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INTERAÇÃO CÉLULA HOSPEDEIRA E A INFECÇÃO PELO HERPESVÍRUS BOVINO 5 (BHV5)

São José do Rio Preto
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SUMÁRIO

RESUMO

O Brasil possui um dos maiores rebanhos de bovinos mundialmente. Estes animais estão expostos constantemente a agentes virais causadores de patologias. Dentre estes, o herpesvírus bovino 1 (BHV1) e 5 (BHV5) apresentam grande importância epidemiológica. O BHV1 causa infecções respiratórias e genitais, enquanto que o BHV5 meningoencefalite. As proteínas de membrana presentes nos herpesvírus os tornam capazes de infectar as células do hospedeiro. Com afinidade a distintas células, desenvolvem sua capacidade de infecção, apresentam mecanismos de sobrevivência à morte celular programada e a resposta imunológica. As interações vírus-hospedeiro são fundamentais no entendimento de aspectos relacionados a transmissão viral, capacidade de infecção e progressão da doença. Para este fim, se faz necessário compreender elementos fundamentais, como a modulação da função mitocondrial e a replicação do BHV, bem como os níveis de apoptose nas células do hospedeiro. De igual importância, é indispensável entender o comportamento de genes apoptóticos que estão relacionados à infecção por BHV5 e seus envolvimentos na viabilidade celular. Estes achados contribuem para o entendimento da imunopatogenia do BHV, auxiliam a evitar a disseminação destes agentes e são de grande valia para o desenvolvimento de vacinas.

ABSTRACT

Brazil has one of the largest cattle hers worldwide. These animals are constantly exposed to pathological viral agents. Bovine herpesvirus 1 (BHV1) and BHV5 have great epidemiological importance. BHV1 is causative agent of respiratory and genital infections, while BHV5 causes meningoencephalitis. Herpesvirus membrane proteins allow them to infect the host cells. They present affinity to different types of cells, develop mechanisms to survive programmed cell death, and immune response. Virus-host interactions are essential to understand aspects related to viral transmission, capacity of infection and disease progression. To this end, it is necessary to understand the fundamental elements related to the modulation of mitochondrial function and replication of BHV, as well as apoptosis levels in the host cells. It is crucial to understand the behavior of apoptotic genes related to BHV5 infection and cell viability. These findings contribute to understanding the BHV immunopathogenesis, help to prevent the spread of these agents, and are of great value to the development of vaccines.

LISTA DE FIGURAS

LISTRA DE ABREVIATURAS

APC	Células apresentadoras de antígeno
Bax	*B-cell lymphoma 2-associated X protein*
BHV	Herpesvírus bovino
CD4	Grupamento de diferenciação 4
CD8	Grupamento de diferenciação 8
CTL	Linfócito T citotóxico
DC	Célula dendrítica
DNA	Ácido desoxirribonucleico
g	Glicoproteína
GM-CSF	Fator estimulante de colônia de granulócitos
HSV1	*Herpes simplex virus 1*
IBR	Rinotraqueíte infecciosa bovina
IFN	Interferon
IL	Interleucina
IPV	Vulvovaginite pustular infecciosa
JAK-STAT	*Janus kinase-Signal Transducer and Activator of Transcription*
Mcl-1	*Induced myeloid leukemia cell differentiation protein*
MHC	Complexo principal de histocompatibilidade
NK	*Natural Killer*
NKT	*Natural Killer T*
NO	Óxido nítrico
OIE	Organização Mundial de Saúde Animal
pDC	Células dendríticas plasmocitóides
Pi	Pós-infecção
PMN	Células polimorfonucleares
SNC	Sistema nervoso central
STAT	*signal transducer and activator of transcription*
Th	T *helper*

TLR	Receptores como Toll
TNF-α	Fator de necrose tumoral alfa
US3	*Serine/threonine protein kinase*
VP8	Proteína viral 8

1 INTRODUÇÃO

1. INTRODUÇÃO

O Brasil possui o segundo maior rebanho de bovinos no mundo, somando 200 milhões de animais. É líder em exportações, com 1/5 da carne comercializada internacionalmente. A produção de carne e leite evidencia a importância econômica do setor por gerar cerca de 67 bilhões de reais para o país (BRASIL, 2017).

Os rebanhos de bovinos são constantemente expostos a agentes que os levam a distintas enfermidades. Estas patologias afetam sua produtividade e são de suma importância para a sanidade animal. Dentre os vírus que podem acometer bovinos, os herpesvírus são responsáveis por gerar perdas econômicas equivalentes a um bilhão de dólares por ano no mundo (DEL MÉDICO ZAJAC et al., 2010). Epidemias causadas por estes agentes levam ao aumento da mortalidade e morbidade, elevam o número de aborto e meningoencefalite, além de levar à diminuição da produção de leite (STATHAM et al., 2015). Os herpesvírus acometem animais adultos e principalmente jovens, e podem estar presentes em animais aparentemente sadios (JONES et al., 2007).

O herpesvírus bovino 1 (BHV1) e o BHV5 são os mais frequentes e disseminados entre estes animais. Ambos pertencem à família *Herpesviridae*, sub-família *Alphaherpesvirinae* e gênero *Varicellovirus*. Suas estruturas virais são compostas de um DNA linear de dupla fita, um capsídeo icosaédrico, o tegumento, e um envelope fosfolipídico contido de glicoproteínas (g) em sua superfície (Figura 1) (WILD et al., 2005). Os BHV1 e BHV5 compartilham cerca de 80% de similaridade genética. As regiões genéticas mais conservadas estão envolvidas na replicação do DNA e no processamento proteico. Por outro lado, as regiões menos conservadas, aparentemente estão envolvidas no controle do ciclo viral, na interação vírus-célula e na neuropatogênese (VOEGEL et al., 2002, DELHON et al., 2003).

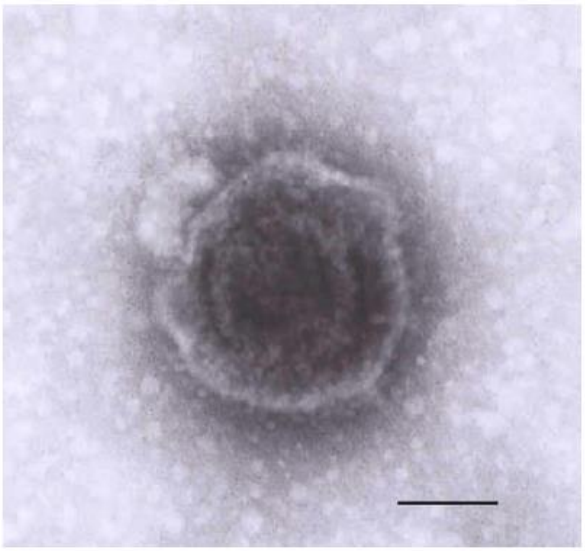

Figura 1. Morfologia do BHV5 visualizada por microscopia eletrônica de transmissão. A partícula viral possui aproximadamente 260 nm. A barra preta indica 100 nm (DEL MÉDICO ZAJAC et al., 2010).

Extensivamente investigado na literatura, o BHV1 é associado à rinotraqueíte infecciosa bovina (IBR), vulvovaginite infecciosa (IPV) e balanopostite. As infecções respiratórias levam a sintomas clínicos que incluem febre, anorexia, tosse, salivação excessiva, descargas nasais, e dispneia. A respeito das infecções genitais, as fêmeas apresentam micção frequente, pápulas vulvares e úlceras na superfície da mucosa. Em machos, lesões semelhantes ocorrem no pênis e prepúcio (JONES et al., 2008). O vírus causa alta morbidade nos animais afetados (GRAHAM, 2013; MUYLKENS et al., 2007; WRATHALL et al., 2006). Devido à sua importância, o BHV1 foi listado pela Organização Mundial de Saúde Animal (OIE) como patologia transmissível de importância socioeconômica e de saúde pública (RATTA, et al., 2014).

O BHV1 tem distribuição mundial, entretanto alguns países da Europa como Dinamarca, Finlândia e Suécia erradicaram com sucesso a doença (COWLEY et al., 2013). No Brasil, os coeficientes de soropositividade variam entre 10,6% e 96%. Além da carência de estudos epidemiológicos, muitos destes trabalhos apresentam um baixo número de

amostras, o que explica a grande variação observada (LOVATO, 1995; VIEIRA, 2003).

O BHV5 é responsável por acometer principalmente o sistema nervoso central de bovinos jovens (DEL MÉDICO ZAJAC et al., 2011). A infecção, usualmente fatal, é associada a lesões necróticas do córtex cerebral, e a inflamação nas substâncias branca e cinzenta do cérebro afetado (ROIZMAN, 1992, CLAUS et al., 2002).

A similaridade genômica compartilhada entre o BHV1 e o BHV5 dificultara a classificação taxonômica e epidemiológica destes vírus (KUNRATH et al., 2004). Como consequência, o BHV5 foi outrora considerado um subtipo do BHV1 (METZLER et al., 1986; TEIXEIRA et al., 1998).

A ocorrência de surtos de meningoencefalite causados por BHV5 foram relatadas na Austrália (JOHNSTON et al., 1962), Europa (BARTHA et al., 1969), bem como na América do Norte (BARENFUS et al., 1963; KIRKLAND et al., 2009). O vírus possui alta incidência em países da América do Sul, como Argentina, e principalmente no centro e sudeste brasileiros (LUNARDI et al., 2009; VOGEL et al., 2002). Casos clínicos de encefalite causados por BHV5 foram a segunda maior causa diagnosticada no Brasil, suplantada somente pela raiva (SANCHES et al., 2000). Além de animais jovens, o BHV5 também possui a capacidade de infectar gametas e ser transmitido a embriões *in-vitro*, apesar de não afetar o desenvolvimento destes (SILVA-FRADE et al., 2010; SILVA-FRADE et al., 2010b).

Não obstante as similaridades entre os vírus, as principais diferenças entre o BHV1 e o BHV5 estão relacionada à sua habilidade de invasão e replicação no sistema nervoso central (SNC) e capacidade de causar enfermidade neurológica (BELKNAP et al., 1994; STUDDERT, 1989). Amostras de *Alphaherpesvirus* identificadas como BHV1 são, geralmente, relacionadas a casos de doença respiratória ou genital e, raramente, causam infecção no SNC. Por outro lado, os herpesvírus isolados de casos

de doença nervosa têm sido identificados como BHV5 (D'OFFAY et al., 1993; SALVADOR et al., 1998; STUDDERT, 1989).

1.1 Patogênese do BHV1 e BHV5

As portas de entrada naturais para o BHV1 são as mucosas do trato respiratório superior ou genital. Portanto, a transmissão do vírus se dá pelo contato direto entre o nariz dos animais infectados e sadios, por meio de aerossóis ou, no momento do coito, por meio do sêmen (MARS et al., 2000). O BHV1 ainda pode estar presente em materiais biológicos como o soro bovino (KNIAZEFF et al., 1975), o fluido folicular (WEBER et al., 2013), em oócitos bovinos (BIELANSKI et al., 1994; TANGHE et al., 2005), assim como nas células do oviduto (GONÇALVES et al., 2015) e no sêmen de touros infectados (WRATHALL et al., 2006).

Quando células susceptíveis são infectadas, as partículas virais se ligam à superfície celular do hospedeiro; o envelope viral se funde com a membrana plasmática celular e o capsídeo é liberado no citoplasma. Em seguida, o capsídeo é transportado para o núcleo; os genes virais são expressos; o DNA viral é replicado; e uma nova partícula viral é sintetizada (MURATA et al., 1999).

Quando o BHV1 infecta estas células susceptíveis, as glicoproteínas possuem um papel fundamental na entrada e fusão vírus-célula. A glicoproteína C (gC) é altamente envolvida na ligação entre o vírus e a célula do hospedeiro (CHOWDHURY, 1997; HAMMEL, 2003; LIANG, 1992; LIANG, 1993;). A glicoproteína D (gD) além de ser usada na penetração viral, também está envolvida no estímulo da proliferação de linfócitos, juntamente com outras proteínas virais, como a proteína viral 8 (VP8). Em comparação com outras glicoproteínas, é mais efetiva em fornecer proteção contra o BHV1 (HUTCHINGS et al., 1990b; VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993; VAN DRUNEN LITTEL-VAN DEN HURK, 1995). No entanto, as diferenças entre as funções da gB e gD nos

Alphaherpesvirus ainda não estão totalmente elucidadas (ATANASIU et al., 2010).

Além do papel de infectar células, as glicoproteínas no BHV5 estão ligadas à neurovirulência (CHOWDHURY et al., 2000; SILVA, et al., 2009). Em modelo animal, BHV5 que não expressam glicoproteína E (gE), apresentam movimentos de transporte dos receptores olfatórios neuronais para o bulbo olfatório restringido. Consequentemente, neurônios secundários e terciários não são infectados (CHOWDHURY et al., 2000). A gE aparenta ser de grande importância, uma vez que o vírus não necessita de glicoproteína I (gI) para desenvolver a neuropatogênese (AL-MUBARAK et al., 2004; FLORES et al., 2009). Vale ressaltar que a gC difere em sua sequência de aminoácidos entre o BHV1 e o BHV5. Esta variação pode contribuir para as diferenças biológicas observadas entre estes tipos virais, entretanto, resultados conclusivos ainda não foram publicados (CLAUS et al., 2005; ESTEVES et al., 2008; TRAESEL et al., 2015).

Após a penetração viral nas células epiteliais, o BHV1 e o BHV5 iniciam o ciclo de replicação lítica. Novas partículas virais produzidas são liberadas no meio extracelular ou infectam outras células diretamente via contato célula-célula. Como consequência da replicação, o efeito citopático causado pelo vírus é caracterizado durante a apoptose (CARDOSO et al., 2015; GARCIA et al., 2013; STEUKERS et al., 2011).

A apoptose consiste em um processo fisiológico que envolve condensação da cromatina, redução do volume celular, perda de aderência da matriz extracelular, fragmentação do DNA e formação de vesículas, ou corpos apoptóticos (ELMORE, 2007). Distintas proteínas, como a US3 (do inglês, *serine/threonine protein kinase*) do BHV5, estão envolvidas no processo de rearranjo e quebra do citoesqueleto celular (LADELFA et al., 2011). Estes corpos são fagocitados por macrófagos antes que ocorram quaisquer processos inflamatórios (SCHWARTZMAN et al., 1993; FABIAN et al., 2005) (Figura 2).

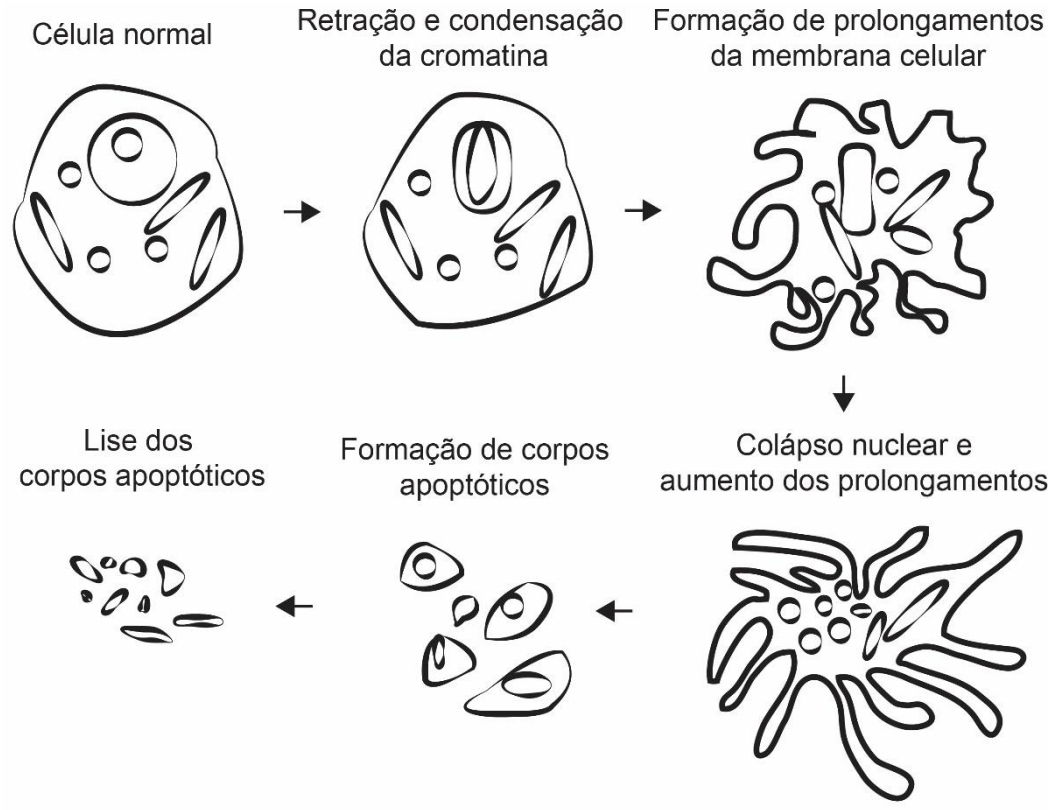

Figura 2. Representação esquemática da apoptose.

No entanto, ainda não é possível estabelecer se a apoptose tem uma consequência direta na replicação viral ou se é mediada por mecanismos indiretos, tais como a liberação de citocinas pró-apoptóticas (BORDI et al., 2006).

Após a replicação na mucosa nasal, o BHV5 invade terminações nervosas, sendo transportado pelas fibras nervosas até o SNC, sítio no qual o vírus pode se replicar e disseminar, podendo levar a manifestações clínicas de meningoencefalite (BELKNAP et al., 1994). Durante a fase inicial de infecção, o aumento da síntese de óxido nítrico (NO) está correlacionado com a disseminação do BHV5 no cérebro de coelhos infectados (DEZENGRINI et al., 2009). Pequenas concentrações de NO inibem a expressão de moléculas de adesão, a síntese de citocinas e quimiocinas, a adesão e migração de leucócitos, e em maiores concentrações, são tóxicas para as células. Logo, não é surpreendente sua relação com a disfunção, neurotoxidade e doença neurológica na infecção por BHV5.

Como o efeito biológico depende do microambiente e dos sinais de transdução e transcrição, o modo de ação do NO ainda não é totalmente esclarecido (GUZIK, 2003).

Quando em latência, o vírus permanece nos neurônios do gânglio trigeminal do SNC, da mesma forma que o BHV1 (PEREZ et al., 2002, DIEL et al., 2005). Várias áreas do cérebro de animais com infecção latente mostram a presença de DNA do BHV5 (VOGEL et al., 2003). Além da via trigeminal, o BHV5 utiliza o tráfego de leucócitos para se direcionar ao SNC (sistema nervoso central), bem como a via olfatória, colaborando para a disseminação viral no cérebro (DIEL et al., 2005; DREVETS et al., 2000; RANSOHOFF et al., 2003). Infecções experimentais realizadas em coelhos (MACHADO et al., 2013), caprinos e ovinos demonstraram que o vírus pode permanecer neste estado por tempo indeterminado, ou sofrer a reativação devido à queda na resposta imunológica do hospedeiro (CARON et al., 2002; DIEL et al., 2007). Quando reativado, o BHV5 causa doença neurológica novamente, podendo levar os animais a óbito (PERES et al., 2002).

1.2 BHV1, BHV5 e a resposta imunológica do hospedeiro

Como em distintos tipos de infecção, os mamíferos dispõem de barreiras não imunológicas de proteção. As secreções do muco, o sistema ciliar, tosses e espirros, a saliva e as junções das células epiteliais são as primeiras barreiras físicas não imunológicas que protegem o hospedeiro destas infecções (ACKERMAN et al., 2010; KEELE et al., 2012).

Ultrapassadas estas barreiras, a resposta imunológica do hospedeiro frente ao BHV1 é iniciada pela resposta imunológica inata (JONES et al., 2009). Esta tem por objetivo reconhecer e resistir à infecção, e eliminar o agente patológico. Caso a infecção persista, a reposta imunológica adaptativa de maior potência e especificidade imunológica é ativada (IWASAKI et al., 2010; SHETNEN et al., 2011).

Na prevenção à infecção, qualquer interferência na ligação entre o vírus e a célula do hospedeiro irá influenciar a patogenicidade do BHV1 e do BHV5 (AL-MUBARAK e CHOWDHURY, 2004; ROMERA et al., 2014). Em infecções secundárias, ou em futuras reativações do vírus, os anticorpos inibem esta ligação, impactando significativamente na disseminação viral. Entretanto, na resposta imunológica inata não há modulação por anticorpos (BABIUK et al., 1996).

A cinética de replicação do BHV1 é considerada relativamente rápida. Seguindo a ligação vírus-célula, a expressão de antígenos na superfície celular se dá com aproximadamente 3 a 4 horas pós-infecção (pi) (BABIUK, 1996). Em seguida, a montagem viral com 6 a 7 horas pi. Posteriormente, o vírus é liberado para o espaço extracelular (Figura 3). Logo, a cascata de reações imunológicas do hospedeiro é estabelecida após os eventos iniciais de infecção (BABIUK et al., 1996). A rápida replicação em cultivo celular é característica dos Alphaherpesvirus e, apesar da escassez de estudos, é sugerido que o BHV5 tenha uma cinética parecida (ENGELS e ACKERMANN, 1996; RISSE et al., 2007).

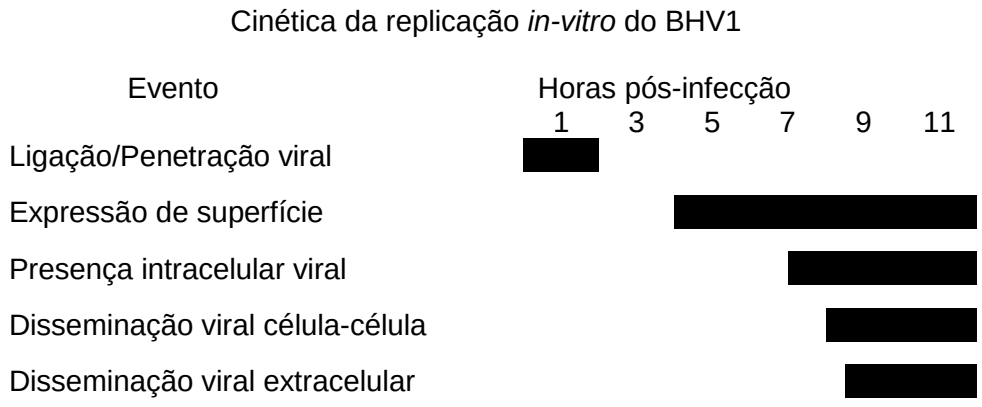

Figura 3. Cinética de replicação *in-vitro* do BHV1. Após interação entre o vírus e a célula do hospedeiro, ocorre uma rápida penetração viral, o início da replicação viral e a expressão dos antígenos de superfície (adaptado de Babiuk et al., 1997).

Uma vez que a infecção primária é estabelecida, a resposta inflamatória celular inata é ativada (ENGELS et al., 1996; OIE, 2010). Com poucas horas pós-infecção, as citocinas de primeiros eventos (*early*

citokines) são produzidas. As produções de interferon alfa (IFN-α) e interferon beta (IFN-β) são iniciadas por células infectadas e não infectadas em reposta à produção dos antígenos virais (TANIGUCHI et al., 2002). Estas moléculas são detectáveis com aproximadamente 5 horas pi e atingem o pico de produção no nariz e no sangue do animal infectado com aproximadamente 36 a 72 horas pi (Figura 4) (BABIUK, 1996).

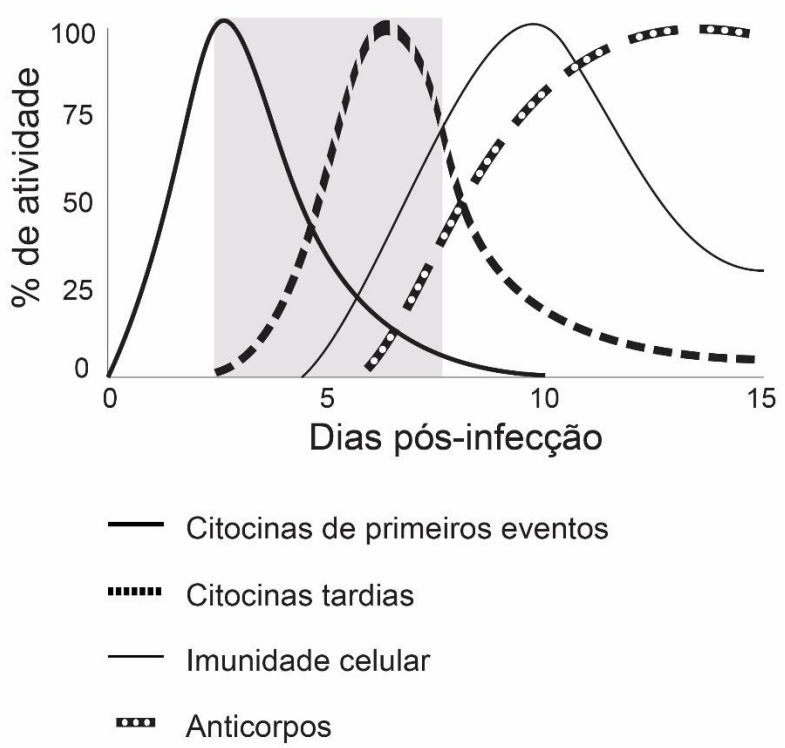

Figura 4. Desenvolvimento da resposta imunológica frente a uma infecção viral por BHV1 (BABIUK et al., 1996).

Estas citocinas permanecem elevadas até que a replicação viral cesse. A ativação dos receptores celulares para IFN-α e IFN-β induzem à modificação de fatores transcricionais e translacionais celulares que induzem as células do hospedeiro a um “estado antiviral” (SAINZ et al., 2002). Os IFN-α e IFN-β ativam a cascata de sinalização *JAK-STAT* (*janus kinase-signal transducer and activator of transcription*). Como consequência, estes interferons do tipo I (IFN I), em células vizinhas não infectadas, induzem a expressão de genes cujos produtos irão interferir

com a replicação viral (estado antiviral) (SCHINDLER et al., 2007). Em células infectadas, induzem a expressão de genes cujos produtos aumentam a susceptibilidade e a capacidade de destruição dos linfócitos T citotóxicos (CTL). Esta ação biológica configura uma das principais atividades antivirais na resposta imunológica inata (ACKERMAN et al., 2010; SCHLINDER, 2007). Estes interferons (IFNs) também aumentam a expressão do complexo principal de histocompatibilidade classe I (MHC I), da apresentação de antígenos, bem como ativam células dendríticas (DC), macrófagos e linfócitos *natural killer* (NK) (MURPHY et al., 2008). Entretanto, somente a produção destas citocinas não é suficiente para inibir a replicação do *Herpes simplex virus 1* (HSV1) *in-vitro*, por exemplo, mesmo que o pré-tratamento com IFN-α e IFN-β reduza a patogenicidade do HSV1 em humanos (CARR et al., 2003). A produção de outras células e citocinas, pró e anti-inflamatórias como a interleucina 1 (IL-1), IL-6 e IL-10, determinarão o *status* da infecção (RISALDE et al., 2011).

A infecção pelo BHV1 leva ao influxo de macrófagos, células polimorfonucleares (PMN) e de leucócitos para o sítio de infecção (OHMANN, 1986). A expressão de moléculas de adesão em células endoteliais promove a aderência de leucócitos (LEY et al., 2007). Com o aumento da permeabilidade vascular, as células de defesa migram para o local da infecção. Agindo em conjunto, a IL-1 e a IL-6 induzem à produção do fator estimulante de colônia de granulócitos (GM-CSF), contribuindo para a diferenciação de macrófagos e fazendo com que estas células produzam o fator de necrose tumoral alfa (TNF-α) (BABIUK et al., 1996). Esta citocina, assim como os IFNs, são de grande importância no controle de infecções virais, uma vez que ratos infectados com HSV1 e que são *knockout* para TNF-α têm sua sobrevivência reduzida em quase 25%, com uma taxa de reativação de aproximadamente 50% (MINAMI et al., 2002). Outras citocinas como a IL-2 agem em conjunto melhorando a resposta contra o BHV1, contribuindo para a ativação e proliferação de linfócitos, e formação da memória imunológica (TURIN et al., 1999).

