection of both human and animal herpesviruses,[37,38,39] the objective of this work was to evaluate the antiviral activity of GCV, lactoferrin and P34 against FHV-1 *in vivo*, using BALB/c mice as experimental models.

Material and Methods

Virus

The strain B927 of FHV-1 was propagated in Crandell-Rees Feline Kidney (CRFK - ATCC® Number: CCL-94™, USA) cells cultivated in Eagle's minimum essential medium (E-MEM – Sigma Aldrich, USA) supplemented with 10% of bovine fetal serum (BFS, Gibco, USA), penicillin (Sigma Aldrich, USA), streptomycin (Vetec, Brazil), amphotericin B (Cristália,

Brazil) and enrofloxacin (Bayer, Brazil), in an incubator at 37 °C. The virus was titrated in monolayers of CRFK cells before use and the titer was considered the highest dilution of the culture that showed cytopathic effect.

Animals

Seventy female BALB/c mice (4 weeks old) were obtained from the Central Bioterium of the Federal University of Pelotas (UFPel). All mice were acclimatized for one week before experimental manipulation and were housed at a temperature of 22-24°C, humidity of 40-60% and a light/dark cycle of 12/12 h in the animal facility of the university. Experimental and control mice were housed separately in mini-isolators. Autoclaved water and comercial pellets of food were given *ad libitum*. All the animal handling was carried out in a bio-safety cabinet and the procedures were approved by the the Ethical Committee for Animal Experimentation (protocol number 23110.009152/2013-59).

Experimental Infection

Fifty six female BALB/c mice (4 weeks-old) were inoculated intranasally with a viral suspension of FHV-1 (10 µL/nostril/animal), with a titer of 10^5 tissue culture infective doses ($TCID_{50}$/100 µL). The negative control group (fourteen animals) received autoclaved distilled water in the same way. Animals were observed daily for clinical signs of disease, body weight changes and mortality. The animals were euthanized within eleven days post-infection (11 dpi) by an anaesthetic overdose and the organs kidney, liver, lung and spleen were collected and stored at -70°C to perform quantitative PCR (qPCR).

Three groups with fourteen animals each were infected and treated with different drugs, once a day, starting one day post-infection (1 dpi). The positive control group (PCG) consisted of fourteen mice experimentally infected with FHV-1 and not treated. The negative control group (NCG) had fourteen not infected mice.

The first group of treated mice was named group 1 (G1). The animals were infected with FHV-1 and 24 h after the infection the treatment with GCV (Ganciclotrat 500 mg – União Química, Brazil) was started. The animals received 25 mg/kg [40] of GCV intraperitoneally once a day, for ten days. After the end of the treatment the animals were euthanized with an anaesthetic overdose and the organs were collected.

Group 2 (G2) was infected with FHV-1 and 24 h after, the treatment with bovine lactoferrin (Sigma-Aldrich, USA) in the dose of 200 μg/animal/day [41] via gavage was started. After ten days of treatment the animals were euthanized with an anaesthetic overdose and the organs were collected.

The last group, named group 3 (G3), was infected with FHV-1 and, at the following day, the animals were treated with the peptide P34 (100 μg/animal/day) [32] via gavage. After ten days of treatment the animals were euthanized with an anaesthetic overdose and the organs were collected.

DNA Extraction and Quantification

DNA extraction was performed from the collected organs with the kit Wizard SV Genomic DNA Purification System (PROMEGA, USA) according to the manufacturer's protocol.

DNA concentrations of each sample were assessed by fluorometry in a QUBIT 3.0 device, with the kit QUBIT ssDNA Assay (Thermo Fisher Scientific, USA), according to the manufacturer's reccomendations. Briefly, an aliquot of 1 to 20 μL of each sample was diluted into a buffer solution with dye supplied by the kit. The reading was performed in ng/μL.

Real Time PCR

The samples were amplified by real time quantitative PCR, using the methodology of absolute quantification.[42]

Primers *forward* 5' GAC GTG GTG AAT TAT CAG CTG AAG 3', *reverse* 5' AAG GTA TGG TGC GGC AAA TC 3' and the probe 5' FAM-TGC TGC CTA TAT CAC CGC CCA CTA TCA A 3'TAMRA, were designed to amplify a conserved fragment of 77 base pairs of the TK gene of FHV-1(GenBank sequence KR296657.1).

PCR reaction was performed using 10 µl of 2x QuantiNova Probe PCR Master Mix (QIAGEN® USA), 1 µl (450 nM) of each primer, 1 µl (250 nM) of the probe, 3 µl of RNAse-free water and 4 µl of the extracted DNA. Each cycle of amplification was composed of an initial denaturation at 95ºC during 2 minutes, followed by 50 cycles at 95ºC during 5 seconds and 30 seconds at 60ºC.

