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Ana Paula Gnocato Mortari

ATIVIDADE DO GANCICLOVIR EM COELHOS INOCULADOS COM O ALFAHERPESVÍRUS EQUINO 1

Santa Maria, RS
2019

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Medicina Veterinária**.

Orientador: Prof. Dr. Eduardo Furtado Flores

Santa Maria, RS
2019

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Santa Maria, RS
2019

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Aos meus pais, Cléber e Isabel
pelos valores e educação recebida,
pelo amor incondicional e
por não medirem esforços
para concretização de mais essa etapa!

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RESUMO

ATIVIDADE DO GANCICLOVIR EM COELHOS INOCULADOS COM O ALFAHERPESVÍRUS EQUINO 1

AUTORA: Ana Paula Gnocato Mortari
ORIENTADOR: Eduardo Furtado Flores

O alfaherpesvírus equino 1 (EHV-1), membro da família *Herpesviridae* e subfamília *Alphaherpesvirinae,* possui distribuição mundial e tem sido associado com abortos, doença respiratória e neurológica em equinos. Como os fármacos anti-herpéticos de uso humano ainda não são rotineiramente utilizados em medicina equina, não há tratamento específico para as doenças associadas com o EHV-1. Assim, o objetivo deste trabalho foi investigar a atividade *in vivo* anti-EHV-1 do ganciclovir (GCV), um análogo de nucleosídeo utilizado no tratamento de infecções herpéticas em humanos. Os testes *in vivo* foram realizados em coelhos, um modelo experimental para a doença respiratória pelo EHV-1. Para isso, dezoito coelhos da raça Nova Zelândia com aproximadamente 30 dias de idade foram alocados em três grupos de 6 animais cada, e cada grupo recebeu um tratamento: (G1) inoculados pela via intranasal (IN) com meio RPMI; (G2) inoculados pela via IN com 10^7 $DICC_{50}$ do EHV-1 cepa Kentucky D e (G3) inoculados com o EHV-1 e tratados com o GCV (via intravenosa, 2,5mg/kg a cada 12h por 7 dias). Após a inoculação, os animais foram monitorados nos aspectos clínicos, virológicos e patológicos durante 15 dias. Todos os animais do G2 apresentaram sinais sistêmicos (apatia, inapetência) e sinais clínicos respiratórios, de severidade variável, entre os dias 3 e 13 pi. Esses sinais consistiram de secreção nasal serosa à mucopurulenta e dificuldade respiratória leve à grave, além de secreção ocular. Além disso, esses animais apresentaram ganho de peso inferior aos grupos controle (G1) e ao grupo tratado com GCV (G3) nos dias 4, 6, 10, 12 e 14 pi ($p<0,05$). Um coelho deste grupo apresentou sinais neurológicos e morreu no dia 3 pós inoculação (pi). A presença do vírus no pulmão deste animal foi confirmada pelo isolamento viral e achados de histopatologia. Ao contrário, os animais do grupo G3 (inoculados com o EHV-1 e tratados com GCV) não apresentaram sinais sistêmicos e apresentaram apenas secreção nasal serosa leve. O ganho de peso desses animais foi semelhante aos animais do grupo controle (G1) e superior aos do grupo G2 nos dias 4, 6, 10, 12 e 14 pi ($p<0,05$). Excreção viral em secreções e soroconversão ao EHV-1 foram observados tanto nos animais do G2 quanto do G3, sem diferenças evidentes em magnitude e duração. Assim, o tratamento com GCV nos coelhos infectados com o EHV-1 (G3) resultou em manutenção do ganho diário de peso, abolição dos sinais sistêmicos e atenuação importante dos sinais clínicos respiratórios. Esses resultados são promissores no sentido da utilização do GCV no tratamento de infecções herpéticas em equinos. Não obstante, estudos mais aprofundados investigando-se diferentes doses, frequência de administração e atividade quando administrado após início dos sinais clínicos são necessários antes de sua utilização nessa espécie.

Palavras-chave: Alfaherpesvírus. EHV-1. Terapia. Ganciclovir. Modelo animal.

ABSTRACT

Activity of ganciclovir in rabbits inoclulated with *Equid alphaherpesvirus 1*

AUTHOR: Ana Paula Gnocato Mortari
ADVISER: Eduardo Furtado Flores

Equine alfaherpervirus 1 (EHV-1), a member of the family *Herpesviridae* and subfamily *Alphaherpesvirinae*, is distributed worldwide and associated with abortions, respiratory and neurological disease in horses. No specific treatment for diseases associated with EHV-1 in horses is available, since human anti-herpetic drugs are not yet routinely used in equine medicine. Thus, the objective of this study was to investigate the *in vivo* anti-herpetic activity of ganciclovir (GCV), a nucleoside analogue drug used in the herpesvirus infections treatment in humans, against EHV-1. *In vivo* tests were performed in rabbits, an experimental model for EHV-1 respiratory disease. For this, eighteen New Zealand rabbits with approximately 30 days of age were alocated into three groups of 6 animals each and each group received a treatment: (G1) inoculated by the intranasal route (IN) with RPMI medium; (G2) inoculated by the IN route with 10^7 $DICC_{50}$ of EHV-1 strain Kentucky D and (G3) inoculated with EHV-1 and treated with GCV (intravenously with 2.5mg / kg every 12h for 7 days). After inoculation, the animals were monitored for clinical, virological and pathological aspects for 15 days. All animals of G2 developed systemic signs (apathy, inappetence) and respiratory signs of variable severity between days 3 and 13 pi. These signs consisted of serous to mucopurulent nasal secretion and mild to severe respiratory distress, as well as ocular secretion. In addition, these animals presented lower weight gain than the control (G1) and GCV (G3) groups at days 4, 6, 10, 12 and 14 pi ($p < 0.05$). A rabbit from this group presented neurological signs and died on day three after inoculation (pi). The presence of the virus in the lung of this animal was confirmed by viral isolation and histopathology. In contrast, animals from the G3 group (inoculated with EHV-1 and treated with GCV) showed no systemic signs and presented only mild serous nasal secretion. The weight gain of these animals was similar to those of the control group (G1) and higher than those of the G2 group at days 4, 6, 10, 12 and 14 pi ($p < 0.05$). Viral excretion in secretions and seroconversion to EHV-1 were observed in both G2 and G3 animals, with no evident differences in magnitude and duration. Thus, GCV treatment in rabbits infected with EHV-1 (G3) resulted in maintenance of daily weight gain, abolition of systemic signs and significant attenuation of respiratory clinical signs. These results are promising for the use of GCV in the treatment of herpes infections in horses. However, further studies investigating different doses, frequency of administration and activity when administered after the onset of clinical signs are necessary prior to its use in this species.