Outras células possuem um papel importante na resposta imunológica primária. Assim como os neutrófilos, células dendríticas plasmocitóides (pDC), por meio de receptores do tipo Toll (TLR) internalizam antígenos virais e produzem IFN-α e -β, TNF-α e IL-12, funcionando como ponte entre o sistema imunológico inato e adaptativo (BARCHET et al., 2005, REIZIS et al., 2011). Linfócitos NK produzem compostos citotóxicos como as perforinas e granzimas que penetram a membrana celular, induzindo à apoptose em infecções virais (ENDSLEY et al., 2006). Sua importância também está ligada ao reconhecimento de células infectadas, nas quais o vírion bloqueou a expressão de MHC I (JUGOVIC et al., 1998). Linfócitos T $\gamma\delta$, linfócitos *Natural Killer T* (NKT) e linfócitos B1 também são estimulados e produzem interferon gama (INF-γ) (SCHOENBORN et al., 2007). Entretanto, quanto a estas células, pouca informação está disponível na literatura e ainda há necessidade de futuros esclarecimentos. Esta onda de citocinas de primeiros eventos inicia a resposta inflamatória e influencia dramaticamente a habilidade do hospedeiro em controlar a infecção (MUYLKENS et al., 2007).

A resposta imunológica adaptativa, por sua vez, é caracterizada pela especificidade dos linfócitos T e B e principalmente pela capacidade de desenvolver memória (FARBER et al., 2016). De forma distinta da resposta imunológica inata, essa resposta ocorre via MHC, sistema já demonstrado em bovinos também (GERNER et al., 2009). Durante a infecção pelo BHV1, as DC, linfócitos B e macrófagos funcionam como células apresentadoras de antígeno (APC) via MHC I ou MHC II, e dessa forma, ativam linfócitos T (TIKOO et al., 1995a).

Os linfócitos T $CD4^+$ são ativados via MHC II, e durante a infecção por HSV1 são considerados essenciais para o *clearance* do vírus (JOHNSON et al., 2008). Estas células são responsáveis por produzir citocinas tanto de perfil T *helper* 1 (Th1) como a IL-2, IL-12, IFN-γ, bem como Th2 como a IL-4, IL-6 e IL-10, estimulando também a produção de anticorpos. Estes linfócitos, além de reconhecerem as gB, gC e gD,

também reconhecem macrófagos infectados *in-vitro* por BHV1 (HUTCHINGS et al., 1990, LEARY et al., 1990, WANG et al., 1998).

Os linfócitos T $CD8^+$ reconhecem peptídeos via MHC I e são responsáveis, como outras células da resposta imunológica inata descritas anteriormente, por produzir perforinas e granzimas (SALTI et al., 2011). Uma vez na célula alvo, as granzimas ativam a apoptose (PARDO et al., 2004). Estes CTL carregam outras moléculas de superfície, como o Fas (CD95), que também ativam a apoptose (SLOAN et al., 2003). As gC e gD do BHV1, também foram descritas na literatura como alvo de reconhecimento dos linfócitos T $CD8^+$ (HART et al., 2011). Esta resposta celular é mediada com cerca de 7 a 10 dias pi (BABIUK et al., 1996).

A produção de IFN-γ é fundamental na resposta Th1 (McNAB et al., 2015). Esta citocina apresenta múltiplas funções antivirais, como a ativação de macrófagos, aumento do tráfego de linfócitos e regulação do reconhecimento do patógeno e do processamento de antígenos (CAMPOS et al., 1989; JAIME-RAMIREZ et al., 2011; SCHOENBORN et al., 2007). O IFN-γ impede a replicação viral por ativar mecanismos de sinalização que induzem à produção de proteínas que, por sua vez, inibem o crescimento celular, induzem a apoptose e promovem a regulação negativa do mRNA viral (SCHRODER et al., 2004; TAKOAKA et al., 2012).

O balanço entre a infecção viral e a resposta imunológica do hospedeiro é uma relação complexa e detalhada (CHAN et al., 2011). Os vírus, se replicam usando a “maquinaria” de síntese proteica do hospedeiro, infectam grandes populações celulares e se utilizam das moléculas normais presentes nas células para a invasão viral (WALSH et al., 2011). Interferem na síntese de proteínas, causam lesão tecidual e levam a infecções latentes que perduram por anos (PALUDAN et al., 2011). E se por outro lado as células possuem grande arsenal imunológico de combate, os vírus respondem novamente com múltiplas estratégias de evasão, iludindo e retardando a resposta imunológica. Logo, as interações entre os BHV e o hospedeiro são fundamentais para entender os aspectos relacionados à latência, transmissão, infecção e doença (WHITE et al., 2012).

Compreender elementos dessa relação como: modulação da função mitocondrial, replicação do BHV, apoptose, genes envolvidos na ativação e seu envolvimento na replicação celular se mostram de extrema importância. Estes resultados contribuirão para elucidação do comportamento de infecção e imunopatogenia, bem como colaboram para o desenvolvimento de vacinas, buscando evitar a disseminação destes agentes virais.

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CAPÍTULO 1

3. CAPÍTULO 1

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Cellular response markers and cytokine gene expression in the central nervous system of cattle naturally infected with bovine herpesvirus 5

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ABSTRACT

The present study reports an investigation on the phenotype of inflammatory and immune cells, cytokine and viral gene expression in the brains of cattle naturally infected with bovine herpesvirus 5 (BHV5). Brain sections of 38 affected animals were analysed for the nature and extent of perivascular cuffs in the Virchow–Robin space and parenchyma.

Histopathological changes were severe in the olfactory bulbs (Obs), hippocampus, piriform, frontal, temporal and parietal cortices/lobes and were characterized by inflammatory infiltrates in Virchow–Robin spaces. The histopathological changes correlated positively with the distribution of BHV5 antigens ($r = 0.947$; $P < 0.005$). Cells of CD3+ phenotype were predominant in areas with severe perivascular cuffs. Viral antigens and genomic viral DNA were detected in the Obs and piriform lobe, simultaneously ($r = 0.987$; $P < 0.005$). Similarly, pro-inflammatory cytokine genes *INFG*, *IL2*, *TNF* and *LTBR* were expressed in the same brain areas ($P < 0.005$). These results provide important information on the inflammatory and immunological events accompanying BHV5 neurological infections. Our findings provide the first evidence for increased immune activation followed by inflammatory cytokine expression, positively correlated with viral replication in the cranial areas of the brain. Taken together, these results suggest that the host immune response and inflammation play a crucial role in the pathogenesis of acute encephalitis by BHV5 in cattle.

Introduction

Bovine herpesvirus 5 (BHV5) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison et al., 2009; Davison, 2010; Del Medico Zajac et al., 2010). A hallmark of the biology of *alphaherpesviruses* is the lifelong latent infection established after acute infection, providing a means for viral reactivation, transmission and spread (Perez et al., 2002; Jones, 2003; Lovato et al., 2003; Jones et al., 2011; St Leger and Hendricks, 2011). The active viral replication during acute infection and after viral reactivation frequently results in neurological disease characterized by tremors, nystagmus, tooth grinding, bruxism, ptyalism, tongue protrusion, circling, ataxia, paddling and, eventually, death (Del Medico Zajac et al., 2010; Marin et al., 2014).

Several reports have described experimental inoculations of BHV5 to reproduce clinical encephalitis and produced variable results (Perez et al., 2002; Del Medico Zajac et al., 2010; Machado et al., 2013). Neurologic signs and microscopic lesions observed during acute phase of BHV5 infection after experimental and/or natural field outbreaks were considered similar (Perez et al., 2002). Moreover, during acute infection, BHV5 would access mostly the rostral areas indicating that viral invasion of the brain occurs more directly along olfactory neurons (Favier et al., 2014). In summary, the types of neural damage produced after BHV5 infection of the brain have been described as neuronal degeneration and inflammatory perivascular cuffs with different degrees of severity (Machado et al., 2013). However, due to the variability of these histopathological patterns, the characterization of lymphocyte recruitment after natural BHV5 infection remains unclear.

Cytokine levels are markers of the inflammatory cascade in the CNS, in which the immune system plays an essential role in the homeostasis (Yshii et al., 2015). A number of neurotropic viruses induce the loss/disruption of the blood–brain barrier (BBB), including Nipah virus, rabies virus, West Nile virus, mouse adenovirus type 1 and herpes simplex virus (HSV1; Sarma, 2014). A similar mechanism has been recently proposed for BHV5 in rabbits (Machado et al., 2013). Inflammatory cytokines or chemokines, such as gamma interferon (IFN-γ), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF-α) and IL-1β, may facilitate virus entry into the CNS by inducing a breakdown in the BBB (Franco and Fernández-Suárez, 2015). Major

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sources of these cytokines include the microglial CNS cells or infiltrating lymphocytes, which move towards the site of infection via chemokine-mediated cell trafficking upon the breakdown of the BBB (Yshii et al., 2015). However, the inflammatory response is an essential component of the host immune system, as observed in HSV1 infection, where T and B lymphocytes remain activated for a long time (Jones, 2003). Lymphocyte T cells also induce the biological activation of astrocytes in experimental and natural cases of BHV5 infection (Cardoso et al., 2010; Machado et al., 2013). Moreover, IFN-γ, produced by a subset of T cells, has been described as a potent activator of astrocytes (Xie and Yang, 2015).

Since many aspects of BHV5-induced neuroinflammation remain obscure, the present study was designed to investigate the phenotype of inflammatory and immune cells, cytokine and viral gene expression in the brains of cattle naturally infected with bovine herpesvirus 5 (BHV5), correlating them with the distribution of viral antigens and genome.

Materials and methods

Animals and ethical statement

Brains from 38 cattle with positive diagnoses for acute BHV5 neurological infection and from 10 healthy cattle were obtained from routine pathological diagnostics at the Laboratory of Animal Virology of University of São Paulo state, Brazil. All applicable institutional guidelines for the care and use of animals were followed (CEEA 2015/09765). In each animal, the whole brain was sampled, sectioned into two halves and divided into 12 regions, as follows: rostral areas: Ob (olfactory bulbs and rostral olfactory peduncles), Pl (Piriform lobe), H (hippocampus), Fc (frontal cortex), Tc (temporal cortex), Pc (parietal cortex); caudal areas: Oc (occipital cortex), T (thalamus), M (mesencephalon), P (pons), Mob (medulla oblongata), Sc (cervical spinal cord). For each region from each brain half, two fragments of approximately 12.5 mm were stored in RNA later-ICE (Ambion, Life Technologies) at −86 °C for molecular analysis, and immersed in 10% formalin for histopathological and immunohistochemical analyses in parallel (Vogel et al., 2003; Marin et al., 2015).

Histochemical and immunohistochemical investigations

Sections from both brain halves, embedded in paraffin wax, in serial sections, 3-µm thick, were submitted to haematoxylin and eosin staining following standard procedures (Ferrari et al., 2007). The numbers of perivascular cuffs were counted by two different pathologists masked to the experimental design, and they were classified as mild (increase in cell nuclei or incomplete ring of cell nuclei within the Virchow–Robin space), moderate (complete ring of cells in the Virchow–Robin space) or severe (two or more complete cell layers in the Virchow–Robin space).

BHV5 antigen levels and the inflammatory cell phenotype were determined according to previously reported methods, with some modifications (Cardoso et al., 2010). The LSAB + Kit was used according to the manufacturer's recommendations (K0690 Dako). BoHV5 monoclonal antibody (BHV5 2F9) was used at a dilution of 1:50 in 1% bovine serum albumin (BSA, Sigma-Aldrich). T lymphocytes were identified and counted using the mouse monoclonal antibody anti-CD3 diluted at 1:100 in 1% BSA. For B-lymphocytes, mouse anti-CD45R/B220 antibody (Sigma-Aldrich) diluted at 1:50 in 1% BSA was applied. Epitope retrieval was performed by immersion of slides in a solution of 0.05% trypsin (Sigma-Aldrich). The reaction was performed according to the instructions of the LSAB + Kit (Dakocytomation). Finally, the slides were counterstained with Harris's haematoxylin and mounted. Negative control sections were prepared by replacing the primary antibodies with 1% BSA (Sigma-Aldrich; Machado et al., 2013). The numbers of T and B cells per perivascular cuff and infiltrating the surrounding parenchyma were assessed using an AxioImager A.1 light microscope connected to an AxioCam MRc (Carl Zeiss). The images were processed using AxioVision 4.8 software (Carl Zeiss) for detecting viral antigens and T and B lymphocytes. Fifteen cuffs (five mild, five moderate and five severe) were analysed for each brain region in each animal.

Isolation of DNA and RNA and synthesis of complementary DNA (cDNA)

Firstly, total DNA was extracted from each brain region in parallel (200 µm) using the DNAzol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). Total RNA was extracted from 200-µm sections of frozen tissue to quantify cytokine transcripts, using the TRIzol reagent protocol (Invitrogen). Genomic DNA contaminating the isolated RNAs was removed using Turbo DNA-free kit according to manufacturer's instructions (Ambion). In addition, isolated RNA was also precipitated by removing proteins using 3 M sodium acetate, pH 5.5 (Ambion), glycogen (5 mg/mL) and ethanol. cDNA was obtained by reverse transcription of 100 ng of purified RNA according to the Superscript III protocol (Invitrogen).

Real-time PCR (qPCR)

The single-tube TaqMan assay (Life Technologies) was used; the customized primers and probes for targeting cytokine genes are detailed in Table 1. The primers and the probe used for viral DNA detection were the same as described previously for BHV5 gC (Diallo et al., 2011; Cardoso et al., 2013).

The quantitative PCR mixtures (50 µL) contained 1.2 µg of viral DNA and cytokine cDNA, and 400 nM primers and 200 nM probes FAM-mGB (5′ region) were used in qPCR initiated by sequential amplification with 40 cycles at 95 °C (15 s) and 60 °C (60 s). The results are expressed as viral DNA detection and as relative cytokine gene expression, indicating the fold change (up- or downregulation) in expression of the cytokine genes. Quantification of cytokine gene expression was performed using the $2^{-\Delta\Delta Ct}$ method, using the bovine histone 2a gene (*H2A*; Bt03252057_g1) as a reference gene as described previously (Cardoso et al., 2013). The reaction was carried out and analysed using the StepOnePlus real-time instrument software (Applied Biosystems). The data were obtained from three replicates of each sample (Livak and Schmittgen, 2001).

Statistical analysis

The perivascular cuff distribution within the brain was assessed by comparing the rostral and caudal regions, and total perivascular cuffs per animal were compared among brain regions using the non-parametric exact Wilcoxon sum rank test. For T and B lymphocytes, significant differences among groups were determined using one-way ANOVA followed by Tukey's multiple comparison test. A value of $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± standard deviation (SD). All statistical analyses were performed using Prism software (GraphPad v. 6.1).

Results

Histopathology and immunohistochemistry findings

Histopathological findings in the brains of 38 cases of acute BHV5 neurological infection were mainly composed of foci of perivascular cuffing with mononuclear cells, mainly lymphocytes (Figs. 1A–D). Perivascular cuffings were observed in the Obs (Fig. 1A), the frontal, temporal and parietal lobes (Figs. 1B, C), and in the piriform lobe (Fig. 1D). These changes ranged in composition, varying from a few cells (Figs. 1C, D) to multiple layers of cell nuclei in the Virchow–Robin spaces (Figs. 1A–C).

Lymphocyte infiltration into the brain parenchyma was rarely observed. Perivascular cuffing was severe in the Obs, piriform lobe, hippocampus, and frontal, temporal and parietal lobes (Fig. 2A). There appeared to be more cuffs in the rostral regions than the caudal

Table 1
Names, descriptions, and locations of the (*Bos taurus*) genes evaluated by qRT-PCR.

Gene symbol	ID	Description	Location
INFAC	281.236	Interferon alpha C (INF-alpha C)	Chromosome 8, AC_000.165.1
INFB1	281.845	Interferon, beta 1, fibroblast	Chromosome 8, AC_000.165.1
INFG	281.237	Interferon, gamma	Chromosome 5, AC_000.162.1
IL2	280.822	Interleukin 2	Chromosome 17, AC_000.174.1
IL4	280.824	Interleukin 4	Chromosome 7, AC_000.209.1
IL6	517.016	Interleukin 6	Chromosome 8, AC_000.162.1
IL1F10	615.702	Interleukin 1, family member 10	Chromosome 11, AC_000.168.1
TNF	280.943	Tumour necrosis factor (TNF alpha)	Chromosome 23, AC_000.180.1
LTBR	280.845	Lymphotoxin beta receptor (TNF superfamily member 3)	Chromosome 5, AC_000.162.1

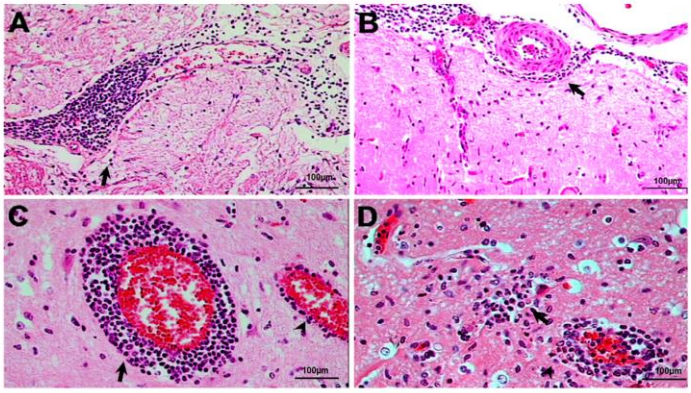

Fig. 1. Photomicrographs of histopathological changes (HE staining) characterized by perivascular cuffing (100 µm): (A) Image of the olfactory bulb showing intense mononuclear infiltration (arrow); (B) Perivascular cuff in the cortical area of the frontal lobe; (C) Perivascular cuffs characterized as severe (arrows) and mild (arrowheads) observed in the frontal, temporal and parietal lobes; (D) Perivascular cuffs considered moderate (arrows) localized in the hippocampus.

regions ($P < 0.005$). Viral antigens were detected in the same regions (Fig. 2B). A significant correlation was observed between severe perivascular cuffs and BHV5 antigen distribution ($r = 0.947$; $P < 0.005$). Viral antigens and genomic DNA were mainly detected in the Obs and piriform lobe, simultaneously ($r = 0.987$; $P < 0.005$).

Inflammatory infiltrates of CD3+ lymphocytes were detected mainly in the Obs, hippocampus, and piriform, frontal, temporal and parietal lobes (Figs. 3A, B). A strong association between CD3+ lymphocytes and BHV5 antigens was observed in the Ob and the frontal, temporal, and parietal lobes (Figs. 4A–D). CD45R+ cells (approximately 10%) were observed only in the temporal and parietal lobes (Figs. 4E, F).

Pro-inflammatory and anti-inflammatory profiles associated with virus distribution

The symbols, identities, descriptions and locations of genes studied are presented in Table 1. In this study, the highest expression levels of the genes *INFG* and BHV5 gC were observed in the Obs and hippocampus and in the piriform, frontal temporal and parietal lobes (Fig. 5A; $P < 0.005$). *INFAC* and *INFB1* expression was similar to control samples (Fig. 5). The genes *IL4*, *IL2* and BHV5 gC were detected and positively correlated in the Obs, hippocampus and piriform lobe (Fig. 5B; $P < 0.005$). The parietal cortex had higher expression of *IL4* than the other cytokines or BHV5 gC, which were detected at low levels (Fig. 5B; $P < 0.005$). In the Obs, piriform lobe, hippocampus and parietal lobe, the expression levels of *TNF*, *LTBR* and BHV5 gC were approximately equal, with lower levels observed in the frontal and temporal cortices (Fig. 5C; $P < 0.005$).

Discussion

This study demonstrated the presence of severe inflammatory infiltrates and the expression of the pro-inflammatory cytokine genes *IFNG*, *IL2*, *TNF* and *LTBR* in the brains of cattle acutely infected with BHV5. These findings were positively correlated with the presence of BHV5 DNA genome and viral antigens in the forebrain region.

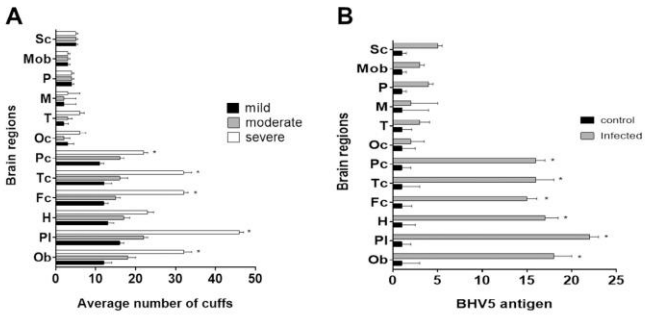

Fig. 2. Detection of perivascular cuffs and distribution of BHV5 antigen. (A) Average number of perivascular cuffs per brain area, divided into mild, moderate and severe; (B) Frequency of detection of BHV5 antigen by immunohistochemistry per brain area. The mean value was statistically significant (*$P < 0.005$).

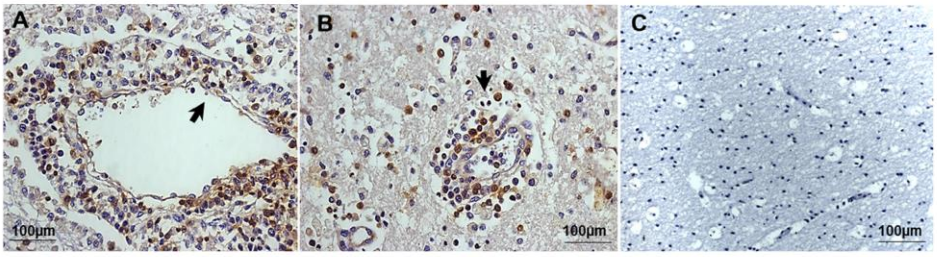

Fig. 3. Immunohistochemistry photomicrographs of CD3+ and CD45R+ lymphocytes. (A) Perivascular cuff graded as severe, mainly composed of CD3+ lymphocytes (brownish deposits) in Virchow–Robin space; (B) CD45R+ lymphocytes detected within a perivascular cuff graded as moderate (100 µm); (C) Control represented by tissue from healthy animals.

The histopathological findings were similar to previous reports on the pathogenesis of BHV5 in experimentally infected rabbits (Chowdhury et al., 1997; Lee et al., 1999; Dezengrini et al., 2009; Machado et al., 2013). However, in contrast to the present study, which examined only brains of acutely diseased animals, some of the BHV5-infected rabbits did not develop neurological signs (Machado et al., 2013). In the rabbit model, microscopic examination revealed histological changes in the cerebral cortices (frontal, temporal, occipital, parietal and piriform lobes) characterized by mononuclear perivascular cuffs (Dezengrini et al., 2009; Machado et al., 2013). In another study, it was postulated that asymptomatic encephalitis in calves experimentally infected with BHV5 might be associated with mild foci of inflammatory cells (Isernhagen et al., 2011). However, in rabbits presenting with neurological signs, BHV5 antigens were also detected in the cerebellum, a finding not observed in the present study. Regarding CD45R+ lymphocytes, 10%

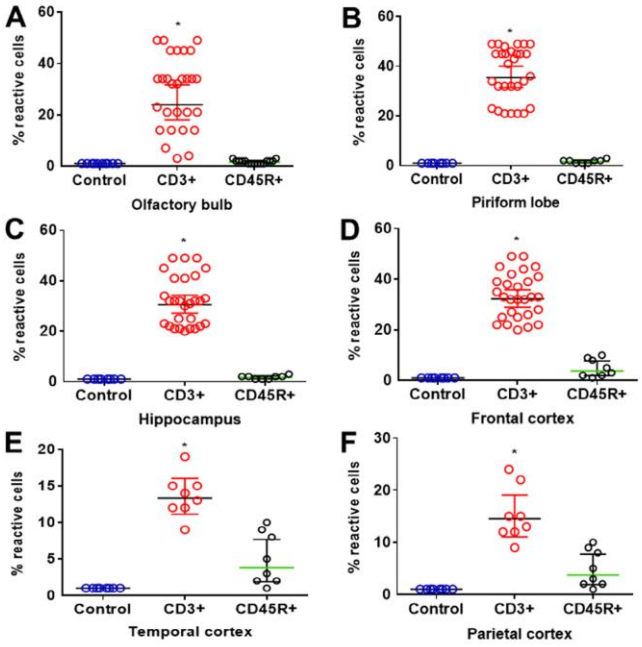

Fig. 4. Quantification of immunohistochemical data. (A–F) Numbers of CD3+ and CD45R+ lymphocytes detected and divided per brain area from BHV5-infected and control animals. The mean value was statistically significant (*$P < 0.005$).

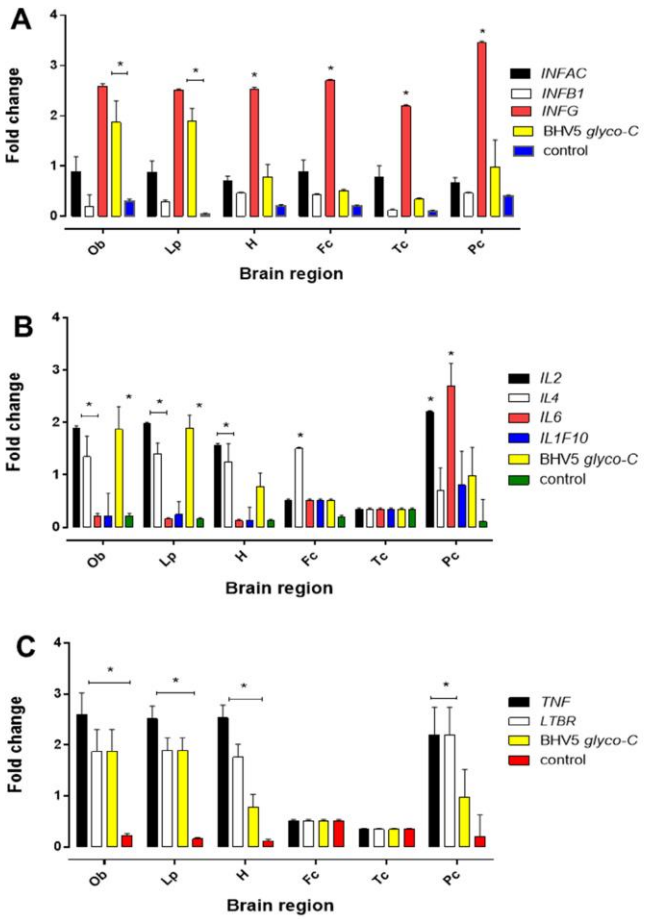

Fig. 5. Bar graph showing cytokine and BHV5 gene expression in each brain area. Values shown are fold changes in expression. Bars indicate the mean (±standard deviation); *$P < 0.005$. (A) Detection of *INFAC*, *INFB1*, *INFG*, BHV5 gC and the respective controls; (B) Detection of *IL2*, *IL4*, *IL6*, *IL1F10*, BHV5 gC and the respective controls; (C) Detection of *TNF*, *LTBR*, BHV5 gC and the respective controls.

of positive cells were observed only in the temporal and parietal lobes, a finding similar to that of a previous study (Machado et al., 2013). Similarly, acute replication of HSV1 in the trigeminal ganglia is largely controlled by the innate immune system, including macrophages and activated lymphocytes (Griffin et al., 2010; St Leger and Hendricks, 2011; Piret and Boivin, 2015).

All animals examined in this study died during the acute phase of BHV5-associated neurological disease. Molecular analysis revealed that the Obs and piriform lobe had the highest level of BHV5 gC gene expression ($P < 0.005$). Previous studies have demonstrated uniform distribution of the BHV5 genomes among brain areas in experimentally infected rabbits (Mayer et al., 2006; Machado et al., 2013). In another study, rabbits acutely infected with BHV5 harboured viral DNA mostly in the trigeminal ganglia, cerebellum, anterior cortex and pons-medulla (Dezengrini et al., 2009). However, in experimentally infected calves, BHV5 DNA was detected in the trigeminal ganglia, midbrain, thalamus and Obs (Vogel et al., 2003). Additionally, similar BHV1 viral genome distribution in the CNS of infected calves has been reported (Silva et al., 2007; Marin et al., 2015). In all animals studied, whether naturally or experimentally infected, BHV5 has most consistently been identified in the Obs. In general, latency and reactivation are critical strategies for the

survival of herpesviruses in nature (Jones, 2003). Typically, most cells infected by both human and animal *alphaherpesviruses* support lytic replication, while only specific cell subsets, especially neurons, harbour latent virus (Jones, 2003; Griffin et al., 2010).