The cycle threshold (C_T) for each sample was determined with the number of the cycle in which the fluorescence produced surpassed the limit of detection. The analysis were performed with the device BIOER LineGene 9600 (Hangzhou Bioer Technology, China).

For the quantification, a standard curve was performed with serial 10 fold dilutions of the positive control, which was the vaccine Feligen® CR/P (Virbac, Brazil) .

Statistical analysis

The statistical analysis was performed with the program Prism (GraphPad Software, USA). The Kruskall-Wallis analythical method was used to compare viral load among the organs of each animal. Values were considered significant when $p < 0.05$.

Results

Clinical Signs of FHV-1 Infection

Daily evaluations of the experimentally infected mice revealed some clinical signs related to the URTD. Animals of the NCG did not present any type of clinical sign. On the other hand,

all the infected mice from the PCG presented clinical signs of FHV-1 infection, that appeared on 3 dpi. Uni or bilateral mild serous ocular discharge and conjunctivitis were observed in 5 animals (35,71%), all the mice presented blepharitis and blepharospasm (100%), ruffled fur was seen in 7 animals (50%) and photophobia was recorded in 6 animals (42,85%). The clinical signs appeared on the 3rd day post-infection and lasted until the day of euthanasia. There was no difference in body weight scores among the PCG, NCG and treated animals ($p>0.05$).

Only three animals (21,4%) from both G1 and G2 presented clinical signs, as blepharitis and serous ocular discharge, on the last day of treatment. Two mice (14,28%) from G3, treated with the peptide P34, presented blepharitis with mild ocular discharge in the penultimate day of the treatment. Mortality was not observed.

qPCR

The animals of NCG presented no viral DNA in the analyzed samples, as expected (Table 1). The mice of PCG presented FHV-1 viral DNA in 100% of the lungs (14/14), 72,42% of the livers (10/14) and 42,85% of the kidneys and spleens (6/14).

FHV-1 viral DNA could be detected in 85,71% of the lungs (12/14), 64,28% of the spleens (9/14), 42,85% of the livers (6/14) and 28,57% of the kidneys (4/14) from the infected and treated mice of G1.

For G2, FHV-1 DNA could be detected in 78,57% of the spleens (11/14), 71,42% of the livers (10/14), and 50% of lungs and kidneys (7/14).

In G3, FHV-1 DNA could be found in 71,42% of the lungs (10/14), 42,85% for livers and spleens (6/14) and 28,57% of the kidneys (4/14).

Table 1. Amount of animals in each group with viral DNA detected per organ.

Group	Spleen	Liver	Lung	Kidney
NCG	-	-	-	-
PCG	6/14	10/14	14/14	6/14
G1	9/14	6/14	12/14	4/14
G2	11/14	10/14	7/14	7/14
G3	6/14	6/14	10/14	4/14

Discussion

Experimental infection models allow studies that are close to the field situation, but raise many ethical issues especially in companion animals such as cats.[43] Mice are relatively inexpensive rodents that can be easily obtained and maintained in statistically sufficient numbers for rapid *in vivo* screening.[44] Several studies were developed using different strains of mice to evaluate the pathogenesis of human and animal viruses and also to verify the efficacy of vaccines [45] and the antiviral properties of drugs.[37,39,41,46]

Since there are no data regarding the experimental infection of mice with FHV-1, comparisons had to be made with other viruses within the same genus, but also using mice as experimental models. The animals of this study presented clinical signs related to URTD, which is in accordance with the observed in clinical and experimental infections of the natural host.[2,47] The animals of all the treated groups presented markedly reduced clinical signs compared to the PCG. All the animals from PCG presented at least one clinical sign of infection, starting on the third dpi, some but few of the treated animals also presented mild clinical signs at the end of the treatment, what may be considered an unspecific finding.

Although there was clinical evidence that the treated mice were markedly less affected than those who were not treated, the viral load did not decrease in any of the treated groups. All the

antiviral compounds tested here, in this dose/frequency/duration regimen were not able to reduce viral load in any of the organs sampled ($p>0.05$).