Keywords: *Alphaherpesvirus*. EHV-1. Therapy. Ganciclovir. Animal model.

LISTA DE ILUSTRAÇÕES

Figura1 - Mean of clinical score obtained daily from control group (G1), and groups of rabbits experimentally infected with equid alphaherpesvirus 1 (EHV-1) (G2) and infected with EHV-1 and treated with ganciclovir (G3).....34

Figura 2 - Mean and standard deviation of the mean of body weight of control group (G1), EHV-1 inoculated rabbits (G2) and EHV-1 inoculated rabbits and treated with ganciclovir (G3).....35

LISTA DE TABELAS

Tabela 1 – Achados clínicos e virológicos em coelhos infectados experimentalmente com o Alfaherpesvírus equino 1 (EHV-1) e tratados com ganciclovir (GCV).**Erro! Indicador não definido.**

SUMÁRIO

1. INTRODUÇÃO 11
2. ARTIGO 16
ABSTRACT. 17
INTRODUCTION 19
MATERIAL AND METHODS 20
DISCUSSION 23
REFERENCES 26
3. REFERÊNCIAS 36

1. INTRODUÇÃO

O alfaherpesvírus equino 1 (EHV-1), é um importante patógeno de equinos e afeta especialmente fêmeas prenhes e animais jovens (GALOSI et al., 2001; MUMFORD & EDINGTON, 1980). A infecção pelo EHV-1 está associada com doença respiratória do trato respiratório superior (DUNOWSKA, 2014), além de rinopneumonite, abortamentos, mortalidade perinatal e mieloencefalopatia (LARA et al., 2010).

O EHV-1 é um vírus DNA pertencente à família *Herpesviridae*, subfamília *Alphaherpesvirinae* e gênero *Varicellovirus* (ICTV, 2018). Assim como os demais alfaherpesvírus, o EHV-1 tem a capacidade de estabelecer infecção latente (AVCI et al., 2014), quando a expressão de genes virais é altamente restrita, resultando na produção de poucas ou nenhuma proteína viral (GULATI et al., 2015). A infecção latente permite a evasão do vírus do sistema imunológico do hospedeiro (BARRANDEGUY et al., 2008), perpetuando a infecção no rebanho. Neurônios dos gânglios do trigêmeo (TG), linfócitos circulantes e tecidos linfoides são os principais locais de latência dos herpesvírus de equinos (PUSTERLA et al., 2012).

Nove alfaherpesvírus já foram identificados em equinos, sendo que cinco deles pertencem à subfamília *Alphaherpesvirinae*, dois pertencem à *Gammaherpesvirinae* e um ainda não foi classificado (ICTV, 2018). Os equinos são hospedeiros naturais do EHV-1, EHV-2, EHV-3, EHV-4 e EHV-5, enquanto que os asininos são hospedeiros do herpesvírus asinino tipo 3 (AHV-3, homólogo ao EHV-1), herpesvírus asinino tipo 1 (AHV-1, homólogo ao EHV-3) e herpesvírus asinino tipo 2 (AHV-2) (PATEL & HELDENS, 2005).

Antigamente, o EHV-1 era classificado em subtipos 1 e 2 (STUDDERT et al., 1981), mas a partir de 1981 foi adotada uma nova classificação, na qual o EHV-1 subtipo 2 foi designado como EHV-4. No entanto, o reconhecimento oficial desta distinção ocorreu somente uma década depois (TELFORD et al., 1998). Esses vírus apresentam reação sorológica cruzada devido ao elevado nível de semelhança na sequência de aminoácidos das glicoproteínas do envelope viral (ARDANS, 2003). A infecção pelo EHV-1 é enzoótica na maioria das populações equinas, e está associada com abortos (WEIBLEN et al., 1994), infecção urogenital, doença respiratória e sinais neurológicos (OSTLUND, 1993). O EHV-4 causa doença respiratória aguda em animais jovens e, em casos raros, infecção em fêmeas gestantes, resultando em abortos (CARVALHO et al., 2000; MORI et al., 2003).

A infecção pelo EHV-1 apresenta distribuição mundial, embora a prevalência dessas infecções não seja bem conhecida (AGUIAR et al., 2008). Devido à semelhança antigênica entre EHV-1 e EHV-4, a interpretação dos dados dos estudos sorológicos foi complicada até o

início dos anos 90, com a criação de um teste para anticorpos específicos (CRABB & STUDDERT, 1993). O EHV já foi detectado sorologicamente em diversos estados brasileiros, como Pará, Amazonas, Minas Gerais, São Paulo, Paraná e Rio Grande do Sul (CARVALHO et al., 2000; DIEL et al., 2006).

A doença respiratória aguda que frequentemente ocorre associada à infecções pelo EHV-1 e/ou EHV-4 é caracterizada por febre, anorexia, secreção nasal de gravidade variável e secreção ocular. A proliferação bacteriana na cavidade nasal pode ser um fator que contribui para o desenvolvimento de rinopneumonite (THOMPSON et al., 1979). Experimentalmente, no entanto, o EHV-1 causa uma doença muito mais grave do que a induzida por EHV-4 (BURROWS & GOODRIDGE, 1973; EDINGTON et al., 1986; PATEL et al., 2003; TEWARI et al., 1993). Os animais podem ser repetidamente infectados por ambos os vírus na natureza, e os sinais de doença tornam-se menos graves com episódios progressivos ao longo dos anos (PATEL & HELDENS, 2005).

A transmissão dos alfaherpesvírus geralmente ocorre por contato direto, principalmente entre mucosas, ou por inalação de aerossóis contendo partículas virais, sendo mais comum em locais com alta concentração de animais (MURPHY et al., 1999). O EHV-1 infecta inicialmente o epitélio respiratório e penetra rapidamente nos tecidos linfoides retrofaríngeos. A partir daí o vírus se difunde sistemicamente por viremia associada a leucócitos, que mostrou ser um requisito para os abortos e paresia, iniciando a replicação do EHV-1 no revestimento de células endoteliais de vasos sanguíneos no sistema nervoso central (SNC) e no útero gestacional (EDINGTON et al., 1986, 1991; SMITH et al., 1992, 1993).

Após a infecção aguda, o vírus estabelece latência no gânglio trigêmeo e, possivelmente, em algumas células imunológicas de vida longa, como os linfócitos T CD5+ / CD8+ definidos como o local predominante da latência do EHV-1 (SMITH et al., 1998; PATEL & HELDENS, 2005). O EHV-1 é reativado do estado latente e é excretado novamente durante situações de estresse e imunossupressão, sendo que nestas ocasiões pode haver manifestações clínicas recorrentes (PELLET & ROIZMAN, 2007).

O diagnóstico presuntivo das infecções por EHV-1 pode ser realizado pela análise dos sinais clínicos associado ao histórico do animal. O diagnóstico definitivo, no entanto, requer o uso de testes laboratoriais como o isolamento viral em cultivo celular (PUSTERLA et al., 2009), reação de polimerase em cadeia (PCR), imunofluorescência (IFA) e imunoperoxidase (IPX), além de técnicas sorológicas como a soroneutralização (SN), sorologia pareada e ELISA.

O controle da infecção é realizado principalmente pelo uso de vacinas. As vacinas EHV disponíveis, incluem vacinas inativadas de componente único para prevenção do aborto em

éguas prenhes [Pneumabort-K® e Duvaxyn-1,4® (Zoetis) e Prodigy® (MSD-Intervet)], vacinas vivas modificadas (MLV) [Rhinomune® nos Estados Unidos (Boehringer Ingelheim Vetmedica) e como Prevaccinol® na Europa (Merck MSD)] e diferentes vacinas inativadas de múltiplos componentes para prevenção de doenças respiratórias (Lexington 8®, Vencofarma,; Fluvac innovator® 6, Fort Dodge; Flu Avert®, Merk; Calvenza®, Boehringer Ingelheim Vetmedica) (GOEHRING et al., 2010; GOODMAN et al., 2006, 2012; MA et al., 2013; WAGNER et al., 2015; WIMER et al., 2018).

A maioria das vacinas contendo antígenos do EHV-1 é comercializada como polivalente, associada com antígenos do *Clostridium tetani*, EHV-4, vírus da influenza equina (EIV), e vírus das encefalites equina venezuelana, leste e oeste (VEEV, EEEV e WEEV). A vacinação não é totalmente protetora, podendo ocorrer surtos da doença mesmo em rebanhos vacinados. Entretanto, sua eficácia na prevenção de viremia, aborto e doenças neurológicas não está bem esclarecida, não existindo vacinas comerciais com esse foco (DIAZ et al., 2015; GOODMAN et al., 2012; KYDD et al., 2006b, 2012).

Não existe tratamento específico de rotina para as doenças causadas pelo EHV-1, sendo indicadas apenas medidas preventivas e de suporte para os animais afetados, com o objetivo de controlar a infecção, transmissão e minimizar os sinais clínicos (CARMICHAEL & GREENE, 2006). Contudo, estudos de atividade antiviral com herpesvírus de animais têm sido ocasionalmente conduzidos (GARRÉ et al., 2007; MAGGS & CLARKE, 2004; MARLEY et al., 2006; MEULEN et al., 2006; SCHWERS et al., 1980), sendo poucos os relatos sobre a eficácia de antivirais frente ao EHV-1 (OLIVEIRA et al., 2018).

Fármacos antivirais são comumente utilizadas nas infecções por herpesvírus em humanos, porém são ainda pouco empregados na medicina veterinária. Entre as drogas anti-herpéticas mais utilizadas em medicina encontram-se o aciclovir (ACV), ganciclovir (GCV), cidofovir (CDV), famciclovir (FAM) e a vidarabina (VID), que são análogos de nucleosídeos (COEN & RICHMAN, 2007). A atividade desses fármacos antivirais ocorre pela sua incorporação na cadeia nascente de DNA viral, impedindo que a DNA polimerase dos herpesvírus complete seu ciclo catalítico (COEN & RICHMAN, 2007; LEVINSON, 2016; WHITLEY & ROIZMAN, 2001).

Em humanos, as infecções por alfaherpesvírus tem sido tratadas com sucesso com medicamentos antivirais (DE CLERCQ, 2008; RAZONABLE, 2011) que reduzem o período de convalescença e a taxa de transmissão para outros indivíduos, diminuindo a disponibilidade de partículas virais capazes de infectar novas células e limitando a excreção viral para o meio ambiente (COEN & RICHMAN, 2007; FLINT et al., 2004). Na medicina equina, o uso de

compostos antivirais pode ser benéfico na diminuição do impacto negativo causado pelas infecções por alfaherpesvírus (CARMICHAEL et al., 2013; GARRÉ, 2008; GLORIEUX et al., 2012; MAXWELL et al., 2009, 2011).

Fármacos antivirais, têm demonstrado eficácia *in vitro* no controle da replicação do EHV-1. A aplicação de compostos como o aciclovir (ACV), ganciclovir (GCV), cidofovir (CDV), valganciclovir (VGC), foscarnet (FOS), entre outros, tem sido estudada contra o EHV-1 (MAXWELL, 2017; OLIVEIRA et al., 2018; VISSANI et al., 2015).

A atividade de seis fármacos antivirais contra o EHV-1 foi avaliada *in vitro* por MUELEN et al. (2006) e GARRÉ et al. (2007), utilizando células de pulmão embrionário de equino (EEL). O Cidofovir (CDV) não foi altamente eficiente na inibição da replicação viral, mas foi capaz de reduzir significativamente o tamanho das placas mesmo em concentrações muito baixas (GARRÉ et al., 2007). No trabalho realizado por MUELEN et al. (2006), o ACV foi eficiente no teste de redução de placas frente o EHV-1, e resultou em EC_{50} 20 vezes maior do que o EC_{50} do GCV, semelhante ao observado por GARRÉ et al. (2007). A atividade do ACV e do Famciclovir (FAM) frente ao EHV-1 foi semelhante, se comparados os valores de $EC_{50,}$ assim como nos relatos de MAXWELL et al. (2008). Porém, em equinos ainda não há estudos de farmacocinética usando o FAM, possivelmente devido ao alto custo do fármaco e à ausência de produtos injetáveis disponíveis (MAXWELL, 2017).

Diversos estudos *in vitro* têm demonstrado resultados satisfatórios de alguns desses fármacos frente ao EHV-1, sendo o GCV o mais eficiente e seguro (VISSANI et al., 2015; OLIVEIRA et al., 2018). Na avaliação da atividade anti-EHV-1 *in vitro*, realizada pelo teste de redução de placas virais, o GCV apresentou-se como um fármaco seguro e eficiente frente ao EHV-1, uma vez que sua dose efetiva foi baixa (EC50: 1,9 μg/mL) e o índice de seletividade foi elevado (IS: 490) (OLIVEIRA et al., 2018). Porém, a cinética e o estudo de dose-efeito do GCV *in vitro* contra o EHV-1 ainda não estão completamente esclarecidos. Além disso, embora a farmacocinética do GCV e do Valganciclovir (VGC) já tenha sido estabelecida em equinos (CARMICHAEL et al., 2013), a atividade anti-EHV-1 *in vivo* não foi sistematicamente avaliada.

A realização de estudos *in vivo* para a avaliação de drogas antivirais em equinos tem sido comprometida devido à dificuldade de se obter animais soronegativos para EHV-1/EHV-4, aos custos e aos problemas éticos do uso de animais em experimentação. Assim, uma alternativa para a realização dos ensaios iniciais para validar protocolos de tratamento para EHV-1 seria o uso de modelos animais. Camundongos têm sido utilizados para investigar diferentes aspectos da infecção por EHV-1, incluindo doenças respiratórias (AWAN et al.,

1990), latência e reativação (BAXI et al., 1996), infecção neurológica (BAXI et al., 1996; FRAMPTON et al., 2004; MORI et al., 2012), falhas reprodutivas (AWAN et al., 1991) e a função de proteínas virais específicas na patogênese viral (FRAMPTON et al., 2004). Esses modelos, no entanto, muitas vezes não conseguem reproduzir aspectos importantes da infecção e da doença em equinos. Por outro lado, foi verificado que coelhos experimentalmente infectados pelo EHV-1 desenvolvem de forma consistente a doença respiratória - e menos frequentemente os sinais neurológicos - após inoculação intranasal (KANITZ et al., 2015). Em geral, a doença desenvolvida por coelhos reproduziu vários aspectos da doença respiratória pelo EHV-1 em equinos (KANITZ et al., 2015).

Dessa forma, o objetivo deste trabalho foi avaliar a atividade anti-EHV-1 do GCV em coelhos infectados experimentalmente, uma vez que este fármaco apresentou significativa atividade antiviral *in vitro* contra o EHV-1(OLIVEIRA et al., 2018).

2. ARTIGO

O GANCICLOVIR ATENUA A DOENÇA RESPIRATÓRIA PELO ALFAHERPESVÍRUS EQUINO 1 EM COELHOS INFECTADOS EXPERIMENTALMENTE

Ana Paula G. Mortari, Bruna P. Amaral, Pablo S. B. de Oliveira, Evelyn K. Dotto, Mariana M. Flores, Juliana F. Cargnelutti, Rudi Weiblen, Eduardo F. Flores

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Ganciclovir attenuates the respiratory disease by equid alphaherpesvirus 1 in experimentally infected rabbits [1]

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ABSTRACT. - Mortari A.P.G., Amaral B.P., De Oliveira P. S. B., Dotto E. K., Flores M.M., Cargnelutti J.F., Weiblen R. & Flores E.F. 2018. **[Ganciclovir attenuates the respiratory disease by equid alphaherpesvirus 1 in experimentally infected rabbits.]** O ganciclovir atenua a doença respiratória pelo alfaherpesvírus equino 1 em coelhos infectados experimentalmente. *Pesquisa Veterinária Brasileira 00(0):00-00.* Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Centro de Eventos da UFSM, Rua Z, prédio 63A, Santa Maria, RS, Brasil, CEP: 97105-900. E-mail: eduardofurtadoflores@gmail.com

Equid alphaherpesvirus 1 (EHV-1) is an important pathogen of horses, associated with respiratory, neurological disease and abortions. As vaccination is not always effective, anti-herpetic therapy may represent an alternative to prevent the losses caused by the infection. We herein investigated the activity of ganciclovir (GCV), an anti-herpetic human drug, in rabbits experimentally infected with EHV-1. Thirty-days-old New Zealand rabbits were allocated in three groups (6 animals each) and submitted to different treatments: G1 (non-infected controls), G2 (inoculated with EHV-1) - 10^7 $TCID_{50}$ intranasally [IN]) and G3 (inoculated IN with EHV-1 and treated with GCV - 5mg/kg/day for 7 days) and monitored thereafter. All animals of G2 developed systemic signs (moderate to severe apathy, anorexia), ocular discharge and respiratory signs (serous to mucopurulent nasal discharge), including mild to severe respiratory distress. Viremia was detected in all rabbits of G2 for up to 11 days (mean duration = 6.5 days). One animal died after severe respiratory distress and neurological signs (bruxism, opistotonus). In addition, these animals gained less weight than the control (G1) and GCV-treated rabbits (G3) from days 4 to 14pi ($p<0.05$). The clinical score of rabbits of G2 was statistically higher than the other groups from days 3 to 6pi ($p<0.05$), demonstrating a more severe disease. In contrast, G3 rabbits did not present systemic signs, presented only a mild and transient nasal secretion and gained more weight than G2 animals ($p<0.05$). In addition, viremia was detected in only 3 rabbits and was rather transient (average of 2.3 days). Thus, administration of GCV to rabbits inoculated IN with EHV-1 resulted in an important attenuation of the clinical disease as demonstrated by full prevention of systemic signs, maintenance of weight gain and by drastic reduction in viremia and in the magnitude of respiratory signs. These results are promising towards further testing of GCV as a potential drug for anti-herpetic therapy in horses.

INDEX TERMS: Ganciclovir, herpesvirus, therapy, EHV-1, animal model.

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RESUMO.- O alfaherpesvírus equino 1 (EHV-1) é um importante patógeno de equinos, associado a doenças respiratórias, neurológicas e abortos. Como a vacinação nem sempre é eficaz, a terapia anti-herpética pode representar uma alternativa para prevenir as perdas causadas pela infecção. Nós investigamos a atividade do ganciclovir (GCV), uma droga humana anti-herpética, em coelhos infectados experimentalmente com EHV-1. Coelhos da raça Nova Zelândia com 30 dias de idade foram alocados em três grupos (6 animais cada) e submetidos a diferentes tratamentos: G1 (controles não infectados), G2 (inoculado com EHV-1) - 10^7 $TCID_{50}$ intranasal [IN]) e G3 (inoculado IN com EHV-1 e tratado com GCV - 5mg/kg/dia por 7 dias) e monitorado posteriormente. Todos os animais do G2 desenvolveram sinais sistêmicos (apatia moderada a grave, anorexia), secreção ocular e sinais respiratórios (secreção nasal serosa a mucopurulenta), incluindo dificuldade respiratória leve a grave. A viremia foi detectada em todos os coelhos do G2 por até 11 dias (duração média = 6,5 dias). Um animal morreu após dificuldade respiratória grave e sinais neurológicos (bruxismo, opistótono). Além disso, esses animais ganharam menos peso que os coelhos controle (G1) e tratados com GCV (G3) dos dias 4 a 14pi ($p<0,05$). O escore clínico de coelhos do G2 foi estatisticamente maior que os demais grupos dos dias 3 a 6pi ($p<0,05$), demonstrando uma doença mais grave. Em contraste, os coelhos do G3 não apresentaram sinais sistêmicos, apresentaram apenas secreção nasal leve e transiente e ganharam mais peso que os animais do G2 ($p<0,05$). Além disso, a viremia foi detectada em apenas 3 coelhos e foi bastante transitória (média de 2,3 dias). Assim, a administração de GCV a coelhos inoculados com EHV-1 resultou em uma importante

atenuação da doença clínica, como demonstrado pela prevenção completa de sinais sistêmicos, manutenção do ganho de peso e pela redução drástica da viremia e da magnitude dos sinais respiratórios. Estes resultados são promissores para testes adicionais de GCV como um potencial medicamento para terapia anti-herpética em equinos.

TERMOS DE INDEXAÇÃO: Ganciclovir, herpesvírus, terapia, EHV-1, modelo animal.

INTRODUCTION

Equid alphaherpesvirus 1 (EHV-1) is an enveloped, double-stranded DNA virus belonging to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* (ICTV 2018). EHV-1 infection is distributed worldwide and is frequently associated with respiratory disease, abortion, perinatal foal mortality and/or neurological disease (equine herpesvirus myeloencephalopathy [EHM]) (Allen & Bryans 1986, Allen et al. 2004, Lunn et al. 2009, Goodman et al. 2012, Ma et al. 2013, Pusterla & Hussey 2014). After acute infection, EHV-1 establishes lifelong latent infections, mainly in sensory nerve ganglia but also in lymphoid cells (Welch et al. 1992, Pusterla et al. 2010). Natural reactivation of latent infection results in virus replication and transmission to susceptible animals, representing a means of virus perpetuation in nature (Patel & Heldens 2005).

The respiratory disease associated with EHV-1 infection is especially important in young racing horses submitted to intensive training since it affects the physical performance (Gilkerson et al. 1999). The reproductive losses by abortions, perinatal foal mortality and neurological disease (EHM) resulting in high mortality, in addition to the restrictions to transportation, contribute to the economic impact of EHV-1 infection (Lunn 2009).

Although vaccines against EHV-1 are available, they are not fully protective such as outbreaks of disease have been reported even in regularly vaccinated herds (Allen et al. 2004, Kydd et al. 2012). Likewise, no specific treatment is available for EHV-1-associated disease and control measures such as support therapy and animal isolation have been indicated to reduce transmission and to minimize clinical signs (Carmichael & Greene 2006). Considering the high economic value of some horses, EHV-1 infection represents an attractive target for testing and, eventually employing of antiviral drugs (Schwers et al. 1980, Maggs & Clarke 2004, Marley et al. 2006, Meulen et al. 2006, Garré et al. 2007).

A number of drugs has been successfully used for the treatment of herpetic infections in humans, yet their use in veterinary medicine is still incipient (De Clercq 2008, Razonable

2011, Maxwell 2017). However, some studies investigated the antiviral action of drugs against bovine herpesviruses (1, 2 and 5) (Dezengrini et al. 2010), felid herpesvirus (Thomasy & Maggs 2016) and herpesviruses of horses (Vissani et al. 2015, Oliveira et al. 2018). These drugs may have important applications in equine medicine by reducing the clinical consequences of herpetic infections (Garré et al. 2008, Carmichael et al. 2013, Glorieux 2012, Maxwell 2008, 2017). Antiviral drugs include the nucleoside analogs acyclovir (ACV), ganciclovir (GCV), cidofovir (CDV), famciclovir (FAM), foscarnet (FOS) and vidarabine (VID) (Coen & Richman 2007). The action of these drugs occurs via their incorporation into the nascent DNA molecule, halting the DNA polymerase from completing polymerization (Whitley & Roizman 2001, Coen & Richman 2007, Levinson 2016). A number of studies *in vitro* has indicated the efficacy and safety of some of these drugs against EHV-1, especially GCV (Vissani et al. 2015, Oliveira et al. 2018), which among six *in vitro* evaluated antiviral drugs was the most effective and safest to *in vivo* studies (Oliveira et al., 2018). Furthermore, the pharmacokinetics of GCV in horses has been already established (Carmichael et al. 2013) and, although it was showed its effectiveness in horses presenting meningoencephalitis (Maxwell, 2017), yet its antiviral activity in the respiratory disease induced by EHV-1 has not been investigated.

GCV was the first antiviral drug used for the treatment of cytomegalovirus (CMV) infections, and currently it is widely used in immunosuppressed patients (HIV infected, transplanted, etc.) that develop secondary CMV infection. GCV is an acyclic analog of the nucleoside guanosine, and when at triphosphate state, it is a competitive inhibitor of deoxyguanosine triphosphate incorporation into DNA and inhibits viral DNA polymerases, serving as a poor substrate for chain elongation, thereby disrupting viral DNA synthesis (Crumpacker 1996).

Thus, in this article we describe an investigation of the antiviral activity of GCV in a rabbit model for EHV-1 respiratory disease. The rabbit model for EHV-1 was previously described by Kanitz et al. (2015). Our results are promising towards further testing of GCV as an anti-herpetic drug for herpetic infections of horses.

MATERIAL AND METHODS

The activity of GCV against EHV-1 *in vivo* was investigated in rabbits inoculated intranasally (IN) with strain Kentucky D. All procedures involving animals were performed under veterinary supervision and conducted following the guidelines by the Brazilian Committee of

Animal Experimentation (COBEA, law nº 6.638, of may 8th, 1979). The animal experiment was approved by the Institutional Committee of Ethic and Animal Experimentation Welfare (UFSM - Comitê de Ética e Experimentação Animal: CEUA nº 3177061217).

Cells and virus. Vero cells (*African Green Monkey kidney*) were used in all procedures of virus amplification, quantitation, isolation from clinical specimens and virus-neutralizing (VN) assays. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (Cultilab, Inc.), added of antibiotics (streptomycin 0.4mg/mL; penicillin 1.6mg/mL) and antifungic (anphotericin B 0.0025mg/mL). The EHV-1 Kentucky D strain was kindly provided by Dr Rodrigo Franco (Instituto Butantan, São Paulo, SP, Brazil).

Animals and virus inoculation. Eighteen New Zealand rabbits (approximately 30-days-old) were allocated in three groups of six animals each according to the respective treatments: G1 (uninfected controls, inoculated IN with RPMI medium); G2 (inoculated with EHV-1); G3 (inoculated with EHV-1 and treated with GCV). Animals from G2 and G3 were inoculated into the paranasal sinuses (Kanitz et al. 2015) with 1 mL of culture supernatant containing 10^7 mean tissue culture infectious doses ($TCID_{50}$) of EHV-1 Kentucky D strain after sedation with ketamine (50mg/kg) and xylazine (5mg/kg). Starting at the day of virus inoculation, animals of G3 received GCV intravenously (5mg/kg/day) during seven days.

Animal monitoring and sample testing. Experimental animals were weighted and monitored in a daily basis for clinical and virological aspects up to day 15 post-inoculation (pi). Clinical monitoring involved a close observation of behavior, food ingestion, alertness, ocular and respiratory signs. A clinical score was determined per group/day by the sum of the values of the following parameters: apathy (presence: 1, absence: 0); ocular secretion (presence: 1, absence: 0), nasal discharge (serous: 1, mucopurulent: 2, absence: 0), respiratory distress (mild: 1, mild to moderate: 2, moderate to severe: 3, absence: 0), neurological signs (presence: 1, absence: 0), death (yes: 1, no: 0).

Nasal swabs were collected in alternate days up to day 15pi and submitted to virus isolation. For virus isolation, swabs collected in 0.5mL of RPMI medium were drained, centrifuged (1,000 x g for 10 min) and the 0.1mL of the supernatant was inoculated onto Vero cell monolayers grown in 24-well plates. Samples were submitted to three passages of four days each for the appearance of cytopathic effect (cpe). Lung fragments collected from rabbit #28 (G3) which died at day 3pi were also submitted to virus isolation in Vero cells.

Viremia was investigated by submitting blood collected in alternate days (50µl/animal) to DNA extraction by phenol-chloroform and a PCR for EHV-1 gB gene according to Kanitz

et al. (2015). Total DNA extracted from uninfected Vero cells and from cells inoculated with EHV-1 was used as negative and positive controls, respectively.

Blood for serology was collected at days 0 and 15pi. Serum samples were submitted to a standard microtiter virus-neutralizing (VN) assay, testing two-fold dilutions of serum against a fixed dose of virus (100-200 $TCID_{50}$). Vero cells were used as indicators and the readings were performed at 96h of incubation. VN titers were considered the reciprocal of the highest serum dilution capable of preventing the production of cpe.

Lungs collected from rabbit # 28 were fixed in 10% buffered formalin, embedded in paraffin, cut at 5µm and stained with hematoxylin and eosin for microscopic examination.

Statistical analysis. The weight values and clinical score obtained daily from all experimental animals were submitted to variance analysis by the Two-way ANOVA method, with Tukey test for multiple comparisons, using the GraphPad Prism (version 6) software. Differences between the groups were considered significant when the *p* values were lower than 5% ($p<0.05$).

RESULTS

The six rabbits inoculated with EHV-1 (G2) developed systemic and respiratory signs between days 2 and 11pi, shed virus in nasal secretions from day 1 to 13pi and seroconverted to EHV-1 (VN titers from 8 to 128) (Table 1). The disease was characterized by moderate to severe apathy and anorexia, serous ocular secretion, nasal discharge (serous to muco-purulent) and mild to severe respiratory distress. Rabbit # 28 died at day 3pi after a course of respiratory distress, bruxism and opistothonus. Infectious virus and histological changes compatible with virus-associated inflammation were observed in the lungs of this animal. Viremia was detected in G2 animals between days 3 and 13pi (mean = 6.5 days). In addition, G2 rabbits presented a reduced weight gain comparing to uninfected controls (G1) and GVC treated rabbits (G3) between days 4 and 14 pi ($p<0.05$). The clinical score was statistically superior in G2 rabbits than in GCV treated animals (G3) on day 3pi (Fig. 1). These results demonstrated the consistent reproduction of systemic and respiratory disease by EHV-1 in experimentally infected rabbits.

In contrast, no systemic signs (changes in alertness, appetite) or overt respiratory signs were observed in rabbits inoculated with EHV-1 and receiving GCV treatment (G3). Furthermore, the clinical score in G3 group was much lower (maximal score was 1) than in non-treated rabbits (G2) (reached score 5, mean around 3), showing an important reduction in the magnitude of clinical signs in animals receiving GCV (G3), at the beginning of the clinical

course (Fig.1). These animals presented only a mild serous nasal secretion between days 5 and 12pi. The weight gain of these animals was comparable to the uninfected control group and higher than the G2 group at days 4, 6, 10, 12 and 14 ($p<0.05$) (Fig. 2). G3 animals shed virus in nasal secretions between days 1 and 15pi and 5/6 seroconverted to EHV-1 at day 15pi, harboring VN titers from 8 to 32 (Table 1). Transient viremia was detected in 3 rabbits and lasted an average of 2.3 days. These results demonstrate that GCV treatment fully prevented the development of systemic signs, preserved weight gain, reduced viremia and resulted in an important attenuation of the systemic and respiratory disease.

No differences in virus shedding (duration) was evident between rabbits from G2 and G3 (Table 1). Uninfected rabbits remained healthy, did not shed virus or presented viremia, gained weight and remained seronegative to EHV-1 throughout the experiment.

DISCUSSION

The administration of GCV to rabbits inoculated IN with EHV-1 resulted in an important attenuation of the clinical disease as demonstrated by full prevention of systemic signs, maintenance of weight gain and by a drastic reduction in viremia and in the magnitude of respiratory signs. As one animal from G2 (# 28) died after severe respiratory and neurological signs, the prevention of mortality may be also attributed to GCV treatment. Interestingly, no evident reduction in virus shedding were noticed between GCV treated (G3) and not treated (G2) groups. These results might pave the way for further studies in rabbits and, eventually in horses, to investigate the real potential of GCV as an anti-herpetic drug for equine medicine. Although antiviral treatment of animals may be expensive due to difficult related to commercial availability of GCV at large scale, the benefits of treating horses against EHV-1 infection may be rewarding.

Anti-herpetic therapy has long been used in human medicine yet its application in veterinary medicine is still incipient. Since vaccination is not always effective in preventing respiratory, neurological disease and abortions associated with EHV-1 infections, antiviral therapy may represent an attractive alternative to reduce the losses caused by these conditions. In fact, human anti-herpetic drugs have been tested for the therapy of diseases caused by EHV-1 and other equine herpesviruses as well (reviewed by Vissani et al. 2015, Maxwell 2017). However, many evidences of anti-herpetic drug efficacy against equine herpesviruses are derived from studies *in vitro* or in animal models, from empirical data, with limited testing and/or clinical experience in horses (Maxwell 2017). In fact, although investigation of anti-viral

therapy has been performed for several equine herpesviruses, only diseases associated with EHV-1 and EHV-5 infections have been treated with anti-herpetic drugs in routine equine practice (Vissani et al. 2015, Maxwell 2017).

The human antiherpetic drugs that are most commonly used for therapy for EHVs are acyclovir and its prodrug, valacyclovir. Oral valacyclovir has been used in therapy of EHV-1 to protect adult horses from myeloencephalopathy. Oral acyclovir is frequently administered for the therapy of equine multinodular pulmonary fibrosis associated with EHV-5 infection. Other anti-herpetic drugs are promising but require further investigation (reviewed by Maxwell, 2017).

GCV has been the drug of choice to replace acyclovir (ACV) in the therapy of herpes simplex virus infections in humans since it is less toxic and more stable (Shen et al. 2009). The efficacy of GCV in attenuating clinical disease has been demonstrated for different human herpesviruses, including Epstein-Barr virus (Egan et al. 2011), CMV in immunodepressed patients (Lalezari et al. 2002), keratitis by HSV-1 (Wang et al. 2015) and encephalitis by HHV-6 (Mookerjee & Vogelsang 1997).

Several studies have shown that GCV is, at least, 10 times more potent than acyclovir and penciclovir against EHV-1 *in vitro* and in mice (reviewed by Maxwell 2017, Oliveira et al. 2018). In addition, GCV presents a favorable pharmacokinetic profile and it is well tolerated for horses (Carmichael et al., 2013). Investigation of GCV anti-EHV-1 activity in cases of neurological disease, however, has yielded conflicting results. GCV administration to experimentally infected horses at the onset of fever was able to reduce the severity of neurological signs, viremia and temperature (Maxwell 2017). On the other hand, GCV treatment of two naturally infected horses developing mioencephalopathy was unable to reduce the severity of clinical disease and death (Estell et al. 2015). The activity of GCV against EHV-1 *in vitro* has been demonstrated, being a potent and selective compound (Oliveira et al. 2018). Regardless, the activity of GCV in the respiratory disease by EHV-1 remained unexploited. In this study we investigated its activity *in vivo*, using a rabbit model for EHV-1 respiratory disease established previously (Kanitz et al. 2015).

The treatment of rabbits inoculated with EHV-1 with GCV starting at the day of virus inoculation was effective in preventing systemic disease, in maintaining weight gain and reducing drastically viremia and the severity of respiratory signs. Interestingly, GCV treatment apparently did not interfere with virus replication in the nasal mucosa since the duration of virus shedding through nasal secretions was similar in G2 and G3 groups. It is conceivable that GCV may have interfered with virus replication in the lower respiratory tract, especially in the lungs,

what would explain the prevention of systemic disease and attenuation of respiratory signs observed in treated animals (G3). The demonstration of infectious virus and inflammatory changes in the lungs of rabbit #28, which died after severe respiratory and neurological signs, reinforce that EHV-1 pathogenesis involves replication in the lower respiratory tract. Indeed, pulmonary inflammation secondary to EHV-1 infection can cause substantial morbidity and mortality in neonatal foals and upper respiratory signs in weanlings (Murray et al. 1998, Brown et al. 2007). The drastic reduction in viremia reinforces that GCV treatment, at some stage, reduced EHV-1 replication and/or spread.

The therapeutic protocol, including drug dose, route and interval of administration and duration was adapted from previous studies of pharmacokinetics and toxicity in rabbits and horses (Hedaya & Sawchuk 1990, Carmichael et al., 2013) and based on previous studies showing antiviral activity *in vitro* (Garré et al. 2007, Oliveira et al. 2018). Implementing GCV administration at the day of virus inoculation, however, was rather unusual and obviously does not reflect the clinical practice. By doing so, we tried to proof the concept of GCV activity *in vivo* against EHV-1-induced respiratory disease. Whether GCV will be capable of attenuating clinical disease by EHV-1 when administered after the onset of clinical signs will demand further studies. In any case, our results demonstrated anti-herpetic activity of GCV *in vivo* and are, therefore, promising towards further studies to define the real potential of GCV as a drug for therapy of herpetic infections in horses.

The antiviral activity of GCV against EHV-1 has been demonstrated *in vitro* (Garré et al. 2007, Oliveira et al. 2018), its pharmacokinetics and toxicity have been studied in rabbits and horses (Hedaya & Sawchuk 1990, Carmichael et al. 2013) and its ability to attenuate EHM by EHV-1 in horses has been studied both in natural and experimental infections (Maxwell 2017, Estell et al. 2015). The availability of a rabbit model for EHV-1 respiratory disease allowed us to evaluate the GCV activity against this EHV-1-induced disease. The establishment of a more realistic therapeutic protocol for horses, however, will initially demand further studies in rabbits, investigating different doses, intervals, duration, etc. and implementation of the protocol after de onset of clinical disease, among others.

In any case, our results are promising towards the use of GCV in the therapy of EHV-1 disease in horses and open the way for further studies of GCV activity against EHV-1 respiratory disease in animal models and, in a second phase, in its natural host.

CONCLUSION

Intravenous administration of GCV at a dose of 2.5mg/kg every 12 hours for 7 days in rabbits inoculated with EHV-1 via the intranasal route was effective in abolishing systemic signs, attenuating respiratory disease and preserving weight gain in animals, when compared to the animals of the untreated group. These results indicate that this drug may be a good alternative for experimental studies in equines, aiming to establish a therapeutic protocol for this species.

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Conflict of interest statement.- The authors have no competing interests.

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FIGURES LEGENDS

Fig.1. Mean of clinical score obtained daily from control group (G1), and groups of rabbits experimentally infected with equid alphaherpesvirus 1 (EHV-1) (G2) and infected with EHV-1 and treated with ganciclovir (G3). * indicate statistical difference between groups G2 and G3 ($p<0,001$); a: indicate statistical difference between groups G1 and G2; b: indicate statistical difference between groups G1 and G3; bar indicate standard error of mean (SEM).

Fig.2. Mean and standard deviation of the mean of body weight of control group (G1), EHV-1 inoculated rabbits (G2) and EHV-1 inoculated rabbits and treated with ganciclovir (G3). Asterisk indicate significant statistical difference ($p<0.05$) between groups. No statistical differences were observed in the weight gain of G1 and G3 groups.

Table 1 – Clinical, virological and serological findings of equid alphaherpesvirus 1 (EHV-1) infected rabbits and treated with ganciclovir (GCV):

Animal #	Viral shedding (dpi[a])	Viremia (dpi)	Clinical signs (dpi)	Neutralizing antibodies 15 dpi
G1 - control				
8	-	-	Healthy	< 2
9	-	-	Healthy	< 2
10	-	-	Healthy	< 2
11	-	-	Healthy	< 2
12	-	-	Healthy	< 2
13	-	-	Healthy	< 2
G2 - EHV-1 infected				
26	3	3-13	Apathy, serous and mucopurulent nasal discharge, socular secretion, mild respiratory distress (3-11)	8
27	1-5	5-13	Apathy, serous and mucopuruelnt ocular and nasal discharge, mild to moderate respiratory distress (3-10)	8
28	1-3*	3	Apathy, serous nasal discharge, severe respiratory distress, bruxism, opisthoton, death (3 dpi)* (2-3)	n.p
29	5-11	3-13	Apathy, serous and mucopurulent nasal discharge, mild respiratory distress (3-10)	128
30	1-13	5-9	Apathy, serous and mucopurulent nasal discharge (3-9)	16
31	1**	13	Apathy, serous and mucopurulent nasal discharge, mild to moderate respiratory distress (3-7)	n.p
G3 – EHV-1 infected and treated with GCV				
20	3-5	-	Mild serous nasal secretion (5-10)	32
21	1-11	-	Mild serous nasal secretion (5-11)	32
22	1-9	-	Mild serous nasal secretion (5-9)	8
23	7-15	7	Mild serous nasal secretion (5-11)	16
24	1-15	3-7	Mild serous nasal secretion (5-12)	< 2
25	1-11	7	Mild serous nasal secretion (7-10)	32

[a]days post-inoculation; - : not-detected; n.p.: not performed; *dead at day 3pi; ** dead at day 14pi

Fig.1

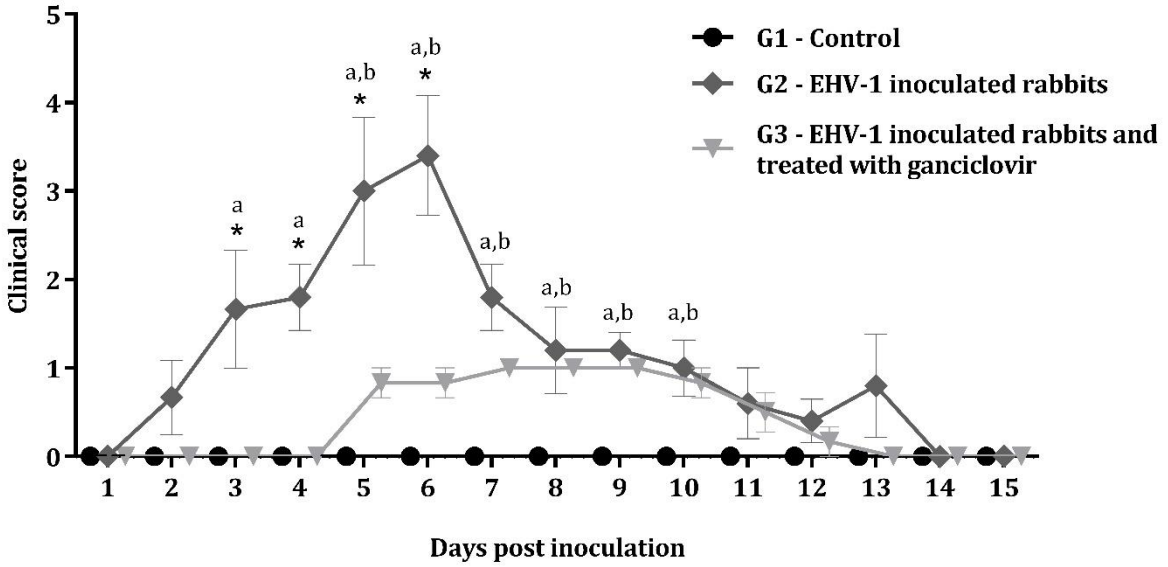
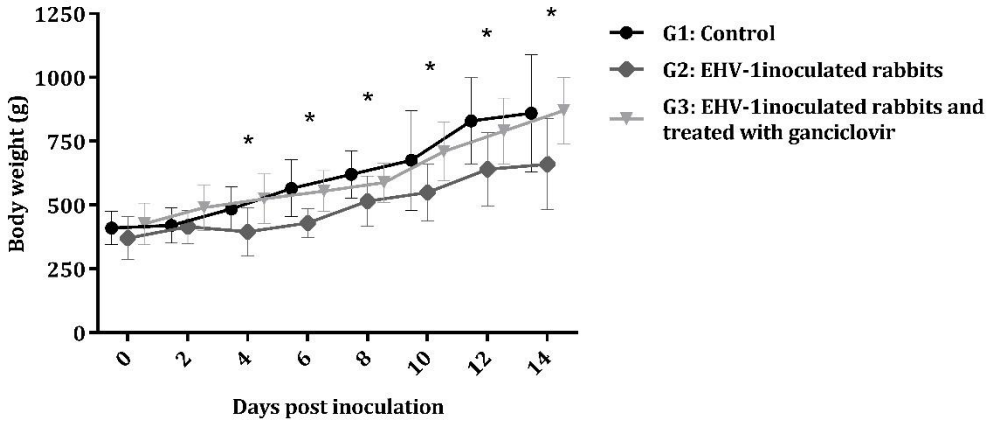

G1 - Control
G2 - EHV-1 inoculated rabbits
G3 - EHV-1 inoculated rabbits and treated with ganciclovir
Clinical score
Days post inoculation
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
a
*
a
*
a,b
*
a,b
*
a,b
a,b
a,b
a,b

Fig.2


1250
1000
750
500
250
0
Body weight (g)
0
2
4
6
8
10
12
14
Days post inoculation
*
*
*
*
*
*
G1: Control
G2: EHV-1inoculated rabbits
G3: EHV-1inoculated rabbits and treated with ganciclovir

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