A recent study described weak T cell infiltration associated with inefficient virus clearance in HSV1 and West Nile virus neuroinflammation (Franco and Fernández-Suárez, 2015). Moreover, cuffs composed of large numbers of T cells could result in cytotoxicity and, as a consequence, in CNS damage (Xie and Yang, 2015). Several reports have demonstrated that viral encephalitis leads to vigorous cytokine expression, an increase in BBB permeability related to IFN-γ release and stimulation of T cell activity, thereby increasing viral clearance (Sarma, 2014). Cytokines play an essential role in modulation of the host immune response, especially in the pathogenesis of many viral infections (Franco and Fernández-Suárez, 2015). Herein, the neuroinflammatory process induced by BHV5 infection was marked by up-regulation of the cytokines *INFG*, *IL2*, *TNF* and *LTBR*.

The importance of interferons (IFNs) in the anti-viral immune response is well known (St Leger and Hendricks, 2011). Instead of a direct antiviral effect, IFNs induce the expression of effector proteins, which, in turn, interfere with or inhibit various stages of viral replication, assembly or release (Piret and Boivin, 2015). The balance between inflammatory and anti-inflammatory cytokines is crucial for avoiding damage to neural tissue (Piret and Boivin, 2015). It is reasonable that the expression of these cytokines would somehow suppress the immune response and contribute to viral spread. Several studies have concluded that a cell-mediated immune response persists in the trigeminal ganglia of mice latently infected with HSV1 or in rodents infected with HSV2 (Jones, 2003; St Leger and Hendricks, 2011). Persistence of immune cells in the trigeminal ganglia is important for maintaining latency, because CD8+ T cells that produce INF-γ can prevent reactivation from latency (Perez et al., 2006; St Leger and Hendricks, 2011).

The expression of *IL2*, a T cell growth factor mainly synthesized by CD4+ lymphocytes and to a lesser extent by CD8+ cells, which also acts as an autocrine growth factor, was observed in this study. Chemokines cause the accumulation and activation of leukocytes in tissues (Yshii et al., 2015), which are essential for inflammation and the host response to infection (Crouse et al., 2015). Recently, the importance of CNS-resident cells in the establishment of an effective inflammatory response to BHV5 infection has been reported (Marin et al., 2014). Moreover, the present study also demonstrated an upregulation of *TNF* (TNF-α) and *LTBR* (TNF-β) gene expression. Both cytokines present similar activity; however, TNF-β stimulates phagocytosis, increasing the synthesis of nitrogen oxide (NO; Xie and Yang, 2015). Although NO was not measured in this study, a previous report demonstrated its production in the CNS of BHV5-infected rabbits (Dezengrini et al., 2009).

Conclusions

These findings provide additional evidence that BHV5 neurological infection in cattle is associated with severe inflammatory infiltration, predominantly by CD3+ cells, followed by pro-inflammatory cytokine expression, that correlates spatially with the distribution of viral DNA and antigens. Regardless, further studies are needed to demonstrate the cytokine profile and involvement in the fatal outcome of BHV5-induced neurological infection in cattle.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Bovine herpesviruses induce different cell death forms in neuronal and glial-derived tumor cell cultures

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Abstract Oncolytic viruses have the ability to infect tumor cells and leave healthy cells intact. In this study, bovine herpesvirus 1 (BHV1; Los Angeles, Cooper, and SV56/90 strains) and bovine herpesvirus 5 (BHV5; SV507/99 and GU9457818 strains) were used to infect two neuronal tumor cell lineages: neuro2a (mouse neuroblastoma cells) and C6 (rat glial cells). BHV1 and BHV5 strains infected both cell lines and positively correlated with viral antigen detection ($p < 0.005$). When neuro2a cells were infected by Los Angeles, SV507/99, and GU9457818 strains, 40 % of infected cells were under early apoptosis and necroptosis pathways. Infected C6 cells were >40 % in necroptosis phase when infected by BHV5 (GU9457818 strain). Blocking caspase activation did not interfere with cell death. However, when necroptosis was blocked, 60–80 % of both infected cells with either virus switched to early apoptosis pathway with no interference with virus replication. Moreover, reactive oxygen species production and mitochondrial membrane dysfunction were detected at high levels in both infected cell lines. In spite of apoptosis and necroptosis blockage, tumor necrosis factor alpha (*TNFA*) and virus transcription were positively correlated for all viral strains studied. Thus, these results contribute to the characterization of BHV1 and BHV5 as potential oncolytic viruses for non-human cells. Nonetheless, the mechanisms underlying their oncolytic activity in human cells are still to be determined.

Keywords Oncolytic viruses · Animal herpesvirus · Apoptosis · Necroptosis · Oxidative stress · Pathogenesis

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Introduction

Bovine herpesvirus 1 and 5 (BHV1 and BHV5, respectively) belong to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison et al. 2009; Davison 2010). BHV1 has been incriminated to induce respiratory and genital disorders whereas BHV5 is the agent of meningoencephalitis (Del Médico Zajac et al. 2010). Oncolytic viruses are defined for their ability to replicate in neoplasic cells and, somehow, to produce their destruction (Cuddington and Mossman 2015). In this respect, BHV1 has been used to infect and destroy human neoplasic cells derived from a variety of mesenchymal and/or epithelial origin (Cuddington and Mossman 2015). Interestingly, BHV1 seems not to infect or cause cytotoxic effect in non-neoplasic human cells (Cuddington and Mossman 2015). Moreover, equine herpesvirus type 1 (EHV-1), another alphaherpesvirus, has been shown to infect human glioblastoma multiforme cells (Courchesne et al. 2011; Cuddington and Mossman 2014).

Programmed cell death or apoptosis is a physiological mechanism implicated, in some cases, in eliminating viral infections (Hay and Kannourakis 2002; Griffin et al. 2010). In contrast, viruses may manipulate anti-apoptotic signals aimed to control/inhibit cell death and, as a consequence, to allow virus replication to proceed (Ohta and Nishiyama 2011). Apoptosis may be activated through two pathways: intrinsic

and extrinsic (Hay and Kannourakis 2002). The intrinsic pathway is characterized by induction of cell stress that activates molecules on the mitochondria surface (Elmore 2007; Scott 2010; Ohnishi et al. 2013; West et al. 2011). The extrinsic apoptotic pathway can be activated by a variety of external stimuli, known as cytokines, toxins, or modulation of cell surface receptors classified as death receptors: tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (Griffin et al. 2010; Dashzeveg and Yoshida 2015; Elmore 2007; Probert 2015).

TNF alpha (TNF-α; *TNFA*) is an inflammatory cascade inducer classified as a pro-inflammatory cytokine (Dashzeveg and Yoshida 2015). This cytokine is important in the innate immunity and crucial against viral infections (Probert 2015). After viral infection, TNF-α production results in fever, cell death, and cachexia and stimulates the inflammatory process (Elmore 2007). In special cases, TNF-α also produces tumorigenesis inhibition and blocks viral replication (Probert 2015) by activation of the caspase cascade, which activates cell apoptosis (Elmore 2007). Necroptosis is a regulated form of primary necrosis that does not involve caspase activation (Silke et al. 2015). Apoptosis and necroptosis mechanisms are rather interconnected and not entirely independent (Silke et al. 2015). To reinforce this, a study demonstrated that *FAS* or *TNF* genes, which normally activate apoptosis through death receptors, can induce necroptosis when caspases are inhibited or deficient, in different susceptible cell lines (Probert 2015; Silke et al. 2015). Although apoptotic mechanisms during viral infections have been well studied, necroptosis remains largely unclear.

In response to pathogens, the innate immune responses recruit and activate phagocytic macrophages, neutrophils, and, during neuronal infections, resident microglia cells (Gonzalez-Dosal et al. 2012). These immune cells eliminate invading pathogens by the production of reactive oxygen species (ROS) (Englezou et al. 2012; Kulahava et al. 2010). ROS production may be either beneficial or harmful to host cells, yet its generation is part of the host immune response against invading microorganisms (Kulahava et al. 2010). In this sense, rabbit brains infected with BHV5 displayed nitric oxide (NO) overproduction, which correlated to virus dissemination (Dezengrini et al. 2009). In mammals, when NO is produced with ROS, it also displays neurotoxicity and may induce apoptotic cell death in different neuronal cell types (Ohnishi et al. 2013). As described, the potential of animal alphaherpesviruses to infect neuronal and glial-derived tumor cells is not fully understood. In this study, we investigated the susceptibility of tumor neuronal and glial cells to BHV1 and BHV5 strains, in order to investigate the potential oncolytic properties of these viruses. For this purpose, apoptosis and/or necroptosis associated with virus infection were investigated. Moreover, the characterization of cell death, *TNFA* transcription, and ROS production were also examined.

Materials and methods

Cell culture, viruses, and infection

Neuro2a (mouse neuroblastoma, ATCC # CCL131) and C6 (rat glial cell tumor; ATCC # CCL107) were used in the experiments. Cells were seeded in 25 cm^2 flasks and 96-culture plates to perform growth curve/viability and virus titration (Nunc™, Rochester, NY, USA). Cell culture conditions have been previously described (Cardoso et al. 2015). BHV1 strains (Los Angeles, Cooper, and SV56/90) (Weiblen et al. 1992; Cardoso et al. 2015) and BHV5 strains (SV507/99 and GU9457818) (Delhon et al. 2003; Ferrari et al. 2007) were used.

Cells were inoculated with BHV suspensions ($10^{2.7}$ $TCID_{50}$/ml) according to previous study (Cardoso et al. 2015). Briefly, viral suspensions were allowed to adsorb for 1 h at 38.5 °C, and following inoculation, infected and uninfected cells were visualized under phase-contrast microscopy for cytopathic effect. For one-step growth assays, cells (5×10^5 per well) were seeded into 96-well plates in duplicate and inoculated with the viruses as described (Cardoso et al. 2015). After 2 h of adsorption, the inoculum was removed and cells were washed twice with medium and further incubated at 37 °C. At 24, 48, 72, 96, and 120 h post-infection (p.i), supernatants and cell-associated virus were recovered and stored at −86 °C. The 50 % tissue culture infectious dose ($TCID_{50}$) of the samples was determined by limited dilution and the data log transformed (Cardoso et al. 2015).

Immunocytochemistry

Immunocytochemistry was performed according to previous study with some modifications (Cardoso et al. 2015). Briefly, infected and control Neuro2a and C6 cells were fixed at 48 h p.i with 4 % paraformaldehyde for 15 min followed by incubation overnight at 4 °C with monoclonal antibodies to BHV1 (MAb 7F12) and BHV5 (2F9 and 1F3) (Varela et al. 2010; Oldoni et al. 2004). After three washes, cells were incubated with the respective goat secondary antibody (1:100) anti-mouse FITC (Sigma-Aldrich®) and nuclear staining was performed with 1 mg/ml of DAPI (4′-6-diamino-2-phenylindole; Sigma-Aldrich®) and 0.1 mg/ml of propidium iodide (PI) was diluted in Fluormount™ aqueous medium. The images were collected under an AxioImager® A.1 light and an ultraviolet (UV) microscope connected to an AxioCam®MRc (Carl Zeiss, Oberkochen, Germany). The images were processed using AxioVision® 4.8 software (Carl Zeiss) for viral antigen detection. Ten fields were analyzed in each condition, and photographs were taken at ×200 magnification by AxioVision™ software (Olympus).

Induction of apoptosis/necroptosis and production of ROS

In order to evaluate apoptosis and production of ROS, 1×10^6 cells/ml were infected with $10^{2.7}$ $TCID_{50}$/ml of each virus strain. In parallel, cells were treated with 1.5 μM of staurosporine (Sigma-Aldrich®) for 6 h to induce apoptosis, as positive control (Cardoso et al. 2015). In another two experiments, both cell lines were pre-treated with 4 μM pan-caspase inhibitor z-vad-fmk (Sigma-Aldrich®); after 1 h, BHV infection was performed. As control, cells were exposed only to pan-caspase inhibitor. In order to evaluate necroptosis, inhibition of receptor-interacting protein kinase was induced with 1 μM of necrostatin 1 (nec-1; cat # N9037, Sigma-Aldrich®). After treatment, BHV strains were inoculated into cell cultures and monitored. The ROS-positive control was composed by cells exposed to 5 % of CO_2 atmosphere during 30 min.

Flow cytometric analysis of apoptosis/necroptosis, ROS production, and mitochondrial membrane potential

The flow cytometric analysis of apoptosis/necroptosis, ROS production, and mitochondrial membrane potential were acquired via an Attune™ acoustic focusing cytometer system at 48 h after virus inoculation (Applied Biosystems®, Foster City, CA, USA) with parameters established in a previous study (Cardoso et al. 2015). Apoptosis/necroptosis was measured using double-staining method with The Vybrant Apoptosis Assay Kit (Molecular Probes™, Life Technologies®) and APO-BrdU™ TUNEL assay Kit (Molecular Probes™, Life Technologies) according to previous study (Cardoso et al. 2015). The differentiation of early apoptotic, secondary necrotic, necroptotic, and viable cells was made according to their phenotype: BrdU +/PI-were considered early apoptotic, BrdU-/PI+ necroptotic, BrdU+/PI+ secondary necrotic, and BrdU-/PI- viable cells. Data were acquired following the Attune equipment parameters described previously (Cardoso et al. 2015). The results were expressed as Δ % of each cell phenotype.

The ROS production was measured by Fluorometric Intracellular ROS™ kit (cat # MAK142, Sigma-Aldrich®) according to manufacturer's instructions. Briefly, 1×10^6 cells/ml were centrifuged, and the resulted pellet was mixed in 40 μl of ROS detection reagent solution. After, data from cells exposed to all experiments were captured.

To perform the mitochondrial membrane potential assay, 2×10^3 infected and uninfected cells were incubated with 10 μg/ml of JC-1 (5,5′,6,6″-tetrachloro-1,1′,3,3′-tetrathylbenzimidazolyl-carbocyamine iodide; Molecular Probes™, Eugene, OR) for 10 min at 37 °C and analyzed by acoustic flow cytometry in the dark according to previous study (Cardoso et al. 2015).

Molecular quantification

Infected and control cell lines were submitted to DNA extraction at 48 h pi using DNAzol™ according to manufacturer's instructions (Invitrogen®). SYBR-Green fluorescence assay-based real-time PCR was used in a total of 100 ng of genomic DNA as described previously (Cardoso et al. 2013). The primers used were: BHV1 forward 5′-GGTACATGTCCAGGGAAAC-3′; BHV1 reverse 5′- GGTACAACATCGTCAACTTC-3′ BHV5 forward 5′-GGTACTTCTTCTTGGTGATG-3′; BoHV5 reverse 5′-TCGGTCTTCGTCAAGTTC-3′ (H2A): forward 5′-GTCTTGGAGTACCTGACCGC-3′; reverse 5′-ACAACGAGGGCTTCTTCTGA-3′ as described previously (Diallo et al. 2011; Silva-Frade et al. 2014).

Total RNA was extracted from cell monolayers using Trizol ™ protocol according to manufacturer's instructions (Invitrogen®). An average of 100 ng of RNA treated with DNase was used for qPCR in order to quantify *TNFA* gene (Bt03259156_m1). The primers and respective probes used were purchased from Applied Biosystems customer service.

The reactions were carried out and analyzed by the software on a StepOnePlus™ real-time instrument (Applied Biosystems®). The data were obtained from three replicates of each sample. The expression of the housekeeping Histone 2a gene was also quantified in a similar way for normalization as described previously (Silva-Frade et al. 2014). The comparative delta-delta C*t* method was used to analyze the results (Cardoso et al. 2013).

Statistical analysis

All experiments were performed at least in duplicate. Descriptive statistics include the mean ± standard deviation (s.d.). A p value <0.005 was considered significant. All statistical analyses were performed using Prism software (GraphPad® v.6, CA, USA). Gene expression values were transformed in logs, and correlations were evaluated with Spearman correlation coefficient. The differences between various groups were examined for a significance using the non-parametric Mann–Whitney U test.

Results

BHV1 and BHV5 replication on Neuro2a and C6 cells

The first experiments were conducted to characterize the replication of BHV1 and 5 strains in the cell lines and their induced cytopathic effects. Virus replication produced floating rounded cells and disruption of the cell monolayer in all BHV strains studied (data not shown). The graphic revealed a peak of virus titers at 48 h p.i. for all BHV strains in both cell lines (Fig. 1a, b). Virus antigen was detected by

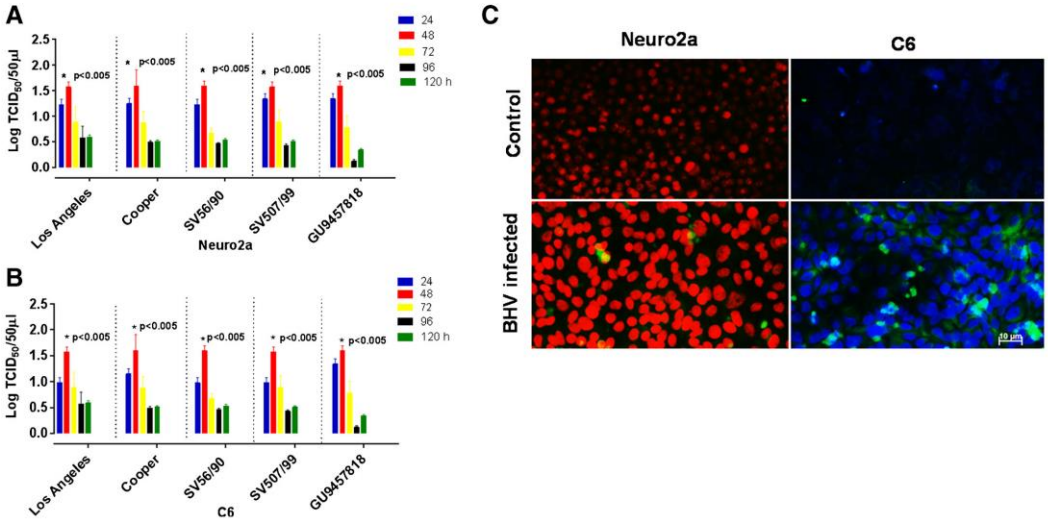

Fig 1 Evaluation of BHV1 and BHV5 titers at different times after infection. **a** Neuro2a and **b** C6 cells both infected with BHV strains ($p < 0.005$). The data were Log transformed corresponded to tissue culture infectious dose 50 % method ($p < 0.005$). **c** Analysis of BHV antigens by immunocytochemistry performed using monoclonal antibodies against BHV1 and 5 (*green*) followed by PI (*red*) and DAPI (*blue*) counterstain. The photomicrography is representative for all BHV strains used. ×40 magnification

immunocytochemistry and confirmed virus replication, as shown by a representative photomicrograph for both cell lines infected by BHV strains at 48 h p.i. (Fig. 1c). A significant correlation was detected ($r = 0.878$) between virus titers and immunostaining ($p < 0.005$).

Determination of apoptosis and necroptosis

To investigate whether apoptosis occurred in the process of virus-induced neuronal death, TUNEL assay was applied at 48 h p.i. In an experiment in which Neuro2a cells were infected by Los Angeles, SV507/99 (BHV5 reference strain), and GU9457818 (BHV5, field isolate), early apoptosis and necroptosis reached the same levels, ≈ 40 % at 48 h p.i. (Fig. 2a). Cooper and SV56/90 infection in the same cells produced lower levels of early apoptosis, followed by increase of viable cells (Fig. 2a). The results observed in C6 cells infected by BHV strains revealed a decrease of early apoptosis followed by an increase of viable cells, except for GU9457818 (BHV5, field isolate) that produced >40 % of necroptotic cells. Likewise, no differences in early apoptosis/necroptosis rates were observed when a pan-caspase inhibitor was applied (data not shown). However, when nec-1 (an inhibitor of necroptosis) was applied, a significant increase of cells under early apoptosis was observed for both infected cells (60–80 %) and all BHV strains (Fig. 3a, b). In summary, these results showed that BHV strains replication directly affect cell metabolism during early apoptosis phase in both tumor cells.

ROS production and mitochondria membrane depolarization

In both cell lines, BHV1 and 5 infection leads to ROS production (Fig. 4a, b). However, Los Angeles and SV56/90 strains induced 76.89 and 86.07 % Neuro2a cells to oxidative stress at 48 h p.i. (Fig. 4b). Cooper, SV507/99, and GU9457818 strains induced 71.05, 76.10, and 91.03 % cells to produce ROS (Fig. 4b). The JC-1 probe emission is illustrated (Fig. 5a, b). All BHV strains produced mitochondrial membrane depolarization at 48 h p.i.; however, Cooper strain revealed the lowest level of cells under mitochondrial damage when Neuro2a cells were infected (Fig. 5a). The BHV5 strains, SV507/99 and GU9457818, produced the highest mitochondria membrane depolarization ($p < 0.005$) compared to BHV1 strains for infected Neuro2a cells (Fig. 5a). Los Angeles strain produced the lowest level mitochondria membrane depolarization in comparison to other BHV1 and BHV5 strains in C6-infected cells (Fig. 5b). Overall, replication of BHV strains in both tumor cell lines was able to activate the oxidative stress pathway to different degrees.

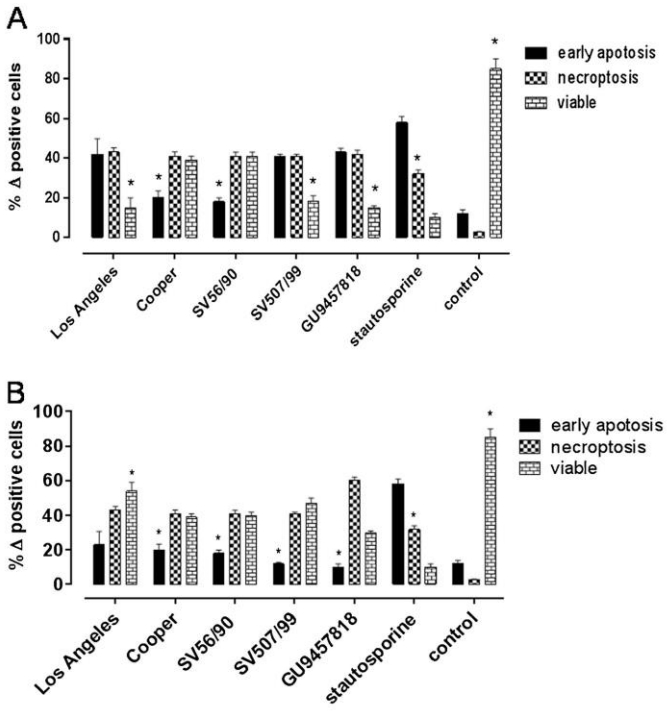

Fig. 2 Percentage of early apoptosis/necroptosis-positive cells after 48 h p.i with BHV1 and BHV5 strains, Neuro2a (**a**) and C6 (**b**) cells; *$p < 0.005$. Negative control corresponded to uninfected cells at the same p.i. Positive control corresponded to both cells submitted to 1.5 μM of staurosporine at 48 h p.i

TNFA expression after BHV infection

The expression of *TNFA* and BHV *glycol-C* genes was determined at 48 h p.i., and a positive correlation was found when Neuro2a and C6 cells were infected by SV56/90 and GU9457818 strains, respectively (Figs. 6 and 7). However, *TNFA* and BHV *glycol-C* gene expression was considered positively correlated for all analyses (Figs. 6 and 7). In spite of apoptosis and necroptosis block, no interference could be observed in the expression of *TNFA* and BHV *glycol-C* during infection on both tumor cell lines. Taken together, these results show that BHV replication in both cell lines activated TNFA transcription with no interference on virus production.

Discussion

The current study pioneered to demonstrate that animal herpesviruses, e.g., BHV, are capable to infect and produce damage in non-human glioma lineage cells, with production of mitochondrial membrane depolarization and ROS production. Numerous viruses have developed different mechanisms to overcome infected hosts defense (Elmore 2007; Geiser et al. 2008). Apoptosis is a cell programmed suicide mechanism essential for development in all metazoan animals (Hay and Kannourakis 2002). In some situations, viruses may take advantages of inducing apoptosis: either to perform the infected cells death, facilitating virus dissemination, or to destroy immune system cells (Egan et al. 2013; Hood et al. 2003; Schachtele et al. 2010).

Most of *alphaherpesviruses* have been shown to induce cell destruction caused by apoptosis (Christensen et al. 2011; Cymerys et al. 2012; Delhon et al. 2002; Geiser et al. 2008; Hood et al. 2003; Montagnaro et al. 2013). Although different apoptotic forms have been described induced by BHV1 and 5 infection (Brenner et al. 2012; Cardoso et al. 2012, 2015; Delhon et al. 2002; Garcia et al. 2013; Geiser et al. 2008; Ladelfa et al. 2013; Silva-Frade et al. 2010, 2014; Zhu et al. 2016), this particular mechanism remains unknown. Likewise, only few reports describe the susceptibility of human tumor cells to BHV1 (Cuddington and Mossman 2014). In this sense, our results demonstrated the ability of BHV1 and 5 to infect neuronal tumor cells with different rates of apoptosis and/or necroptosis. In fact, the cytopathic effect revealed a cytolysis fairly different to what has been demonstrated for neuron-like cells, whereas Cooper strain (BHV1) did not produce complete cell lysis (Cardoso et al. 2015). In

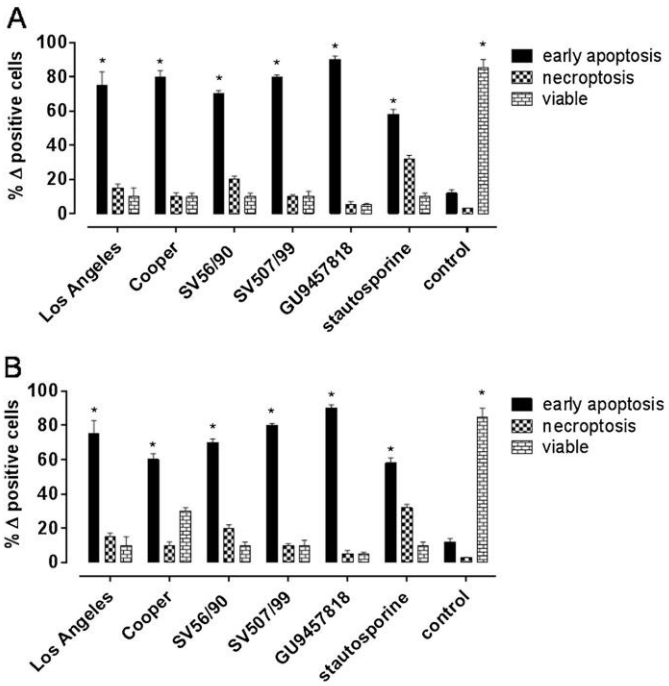

Fig. 3 Percentage of early apoptosis/necroptosis positive cells after 48 h p.i, pre-treated with nec-1 and after infected with BHV1 and BHV5 strains, Neuro2a (**a**) and C6 (**b**) cells; *$p < 0.005$. Negative control corresponded to uninfected cells at the same p.i. Positive control corresponded to both cells submitted to 1.5 μM of staurosporine at 48 h p.i

fact, BHV establishes latent infection in sensory neurons and transcription from the latent-related gene is readily detected in neurons from calves and rabbits (Jones et al. 2011). The phenomenon of latency has not been described in transformed cells infected by BHV1 (Cuddington and Mossman 2014, 2015).

Neurons have a high-energy demand and require a high density of mitochondria. Oncolytic viruses selectively replicate in and kill tumor cells while sparing normal cells. This ability is either natural (wild type) or may be induced through genetic manipulation (Cuddington and Mossman 2015). Although human viruses have shown efficacy as oncolytic agents in clinical trials (Pol et al. 2014), pre-existing immunity presents a barrier to systemic delivery and metastasis treatment. In this respect, a screen on BHV1 replication and cytotoxicity in a panel of human tumor cells revealed 72 % of these cells being permissive to virus infection (Cuddington and Mossman 2014). It is known that mutations on KRAS (rat sarcoma superfamily oncogene that includes Kirsten-KRAS) are associated with lung, colon, and prostate tumor types and have been shown to interfere in tumor progression and treatment (Cuddington and Mossman 2014). There are various forms of cell death, which are classified by several morphological and functional criteria (Elmore 2007; Gonzalez-Dosal et al. 2012). In fact, necroptosis is induced through kinase activity (Silke et al. 2015). Notably, in this

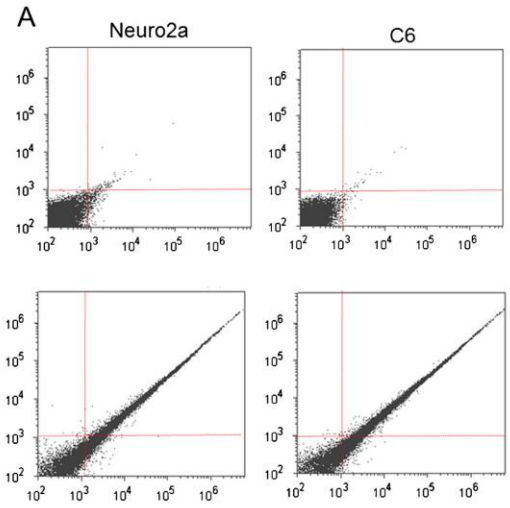

Fig. 4 Detection of ROS production in Neuro2a and C6 cells infected with BHV1 and BHV5 strains (**a**) and respective controls, uninfected (*upper graphs*) and treated CO_2 atmosphere (*lower graphs*); (**b**) by flow cytometry analysis. The data is illustrated by dot plot graphic whereas *x*-axis corresponds to linear amplified ROS fluorescence and *y*-axis logarithmic ROS fluorescence. The fluorescence emission was acquired by BL3A filter excluding auto-fluorescence by global compensation tool ($>10^3$), continuous line using Attune™ acoustic focusing cytometer

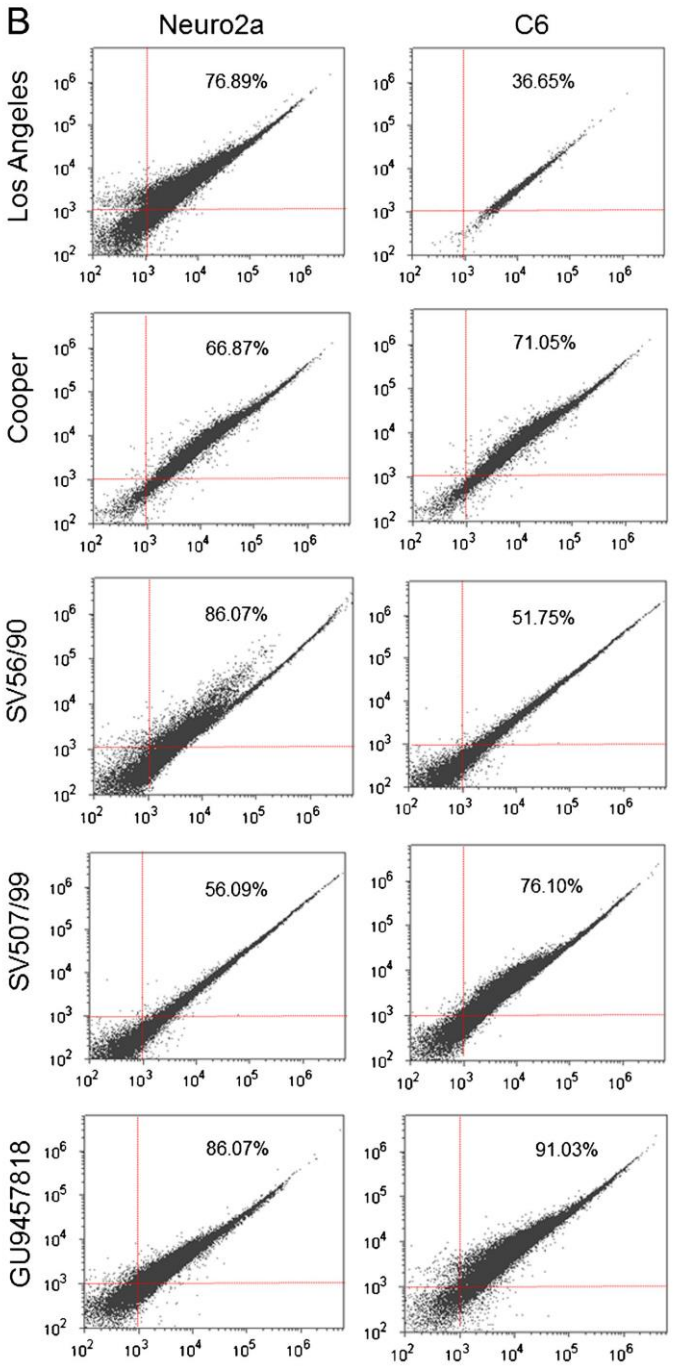

Fig. 4 (continued)

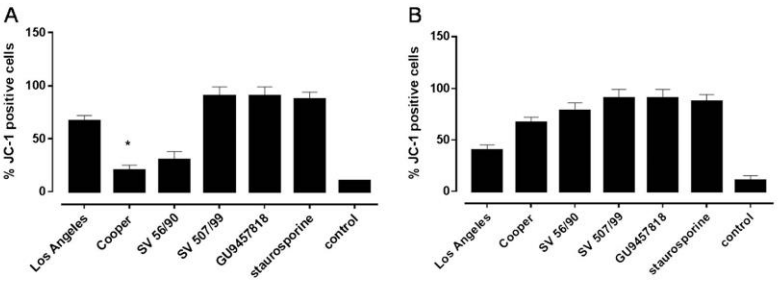

Fig. 5 Acoustic focusing cytometer analysis performed to measure JC-1 emission at 48 h p.i. **a** Neuro2a-infected cells and **b** C6-infected cells. The data were obtained from four different experiments and processed using Attune acoustic focusing cytometer, and results were presented as % JC-1-positive cells ($p < 0.005$)

study, necroptosis seemed to be the preferred pathway of tumor cells observed by inhibition of kinase activity. In this study, however, ROS production seemed to be independent of Neuro2a and C6 apoptosis or necroptosis after BHV1 and BHV5 infection. The method by which BHV1 elicits death in human tumor cells is unknown. At least in this study, infected Neuro2a and C6 cells by BHV1 and 5 strains displayed high production of ROS, *TNFA* high expression, and levels of apoptosis and necroptosis. A previous study has shown that transient blockage of TNF alpha-blocking antibodies significantly enhanced virus replication and survival in glioblastoma intracranial tumors (Meisen et al. 2015). However, BHV1 and varicella-Zooster virus induce cell death, depending on the cell type (Geiser et al. 2008). In a recent study, BHV1 (Colorado strain) infection of a non-tumoral epithelial cell line induced an increase of cellular ROS and directly interfered in mitochondrial metabolism (Zhu et al. 2016).

It is known that neurons have a high-energy demand and require a high density of mitochondria (Englezou et al. 2012; Hood et al. 2003; Kulahava et al. 2010; Schachtele et al.

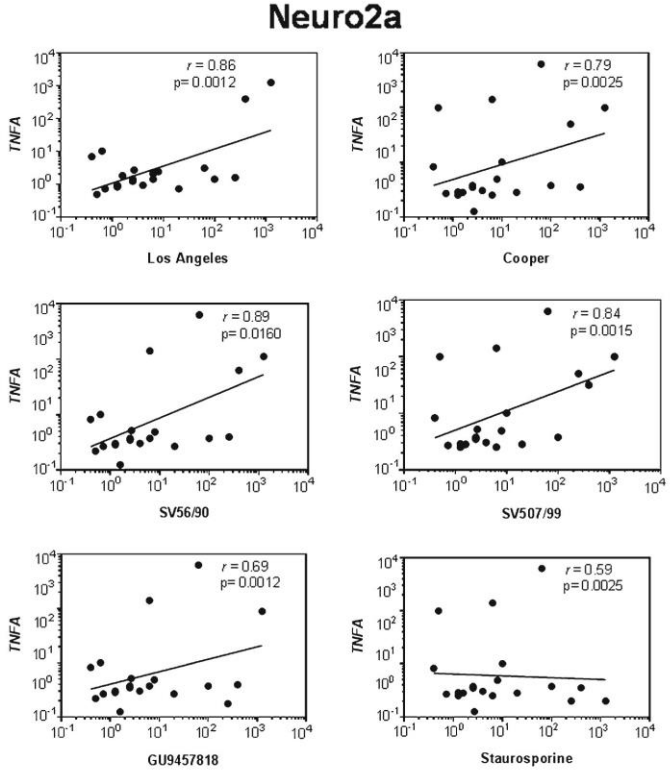

Fig. 6 Correlation between *TNFA* and BHV1 and 5 *Glyco-C* gene expression in Neuro2a cells infected by BHV strains at 48 h p.i. in five different experiments. The correlation coefficient (r) was calculated according to the Spearman analysis and $p < 0.005$ was considered significant

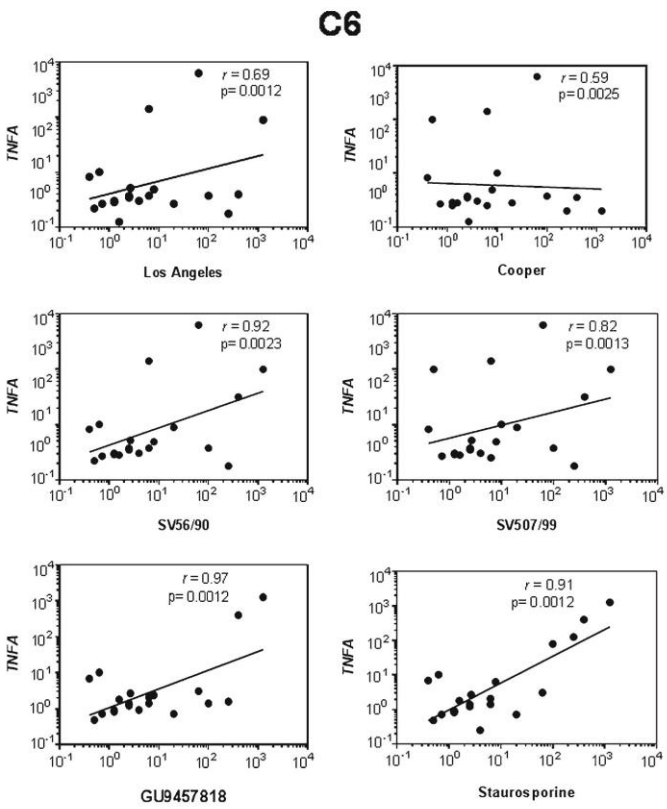

Fig. 7 Correlation between *TNFA* and BHV1 and 5 *Glyco-C* gene expression in C6 cells infected by BHV strains at 48 h p.i. in five different experiments. The correlation coefficient (r) was calculated according to the Spearman analysis and $p < 0.005$ was considered significant

2010). In fact, Neuro2a cells have been described as a model of neuronal death studies upon infection with varicella-zoster virus (Christensen et al. 2011), equid herpesvirus 1 (Cymerys et al. 2012), and caprine herpesvirus 1 (Montagnaro et al. 2013). All these studies revealed susceptibility of neuronal tumor cells to animal *alphaherpesviruses* and their deleterious effects. However, to our knowledge, only BHV1 and EHV1 have been mentioned as potential oncolytic viruses for cancer therapy up to date (Cuddington and Mossman 2014, 2015; Courchesne et al. 2011).

Most of the time, mitochondrial dysfunction induces ROS generation associated with oxidative stress (Dashzeveg and Yoshida 2015; Englezou et al. 2012; Gonzalez-Dosal et al. 2012; Kulahava et al. 2010; Ohta and Nishiyama 2011; Schachtele et al. 2010; Scott 2010; West et al. 2011). In this study, mitochondrial membrane potential was also increased in BHV-infected cells. In fact, mitochondrial membrane potential is essential for cellular ATP production and increase ROS production (Englezou et al. 2012; Schachtele et al. 2010). Rabies virus, a neurotropic virus that infects human and animals, has been incriminated to induce neuronal death by ROS formation (Alandijany et al. 2013). Previous studies revealed that mitochondrial membrane potential can be altered without affecting non-transformed cellular cycle after infected by BHV5 (Brenner et al. 2012; Garcia et al. 2013). The expression of anti-oxidant protein-like 1 (AOP-1), a protective pathway, has been detected in bovine embryos and brain histological sections, both infected by BHV5 (Brenner et al. 2012; Cardoso et al. 2010). Furthermore, increase of mitochondrial membrane potential could be associated to virus replication and not always associated to cell death (Hay and Kannourakis 2002). Recent studies have shown the benefit of manipulating oncolytic viruses with therapeutic genes. Generating a safer potent oncolytic HSV-1 (human simplex herpesvirus) may be an alternative to treat human cancer (Miao et al. 2014).

The results described here strongly suggest that mitochondrial dysfunction is an important cause of oxidative stress in neuronal and glial tumor cells infected by BHV1 and BHV5 at different levels. Moreover, further investigations are required to support this hypothesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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REGULAR ARTICLE

Isolation, characterization and immunomodulatory-associated gene transcription of Wharton's jelly-derived multipotent mesenchymal stromal cells at different trimesters of cow pregnancy

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Abstract The possibility of isolating bovine mesenchymal multipotent stromal cells (MSCs) from fetal adnexa is an interesting prospect due to the potential use of these cells in biotechnological applications. However, little is known about the properties of these progenitor cells in bovine species. Wharton's jelly (WJ) MSC cells were obtained from the umbilical cord of bovine fetuses at three different stages of pregnancy and divided into groups 1, 2 and 3 according to gestational trimester. Cell morphology, from the three stages of pregnancy, typically appeared fibroblast-like spindle-shaped, presenting the same viability and number. Moreover, the proliferative ability of T-cells in response to a mitogenic stimulus was suppressed when WJMSC cells were added to the culture. Multilineage properties were confirmed by their ability to undergo adipogenic, osteogenic/chondrogenic and neurogenic differentiation. Mesenchymal phenotyping, CD105+, CD29+, CD73+ and CD90+ cell markers were detected in all three cell groups, yet these markers were considered more expressed in MSCs of group 2 ($p < 0.005$). Expression of cytokines *IL2*, *IL6RR*, *INFAC*, *INFB1*, *IFNG*, *TNF* and *LTBR* were downregulated, whereas *IL1F10* expression was upregulated in all tested WJMSCs. The present study demonstrated that WJMSCs harvested from the bovine umbilical cord at different gestational stages showed proliferative capacity, immune privilege and stemness potential.

Keywords Stem cells · Umbilical cord · Immunomodulation · Bovine

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Introduction

Wharton's jelly (WJ) is the primitive mucous, connective tissue of the umbilical cord, lying between the amniotic epithelium and umbilical vessels (Troyer and Weiss 2008; Taghizadeh et al. 2011). First described by Thomas Wharton in 1656, this structure is composed mainly of proteoglycans and collagen (Cremonesi et al. 2011; Corrao et al. 2013). In 2006, in order to pursue standardization, the Mesenchymal and Tissue Stem Cell Committee (ISCT) proposed that: mesenchymal cells must be designed as multipotent mesenchymal stromal cells (MSC); be adherent to plastic culture ware; show specific surface antigen expression; and multipotent differentiation potential (Dominici et al. 2008; Calloni et al. 2014; Iacono and Merlo 2015).

The MSC population in WJ of the umbilical cord (UC) present properties that make it of interest (Pham et al. 2016).

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For example, these cells are easy to harvest by non-invasive procedures, provide large number of cells without risk to the donor and can be expanded, genetically manipulated and differentiated in vitro (Troyer and Weiss 2008; Cremonesi et al. 2011; Corrao et al. 2013; Calloni et al. 2014). The immunogenicity of WJMSC has been proposed but is still not clear at present, although immunossupressive effects of bone marrow MSC have been extensively studied and tested in several animal species (Weiss et al. 2008; De Miguel et al. 2012; Mukonoweshuro et al. 2014). Several studies have described WJMSC properties harvested from pregnant women at birth (Prasanna et al. 2010). Nonetheless, WJMSC from bovines (Cardoso et al. 2012) and from buffaloes have been reported only recently (Singh et al. 2013). However, bovine MSCs derived from umbilical cord blood (UCBMSCs), amniotic fluid (AFMSCs) and bone marrow (BMMSCs) have been described (Lu et al. 2011; Raoufi et al. 2011; Corradetti et al. 2013; Cortes et al. 2013). Despite the importance of bovine species as a model for in vitro studies because bovine pregnancy lasts 280 days, as in human beings, there is a lack of information about WJMSCs isolated during cow pregnancy. In contrast to MSCs from different sources, the characterization of bovine MSCs is far from being completely understood, and contradictory information emerges from the literature.

The aim of this study was to isolate and propagate bovine WJMCS cells collected from umbilical cords at three trimesters of pregnancy. Additionally, comparisons were made regarding cell viability, T-cell inhibition, telomerase activity, cell proliferation, phenotype, multipotency and immunomodulatory gene expression. This study highlights a possible potential source of multipotent MSCs and may support their therapeutic and biotechnological use in large animals.

Materials and methods

Isolation of WJMSC from bovine umbilical cord at different stages of pregnancy

Bovine UC were harvested at a slaughterhouse from pregnant Nelore cows ($n = 18$). The bovine pregnancy was divided into three trimesters, as done in humans and gestational periods were estimated by measuring the crown rump length of the fetuses. The first trimester corresponded from 0 to 93 days ($n = 6$ UC; group1 ; Fig. 1a), the second trimester from 94 to 187 days of pregnancy ($n = 6$ UC; group 2; Fig. 1b) and a further 6 umbilical cords were from the third trimester, with the 188-term of pregnancy corresponding to group 3 (Fig. 1c). UCs were collected according to the Animal Care Committee at the University of São Paulo State, Brazil and were conserved at room temperature in sterile phosphate-buffered saline (PBS) supplemented with a penicillin/streptomycin solution containing penicillin 100 μg/ml, streptomycin 10 μg/ml and amphotericin B 250 μg/ml (Sigma-Aldrich, St Louis, MO, USA) until use (within 3 h). Bovine umbilical segments were sectioned longitudinally to expose the WJ after 3 h. Some incisions were made on the matrix and UC fragments were transferred to 25-cm^2 tissue culture flasks (TPP®, Zollstr, SW, Brazil). The initial culture and cell expansion were performed as described previously (Cardoso et al. 2012). Images were taken to observe cell morphology for each group at passage 6 (P6) (Fig. 1d–f), harvested and expanded until they reached subconfluence and then analyzed for their capacity for colony-forming, viability, T-cell proliferation, telomerase activity, phenotype and differentiation as well as for immunomodulatory transcripts.

Cell viability, T-cell proliferation, telomerase activity and cell expansion

Assays for cell viability, T-cell proliferation and telomerase activity were performed according to a previous study (Cardoso et al. 2012). Briefly, cell viability analysis was performed using the In Vitro Toxicology Assay® Kit, MTT-based assay (TOXI-1 Kit; Sigma-Aldrich) following the manufacturer's instructions. In order to evaluate T-cell proliferation, culture and stimulation were performed according to a previous study (Cardoso et al. 2012). Lymphoproliferation was evaluated as counts per minute by a Matrix9600 beta counter (Packard Instrument, Meridien, CT, USA). The ConA was used at 5 μg/ml and PMA and ionomycin at concentrations of 50 ng/ml and 1 μg/ml, respectively (Sigma-Aldrich). A TRAPeze® Telomerase Detection Kit (Millipore, CA, USA) was used to assess the telomerase activity in all groups (Cardoso et al. 2012). The samples were considered positive when the optical density (OD) was ≥0.2 and negative when OD was ≤0.2. All reported values are means of triplicate samples.

Doubling time for passages 1–10 was performed following the procedure described previously (Corradetti et al. 2013). Data representative of three independent experiments were recorded.

In vitro multilineage differentiation assay

The differentiation potential of bovine-derived WJMSC cells was examined using cells at passage number 6 (P6) in all cell groups according to a previous study (Cardoso et al. 2012; Silva et al. 2016). For all procedures 2×10^5 cells/ml were submitted to osteogenic, chondrogenic, adipogenic and neurogenic differentiation according to the manufacturer's instructions (STEMPRO® differentiation medium; Invitrogen). The neurogenic differentiation was adapted from previous studies (Oda et al. 2013). For osteogenic/chondrogenic differentiation, 2 ml of STEMPRO® osteogenic/chondrogenic

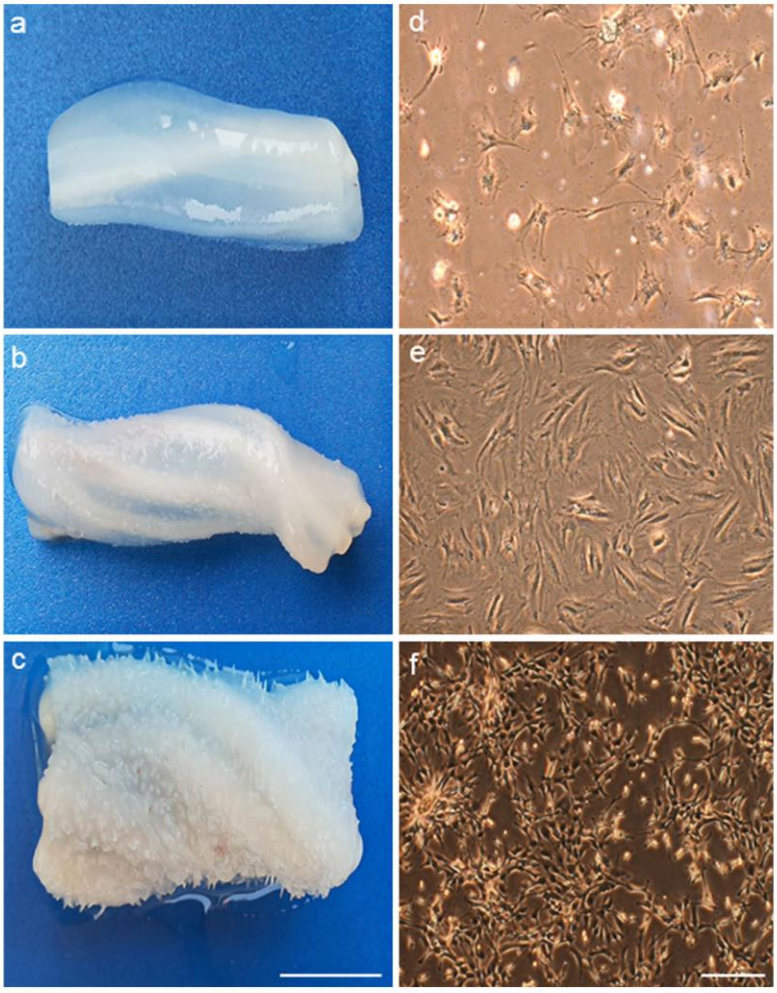


Fig. 1 Bovine umbilical cords analyzed in this study. **a** 0–93 days corresponding to group 1; **b** 94–187 days corresponding to group 2; **c** 188-term of pregnancy corresponding to group 3, *bar* 500 μm. **d** Ex vivo cultured cell obtained from Wharton's jelly zone corresponded to group 1, **e** group 2 and **f** group 3. After P6, spindle-shaped fibroblast-like appearance can be observed under phase contrast microscopy. *Bar* 40 μm

differentiation medium was added to undifferentiated cultures comprising osteogenic and chondrogenic commercial inducers (STEMPRO®). After 15 days of differentiation, cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich). For osteogenic differentiation, Alizarin Red staining (Sigma-Aldrich) was performed (Yang et al. 2015); and for chondrogenic differentiation 0.5 % toluidine blue solution was added (Cardoso et al. 2012).

The adipogenic differentiation followed the described protocol (Cardoso et al. 2012; Silva et al. 2016). In order to verify adipocytes, Oil Red staining was performed (Cardoso et al. 2012). The differentiation of bovine-derived WJMSC cells into neural-like cells followed the procedure described previously, with some modifications (Cardoso et al. 2012; Oda et al. 2013; Silva et al. 2016). The neuronal differentiation was confirmed by immunofluorescence for GFAP and nestin cell markers, as described previously (Cardoso et al. 2012). From each experiment, samples from cell differentiation and undifferentiated cells were harvested for multilineage gene transcription among the three studied groups as described below.

Flow cytometry

Briefly, 2×10^5 cells at P6 were harvested, washed in PBS and incubated for 18 h at 4 °C with monoclonal antibodies: CD34 (hematopoietic precursor cells and MSCs); CD45 (anti-bone marrow lymphoid cells); CD90 (anti-THy1 antigen); CD105

Table 1 Specifications of (*Bos taurus*) cattle gene name, description and location searched by microarray

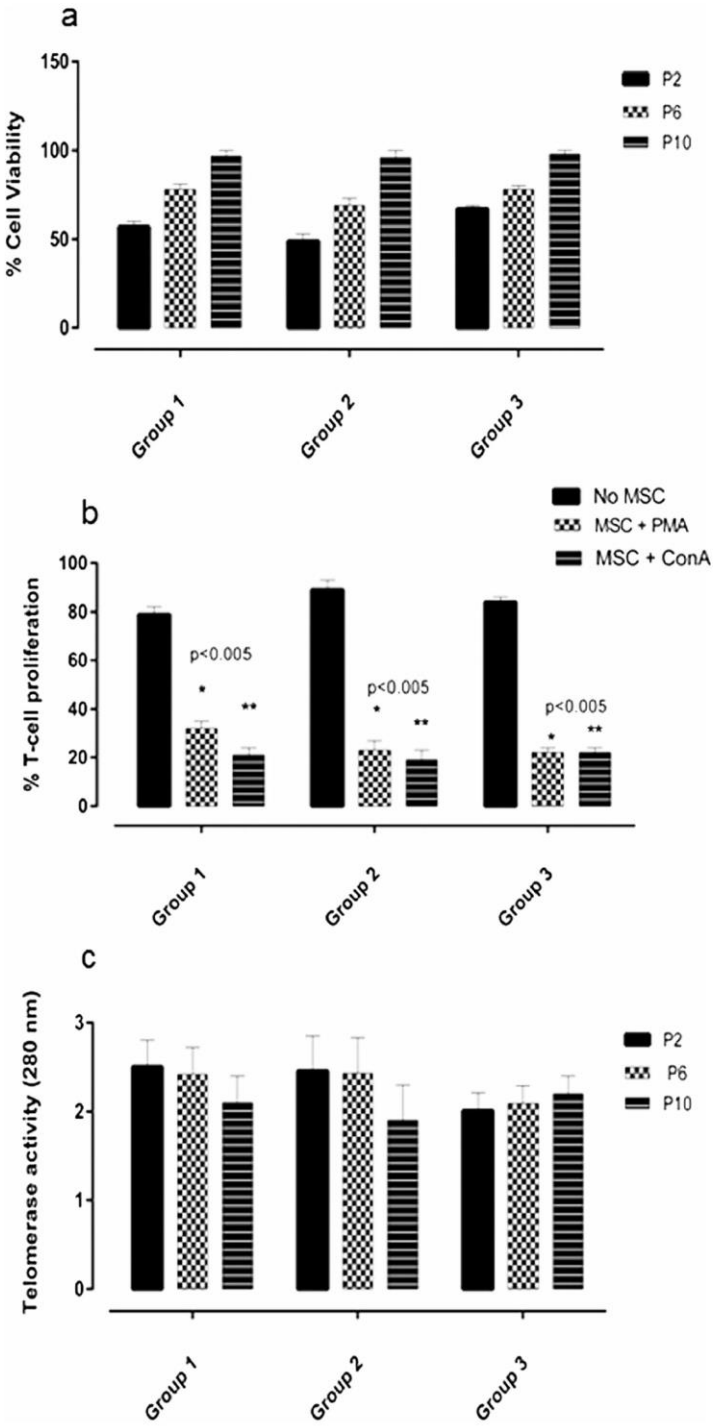
Gene symbol/ID		Description	Location
Positive markers of MSCs			
ENG	615.844	Endoglin (CD105)	Chromosome 11, AC_000.168.1
ITGB1	281.876	Integrin, beta 1 (CD29)	Chromosome 13 AC_000.170.1
NT5E	281.363	5′nucleosidase ecto (CD73)	Chromosome 9, AC_000.166.1
THY1	614.712	Thy-1 cells surface antigen (CD90)	Chromosome 15, AC_ 000.171.1
CD34	281.051	Hematopoietic progenitor cell antigen (CD34)	Chromosome 16, AC_000.954.1
PTPRC	407.152	Protein tyrosine phosphatase, receptor type C (CD45)	Chromosome 16, AC_000.173.1
JSP.1	407.173	Major histocompatibility complex class I (MHCI)	Chromosome 23, AC_ 000.180.1
DSB	618.722	Major histocompatibility complex class II, antigen DS beta (MHC II)	Chromosome 23, AC_000.180.1
Immune-related genes			
IFNAC	281.236	Interferon alpha C (INF-alpha C)	Chromosome 8, AC_000.165.1
INFB1	281.845	Interferon, beta 1, fibroblast	Chromosome 8, AC_000.165.1
IFNG	281.237	Interferon, gamma	Chromosome 5, AC_000.162.1
IL2	280.822	Interleukin 2	Chromosome 17, AC_000.174.1
IL6R	507.359	Interleukin 6 receptor	Chromosome 5, AC_000.162.1
IL1F10	615.702	Interleukin 1, family member 10	Chromosome 11, AC_000.168.1
TNF	280.943	Tumor necrosis factor (TNF alpha)	Chromosome 23, AC_000.180.1
LTBR	280.845	Lymphotoxin beta receptor (TNF superfamily member 3)	Chromosome 5, AC_000.162.1
Positive markers of MSCs multipotency			
LEP	280.836	Leptin	Chromosome 4, AC_000.161.1
FABP4	281.759	Fatty acid binding protein 4, adipocyte	Chromosome 14, AC_000.171.1
PPARD	353.106	Perixome proliferator-activated receptor delta	Chromosme 23, AC_ 000.180.1
COL1A1	282.187	Collagen type 1, alpha 1	Chromosome 19, AC_000.176.1
SOX9	353.115	SRY (sex determining region Y)-box 10	Chromosome 5, AC_000.162.1
GFAP	281.189	Glial fibrillary acidic protein	Chromosome 19,AC_000.176.1
NES	522.383	Nestin	Chromosome 3, AC_000.160.1
OMD	280.885	Osteomodulin	Chromosome 8, AC_000.0149.1
POST	281.960	Osteoblast specific factor	Chromosome 12, AC_000.034.1
OSTF1	281.961	Osteoclast stimulating factor 1	Chromosome 8, AC_000.0610.1

(anti-endoglin); CD29 (anti-integrin β1) all diluted at 1:50; CD73 (anti-nucleotidase) diluted at 1:25 (Sigma-Aldrich). Next, cells were washed three times with PBS plus 0.1 % Triton X-100 and 1:50 dilution of the secondary antibody, represented by goat anti-mouse labeled to FITC (Sigma-Aldrich), was added to 100 μl of cell suspension and incubated at 37 °C for 30 min. The cell suspension was washed as previously described and after the final wash, cells were fixed with 4 % paraformaldehyde. Data were captured with the Attune™ acoustic focusing cytometer system (Applied Biosystems, Foster City, CA, USA). The equipmental settings were defined as an initial threshold: 2.500 events/s, BL1A filter (488 nm emission) 300 voltage, SSC (scatter complexity) 250 V and FSC (forward scatter) 220 V. After the first acquisition, a dot plot graph was obtained and a global compensation was performed to exclude unspecific signals and cell debris (>10^3 cells were excluded). Only one fluorophore was used in this analysis, so these parameters could be applied in all analyses. The data were expressed in histograms.

Microarray analysis

Total RNA was isolated from all groups, corresponding to WJMSCs (5×10^5 cells) in triplicate after trypsinization, according to the Qiagen RNeasy System™ (Qiagen, Hilden, Germany) manufacturer's guide. The total RNA was treated

Fig. 2 Cell viability, inhibition of T-cell proliferation and telomerase activity. **a** Viability of WJMSC cells measured by MTT based assay at 2, 6 and 10P. Data are expressed as mean ± standard deviation (SD) of values obtained from four different experiments. **b** Ability of bovine WJMSCs cells to inhibit T-cell proliferation in response to mitogens at 6P, $P < 0.005$ obtained from four different experiments. **c** Telomerase repeat amplification results obtained from four different experiments. *Bars* represent all groups at 2P, 6P and 10P ▶


a
% Cell Viability
150
100
50
0
P2
P6
P10
Group 1
Group 2
Group 3
b
% T-cell proliferation
100
80
60
40
20
0
No MSC
MSC + PMA
MSC + ConA
p<0.005
p<0.005
p<0.005
*
**
Group 1
Group 2
Group 3
c
Telomerase activity (280 nm)
3
2
1
0
P2
P6
P10
Group 1
Group 2
Group 3

with DNAse and reverse transcribed into cDNA using a reverse transcriptase (Superscript III; Life Technologies, Carlsbad, CA, USA). The Axiom® Genome-Wide BOS 1 Array was used for transcriptome analysis (Affymetrix, Santa Clara, CA, USA). This commercial array was designed to maximize genetic coverage of commercially important cattle breeds, including *Bos taurus*, *Bos indicus* and dairy and beef cattle breeds. The array covers more than 640,000 validated transcript markers representing the genetic diversity of approximately 3 million from the Affymetrix Bovine Genomic Database. After hybridization, the gene chips were washed and stained with SA-PE and read using an Affymetrix Gene Chip fluidic station and scanner. Analyzed genes, corresponding to positive markers of MSCs, immune-related genes and MSCs multipotency, are detailed in Table 1. The average expression was calculated and $\log_2$-transformed for each gene by Affymetrix Microarray Suite 5.0.

Statistical analysis

All statistical analyses were performed using the SAS 9.1.2 software package (SAS Institute). Data are presented as mean ± SD. Three replicates for each experiment were performed and the results represent these replicates. We executed one-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student's *t* test, whenever applicable by GraphPad Prism 6.05. A level of $P < 0.005$ was accepted as significant.

Results

Isolation and characterization of WJMSCs

WJMSCs were cultured individually only during the first passage. After P1, all WJMSCs from each group were transferred to a unique culture flask and proceeded as lineages. The formation of fibroblast-like cells was observed around the second day and in vitro cell expansion was performed until 10 consecutive passages for all groups (Fig. 1d–f).

The culture conditions were able to promote good cell viability, >80 % after 10 P for all groups; induced inhibition of T-cell proliferation; telomerase activity at satisfactory levels (Fig. 2a–c, respectively). The percentage of living cells was maintained approximately constant when the passage number remained at a constant level of 10 (Fig. 2a). The addition of WJMSC cells to blood monocytes stimulated with ConA or PMA/ionomycin inhibited their proliferation less than 20 % in comparison to no addition of WJMSC cells, whereas 80 % of proliferation for all groups was observed ($P < 0.005$). In addition, the activity of telomerase was verified and in all groups after 6P the same activity could be observed (Fig. 2c). The doubling time was measured, calculated and drawn as a graph, where a consistent increasing rate of growth at P6 was observed for each group, respectively (Figs. 3a–c and 4).

Multilineage differentiation and phenotypic characterization

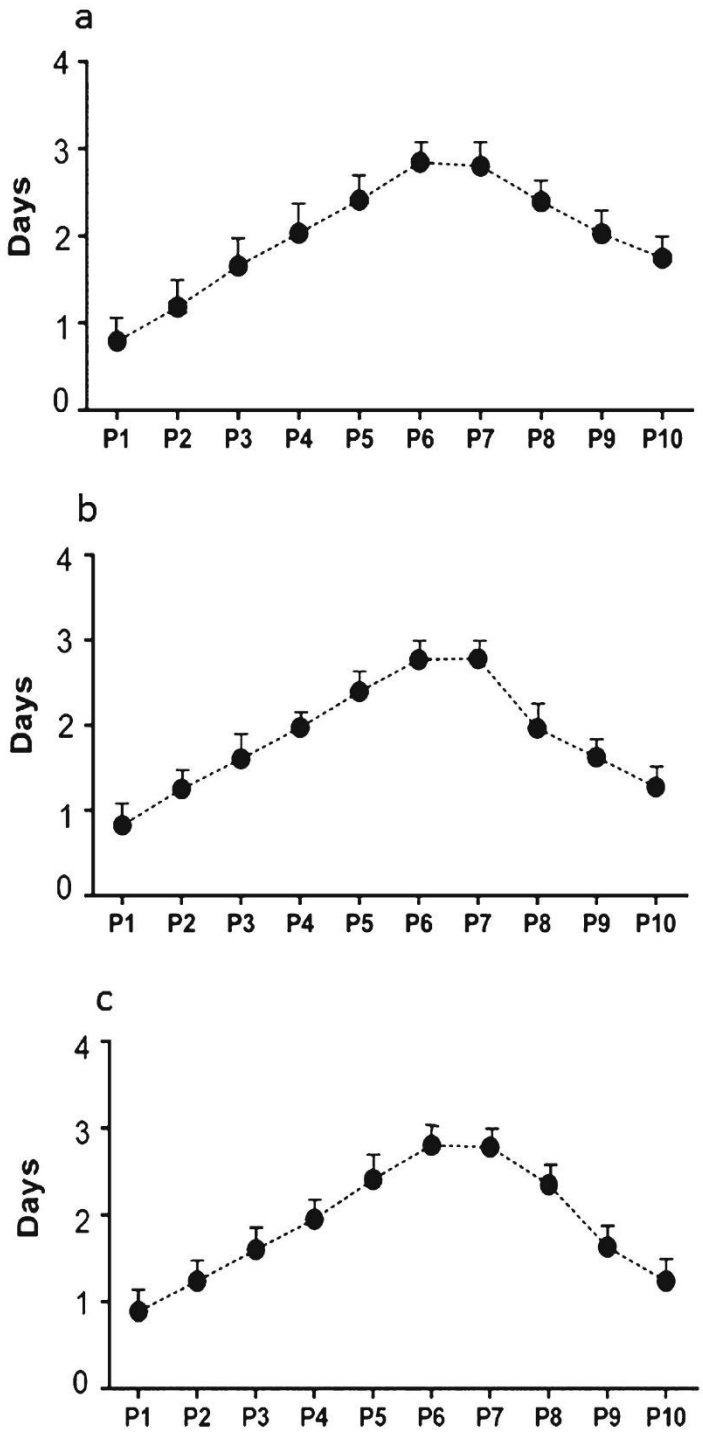
Pluripotency was confirmed by the ability of WJMSCs cells to differentiate into osteocytes, adipocytes, chondrocytes and neuron-like cells. Undifferentiated cells were included in all analyses (Fig. 3a, e, i, corresponding to groups 1, 2 and 3, respectively). Osteogenic differentiation was detected by the matrix calcification shown by Alizarin Red staining (Fig. 3b, f, j, corresponding to groups 1, 2 and 3, respectively). After induction, adipogenic differentiation with a high number of very small lipid vacuoles that stained positively using Oil Red solution was visualized in groups 1, 2 and 3 (Fig. 3c, g, l, respectively). Chondrogenic differentiation was confirmed by blue deposits representing glycosaminoglycans (Fig. 3d, h, m, corresponding to groups 1, 2 and 3, respectively). The neurogenic induction was confirmed by positive staining for GFAP and nestin neuro markers in group 1 (Fig. 5b, c), group 2 (Fig. 5e, f) and group 3 (Fig. 5h, i). Undifferentiated cells were also included in groups 1, 2 and 3 as control (Fig. 5a, d, g, respectively).

The phenotype of WJMSCs obtained from three groups was characterized using flow cytometry (FC) analysis (Figs. 6, 7 and 8 corresponding to groups 1, 2 and 3, respectively). All WJMSCs revealed negative results for CD45 and CD34 surface markers at FC analysis in groups 1, 2 and 3 (Figs. 6a, b, 7a, b and 8a, b, respectively). However, positive results for CD105, CD29, CD73 and CD90 surface markers were recorded at the same rate of 10^4 cells for all groups (Figs. 6, 7, 8). In group 1 (Fig. 6c), CD105-positive cells were considered at a lower rate (45 %) when compared to group 2 (81 %; Fig. 7c). However, in groups 2 and 3 (Figs. 7e, f, and 8e, f, respectively), CD73 (92 % and 78 %; $P < 0.005$) and CD90 (96 % and 79 %) were more highly expressed, respectively ($P < 0.005$). In comparison, group 2 revealed superior expression of positive markers of MSCs in this study (Fig. 7a–f).

Gene expression profile

Genes described as being involved in MSCs characterization revealed consistent results with flow cytometric analysis. High transcription levels for MSCs markers *THY1* (CD90), *NT5E* (CD73), *ITGB1* (CD29), *ENG* (CD105) and low levels for *CD34* and *PTPRC* (CD45) were found among WJMSCs

Fig. 3 Doubling time over 10 passages during cell culture from group 1 (**a**), 2 (**b**) and 3 (**c**). *X-axis* is represented by number of cell passage and *Y-axis* by days of culture. Data are expressed as mean ± standard deviation (SD of values obtained from four different experiments). $^*P < 0.05$


a
Days
4
3
2
1
0
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
b
Days
4
3
2
1
0
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
c
Days
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3
2
1
0
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10

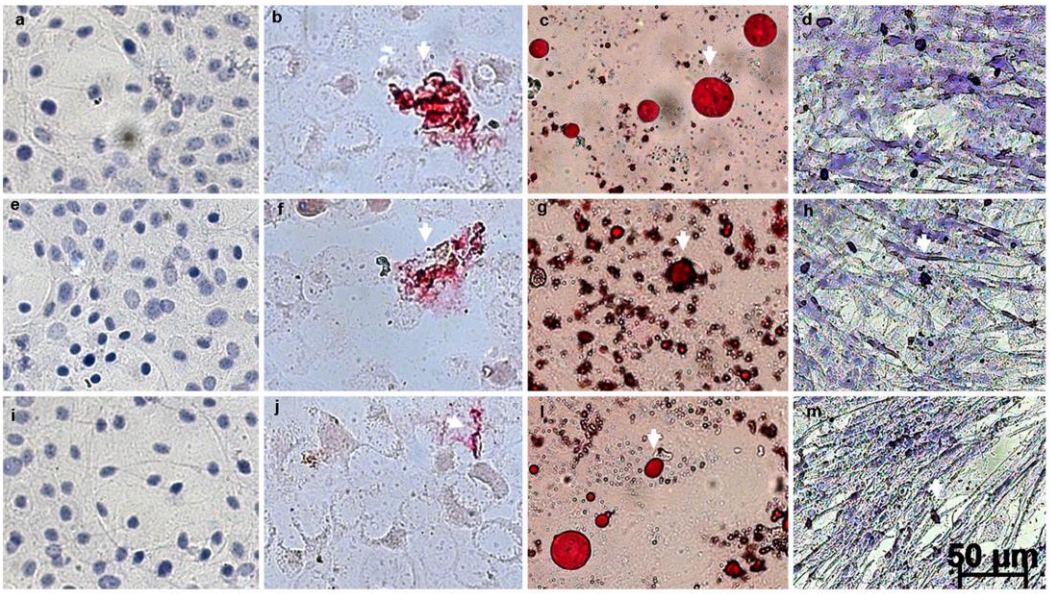

Fig. 4 Photomicrographs representative of the morphological appearance. For each differentiation protocol, undifferentiated cells were kept as controls for group 1 (**a**), 2 (**e**) and 3 (**i**). Osteogenic differentiation was confirmed after Alizarin Red staining (group 4 **b**, group 4 **f** and group 4 **j**; *arrows*), adipogenic differentiation after Oil Red staining (group 4 **c**, group 4 **g** and group 4 **l**; *arrows*) and chondrogenic differentiation after Toluidine blue staining (group 4 **d**, group 4 **h** and group 4 **m**; *arrows*) and differentiation of bovine-derived WJMSCs cells at P6

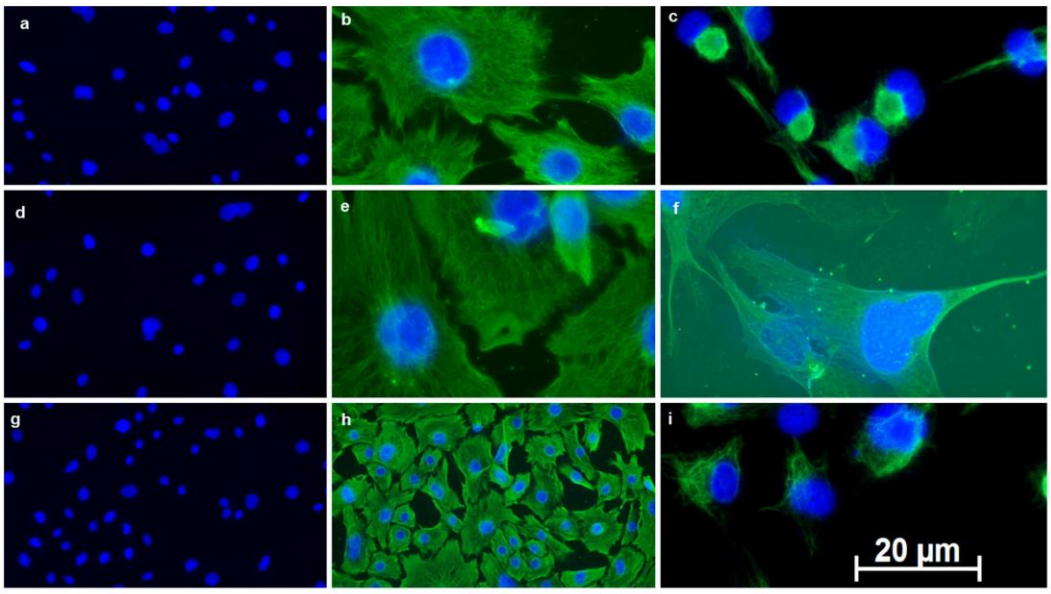

Fig. 5 Photomicrographs representative of the morphological appearance of neuro-like cells after neurogenic induction visible under immunofluorescence microscopy positive GFAP (group 1 **b**; group 2 **e**; group 3 **h**) and Nestin (group 1 **c**; group 2 **f** and group 3 **i**) cell markers (*scale bar* 20 µm). Undifferentiated cells were kept as controls for groups 1 (**a**), 2 (**d**) and 3 (**g**)

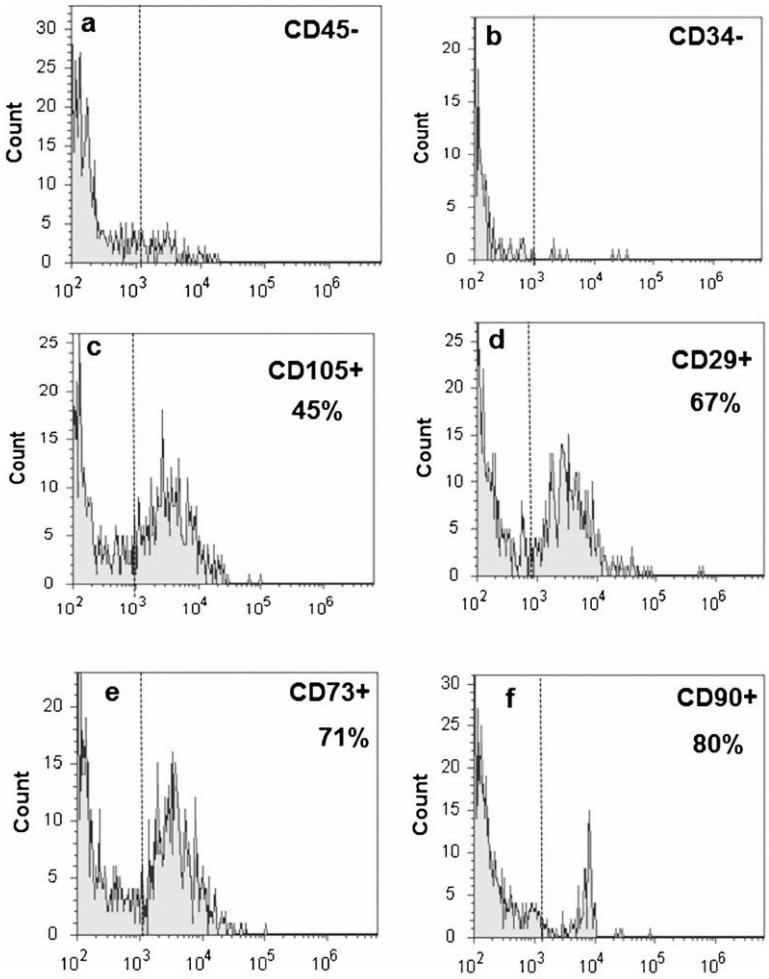

Fig. 6 Flow cytometry analysis of WJMSCs surface markers corresponded to group 1. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 1). For CD105, CD29, CD73 and CD90 the results are illustrated in (**c**–**f**)

(Fig. 9a–c). When stimulated to differentiate towards adipogenic, chondrogenic and neurogenic lineages, WJMSCs showed substantial transcriptional expression of *LEP*, *FABP4*, *PPARD*, *COL1A1*, *SOX9*, *GFAP* and *NES* for all WJMSCs (Fig. 9a–c). The WJMSCs potential to undergo chondrogenesis showed a higher pattern of *COL1A1* gene expression at group 2 in comparison to groups 1 and 3 (Fig. 9b). From the factors measured in this study, *IL2*, *IL6R*, *INFAC*, *INFB1*, *INFG*, *TNF* and *LTB*, considered pro-inflammatory cytokines, were genetically downregulated in all WJMSCs tested (Fig. 9ac; $P < 0.005$). However, *IL1F10* was noticeably upregulated, in microarray analysis (Fig. 9a). The lack of *JSP.1* and *DSB* (MHCI and II) expression could be observed in this study amongst WJMSCs from all groups (Fig. 9a–c).

Discussion

Extra gestational tissues have been widely suggested as ideal sources of mesenchymal cells due to their non-invasive harvest and most of the time being discarded biological material (Troyer and Weiss 2008; Iacono and Merlo 2015). Currently, limited reports are available regarding the isolation and characterization of Wharton's jelly-derived MSCs in farm animal species (Carlin et al. 2006; Corradetti et al. 2008; Cardoso et al. 2012; Singh et al. 2013). WJMSCs demonstrated, at all gestational periods, satisfactory telomerase activity, viability and MSCs surface markers according to previous studies (Cardoso et al. 2012). In spite of the fact that non-

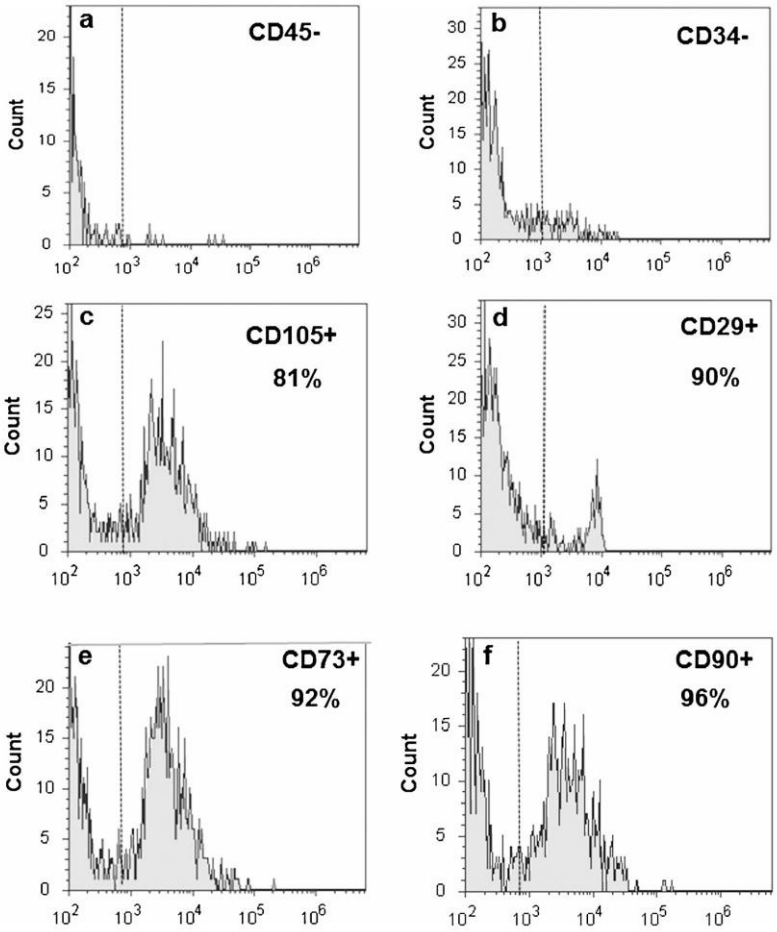

Fig. 7 Flow cytometry analysis of WJMSCs surface markers corresponded to group 2. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 2). For CD105, CD29, CD73 and CD90, the results are illustrated in (**c**–**f**)

bovine monoclonal antibodies were used here, several reports have described cross-reaction between animals and human MSCs epitopes (Godoy et al. 2014). Moreover, *THY1* (CD90), *NT5E* (CD73), *ITGB1* (CD29) and *ENG* (CD105) transcripts were confirmed by flow cytometric analysis, confirming the stem cell potential of WJMSCs in this study.

Studies on the isolation and characterization of MSCs from fetal adnexa in humans are advancing rapidly (Troyer and Weiss 2008; Weiss et al. 2008; Corrao et al. 2013; Gottipamula et al. 2013). Some authors have reported that MSCs isolated from human umbilical cord matrix, precisely from Wharton's jelly, could be used for therapy of some diseases such as amyotrophic lateral sclerosis and Parkinson's disease, even in cancer treatment (Troyer and Weiss 2008; Corrao et al. 2013). However, studies in animal models are still in their infancy. MSCs have been isolated from umbilical cord matrix of cattle, pigs, goats, horses and dogs (Uranio et al. 2011; Cremonesi et al. 2008; Cardoso et al. 2012). The bovine model could have a critical role in studying fetal adnexa MSC sources, mainly through the similarity to human gestational time. Our study aimed specifically to isolate, expand in vitro, and characterize WJMSCs harvested at different gestational stages. Previous studies have reported the isolation of WJMSCs from bovine umbilical cord at birth, successfully growing them in culture without fetal calf serum showing pluripotency capacity (Cardoso et al. 2012; Silva et al. 2016). Moreover, a recent study demonstrated that bovine MSCs isolated from amniotic fluid and adipose tissue could be an alternative for nuclear

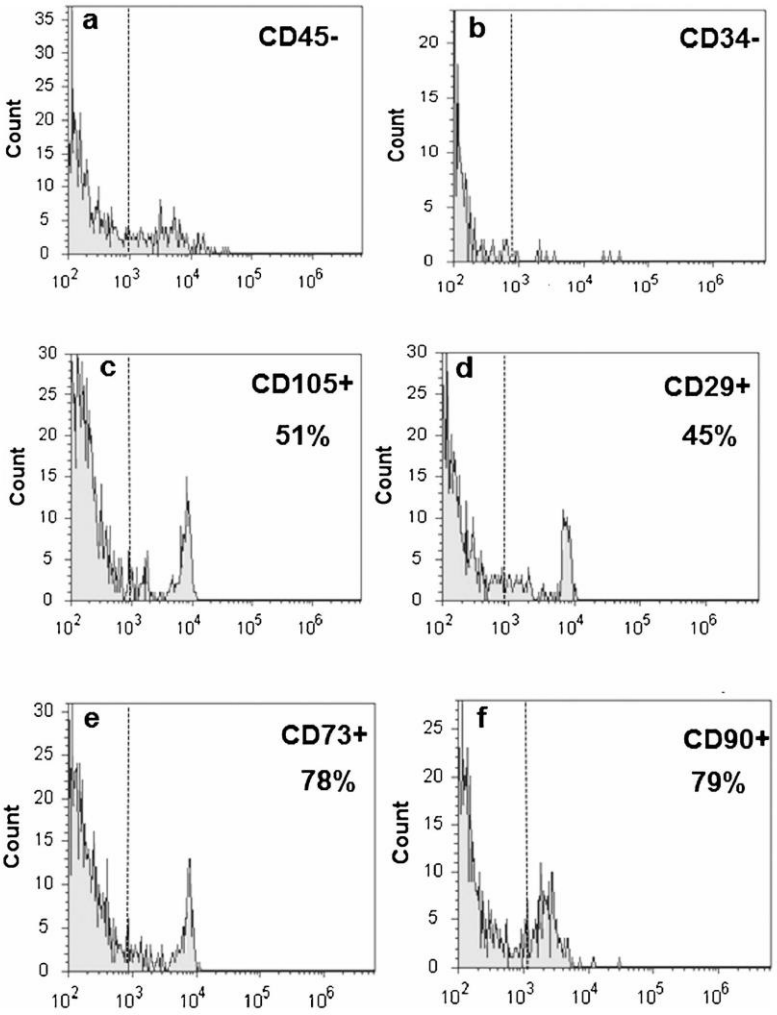

Fig. 8 Flow cytometry analysis of WJMSCs surface markers corresponded to group 3. *X-axis* corresponds to the number of positive labeled cells detected by the BL1A filter (488 nm). *Y-axis* is cell count (log scale). The data obtained from four different experiments were processed using an Attune™ acoustic focusing cytometer. For CD45 and CD34, data are illustrated in (**a**, **b**) (group 3). For CD105, CD29, CD73 and CD90, the results are illustrated in (**c**–**f**)

transfer (Silva et al. 2016). In both studies, the size of bovine MSC-derived WJ cells was found to be smaller when compared to human studies (Taghizadeh et al. 2011; Gottipamula et al. 2013; Pham et al. 2016) and ruminant MSCs were derived from different fetal adnexa sources (Cardoso et al. 2012; Corradetti et al. 2013; Cortes et al. 2013; Raoufi et al. 2011; Somal et al. 2016). This finding must be investigated in future studies.