FHV-1 replicates tipically in the epithelial cells of the conjunctiva and the upper respiratory tract (URT), infecting local neurons and establishing latency in the trigeminal, pterygopalatine and cranial cervical ganglia.[48] As for other alphaherpesviruses, peripheral nervous system infection leads to lifelong latency after primary infection.[2] Although viral replication is usually limited to the URT and conjunctiva, viremia was detected during the acute phase of infection[49,50] and, there are reports of nonsuppurative meningoencephalitis [51] and pneumomediastinum with subcutaneous emphysema in domestic cats [52], suggesting that FHV-1 has the ability to cause a more invasive disease.

GCV is an acyclic analogue of guanine and, as acyclovir (ACV), its first phase of phosphorilation is mediated by the viral TK.[40] Researchers have demonstrated that GCV has two-fold superior efficacy when compared to ACV against FHV-1 *in vitro*,[18] and Garré et al. (2007) [53] have found that GCV is twenty fold superior to adefovir and ten-fold superior to acv and PMEDAP *in vitro* against EHV-1. Systemic GCV may be administered in intravenous, oral, and vitreous implant forms.[13] As GCV has poor oral bioavailability[11], we opted to administer the drug to the animals of G1 intraperitoneally. Even then, our treatment did not result in reduction of the viral load among the organs. It may be explained by the fact that the drug was administered only once a day and in a low dosage. In the study conducted by Duan et al. (1998)[44] mice were infected with murine cytomegalovirus (MCMV) and received GCV treatment (10 to 160 mg/kg) once a day, subcutaneously, but the first administration was 3 h post-inoculation, while our treatment started 24 h post-inoculation. The higher dosage used in this study was toxic for the mice, but the best in terms of antiviral activity, so the optimal dose used for that experimental regimen was 80 mg/kg,[44] more than three times higher than the used in G1. In another study, mice were infected intraperitoneally with MCMV and treated

with 40 mg/kg of GCV 24 h post inoculation, once a day. The treatment was able to reduce viral titers of the spleens from the infected animals.[40]

A study conducted by Hussein et al. (2008) [54] tested the susceptibility of a recombinant FHV-1 TK expressed in *E. coli*. The recombinant exhibited relatively lower affinity for the guanosine analogue substrates penciclovir (PCV), GCV and ACV. PCV was most efficiently phosphorylated, followed by GCV, with approximately twofold reduction in the phosphorylation rate.[54] The lowest phosphorylation rate was recorded for ACV, suggesting that FHV-1 TK has a relatively narrow substrate specificity. GCV is structurally related to PCV, differing only in the presence of an O-atom in the acyclic side chain.[54] However, the FHV-1 TK model did not reveal any significant differences in the binding of these two substrates to the active site and the fact that PCV was more efficiently phosphorylated than GCV by the FHV-1 TK remains unexplained.[54]

Lactoferrin is a glycoprotein produced by the mucosal epithelium of several mammal species having antibacterial, anti-fungal, antiparasitic and antiviral properties.[55] The antiviral activity occurs against many different viruses, but always involves interference with early events of the viral infectious cycle, either by a direct interaction with the viral particle or by inhibiting viral attachment/entry.[56] A study demonstrated that lactoferrin exerts a very potent antiviral effect on the replication of FHV-1 *in vitro* and this effect appears to be mediated by the inhibition of the adsorption step of FHV-1 on the cell surface and/or by preventing its penetration in the cell.[19] Fujihara et al. (1995) [57] reported that topical ophthalmic administration of 1% lactoferrin solution significantly decreased HSV-1 titers *in vivo* using a mouse corneal infection model.

To verify if its antiviral action would occur *in vivo*, mice were infected with FHV-1 and tretated with lactoferrin 24 h after inoculation. Unfortunately, treatment did not result in a

decrease of the viral load in any of the organs sampled, when compared to the PCG. Maybe low dosage and frequency of lactoferrin administration might have contributed to this result. Fujihara et al (1995) [57], verified that the mice infected with HSV-1 and treated 24 h after had no viral inhibition, on the other hand, mice that received lactoferrin 30 minutes prior to, simultaneous with, and treated 4 times daily had 90% of viral inhibition. In the study conducted by Shestakov et al. (2012) [41], mice were infected intravaginally with HSV-2 and the treatment with lactoferrin was administered locally together with the virus, immediately before or after infection. Despite a strong anti-HSV-2 activity *in vitro*, lactoferrin did not have any effect in the genital *in vivo* HSV-2 model, but the contrary is true for lactoferricin. Our results may be due to the dosage of lactoferrin used, as studies about the antiviral activity of lactoferrin against enterovirus and CMV were successful when using a dose of 5-10 mg/mouse.[41] When lactoferrin was given orally to mice prior to infection with HSV-1 it had beneficial effects on body weight and Th1 cytokine responses, but it did not affect susceptibility to or clearance of the virus.[58]