The in vitro differentiation results support the findings already reported for bovine MSC (Lu et al. 2011; Raoufi et al. 2011; Corradetti et al. 2013; Cortes et al. 2013). All WJMSCs show high plasticity, being able to differentiate into multiple germ layers, mesoderm and ectoderm. This is in agreement with all studies regarding bovine MSC and other species (Cremonesi et al. 2008; Uranio et al. 2011; Oda et al. 2013). WJMSCs, when stimulated to differentiate toward adipogenic lineage, expressed high levels of the *LEP* gene that is regarded as an intermediate and late marker of adipocyte differentiation, which may lead to a distinct differentiation characteristic of stem cells in all gestational periods. The WJMSCs potential to undergo osteogenesis/chondrogenesis showed a higher pattern of expression of *OMD*, *POST*, *OSTF1* and *COL1A1* in all groups. As demonstrated previously, WJMSCs have a proven ability to undergo astrocyte differentiation, confirmed by *GFAP* expression already demonstrated for other species (Oda et al. 2013). Taking these results together, it seems that

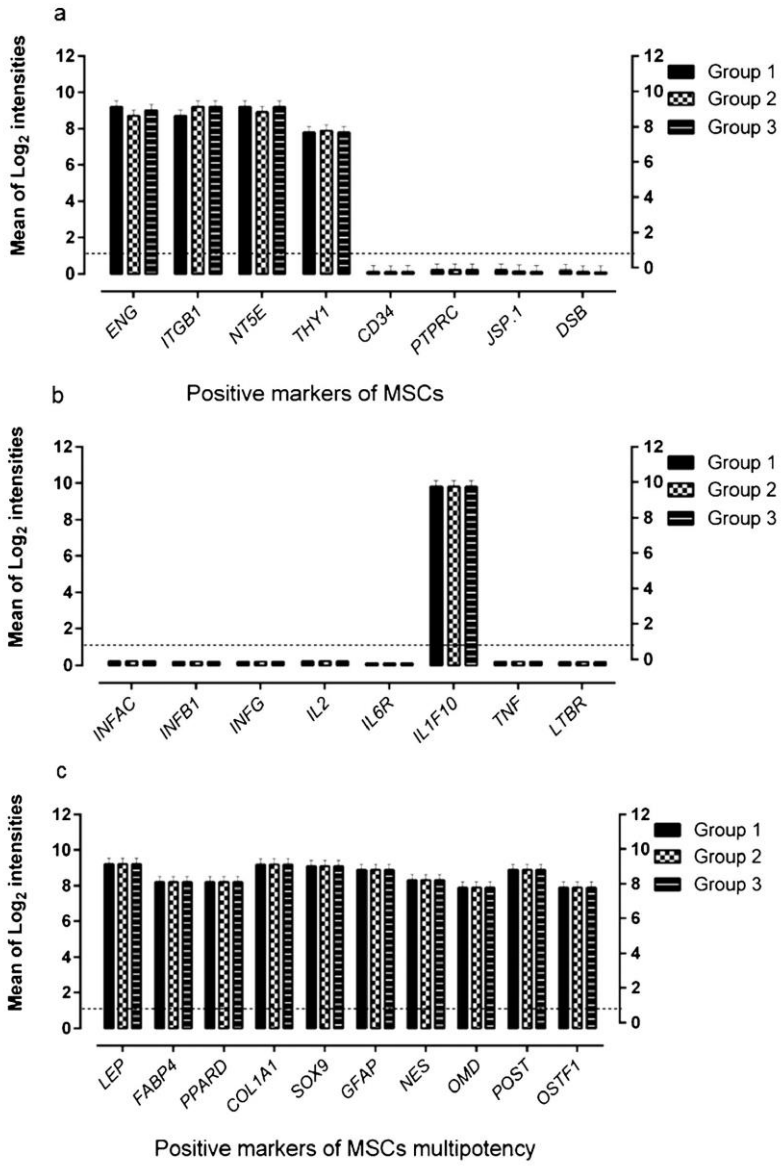

Fig. 9 Transcriptome analysis of WJMSCs surface markers, multipotency differentiation and immune related genes from groups 1, 2 and 3. Relative gene expression are represented as mean + SD obtained from four different experiments. *Unbroken line* is positioned on the average negative known markers of MSCs. Genes with relative expression values above this line were considered upregulated and were analyzed in comparison to control comprising the bovine T-lymphocyte population. **a** Group 1, **b** group 2 and **c** group 3

all gestational periods have provided WJMSCs with stemness attributed to MSCs according to the International Society for Cellular Therapy (Dominici et al. 2008).

Since MSCs are trapped within the Wharton's jelly between days 4 and 12 of embryonic development and reside there for the whole gestation, they can be harvested after the birth of the newborn and during pregnancy (Taghizadeh et al. 2011). Therefore, WJMSCs that formed during the earliest ontogenic period result in a significant expansion potential compared to bone marrow mesenchymal cells (Troyer and Weiss 2008).

There is considerable controversy in the literature regarding the immunogenicity of human MSCs (Weiss et al. 2008; De Miguel et al. 2012; Mukonoweshuro

et al. 2014) and a lack of information about bovine MSCs. However, porcine umbilical cord-derived stem cells did not induce a considerable immune response in vivo but stimulation with interferon gamma or injection in an inflamed region resulted in immunogenicity (Poncelet et al. 2007). Inflammatory situations prevail during any injury and MSCs could be exposed to such stimuli in many clinical conditions. Not only neighboring cells but also environmental factors like systemic or local inflammation can influence the immune behavior of MSCs (Poncelet et al. 2007). In fact, recent reports indicate the role of inflammatory cytokines in affecting functions of mouse MSCs (Mukonoweshuro et al. 2014).

From the factors measured in this study, *IL2*, *IL6R*, *INFAC*, *INFB1*, *INFG*, *TNF* and *LTBR*, considered pro-inflammatory cytokines, were genetically downregulated in all the bMSCs tested. IL10 cytokyne is produced by both myeloid and lymphoid cells. However, it is a good immune suppressor, although some stimulatory effects have been described. Therefore, IL10 cytokine is recognized by its effect on T-cells, macrophages and monocytes in suppressing inflammation processes (Prasanna et al. 2010; Mukonoweshuro et al. 2014). However, when *IL1F10* is expressed it will downregulate *JSP.1* (MHC I) as revealed in WJMSCs cultures. These findings are in accordance with what has been described in human MSCs (Weiss et al. 2008). The results described previously revealed that MSC possess immunosuppressive properties; however, they might not be immunoprivileged (De Miguel et al. 2012). The lack of *DSB* and low *JSP.1* expression observed in this study is thought to be, in part, responsible for their WJMSC immunoprivileged status, which would mean allogeneic bovine MSC could be used without the risk of immune rejection, a scenario that is attractive for tissue comparative studies.

These findings demonstrated the complexity of studying the immunological properties of WJMSCs in vitro, as well as the difficulty of distinguishing between optimal gestational stages in order to collect Wharton's jelly cells with optimal stemness properties. Finally, WJMSCs collected from bovine umbilical cord at all gestational periods showed similar stemness properties.

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Author's contributions TCC participated in the design of the study, performed the cell culture in all steps and flow cytometric analysis. LHO and JCB participated in the preparation of respective umbilical cords. RG and HLF participated also in the design of the study, performed the statistical analysis and drafted the manuscript. MM and EFF drafted the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors indicate no conflicts of interest.

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ORIGINAL ARTICLE

Comparative analysis of the replication of bovine herpesvirus 1 (BHV1) and BHV5 in bovine-derived neuron-like cells

Tereza C. Cardoso [1] · Helena. L. Ferreira [3] · Lucas H. Okamura [1] · Bruna R. S. M. Oliveira [1] · Ana Carolina G. Rosa [1] · Roberto Gameiro [2] · Eduardo F. Flores [4]

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Abstract Members of the subfamily Alphaherpesvirinae use the epithelium of the upper respiratory and/or genital tract as preferential sites for primary replication. However, bovine herpesvirus 5 (BoHV5) is neurotropic and neuroinvasive and responsible for meningoencephalitis in cattle and in animal models. A related virus, BoHV1 has also been occasionally implicated in natural cases of neurological infection and disease in cattle. The aim of the present study was to assess the in vitro effects of BoHV1 and BoHV5 replication in neuron-like cells. Overall, cytopathic effects, consisting of floating rounded cells, giant cells and monolayer lysis, induced by both viruses at 48 h postinfection (p.i.) resulted in a loss of cell viability and high virus titres (= 0.978). The BoHV1 Cooper strain produced the lowest titres in neuron-like cells, although viral DNA was detected in infected cells during all experiments. Virus replication in infected cells was demonstrated by immunocytochemistry, flow cytometry and qPCR assays. BoHV antigens were better visualized at 48 h p.i. and flow cytometry analysis showed that SV56/90 and Los Angeles antigens were present at higher levels. In spite of the fact that BoHV titres dropped at 48 h p.i, viral DNA remained detectable until 120 h p.i. Sensitive TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) and annexin V assays were used to identify apoptosis. BoHV5 induced death in approximately 50 % of cells within 24 h p.i., similar to what has been observed for BoHV1 Los Angeles. Infection with the BoHV1 Cooper strain resulted in 26.37 % of cells being in the early stages of apoptosis; 63.69 % of infected cells were considered viable. Modulation of mitochondrial function, as measured by mitochondrial membrane depolarization, was synchronous with the virus replication cycle, cell viability and virus titres at 48 h p.i. Our results indicate that apoptosis plays an important role in preventing neuronal death and provides a bovine-derived in vitro system to study herpesvirus-neuron interactions.

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Introduction

Bovine herpesvirus 1 (BoHV1) and BoHV5 are genetically and antigenically related viruses in the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus. BHV1 is associated with respiratory and genital diseases and is widespread in cattle populations, with the exception now of a few European countries that have eradicated BHV1 [5–7]. BoHV5 infection is associated with neurological disease and appears to have a more restricted geographic distribution. BoHV5, which was previously considered a subtype of BoHV1 with neurogenic potential, has since been recognized as a distinct virus type [7]. Although neurotropism and neurovirulence are most commonly attributed to BoHV5, BoHV1 has also been occasionally implicated in neurological infection and disease [24].

Studies in vivo, in the natural hosts and in a rabbit model, showed that BoHV5 replicates in the neurons of the trigeminal ganglion and olfactory bulb, after which it establishes latent infection. The virus also invades the brain, where it replicates extensively in several areas [5, 6]. Virus replication in neural tissue is accompanied by neuronal degeneration, but the exact mechanism of this process remains unknown. One possibility could be the induction of programmed cell death, which has already been demonstrated for other alphaherpesviruses [4, 14, 16, 17, 20]. Studies in vitro have shown that, following BoHV1 infection of cultured neurons obtained from rabbit ganglionic tissue, hallmarks of apoptosis such as chromatin condensation, DNA fragmentation and membrane blebbing are detected [8]. Moreover, latency-related (LR) RNA transcripts are the only abundant viral transcripts detected in latently infected neurons, and the antiapoptotic properties of the LR gene play an important role during establishment oflatency [17].

Apoptosis is one of the main mechanisms that animals use to eliminate viral infections [12, 13]. By inducing cell death, infected cells and viruses contained within them are eliminated [22]. Herpesviruses produce numerous antiapoptotic factors that delay or prevent apoptosis to allow production of the maximal number of virus progeny [27]. Apoptosis can be triggered in two ways, intrinsic and extrinsic [13]. The extrinsic pathway can be activated by a variety of external factors such as cytokines, toxins, or ligand binding to apoptotic receptors on the cell surface [13]. The intrinsic apoptosis pathway is triggered by cell stress, such as viral proteins, DNA damage, or oxidative stress, leading to the activation of molecules on mitochondrial membranes [10, 13, 15, 21, 29].

Mitochondria are also directly involved in several host and viral responses. These organelles participate in major early antiviral immune responses through changes in their metabolism [29]. Viral infections may interfere with mitochondria bioenergetics by affecting cellular respiratory function [12]. Moreover, viral proteins inserted in mitochondrial membranes have anti- and/or pro-apoptotic effects, affecting cell survival/death pathways [12, 29]. It has been demonstrated in bovine gametes that experimental exposure to BoHV5 increases mitochondrial membrane depolarization, inhibits the respiratory chain, and increases oxygen consumption without any adverse physiological effect on virus production, an important characteristic of the viruses in the familyHerpesviridae [1, 25, 26].

In this study, the susceptibility of neuron-like cells to BoHV1 and BoHV5 strains was examined. For this purpose, virus infection, cytopathic effects, cell viability and growth kinetics were examined. Moreover, characterization of neuronal death and depolarization of the mitochondrial membrane were also analyzed.

Materials and methods

Cell culture and viruses

Bovine neuron-like cells were characterized at the Laboratory of Animal Virology and Cell Culture, Veterinary College, UNESP, Sa˜o Paulo, Araçatuba, Brazil [2]. These cells were generated by inducing differentiation with a defined neuron-specific medium and were mainly characterized as being of the neuronal/glial phenotype [2]. Neuron-like cells were cultured in 25-cm^2 flasks for the growth curve/viability assay, on Lab-Tek® well chamber slides for immunostaining, and in 96-well culture plates for virus neutralization test (Nunc, Rochester, NY, USA). For all culture conditions, Neurobasal® medium supplemented with B27 was used (Invitrogen, Life Technologies, Carlsbad, CA, USA). Cultures were incubated at 38.5 °C in 5 % CO_2 with 95 % humidity.

Three BoHV1 strains were used: Los Angeles (reference strain), Cooper, and SV56/90, isolated from an outbreak of balanoposthitis in southern Brazil [22]. Two BoHV5 strains were used: SV507/99 (reference strain), was isolated from a cow with neurological disease in southern Brazil, and its genome was sequenced previously [28]. The other BoHV5 strain (GU9457818, GenBank accession number xxxx) was isolated from a case of neurological disease in Sa˜o Paulo State [11]. All virus suspensions were prepared by infecting MDBK cells according to standard procedures [19]. Except for BHV5 GU9457818, all of the viruses were supplied by Prof. Eduardo Furtado Flores, UFSM, Santa Maria, RS, Brazil.

Virus infection and cytopathic effect

Neuron-like cells were grown to 80 % confluence in Lab-Tek® chamber slides (Nunc™) and inoculated with 100 µl of BHV suspensions ($10^{3.2}$ $TCID_{50}$/ml), corresponding to a multiplicity ofinfection (m.o.i.) of approximately 0.1. Viral suspensions were allowed to adsorb for 1 h at 38.5°C, followed by inoculum removal and addition of culture medium. Infected and uninfected cells were visualized under phase-contrast using an Olympus IX-70 microscope. Approximately 10 fields were analyzed for each condition, and photographs were taken at 200x magnification using cell Sens™ software (Olympus).

Immunocytochemistry

Monolayers were fixed at 48 h p.i with 4 % paraformaldehyde for 15 min in Lab-Tek® chamber slides. The cells were permeabilized for 10 min at room temperature in 0.4 % Triton X-100 diluted in phosphate-buffered

saline (PBS). The fixed neuron-like cells were incubated overnight at 4 °C with each of the primary antibodies described for virus quantification. The next day, after three washes, cells were incubated with goat anti-mouse FITC-conjugated antibody (Sigma-Aldrich) at a dilution of 1:100. For nuclear staining, DAPI (4′-6-diamino-2-phenylindole; Sigma-Aldrich®) was diluted to 1 mg/ml in Fluormount™ aqueous medium and incubated with the cells for 15 min. Images were collected using an AxioImager® A.1 light and an ultraviolet (UV) microscope connected to an AxioCam®MRc (Carl Zeiss, Oberkochen, Germany). The images were processed using AxioVision® 4.8 software (Carl Zeiss) for viral antigen detection. The uninfected cells were subjected to the same staining procedure.

Viability assay and viral growth kinetics

Cell proliferation analysis was performed using an In Vitro Toxicology Assay® Kit, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based assay on both infected and uninfected neuron-like cells at 24, 48, 72, 96 and 120 h postinfection (p.i.) (TOXI-1 Kit; Sigma-Aldrich, St. Louis, MO, USA). For each time point, the culture supernatant was removed and 2 ml of MTT (tetrazolium salts) was added following the manufacturer's recommendations (Sigma-Aldrich). Absorbance was measured at 600 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). All reported values are means of triplicate samples. A one-step growth curve of virus replication in neuron-like cells was generated in a multiplication kinetics experiment, followed by infection at 80 % confluence at an m.o.i. of 0.1. Adsorption was allowed for 90 min at 38.5°C, the inoculum was removed, and fresh medium was added. The neuron-like cells were incubated for 24, 48, 72, 96 and 120 h p.i. After incubation, both supernatant and lysate cells were harvested and assayed for virus by infecting Madin-Darby bovine kidney (MDBK) cells as described previously, and cytopathic effect was documented [28]. All experiments were performed at four different time points. Infectious virus titres were calculated according to the Spearmann-Kaerber method and expressed as $\log_{10}$ $TCID_{50}$/ml [19].

Virus quantification

Infected cells were harvested at 48h p.i after detachment with 0.25 % trypsin (Sigma-Aldrich), washed with PBS, and incubated for 18 h at 4°C with anti-BHV1 monoclonal antibody (MAb) 7F12 and anti-BHV5 MAbs 2F9 and 1F3 [24, 28]. After incubation with primary antibodies at 1:50, the cells were washed three times with PBS plus 0.1 % Triton X-100. Next, a 1:50 dilution of the secondary antibody was added to 100 µl of cell suspension and incubated at 37 °C for 30 min. The cell suspension was washed as described previously, and after the final wash, the cells were fixed with 4 % paraformaldehyde. Data were acquired using an Attune™ acoustic focusing cytometer system (Applied Biosystems, Foster City, CA, USA), and at least 50,000 events were counted. The negative pattern was examined by applying the same cell suspension with the first incubation, and the result was included in the global compensation to exclude autofluorescence. A BL1-A (488 nm) filter was used in each analysis. The parameters used were as follows: 799 events/second; flow rate, 11 µL per min; stop gate, 500,000 total cells; BL1-A voltage of a 488 laser; SSC threshold, 300.

Viral nucleic acid detection by quantitative real-time polymerase chain reaction (qPCR)

Upon harvesting at 24, 48, 72, 96 and 120 h p.i., DNA from inoculated cell culture monolayers was extracted using DNAzol™ according to the manufacturer's instructions (Invitrogen). An average of 100 ng of genomic DNA was used for qPCR as described previously [2]. The following primers and probes were used: BoHV1 forward (5′-GGTACATGTCCAGGGAAAC-3′); BoHV1 reverse (5′-GGTACAACATCGTCAACTTC-3′) and BoHV1 probe FAM-TAATCTTCACCTTGCTC-MGB; BoHV5 forward (5′-GGTACTTCTTCTTGGTGATG-3′); BoHV5 reverse (5′-TCGGTCTTCGTCAAGTTC-3′); and BoHV5 probe FAM-AGGTCTTCTCGCACTC-MGB [9]. The reaction was carried out and analyzed using the software on a StepOnePlus™ real-time instrument (Applied Biosystems). The data were obtained from three replicates of each sample.

The expression of the housekeeping gene for histone 2a (H2A) was also quantified in a similar way for normalization [26]. The viral DNA level at the corresponding time p.i. in infected and uninfected cells was quantified in comparison to the housekeeping gene. The comparative delta-delta Ct method was used to analyze the results.

Flow cytometry for characterization of cell death and mitochondrial membrane potential

Apoptosis/necrosis was measured using a double staining method with a Vybrant Apoptosis Assay Kit (Molecular Probes, Life Technologies) and an APO-BrdU™ TUNEL assay Kit (Molecular Probes, Life Technologies). After infection at 48 h p.i, cells were processed according to manufacturer's instructions. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrathylbenzimidazolyl-carbocyamine iodide (Molecular Probes, Eugene, OR) is a mitochondrion-specific probe. In its monomeric form, the probe emits at 527 nm (red) after excitation at 490 nm (green).

Depending on the mitochondrial membrane potential, JC-1 forms aggregates that are associated with large shifts in emission, and the color dye changes reversibly from red to green as mitochondrial membranes become depolarized. To perform this assay, 29 10^3 infected or uninfected cells at 48 h p.i were trypsinized, washed, and incubated with 10l g of JC-1 per ml for 10 min at 37°C in the dark. After this period, cells were immediately analyzed by acoustic flow cytometry using BL1-A (488 nm) and BL2-A (575 nm) filters. Data were acquired via the Attune™ acoustic focusing cytometer system (Applied Biosystems). Data were expressed as a percentage of the emission from JC-1 cells. The parameters used were as follows: 2,500 events/second; flow rate, 1 L per min; stop gate, 500,000 total cells; BL1-A voltage of a 488 laser; SSC threshold, 300.

Statistical analysis

All experiments were performed at least in quadruplicate. Descriptive statistics include the mean± standard deviation (s.d.). A p-value \ 0.005 was considered significant. All statistical analyses were performed using Prism software (GraphPad, CA, USA).

Results

Characterization of BoHV1 and BoHV5 replication in neuron-like cells

The first set of experiments was conducted to characterize the replication of BoHV1 and BoHV5 strains and their induced cytopathic effects. The CPE was better documented at 48 h p.i. with the BoHV5 strains (GU9457818 and SV507/99). Virus replication produced floating rounded cells and disruption of the cell monolayer (Fig1). A similar CPE was produced by the BoHV1 Los Angeles strain in neuron-like cells at 48 h p.i (Fig.1). Cell fusion followed by giant-cell formation without disruption of the cell monolayer was another form of CPE that was observed, mainly in neuron-like cells infected with the BHV1 Cooper strain (Fig. 1). The BoHV5 SV56/90 strain induced only a few floating rounded cells that were difficult to distinguish from uninfected cell monolayers except by visual assessment of cell integrity (Fig.1). Moreover, an immunocytochemistry assay confirmed virus replication by detecting viral antigens in infected cells (Fig2).

CPE by all viruses was more pronounced at 48 h p.i., whereas cell viability was at the cutoff point (dead cells) (Fig. 3a). At this time, virus titres were considered to be at the highest level, dropping at 72 h p.i. (Fig3b). These findings were in agreement with those obtained by flow cytometry analysis (Fig. 4a and b). The histogram graphic revealed a peak of virus antigen detection at the same time postinfection, with no measurement during subsequent periods. A significant correlation was detected r (= 0.978) among cytopathic effect, cell viability, and virus titres (p \ 0.005). Regarding viral DNA, all BHV strains produced levels of viral DNA at all times p.i. (Fig. 3c).

Mitochondrial membrane depolarization and neuronal apoptosis

The JC-1 probe emission is illustrated (Figs.5a and b) by histogram graphic where a BL1-A filter (488 nm, green) was applied. All BHV strains produced mitochondrial membrane depolarization at 48 h p.i. (Fig5a and b). No evidence of this phenomenon could be observed from this time measured by acoustic focusing flow cytometry analysis (data not shown). The BoHV5 strains, SV507/99 and GU9457818, produced the lowest mitochondria membrane depolarization (p \ 0.005) compared to SV56/90, which produced the highest values. The Los Angeles and Cooper strains produced the same mitochondria membrane depolarization at the same time (Fig.5a and b).

To investigate whether apoptosis occurred in the process of BoHV-induced neuronal death, annexin V and PI staining and TUNEL assay were applied. Infection with Los Angeles, SV507/99 (BoHV5 reference strain) and GU9457818 (BHV5, field isolate) resulted in 45.8 %, 49.9 % and 57.8 % dead cells at 48 h p.i. in both assays (Table 1). Likewise, the same strains also produced late apoptotic/necrotic cells in& 30 % of the infected cell population at 48 h p.i. (Table1). SV56/90 infection resulted in 50.4 % neuron-like cells in the early apoptotic phase at 48 h p.i. In contrast, the Cooper strain produced 12.7 % dead cells and almost same proportion of neuron-like cells in the early/late apoptotic phase (Table 1). In the TUNEL assay analysis, (cutofflevels of 10^3 and 10^4 counted events), similar results were obtained (Fig.6). Cooper strain infection induced 26.37 % apoptosis, followed by 63.69 % viable cells, similar to what was observed in the control (Fig6). In contrast, the Los Angeles and SV56/90 strains showed 41.93 % and 24.49 % infected cells in the late apoptotic/necrotic phase. Two BoHV5 strains produced similar necrotic effect, whereas 46.74 % (SV507/99) and 45.39 % (GU457818) ofinfected neuron-like cells were dead (Fig. 6).

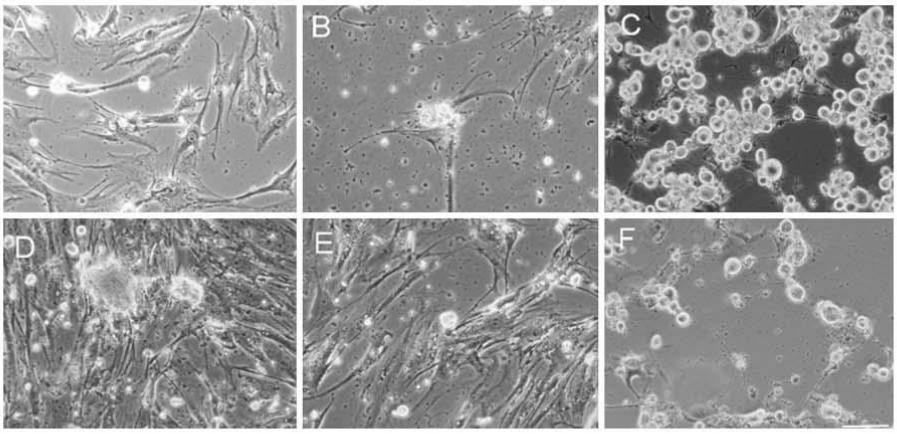

Fig. 1 Cytopathic effects in BoHV-neuron-like infected cells at 48 h p.i. assessed by phase-contrast microscopy. A) Uninfected neuron-like cells used as controls, B) BoHV5 cytopathic effect characterized as lysis and some rounded cells, C) floating and rounded cells produced by BoHV5 strain (SV507/99), D) Cooper strain cytopathic effect showing giant cells but no disruption of the monolayer, E) a few rounded cells produced by the SV56/90 strain, F) cytopathic effect similar to that caused by the Los Angeles strain of BoHV5 (SV507/99). Bar, 20 l m. The data were obtained from four different experiments

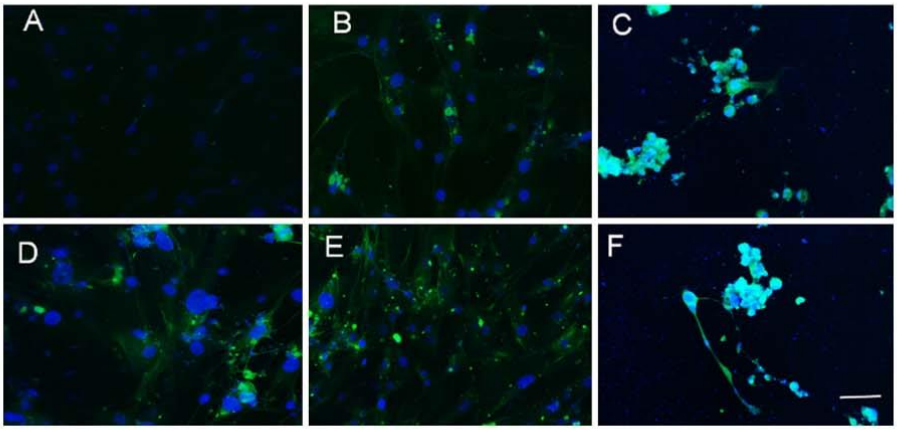

Fig. 2 Analysis of expression of BoHV antigens in neuron-like cells by immunocytochemistry at 48 h p.i. A) Uninfected neuron-like cells (control); B-C) BoHV5 antigen-positive cells GU9457818 and SV507/99, respectively; D) BoHV1 Cooper strain antigen-positive cells; E) BoHV1 (SV56/90) antigen-positive cells; F) BoHV1 Los Angeles antigen-positive cells. Bar, 20 l m; immunocytochemistry performed using monoclonal antibodies against BoHV1 and BoHV5 (green), followed by DAPI counterstain (blue). The data were obtained from four different experiments

Discussion

In this study, we investigated changes in neuron-like cells in response to infection with different BoHV1 and BoHV5 strains to better understand the possible mechanisms underlying its enhanced infectivity and apoptosis. This in vitro model presents ethical and economic advantages over in vivo models [18, 25]. For this purpose, suitable in vitro systems resemblingin vivo situations and implementing the "Three Rs Principle" of Russell and Burch [?REFERENCE?] are needed to study primary host-virus interactions [27].

It has been proposed that viruses are metabolic engineers because of their ability to alter host energy

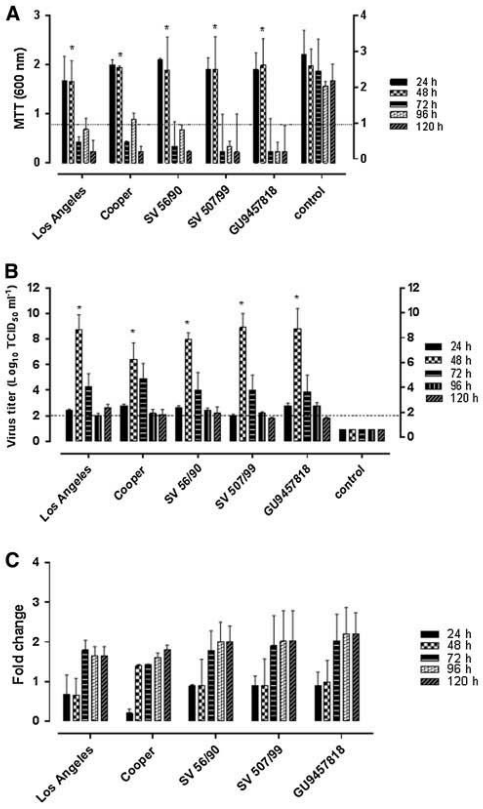

Fig. 3 Evaluation of viability of neuron-like cells, BoHV titres at 48 h p.i., and viral DNA expression. A) MTT values indicate loss of cell viability at 72 h p.i. B) Virus titres were considered low at 72 h p.i. (*, p\ 0.005) expressed as $TCID_{50}$. The data were obtained from four different experiments. C) Real-time PCR for detection of viral DNA. Fold changes were calculated from 24 to 120 h p.i.p(\ 0.005)

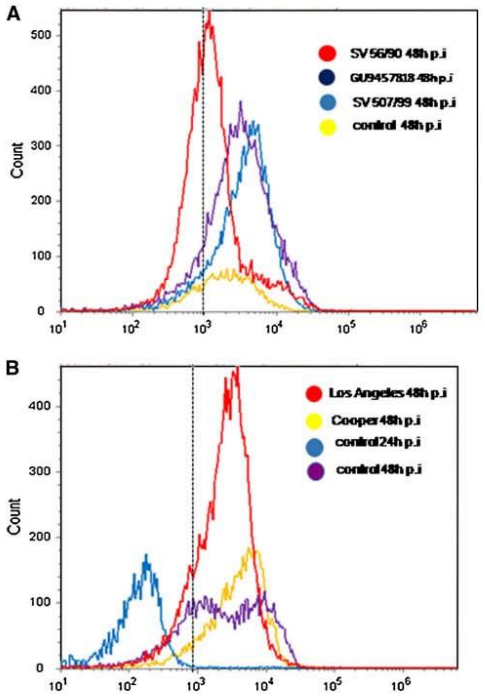

Fig. 4 Analysis of BoHV antigen expression by flow cytometry of infected neuron-like cells at 48 h p.i. The x-axis corresponds to the number of positive labeled cells detected using a BL1A filter (488 nm). The y-axis is on a log scale. The data obtained from four different experiments were processed using an Attune acoustic focusing cytometer, and auto-fluorescence was excluded using a global compensation tool(10^3, discontinuous line)

metabolism to favor replication [3]. Although mitochondrial involvement in BoHV neuron infection was first documented in this study, previous studies revealed that the BoHV5 GU9457818 strain promotes survival of bovine embryos and does not interfere with in vitro development [1, 25, 26]. All BHV1 and BHV5 strains produced JC-1 emission at a high level, with the exception of the BoHV1 Cooper strain. The uptake of JC-1 and subsequent formation of J-aggregates in living cells is caused principally by the mitochondrial membrane potential[1]. Our results demonstrated a positive correlation between virus production and mitochondrial membrane potential, which is probably a result of production of progeny virus.

We also showed that the CPE of the Los Angeles strain was similar to that induced by GU9457818 (a BoHV5 field isolate) and SV507/99 (a standard BoHV5 strain) and was produced at comparable levels in bovine neuron-like cells. It has been demonstrated that the reference strains Los Angeles strain (LA38) and BoHV5 97/613 both produce high virus titres in MDBK cells when compared to what was observed in this study [9]. In fact, a previous study demonstrated that rabbit ganglionic neurons, when infected with the Cooper strain (BoHV1), produced the highest viral titres between 24 and 48 h p.i., similar to what was observed here [8].

Like other alphaherpesviruses, BoHV establishes latent infection in sensory neurons and induces apoptosis in lymphoid cells in vivo and in epithelial cell lines [16, 17]. Transcription from the LR gene is readily detected in

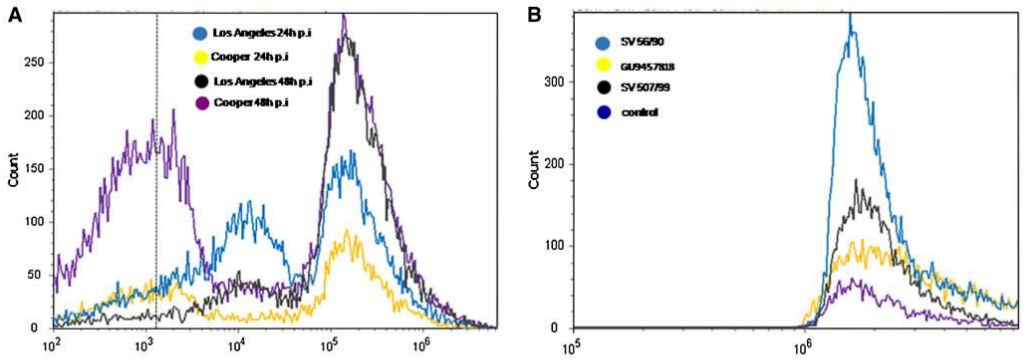

Fig. 5 Acoustic focusing cytometry for measuring JC-1 emission at 48 h p.i. The x-axis corresponds to JC-1 probe emission, and the y-axis is on a log scale. The data were obtained from four different experiments and processed using an Attune acoustic focusing cytometer, and auto-fluorescence was excluded using a global compensation tool[(10^3; discontinuous line)

Table 1 Proportion of annexin-V-FITC/PI-labeled neuron-like cells 48 h after infection with different BoHV subtypes

	Dead (% ± sd) (annexinV-FITC-/PI ?)	Late apoptotic/necrotic (%± sd) (annexin V-FITC ? /PI?)	Early apoptotic (% ± sd) (annexin V-FITC ? /PI-)	Viable (% ± sd) (annexin V-FITC-/PI-)
Los Angeles	45.8 ± 4.7	31.3 ± 2.9	10 ± 1.5	12.9 ± 3.7
Cooper	12.7 ± 3.8	9 ± 1.0	10.7 ± 1.3	67.6 ± 6.0
SV56/90	16.8 ± 4.9	9 ± 0.9	50.4 ± 3.9	23.8 ± 7.9
SV/507/99	49.9 ± 5.3	30.6 ± 2.3	6.0 ± 0.8	13.5 ± 2.0
GU9457818	57.8 ± 6.2	30 ± 2.2	2.4 ± 0.7	9.8 ± 1.6
Control	5.6 ± 1.2	0.5 ± 0.1	5.0 ± 0.6	88.9 ± 7.8

neurons of trigeminal ganglia of calves and rabbits latently infected with BoHV1 [16]. Higher levels of apoptosis occur in trigeminal ganglia neurons of calves infected with the LR mutant virus when compared to wild-type BoHV1, indicating that activation of antiapoptotic factors is directly associated with the LR gene [16, 17]. In spite of the fact that the BHV1 Los Angeles strain has not been associated with neurological disease, after 48h ofinfection, 45.8 % of neuron-like cells were dead. Similar results were obtained with the other two BoHV5 strains, which are considered neurotropic and neurovirulent viruses. Previous studies with varicella-zooster virus demonstrated that neurotropism and growth is not associated with neuronal death [3, 14, 23]. Another study demonstrated that the BHV1 Cooper strain produced high viral titres and CPE but no evidence of apoptosis in infected rabbit neuron cultures[8]. In fact, the Cooper strain produced 67.6 % neuron-like viable cells after 48 h p.i. in this study. Moreover, BHV1 SV56/90, another non-neurotropic virus, produced 50.4 % neuron-like cells in the early apoptosis phase. It was expected that BoHV5 strains would produce high levels of neuronal death. However, the Los Angeles strain behaved in the same way and is not currently considered a neurovirulent virus.

Recently, it has been shown that caprine herpesvirus 1 (CpHV-1) infecting a neuronal cell line triggered death-receptor and mitochondrial pathways separately and in parallel [20]. However, murine neurons infected with equine herpesvirus 1 (EHV1) showed no changes, and cells survived for more than eight weeks in culture[4]. This study concluded that EHV1 activated some mechanism of protection during infection, and the same phenomenon has been observed for BoHV5-infected bovine embryos[1, 25, 26].

Alphaherpesviruses establish lifelong latent infections [5]. Several days after primary infection, some neurons are productively infected (acute phase), whereas other neurons become latently infected[2, 16]. In the present study, the absence of floating neuron-like cells after infection with BHV1 Cooper and BHV1 SV56/90 was associated with a low rate of neuron death. In addition, the BoHV5 GU9457818 strain induced cell lysis and 57.8 % neuronal

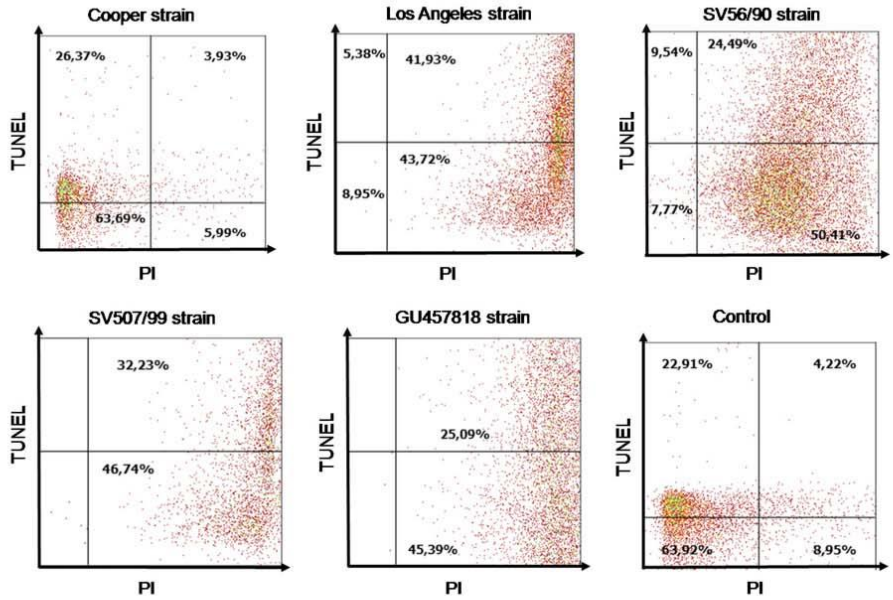

Fig. 6 Acoustic focusing cytometry for detecting TUNEL-positive neuron-like cells at 48 h p.i, illustrated by a density dot-plot graphic. The x-axis corresponds to propidium iodide (PI) emission using a BL2A filter (575 nm), and the y-axis corresponds to TUNEL (BL1A; 488 nm) emission. The data were obtained from four different experiments and processed using an Attune acoustic focusing cytometer, and autofluorescence was excluded using a global compensation tool[(10^3 and 10^4 events counted)

death, which is different from what has been observed before in a similar experiment [2]. Taken together, our results contribute to the understanding of the mechanisms of neurotropism and neurovirulence of alphaherpesviruses. Our findings also suggest that differential modulation of the host response by different BHV1 and BHV 5 strains might be an important factor influencing viral pathogenesis.

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Short communication

Programmed cell death-associated gene transcripts in bovine embryos exposed to bovine *Herpesvirus* type 5

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ABSTRACT

In vitro-produced bovine embryos become infected after exposure to bovine *Herpesvirus* type 5 (BoHV-5), yet no changes in developmental rates, mitochondrial activity and inhibition of apoptosis are detected in comparison to unexposed embryos. Thus, the aim of the present study was to assess the transcription of mitochondria-mediated apoptosis genes using TaqMan real-time polymerase chain reaction. Transcripts of mcl-1, caspase-2, -3, Apaf-1 and Bax genes were measured after exposure to BoHV-5 *in vitro*. Mitochondrial dehydrogenase activity was evaluated by MTT test and compared between groups of exposed and unexposed embryos, at day 7 of development. The rate of oocyte maturation was assessed by the extrusion of the first polar body. In summary, BoHV-5 exposed embryos retained their viability, mitochondrial dehydrogenase activity and displayed up-regulation of transcription of survival mcl-1 gene and down-regulation of Bax transcription in relation to mitochondria-mediated pathway which might improve embryo viability. These findings demonstrate that BoHV-5 exposed embryos maintain their viability and mitochondrial dehydrogenase activity with no compromise of embryos produced *in vitro*.

Bovine *Herpesvirus* type 5 (BoHV-5) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* and is associated with meningoencephalitis in cattle and, to a lesser extent, also incriminated in reproductive disorders [1]. BoHV-5 replication *in vitro* is characterized by a rapid lytic cycle especially in Madin–Darby bovine kidney (MDBK) cells. In vivo, acute BoHV-5 replication in the nasal mucosa is followed by establishment of latent infection in sensory nerve ganglia [2]. Different virus replication behavior *in vitro* has been described for different field isolates [3,4].

Several mammalian DNA and RNA viruses have been shown to be associated with induction of cell apoptosis [5]. Apoptosis is a genetic and biochemical process that plays an essential role in morphogenesis, host defense and homeostasis of cell tissues. Caspases are a family of cysteine proteases that mediate apoptosis induced by a variety of stimuli [6]. Based on their structure and order in cell death pathways, caspases can be divided into initiators (such as caspase-2, -8, -9, -10, and -12) and effectors (such as caspase-3, -6, and -7) [6]. Viruses possess various biochemical and genetic mechanisms to evade and/or to induce apoptosis modulation through virus-encoded proteins [7,8]. Two death pathways, intrinsic and extrinsic, have been identified in most cases of caspase-dependent apoptosis. The intrinsic death pathway involves mitochondrial release of cytochrome *c* which interacts with Apaf-1 and dATP to promote procaspase-9 auto activation, which in turn, activates downstream effectors such as caspase-3, -6, and -7. The extrinsic death pathway is initiated by engagement of cell surface death receptors (CD95/Fas/APO-1) and tumor necrosis factor receptor [9]. The complex of death receptors and ligands leads to the recruitment of the adapter molecule FAAD and the activation of caspase-8 [6]. In some cells, active caspase-8 is sufficient to lead to caspase-3 activation by cleaning the proapoptotic BCl-2 family members, including mcl-1, which induces mitochondrial cytochrome c release and, thereby, links the two pathways [10,11]. After activation, caspases cleave various cellular substrates resulting in membrane blebbing, chromatin condensation and formation of apoptotic bodies [12]. Modulation of apoptosis seems to be a key step in *herpesviruses* pathogenesis [12,13].

Recently, we demonstrated that experimental exposure of bovine gametes to BoHV-5 led to the infection of *in vitro*-produced embryos without interference in embryonic development. In addition, derived embryos revealed less apoptotic cells detected by annexin-V and TUNEL analysis [14,15]. Moreover, in another study,

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BoHV-5 exposed bovine blastocysts seemed to display an increase on mitochondrial membrane potential [16]. Determining how BoHV-5 interferes with cell-death pathways will not only improve our knowledge of viral pathogenesis but also has the potential to advance our understanding of the processes that control cellular death pathways. Besides, BoHV-5 has been, in some cases, associated with reproductive disorders worldwide [17–22].

The objective of this study was to investigate the role of mitochondrial intrinsic pathway of apoptosis *in vitro* produced bovine embryos exposed to BoHV-5. Caspase-2, -3, mcl-1, Bax and Apaf-1 expression was investigated by assessing gene transcription. Both mitochondrial dehydrogenase activity and embryo development were also investigated after virus exposure.

BoHV-5 isolates obtained from animals affected during an outbreak in Araçatuba, SP, Brazil in 2007 were propagated in "Madin Darby Bovine Kidney" cells (MDBK, ATCC CCL-2). Cells were grown under the culture conditions described previously [2]. After virus amplification, virus titers were calculated and expressed as mean tissue culture infective dose per 50 μl ($TCID_{50}$). Aliquots of virus stock (100 μL) with $10^{3.4}$ $TCID_{50}$/50 μL were frozen at −86 °C until embryo exposure. Bovine oocytes used were collected from ovaries of crossbred cows obtained in local slaughterhouses. Ovaries were transported to the laboratory within 1 h *post mortem* in physiological solution at 37 °C. The *cumulus*-oocytes complexes (COC) were recovered by aspiration of the follicles with a diameter of up to 2.6 mm, with the aid of a syringe (10 ml) and needle (40 × 12). Only the COC with 3–4 or more cell layers of compact *cumulus* and homogeneous cytoplasm were selected.

The oocytes were divided into two groups, depending on the treatment: unexposed ($n = 357$) and exposed ($n = 388$) to BoHV-5. Before virus exposure the oocytes were conditioned in cryotubes containing 400 μL of maturation medium (MM). The unexposed group received 40 μl of fresh MEM (Sigma–Aldrich®, St. Louis, MO, USA) and the exposed group received 40 μl of viral suspension, derived from the virus stock containing $10^{3.4}$ $TCID_{50}$/ml. Virus adsorption was carried out at humid atmosphere and 5% of CO_2 for 1 h at 38.8 °C. After virus adsorption, oocytes were washed three times in MM and allocated in drops of 70 μl of MM, distributed in Petri dishes (35 × 10 mm) under mineral oil, with each drop containing a maximum of 20 oocytes. A drop of culture was the experimental unit of work, there were 23 replicates, or 23 drops (experimental units), for each group. At each step the same number of drops was made for each group, and the dishes were kept under the same conditions described before for a period of 23 h. After 23 h of maturation, 100 oocytes from each group (unexposed and exposed) were washed three times in droplets 400 μl of PBS solution with hyaluronidase 5 mg/ml (Sigma–Aldrich®). The oocytes were pipetted successively to promote complete removal of cumulus cells in order to analyze the first polar body extrusion by inverted microscope (IX70, Olympus, Tokyo, Japan). Polar bodies were counted in both unexposed and exposed oocytes.

Frozen bovine semen from a single bull and in the same sample was used in the *in vitro* fertilization (IVF) in 0.5 ml vanes. The semen was centrifuged in a Percoll gradient 700× g for 20 min. The spermatozoid pellet was washed in TALP-FERT (Tyrode albumin, lactate and pyruvate) media, supplemented with 6 mg/ml of BSA, 30 μg/ml of heparin and PHE solution (2 mM of penicillamine, 1 mM of hipotaurine and 250 mM of epinephrine) and centrifuged at 200× g for 5 min. The pellet was diluted in TALP-FERT medium to a final concentration of 1×10^5 sperm/ml in drops of 100 μl. After 24 h of maturation, oocytes were washed and transferred to drops with 100 μl of TALP-FERT medium. In the IVF, oocytes and sperm were co-incubated for 20 h. After this period, presumptive zygotes (pz) were denuded by repeated pipetting and placed on drops with 100 μl of *in vitro* culture medium SOFm (synthetic oviduct fluid modified) until day seven, being considered day zero the day of fertilization. The cleavage rate was evaluated at day three, and the feeding realized for removal and addition of 50 μl of fresh medium of the same composition. The procedure of feeding was repeated on day five. IVF and culture were carried out under the same conditions described for IVM. Embryo development was monitored, and cleaved oocytes and blastocyst stages (BL), expanded blastocyst (BX) and hatched blastocyst (BE) were evaluated at days three and seven, respectively. On day seven, only embryos of grade 1 (excellent or good) or grade 2 (good or reasonable) according to the IETS instructions (1998), were stored for further analysis. All animal reagents used in this study were tested for the presence of BoHV-1 and 5 [23] in order to ensure the absence of non-experimental viral contamination.

Table 1
Effects of experimental exposure of bovine oocytes to BoHV-5 during *in vitro* embryo development.

Groups	Oocytes (n)	Embryo development — n (%)	
		Cleaved 72 h (mean ± S)	bl/bx/be 168 h (mean ± S)
Unexposed	357	313 ± 6.5 (87.7)[a]	195 ± 3.2 (54.6)[a]
Exposed	388	328 ± 8.9 (84.5)[b]	193 ± 3.2 (49.7)[a]

[a,b]Values followed by the same letter, in the column, do not differ by t test ($p < 0.05$).
blastocyst stages (BL), expanded blastocyst (BX) and hatched blastocyst (BE).
h = hours post *in vitro* fertilization.

Mitochondrial activity was assessed using the "*In vitro* toxicology assay kit based MTT" (Sigma–Aldrich®) using spectrophotometry. The system measures the MTT activity of viable cells through detect the mitochondrial dehydrogenases activity. The key component is (3 − [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide) or MTT. The MTT solution dissolved in saline balanced salt solution without serum or phenol red shows a yellowish color. The mitochondrial dehydrogenases activity of viable cells cleave tetrazolium ring to form formazan crystals, which have purple and are insoluble in water. The crystals are dissolved in acidified isopropanol, resulting in a purple solution is measured by spectrophotometry at 600 nm.

Total RNA, from unexposed/exposed embryos was extracted using TRIzol® reagent (Invitrogen®, California, USA) following the manufacturer's recommendations. A total of 2 ng of each RNA sample was treated with 1 μl of DNAse (Sigma–Aldrich®, DNAse 1 mg) and reverse-transcribed using the kit high capacity RNA-to-cDNA™ (Applied Biosystems™). The expression of apoptotic and anti-apoptotic regulating genes was quantified using software on a StepOnePlus® real time instrument (Applied Biosystems™). The real time PCR mix (50 μl) contained 1.2 μg of cDNA, 400 nM primers and 200 nM probes FAM-MGB (5′region) customized for Apaf-1 (Bt03210919_g1), Bax (Bt03211776_m1), caspase-2 (Bt03817113_m1), caspase-3 (Bt03250955_g1) and mcl-1 (Bt03276965_g1), bovine sequences (Applied Biosystems™). The PCR was initiated by 40 cycles of amplification at 95 °C (15 s) and 60 °C (60 s). The results were obtained from three replicates of each sample to ensure representative and accuracy pipetting. The expression of bovine histone 2a gene was quantified in a similar way. The comparative delta–delta C_t method was used to analyze the results with the expression level of the respective target genes at the corresponding time point in exposed/unexposed embryos and in comparison to histone 2a C_t values described previously [24].

Cleavage, embryo development, mitochondrial viability and expression of genes mcl-1, caspase-2, caspase-3, Apaf-1 and Bax were analyzed by unpaired t test, with differences considered significant at $p < 0.05$. The maturation rate was evaluated by χ^2 test. The analysis of reference genes was performed by linear correlation coefficient and determination of the.

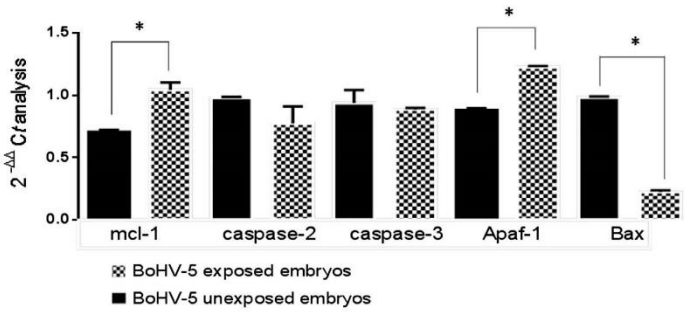

Fig. 2. Relative amounts of mRNA transcription of mcl-1, caspase-2, caspase-3, Apaf-1 and Bax genes in bovine embryos, exposed and unexposed to $10^{3.4}$ $TCID_{50}$/ml of BoHV-5. Differences were observed in mcl-1, Apaf-1 and Bax gene expression between the two groups ($^{*}p < 0.05$).

Apoptosis is essential during embryonic development [6]. Neither caspase-2 nor -3 were affected by BoHV-5 exposure during bovine embryo development in this study. In many cases, caspases are required to propagate the signal to commit suicide. In a previous study, incubation of specific caspase inhibitors significantly enhanced the MDBK viability after BoHV-1 infection [28]. This observation might explain the bovine embryo viability and developmental performance at good rates after BoHV-5 exposure in this study. Moreover, previous study also demonstrated inhibition of apoptosis in virus-exposed embryos [14].

Release of cytochrome *c* from BoHV-1 infected- MDBK cells and increase of Apaf-1 have been proposed to be critical steps during apoptosis [28]. Here, Apaf-1 transcripts were detected in higher amount among BoHV-5 exposed embryos; however cytochrome *c* was not detected (data not shown). Further studies are necessary to understand these findings.

Mitochondria-mediated apoptosis is strictly controlled by Bcl-2 family members, which include mcl-1 as anti-apoptotic protein, and Bax as apoptotic regulator. In contrast to what has been described for BoHV-1, at least among exposed BoHV-5 embryos, the transcripts unbalance between mcl-1 and Bax favored embryo viability [28]. These results are consistent with the observation in other herpesviruses, which suggest that modulation of anti-apoptotic proteins is a key step for induction of cytopathic effect and eventually apoptosis [29]. Thereafter, induction of apoptosis accelerates reactivation of latent *Herpesvirus simplex* type 1 in ganglionic organ cultures and replication in cell cultures [30]. The results presented herein may open new perspectives on latency cycle in bovine virus-exposed embryos, based on the fact that apoptosis has a negative impact on the virus life cycle, demonstrated previously [29,30]. It is clear that *alphaherpesvirus* induces cell death in a cell-type-dependent fashion, and this is probably because virus–host interactions are important for apoptosis in different cell types [31,32].

In summary, the results presented here confirmed previous observations that BoHV-5 exposed embryos did not lose viability, mitochondrial dehydrogenase activity and, more importantly, revealed an increase on survival of *in vitro* produced embryos by overexpression of mcl-1 transcripts related to mitochondria-mediated pathway.

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Short communication

Validation of a reference control for an SYBR-Green fluorescence assay-based real-time PCR for detection of bovine *herpesvirus* 5 in experimentally exposed bovine embryos

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ABSTRACT

The objective of this study was to optimize an internal control to improve SYBR-Green-based qPCR to amplify/detect the BoHV-5 US9 gene in bovine embryos produced *in vitro* and experimentally exposed to the virus. We designed an SYBR-Green-based binding assay that is quick to perform, reliable, easily optimized and compares well with the published assay. Herein we demonstrated its general applicability to detect BoHV-5 US9 gene in bovine embryos produced *in vitro* experimentally exposed to BoHV-5. In order to validate the assay, three different reference genes were tested; and the *histone 2a* gene was shown to be the most adequate for normalizing the qPCR reaction, by considering melting and standard curves ($p < 0.05$). On the other hand, no differences were found in the development of bovine embryos *in vitro* whether they were exposed to BoHV-5 reference and field strains comparing to unexposed embryos. The developed qPCR assay may have important field applications as it provides an accurate BoHV-5 US9 gene detection using a proven reference gene and is considerably less expensive than the TaqMan qPCR currently employed in sanitary programs.

The development of assisted reproduction biotechnology, including *in vitro* embryo production (IVP) and cryopreservation of gametes and embryos, has enabled an increased international trade of bovine genetic material [1,2]. Over the past few years, Brazil represents the world's main producer of IVP bovine embryos, however this status has raised concerns regarding the risk of transmission of infectious agents through reproduction biotechniques.

Bovine *herpesvirus* type 5 (BoHV-5) is a neurovirulent *alphaherpesvirus* usually associated with necrotizing encephalitis in cattle [3]. BoHV-5 infection has been occasionally described in several countries including Australia, United States and European countries, however the disease is noticeably more prevalent in South America, especially Brazil and Argentina [4–10]. Bovine *herpesvirus* type 1 (BoHV-1) and BoHV-5 are genetically and antigenic closely related viruses [11]. Both viruses establish latency in sensory neurons, where their genomes persist for the host's lifetime, awaiting reactivation into a new productive lytic cycle [12].

Interestingly, natural transmission of BoHV-5 by contaminated semen was described in Australia and afterwards a similar event was described elsewhere [4–7,10]. Even though the significance of contamination of bull semen with BoHV-5 is still unknown, an association of veneral disease in cows due to artificial insemination with contaminated semen has been described [5]. In addition, previous studies have demonstrated the risk of contamination of bovine embryos exposed to BoHV-5 [13,14]. As a consequence, bovine semen destined for export should be screened for BoHV-5, in addition to BoHV-1.

In the recent years, real-time polymerase chain reaction (qPCR) has been applied successfully in microbiology laboratories in general and in veterinary virology in particular, and has proven to be very useful for the quantification of viral pathogens [16]. Many studies have been conducted to detect BoHV-1 and BoHV-5 in clinical specimens, including semen, using the monoplex, duplex and/or multiplex qPCR [17–22]. In spite of these achievements, the search for this agent in bovine embryos has not been considered to date.

This article reports the validation of three reference genes as targets in qPCR assays, using the inexpensive SYBR-Green technology on bovine embryos experimentally exposed to BoHV-5. Oocyte collection, *in vitro* maturation (IVM), sperm and *in vitro* fertilization procedures were performed as previously described [13,14]. The study was conducted with presumptive zygotes produced from

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oocytes exposed or unexposed to BoHV-5. Frozen-thawed sperm for IVF were derived from 0.5 mL straws of bovine (*Bos indicus*) semen collected from a single bull. Semen was centrifuged on a Percoll (Nutricell®, Campinas, SP, Brazil) gradient at 700× *g* for 20 min. The resulting sperm pellet was washed in TALP medium (Tyrode medium with added bicarbonate buffer and supplemented with 6 mg BSA per milliliter) and centrifuged at 200× *g* for 5 min. The pellet was diluted in IVF medium (TALP medium supplemented with 3 mg/mL heparin and PHE solution: 2 mM penicillamine, 1 mM hypotaurine, and 250 mM epinephrine) to a final concentration of 1×10^5 sperm/mL in drops of 100 μL. After 24 h of maturation, the oocytes were transferred to drops containing IVF medium. For IVF, oocytes and sperm were co-incubated in the IVF medium for 20 h under the same conditions used for IVM. After, presumptive zygotes (PZ) were placed in *in vitro* culture medium (IVC) up to day 7 (day 0 = day of fertilization). After blastocyst production on day 7 post-fertilization, only embryos graded as Code 1 (Excellent or Good) or Code 2 (Fair) following the IETS guidelines [1] were used. Similarly, only oocytes and presumptive zygotes classified as good quality were used. All uninfected cells and reagents used in this study were assayed for bovine *Herpesvirus* types 1 and 5 (BoHV-1 and 5), bovine viral diarrhea virus (BVDV), and Mycoplasma [13].

Reference BoHV-5 strain SV-507/99 gently supplied by Eduardo Furtado Flores and wild-type BoHV-5 strain (AY06417) were used in the experiment. Both strains were propagated in Madin–Darby bovine kidney cells (MDBK, ATCC CCL-2), which were cultured in minimum essential medium (MEM) [15]. The tissue culture infective dose per 50 μL ($TCID_{50}$) of stock virus was determined by virus titration infection of confluent monolayers of MDBK cells [15] at a multiplicity of infection (MOI) of 1. Aliquots of stock virus (100 μL) with $10^{3.3}$ $TCID_{50}$/50 μL were frozen at −86 °C prior to use. Only COCs with several layers of compact cumulus cells and homogeneous cytoplasm were used, divided into drops of 15–20 oocytes each for experimental use. The culture consisted of oocytes maintained in 100 μL TCM-199 (GIBCO-BRL™, Grand Island, NY, USA), supplemented with 10% FBS, 2.2 mg/mL sodium bicarbonate, 0.02 mg/mL sodium pyruvate, 0.05 mg/mL gentamicin sulfate, 0.5 μg/mL FSH (Pluset, Calier, Barcelona, Spain), and 50 μg/mL LH (Lutropin-V, Bioniche Inc., Belleville, ON, Canada) for 24 h at 39 °C in 5% CO_2-air. Selected oocytes were washed in maturation medium (MM) and transferred to drops containing 100 μL of MM. Oocytes were experimentally infected by co-incubation with 10 μL BoHV-5 strains ($10^{2.3}$ $TCID_{50}$ corresponding to 1 MOI) for 1 h at 39 °C in 5% CO_2-air. The oocytes were subsequently washed three times and transferred to new, virus-free maturation drops for further *in vitro* development, as previously described [13,14].

DNA from exposed and unexposed embryos was extracted by PureLink™ Viral RNA/DNA Mini Kit (Invitrogen™, cat # 12280-050) following the manufacturer's recommendations (Applied Biosystems™, CA, USA). After, total DNA isolation the purity and concentration was determined and set up as 100 ng per reaction. The qPCR was performed using an OneStepPlus™ real-time PCR system in the presence of SYBR-Green detection system (Applied Biosystems™). The optimization of qPCR reaction was performed according to the manufacturer's instructions (Applied Biosystems™, User Bulletin 2 applied to the reagent protocol). The reaction consisted of 25 μl of: 12, 5 μl SYBR-Green master mix, 100 μM of each primer (Table 1), 100 ng of template and ultra-pure DNA/RNAse free water (Invitrogen™). qPCR conditions consisted of an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 10 min. The three reference controls (*histone* 2a, *β-actin* and *GAPDPH* [glyceraldehyde-3-phosphate dehydrogenase] genes) were tested in separate reactions in order to verify melting temperature and standard curve. To perform the standard curve, the template was diluted into 100 ng, 20 ng, 4 ng, 0.8 ng and 0.16 ng. The comparative delta–delta C_t method was used to adjust the level of US9 gene considering the level for each internal control in infected and uninfected embryos in comparison to *GAPDH*, *histone 2a* and *β-actin* C_t values. All of the experiments were performed at least in triplicate. Results of representative experiments are presented. Descriptive statistics include the mean ± standard deviation (s.d.). Statistical analysis was performed using ANOVA (GraphPad Prism® v. 6.01 Software) to evaluate the results. Student's *t*-test was used for comparison of uninfected (control) and BoHV-5 infected embryos. A variety of BoHV-1 isolates/strains from different sources were used in this study for assessment of the specificity. In addition, bovine viral diarrhea virus (BVDV) was tested to evaluate the specificity of US9 primers used in the assay. Two-tailed *p* values < 0.05 were considered significant.

The results of embryonic development upon exposure to BoHV-5 comparing to unexposed controls are shown on Table 2. No

Table 1
Real-time nucleotide primers sequence design on Primer Express® software for this study (Applied Biosystems™).

	Forward 5′-3′	Reverse 5′-3′
US9 BoHV-5 (GU947818.1; Gene Bank accession number)		
Sequence	ACACAGCGTCGTCAACGAAA	TGGTGAGCTCGAGGATGGG
Size	20	19
Melting temperature	57.4 °C	56.8 °C
% CG	50%	59.2%
Product	110 bp	
	Forward 5′-3′	Reverse 5′-3′
Bovine beta actin (NM_173979.3, Gene Bank accession number)		
Sequence	AGGTCATCACCATCGGCAAT	TGAATGCCGCAGGATTCCAT
Size	20	20
Melting temperature	56.7 °C	57.3 °C
% CG	50%	50%
Product	88 bp	
	Forward 5′-3′	Reverse 5′-3′
Histona 2a (AW461431, Gene Bank accession number)		
Sequence	GTCTTGGAGTACCTGACCGC	ACAACGAGGGCTTCTTCTGA
Size	20	20
Melting temperature	56 °C	56 °C
% CG	50%	50%
Product	101 bp	
	Forward 5′-3′	Reverse 5′-3′
GAPDH (XM_618013, Gene Bank accession number)		
Sequence	TTCAACGGCACAGTCAAGGA	ACATACTCAGCACCAGCATC
Size	20	20
Melting temperature	56.7 °C	57.3 °C
% CG	50%	50%
Product	88 bp	

Table 2
Comparison of the developmental rates of bovine embryos subjected to BoHV-5 strains exposure.

Groups	Oocytes (*n*)	Embryo development – *n* (%)	
		Fertilized 72 h	Expanded blastocyst 168 h
Unexposed	357	313 (87.7)[a]	195 (54.6)[a]
Wild-type	388	328 (84.5)[b]	193 (49.7)[a]
Reference strain	398	326 (84)[c]	193 (59.2)[a]

[a,b]Within a column, the values without a common superscript are significantly different ($p < 0.05$).

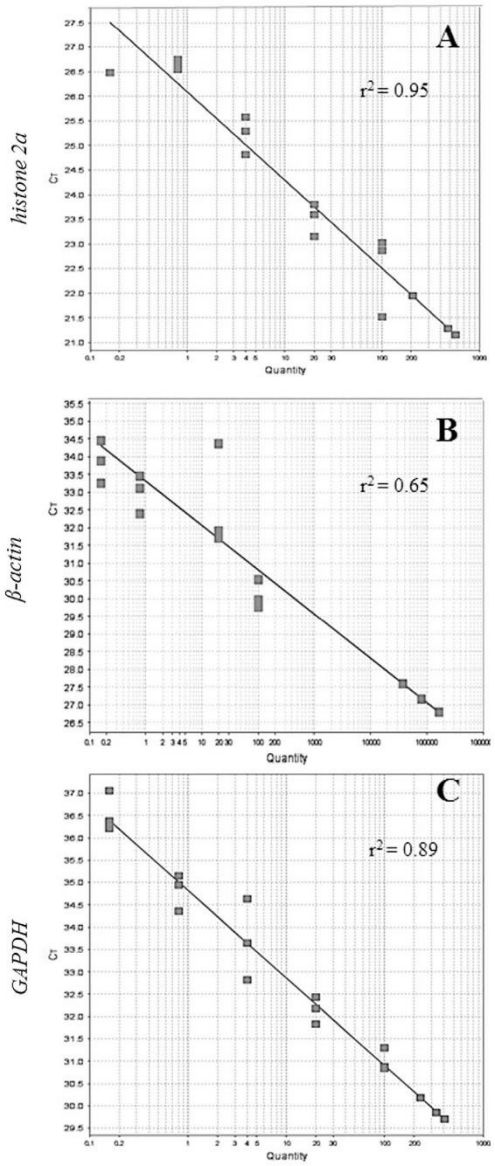

Fig. 1. Comparison of resulting qPCR standard curves generated by plotting the log of target DNA: A) histone 2a, B) β-actin and C) GAPDH versus threshold cycle (*Ct*). Serial dilutions of unexposed bovine embryos DNAs ranging 100 ng–0.16 ng were used to generate standard curves. Each point is an average of triplicate under independent amplifications.

differences were observed on embryos development after virus exposure with both strains. However, the number of oocytes that were cleaved into blastocytes was higher in the exposed oocytes in comparison to unexposed group (Table 2; $p = 0.0031$). There was no significant difference on the effects of exposure to different BoHV-5 strain, regarding to embryonic development, including the rate of oocytes developing into blastocysts. The quality of the exposed and unexposed embryos was similar, and both groups were rated as Code 1 (excellent or good). To confirm virus infection, BoHV-5 antigens were identified by indirect immunofluorescence assay (IFA) and PCR amplification in all exposed embryos (data not shown). No evidence of BoHV-5 infection was observed among the unexposed group. Thus, experimental infection of bovine embryos with BoHV-5 in this study appeared not to interfere with *in vitro* quality and production of embryos, as has been demonstrated before [13,14]. These results also showed that BoHV-5 strains (reference and wild-type) had no impact on the development *in vitro* of bovine embryos [13,14]. These observations contrast with previous findings that BoHV-1 exposure directly exerted influence on the fertilization process, supported by the observation of a strong decrease in the embryonic developmental rate when bovine IVF was performed in the presence of BoHV-1 [24]. Therefore, the potential of BoHV-5 to induce bovine reproductive disorders should not be underestimated, and a review of the sanitary measures required for international marketing of biological products should be considered [9]. In a recent report, attempts to detect BoHV-5 by molecular methods in bovine follicular fluid pools, both to BoHV-1 and BoHV-5 viruses were fruitless [23]. Currently used biological materials collected from cattle of unknown health status compromise the sanitary security IVP procedures [2]. In spite of the use the nested PCR to search for BoHV-1 and BoHV-5 in prenucleic acid procedure, no internal control was applied to validate the negative results, as usually applied on conventional PCR elsewhere [23].

The use of qPCR reagents and cycling parameters followed the manufacturer's instructions of SYBR Green. The primers designed for the present study (Table 1), and magnesium chloride were titrated in chequerboard assays to determine the most suitable concentrations. The qPCR read-out is given as the number of PCR cycles (cycle threshold *Ct*) necessary to achieve a given level of fluorescence. These were defined as the maximum fluorescence values combined with the earliest *Ct* values. The values were calculated using the ΔCt method ($\Delta Ct = Ct$ of the target $- Ct$ of the reference gene) [22].

Optimal design of the PCR primers is essential for accurate and specific quantification using qPCR. For the assay described herein, detection is based on the binding of the SYBR-Green dye into double stranded PCR products, which is a sequence independent process [22]. For all experiments described in this report, each pair of primers was designed following the manufacturer's instructions to amplify products from genes ranging from 81 to 122 bp [22]. In a recent study, a real-time 5′nuclease assay using specific probes to differentiate BoHV-1 and BoHV-5 was proposed [19]. However, the *GAPDH* gene was used to normalize the comparative analysis [24]. Due to the fact that BoHV-5 infection may interfere on apoptosis pathway in bovine embryos during maturation [14], the use of *GAPDH* gene as standard should be avoided [25]. GAPDH is an enzyme that catalyzes glycolysis breaking down this sugar for energy production and carbon molecules. In addition to this long established metabolic function, *GAPDH* has recently been implicated in several non-metabolic processes, including transcription activation and initiation of apoptosis [26,27]. Viral infection typically results in the perturbation of cellular processes that may trigger cell death via the mitochondrial pathway [28]. Successful replication of many viruses, therefore, depends on the ability of the virus to prevent apoptosis induced by the mitochondrial pathway [28]. In 2005, a study showed that activation of *GAPDH* initiates

apoptosis [27]. Recently, it has been demonstrated that some metabolic changes in oxidative stress and apoptosis pathways are changed in BoHV-5 exposed embryos [29]. Because the *GAPDH* gene is often stably and constitutively expressed in most tissues and cells, it is used as standard for many conventional and qPCRs assays applied on veterinary diagnosis [25]. However, different regulation of *GAPDH* under specific conditions has been reported and, this needs to be taken in consideration when normalizing standard curves [25–27].

The best results obtained by using *histone 2a* gene, in terms of correlation and specificity, candidates this gene as an option for *GAPDH* as a standard gene in such assays. Moreover, in DNA quantification in exposed and unexposed embryos, the best reference gene was the *histone 2a* based on the linear regression analysis with (Fig. 1). In biology, histones are highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes [30]. These proteins are among the most highly conserved proteins in eukaryotes, emphasizing their importance in the nuclear biology [30].

The sensitivity of the detection with SYBR-Green may be compromised by the formation of primer-dimers, lack of specificity of the primers, primer concentration and the formation of secondary structures in the PCR products [22]. All these factors may lead to generation of unexpected double-stranded DNA products, which would incorporate SYBR-Green and yield a fluorescent signal. The melting curves revealed primer-dimers for *histone 2a*, *β-actin* and *GAPDH* genes (Fig. 2A–C). The 5′nuclease assay using TaqMan probes would also be compromised by primer dimer formation, yet these products are not detected and are not expected to affect the amplification efficiency [22].

Many studies describing the conventional PCR methodology emphasize the importance of bovine *β-actin* reference gene [7,17]. In our study, *β-actin* presented the worst performance as a control gene (Fig. 1B). BoHV-5 infection has been implicated in reorganization of

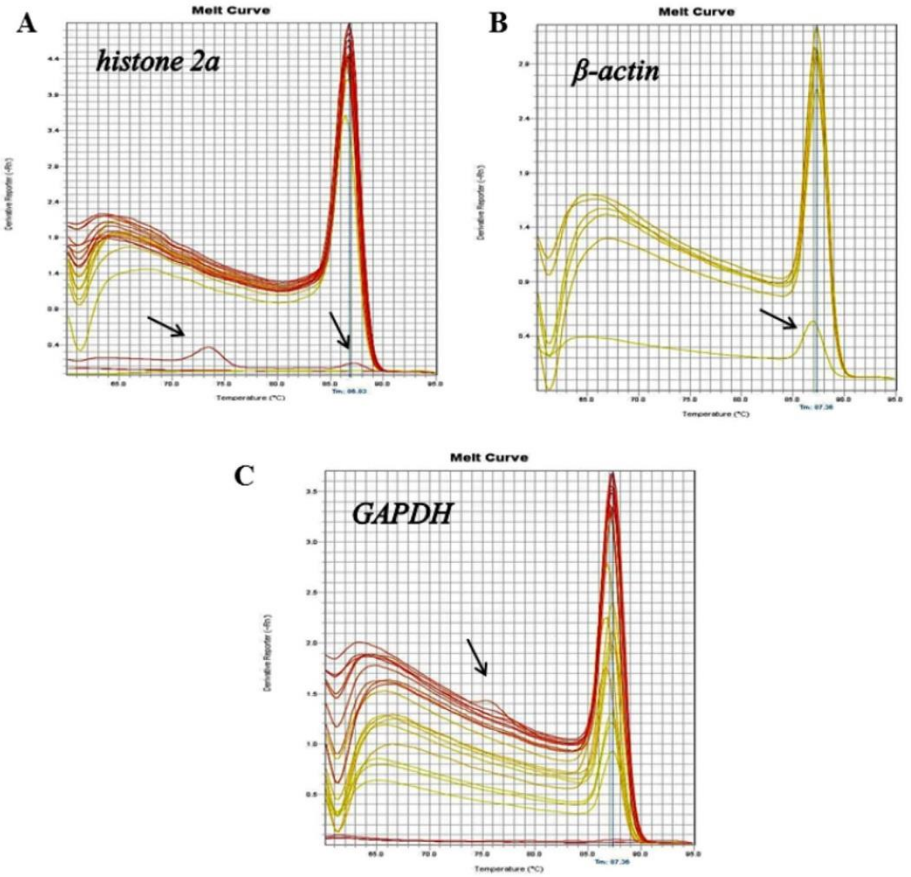

Fig. 2. Validation of qPCR primers by melt curve analysis. A) Single peak on the melt curve indicating a PCR product from the use of histone 2a primers; small unique peak (arrow) indicating a primer dimer; B) Two peaks inside the optimal melt temperature (arrow) indicating primer dimer during amplification for β-actin primers; C) Various peaks outside and inside the melting temperature for GADPH primers (arrows).

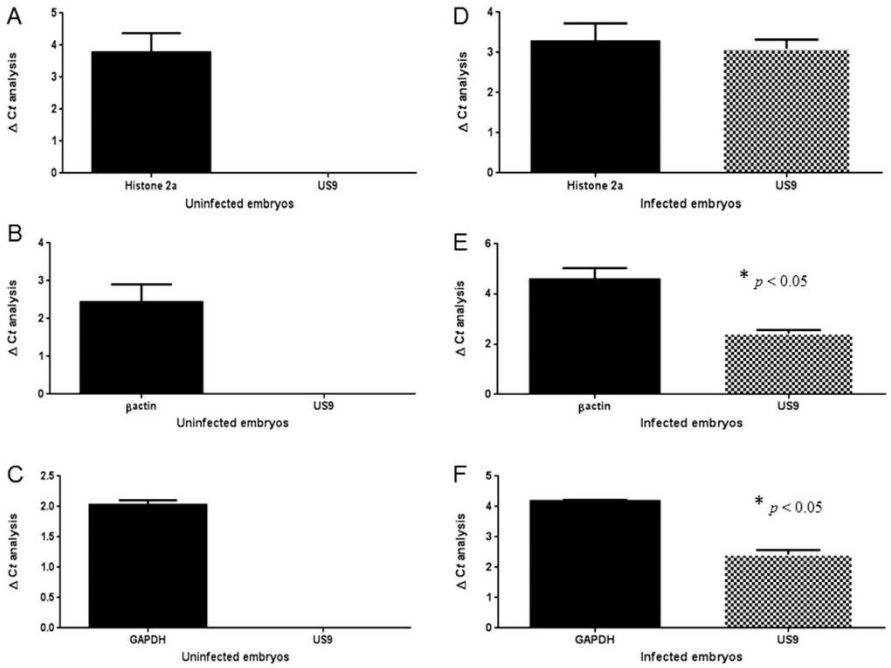

Fig. 3. Relative abundance (mean ± s.d) of histone 2a, β-actin, GAPDH and BoHV-5 US9 genes of *in vitro* produced bovine embryos. Bars generated by plotting the Δ*Ct* values (Δ*Ct* = *Ct* of the target−*Ct* of the reference gene). A, B and C corresponding to amplification of histone 2a, β-actin, GAPDH in relation to US9 on unexposed bovine embryos; D, E and F represents the efficiency of each control gene to normalize the US9 amplification on virus-exposed bovine embryos. Bars with * differ statistically within a group ($p < 0.05$).

cellular cytoskeleton due to syncytium formation on MDBK and bovine testicle cells [31]. The most desirable quality of a control gene would be the ability to not modify gene expression during the experimental procedures [31]. Therefore, if BoHV-5 infection does interfere on the expression of cytoskeleton filaments, the use of related genes as standards in qPCR should be avoided [22].

Recent reports have described the advantages of qPCR to test large number of samples in short periods of time, especially in artificial insemination (AI) centers. In this respect, the choice of 5′nuclease assay using the *GAPDH* as reference amplification target should be carefully reexamined both for semen and oocytes/and/or embryos. The reasons for this come from two recent reports describing that experimental exposed bovine semen and oocytes/embryos developed metabolic changes and initiated some pathways of apoptosis [29].

Regarding to US9 gene amplification, the *histone 2a gene* yielded the best performance in comparison to *GAPDH* and *β-actin* genes (Fig. 3). There was statistical difference among three reference genes ($p < 0.05$) in quantifying US9 expression. In fact, the US9 gene has been proven to be useful target in brain samples and also in experimental procedures using bovine semen and oocytes/embryos [12–14,16]. The US9 gene was generated to amplify only BoHV-5 sequences [17]. This mean an important issue in epidemiological studies since it has been documented the interspecific recombination between BoHV-1 and 5 under *in vitro* assay [32].

In summary, our findings may have important applications for the quality and safety of *in vitro* production of bovine embryos, since embryos exposed to BoHV-5 present development similar to unexposed, healthy embryos, leading to viral spread, potentially leading to low reproductive efficiency and reproductive failure. In this regard, the qPCR based SYBR-Green could be a valuable tool to attest that bovine embryos are free of BoHV-1, BoHV-5 and other bovine viruses as well.

Acknowledgments

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4. CONSIDERAÇÕES FINAIS

Os vírus bem-sucedidos na infecção se utilizam de múltiplos mecanismos de alterações das funções celulares do hospedeiro e as revertem em vantagens. Os Alphaherpesvirus desenvolveram estes mecanismos com objetivo de inibir a morte celular programada. Como consequência, células-alvo de infecções são protegidas da apoptose por CTL, por exemplo. O mecanismo evoluiu, provavelmente, de organismos unicelulares primitivos, nos quais as células infectadas por um vírus ativavam sua morte programada com o objetivo de proteger outros membros de sua população, de forma parecida àquela observada nos organismos multicelulares.

Uma das maneiras de elucidar a relação entre os BHVs e o hospedeiro, seria isolar e agrupar animais em situações e condições padronizadas para um teste fidedigno de infecção viral. Entretanto, questões éticas e de financiamento torna sua realização inviável.

Logo, a aplicação de sistemas *in-vitro* que simulem situações *in-vivo* se fazem necessárias para estudar esta interação multifacetada. De fato, as padronizações dos experimentos laboratoriais e o uso de populações celulares têm contribuído para a redução de uso de animais em laboratório, bem como proporcionam, dentro de seus limites, resultados que mimetizam a complexa relação entre hospedeiro e vírus.

Apesar das similaridades entre os genomas do BHV1 e do BHV5, as diferenças observadas nas regiões de menor conservação aparentemente refletem na interação dos vírus com as células do hospedeiro.

Quanto à apoptose, o aumento da viabilidade celular e consequentemente sua contribuição para a disseminação pode ser considerada estratégia do BHV5 no hospedeiro. Distintos mecanismos estão ligados a esta hipótese. Um destes mecanismos está relacionado ao envolvimento de genes, como o US3 e US9. Outro mecanismo está relacionado a família de proteínas Bax, que podem favorecer a viabilidade

de embriões, diferentemente do que se observa com o BHV1. Logo, células infectadas sobrevivem por tempo suficiente para suportar a replicação viral.

O BHV5 estabelece latência em neurônios sensoriais, em neurônios do gânglio trigeminal. No entanto, nossos resultados também demonstraram que determinadas cepas do BHV1 foram responsáveis por eliminar células neuronais *in-vitro*, mesmo que a cepa não tenha ligação com a meningoencefalite. Estes resultados sugerem que, assim como o BHV5 possui capacidade de infectar animais, mas não seja necessariamente causador de patologia, o mesmo pode acontecer com o BHV1. Ademais, outros fatores desconhecidos, além da implicação das glicoproteínas, possam estar envolvidos na capacidade de penetração e/ou de morte celular como os receptores Fas, TNF e DR3 (*death receptor 3*) e DR4.

Assim como na infecção por HSV1, nossos resultados demonstram que a resposta imunológica do hospedeiro frente ao BHV5 é predominantemente pró-inflamatória. As mesmas citocinas expressas nesse tipo de resposta podem ser responsáveis por lesar a barreira hematoencefálica, facilitando a entrada do vírus no CNS. Uma vez que a imunidade tem uma regulação multifacetada, estudos futuros são necessários para entender o perfil de citocinas expressas e sua consequência em relação ao BHV5 e as manifestações neurológicas.

Há de se considerar que o ambiente *in-vitro*, por não ser um modelo totalmente fiel ao que ocorre na natureza, pode favorecer este tipo de interação celular. Apesar dos resultados obtidos ajudarem a elucidar questões remanescentes sobre neurotropismo, neurovirulência e apoptose nos Alphaherpesvirus, estas diferenças podem estar relacionadas também àquelas observadas na patogenia e resposta imunológica do hospedeiro.