Although many strains of *Bacillus* have been safely used in food and pharmaceutical industry, there are relatively few specific studies on the toxicity of antimicrobial peptides derived from *Bacillus*.[31] Toxicological studies involving animals are a major component of safety assessment of bacteriocins,[59] like P34. The investigation of oral acute toxicity of P34 in mice was performed with a single dose administration of the peptide in the concentrations of 82.5, 165, 247.5 and 330 mg/kg, and no death was observed.[32] As in our study antiviral activity could not be detected, we suppose that again, the dosage and frequency of treatment had a direct interference in the result, as the proteinaceous nature of bacteriocins implies a putative degradation in the gastrointestinal tract of man and animals,[60] requiring the administration more than once a day.

The objectives of this study were to verify if BALB/c mice would be able to reproduce FHV-1 infection and test the antiviral activity of GCV, lactoferrin and P34. To verify if FHV-1 would spread systemically, internal organs were selected to perform the molecular analysis. Nevertheless, the animals presented clinical signs consistent with acute URTD, then it would be advisable to perform another study with similar conditions, but sampling the upper airways (nasal planum/nares, nasal turbinates, trachea and mandibular/rectropharyngeal lymph nodes) and the eyes (palpebral conjunctiva, third eyelid and corneas), as the primary replication sites of FHV-1 in the cat include mucosae of the nasal septum, turbinate, nasopharynx, conjuntivae, tonsils, mandibular lymph nodes and upper trachea.[52] Possibly, in those tissues a decrease in viral load could be found in animals treated with these drugs, but it will require future detailed studies to be elucidated.

Alphaherpesvirus are capable of infecting a great number of species and an important feature of their biology is that acute infection is followed by lifelong persistence of the viral genome in latent form in nervous and lymphoid tissues.[52] Data generated in HSV-1-infected mice treated topically with lactoferrin suggest that treatment early in the course of primary infection is more likely to be effective,[57] it is possible that the same could be assumed for the infection with FHV-1 and both treatments: lactoferrin and P34. As the phosphorilation of GCV by FHV-1 TK is not enough efficient, maybe this drug is not active *in vivo* as it was *in vitro* against FHV-1. However, these propositions can only be confirmed with extensive research, perhaps using the cat itself.

Conclusions

Data from this study are extraordinary and confirm that BALB/c mice could be infected with FHV-1, producing clinical signs of disease apart from local and systemic infection. The antiviral therapies tested here, at this frequency and dose regimen, did not have a beneficial

effect in reducing the viral load in the animals, despite the improvement of clinical signs in all the treated mice. However, another studies should be performed testing the same drugs in a more elaborated regimen of administration to clarify the real antiviral activity of these compounds.

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3 Considerações Finais

A partir do estudo realizado com os camundongos da linhagem BALB/c, provenientes do Biotério Central da Universidade Federal de Pelotas, é possível concluir que tais animais são capazes de se infectar com o FHV-1 e produzir doença local e sistêmica. Até o momento este é o primeiro relato de infecção experimental de camundongos com o FHV-1 e, portanto, nossos dados são inéditos. Embora muito ainda tenha que ser pesquisado e descoberto sobre a patogenia do FHV-1 nesse modelo animal, a quantidade de informações produzidas neste trabalho são primordiais para infecções experimentais de camundongos com vírus da espécie felina. As terapias antivirais testadas neste trabalho não foram capazes de reduzir a carga viral dos órgãos dos animais experimentalmente infectados (nestas doses, frequência e vias de administração), apesar de uma melhora clínica nos camundongos tratados ter sido observada. Outros estudos deverão ser conduzidos futuramente para testar as mesmas drogas, na mesma espécie, mas em regimes de administração mais elaborados para avaliar a real eficácia antiviral destes compostos, bem como fazer uma avaliação mais completa em busca de DNA viral e lesões histopatológicas em outros órgãos e tecidos, incluindo principalmente o trato respiratório superior, local pelo qual o FHV-1 tem maior tropismo.

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Anexos

Anexo I

Parecer da Comissão de Ética em Experimentação Animal (CEEA) - UFPel

Pelotas, 12 de dezembro de 2013

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professora Silvia de Oliveira Hübner

Faculdade de Veterinária

Senhora Professora:

A *CEEA* analisou o projeto intitulado: **"Infecção experimental de camundongos com Herpesvírus felino tipo 1 (FHV-1) e avaliação do potencial terapêutico de diferentes compostos antivirais",** processo n°23110.009152/2013-59, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro n° **CEEA 9152**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA