Short Communication

Bovine Herpesvirus 5 promotes mitochondrial dysfunction in cultured bovine monocyte-derived macrophages and not affect virus replication

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A B S T R A C T

Bovine alphaherpesvirus 1 (BHV1) and 5 (BHV5) are known to establish latent infections in sensory neurons of the trigeminal ganglion, yet leukocytes and tonsils have also been described as sites of latency in experimentally infected cattle. Little information is available on which immune cells are susceptible to BHV5 infection and how the infection may affect cell bioenergy. The aim of this study was to determine whether primary bovine monocyte-derived macrophages are susceptible to BHV5 infection and to evaluate parameters such as cell survival, virus replication and nitric oxide (NO) production. In addition, production of reactive oxygen species and mitochondrial damage were also analyzed. BHV5 infected cells underwent low rates of apoptosis but activated mitochondrial membrane depolarization and complex I. Additionally, production of high NO levels upon monocyte derived-macrophage infection did not interfere with the production of progeny virus. Overall, our findings revealed that primary cultures of bovine monocyte-derived macrophages support BHV5 replication *in vitro* and that mitochondrial dysfunction induced by infection apparently does not interfere with virus replication.

1. Introduction

Bovine alphaherpesvirus 1 (BHV1) and 5 (BHV5) are genetically and antigenically related, belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison et al., 2009; Davison, 2010). An important biological property of these viruses is the ability to establish latent infections, mainly in sensory nerve ganglia, but also in peripheral blood leukocytes (Del Médico Zajac et al., 2010; Favier et al., 2014; Pérez et al., 2005; Winkler et al., 2000). These immune cells, including phagocytic and other cells expressing pathogen recognition receptors (PRRs), can recognize pathogen-associated molecular patterns (PAMPs) (Griffin et al., 2010), activating macrophages, peripheral blood mononuclear and dendritic cells (Griffin et al., 2010).

Apoptosis is a major mechanism used by vertebrates to combat viral infections, thereby leading to cell death and pathogen elimination (Elmore, 2007). Apoptosis is triggered in two ways: intrinsic and extrinsic pathways (Zhou et al., 2017). Extrinsic pathway activation depends on a variety of external factors, such as cytokines, toxins, or ligand binding to the death receptor on the cell surface. The intrinsic apoptosis pathway is triggered by cell stress, such as the presence of

viral proteins, DNA damage or oxidative stress, leading to the activation of molecules on the mitochondrial membrane (El-Bacha and Da Poian, 2013; Zhou et al., 2017).

Mitochondria are directly implicated in several host-viral responses and represent organelles that participate in major early anti-viral immune responses through changes in their metabolism (Ohta and Nishiyama (2011); West et al., 2011). The association among viral infection, mitochondrial dysfunction and apoptosis has been investigated. Viral infection may interfere with mitochondria bioenergetics by affecting cellular respiratory function, and in some cases, viral proteins can be inserted in mitochondrial membranes leading to anti-and/or pro-apoptotic effects, directly affecting cell survival and/or death (Galluzzi et al., 2008; Ohta and Nishiyama (2011); West et al., 2011).

This study investigated the susceptibility of bovine macrophages to BHV 5 infection, by studying cell viability and cell growth kinetics upon *in vitro* infection. In addition, characterization of cell survival, nitric oxide and production of reactive oxygen species (ROS) associated with mitochondrial dysfunction were analyzed.

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2. Materials and methods

2.1. General information and animals

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA), Thermo Fisher Scientific (Carlsbad, CA, USA) and Applied Biosystems (Foster City, CA, USA). All culture dishes and flasks were purchased from BD Falcon (Bedford, MA, USA) unless otherwise specified. Five healthy Nelore cows (*Bos indicus*) aged 5 years were used for blood collection. Work involving animals was conducted in accordance with the recommendations in the institutional guidelines and approved animal care and use committee (FMVA – 154829-8) protocols at São Paulo State University, Brazil. The other experiments were carried out in accordance with the COBEA approval. All animals were maintained under the same environmental conditions and nutritional regimes. Blood sampling was performed by two veterinarians and tested for BHV1 and BVH5, and BVD pathogens by molecular assay. A total of four hundred ml of blood collected from jugular vein of each animal were stored in glass bottle with sodium citrate as anticoagulant. The monocytes isolation was performed as described below.

2.2. Monocytes isolation and monocyte-derived macrophage generation

Bovine peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Percoll density gradient as described (Bannantine et al., 2015). Briefly, blood was layered onto 50 ml conical tubes containing Histopaque 1077 (Sigma-Aldrich) following density gradient centrifugation ($500 \times g$ for 20 min) at room temperature and PBMCs were collected. Thereafter, PBMCs were washed twice, stored diluted in sterile phosphate buffered saline (PBS; Invitrogen) and submitted to CD14+ cells separation following the procedure described below.

CD14+ cells were extracted by positive selection of monocyte that react using mouse anti-human CD14 antibody according to the manufacturers' instructions (Miltenyi Biotec Ltd., San Diego, USA). Purity of selected cells was verified by flow cytometry by sorting positively selected cells with Cy3.3-labeled conjugated anti-mouse IgG2a and mouse anti-bovine fluorescein-labeled MHC II antibody (Bio-Rad laboratories, Inc., Hercules, CA, USA) according to manufacturer's instructions. For monocyte derived-macrophage generation, purified monocytes were seeded at 2×10^6 per well in 12-well tissue culture plates containing Dulbecco's Modified Eagle medium with high glucose (Invitrogen) with 10% heat inactivated fetal calf serum (Sigma-Aldrich), gentamicin (5 mg/ml; Sigma Aldrich), 100 ng/ml GM-CSF (granulocyte-macrophage colony-stimulating factor from mouse; Sigma-Aldrich) and 1 mM β-mercaptoethanol (Sigma-Aldrich). The phenotype of monocytes that differentiate into early stage non-adherent derived-macrophages phenotype was characterized by flow cytometry analysis searching CD14+ and MHCII + positive cells (Cardoso et al., 2015). Besides, identity of monocytes derived-macrophages was also verify by phase contrast microscopy and immunocytochemistry using Cy3.3-labeled conjugated anti-mouse IgG2a and general procedure was followed previous study (Cardoso et al., 2015).

2.3. BHV5 Infection, antigen detection, virus titration and partial viral DNA amplification

After three consecutive passages, confluent monocyte-derived macrophages were used in all described experiments. BHV5 strain used for monocyte derived-macrophage infection BHV5 SV507/99, was supplied by Prof. Eduardo Furtado Flores, UFSM, Santa Maria, RS, Brazil. Cells were cultured to 80% confluence in Lab-Tek® chambers slides (BD Falcon™) and inoculated with 100 μl of BHV suspension ($10^{3.2}$ TCID$_{50}$/ml), corresponding to a multiplicity of infection (m.o.i.) of approximately 1. Viral suspensions were allowed to adsorb for 1 h at 38.5 °C, followed by inoculum removal and addition of culture medium.

Uninfected cells were used as control and non-adherent cells and media were harvested at 24, 48 and 72 h p.i, by treatment with 0.25% trypsin (Sigma-Aldrich) for all experiments performed in triplicate. The effectiveness of BHV5 replication was assessed by immunocytochemistry, virus recovery and viral genome amplification.

First, immunocytochemistry was performed with BHV5 monoclonal antibody 2 F9 produced against *g*C BHV5 structural protein as described previously (Varela et al., 2010; Cardoso et al., 2015). The cells were fixed with 4% of paraformaldehyde, incubated with primary antibody at 1:50 and washed three times with PBS plus 0.1% Triton X-100. Next, a 1:50 dilution of the secondary antibody was added to100 μl of cell suspension, and was then incubated at 37 °C for 30 min. In order to quantify BHV5 positive monocytes derived-macrophages the flow cytometer analysis was performed according to protocol described previously for the same purpose (Cardoso et al., 2015). The data were expressed as media (Δ) of variation (%) mean ± standard deviation (sd) according to Attune™ acoustic cytometer software (Applied Biosystem).

For nuclear staining, 1 mg/ml of DAPI (4′-6-diamino-2-phenylindole; Sigma-Aldrich) was diluted in Fluormount™ aqueous medium and loaded onto samples for 15 min. 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. Uninfected cells underwent the same staining procedure and served as controls.

The growth curve was assayed by infection of monocyte derived-macrophage monolayer confluence at 80% and multiplicity of infection of 1. Virus adsorption was allowed for 90 min at 38.5 °C, then the inoculum was removed and fresh medium was added. After incubation at 24, 48 and 72 h p.i. supernatant and non-adherent cells were harvested. The BHV5 recovery was performed by infecting MDBK cells as described previously (Cardoso et al., 2015). All experiments were repeated three times and infectious titres were calculated according to the Spearmann-Kärber method described before (Cardoso et al., 2015).

Upon harvesting at 24, 48 and 72 h p.i., total DNA from infected and non-infected monocyte derived-macrophage cultures was extracted using DNAzol ™ according to manufacturer's instructions (Invitrogen). An average of 100 ng of genomic DNA was used for qPCR as described previously (Silva-Frade et al., 2014). The primers, respective probes and all procedures were followed as described previously (Diallo et al., 2011; Silva-Frade et al., 2014). The reaction was carried out and analyzed by the software on a StepOnePlus™ real time instrument (Applied Biosystems). The expression of the housekeeping Histone 2a gene (*H2 A*) was included for normalization (Cardoso et al., 2016a). The viral DNA level at corresponding time point in infected and uninfected cells was quantified in comparison to the housekeeping gene. The comparative delta-delta C*t* method was used to analyze the results.

2.4. Proliferation and viability MTT assay

Cell proliferation/viability analysis was performed using an *In Cell Proliferation* Assay® Kit (Sigma-Aldrich), an MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]– colorimetric based assay. Infected and non-infected monocyte derived-macrophage cells and respective medium were harvested at 24, 48 and 72 h post-infection (p.i.) and 2 ml of MTT (tetrazolium salts) was added following the manufacturer's recommendations (Sigma-Aldrich). Absorbance was measured at 600 nm with a Biophotometer (Eppendorf®, Hamburg, Germany). All reported values are means of triplicate samples.

2.5. Determination of nitric oxide production and apoptosis

After 24, 48 and 72 h of BHV5 infection, nitrite (i.e., the stable by-product of nitric oxide generated by phagocytes) was measured in all cell culture supernatants according to Nitrite/Nitrate assay kit (Sigma-
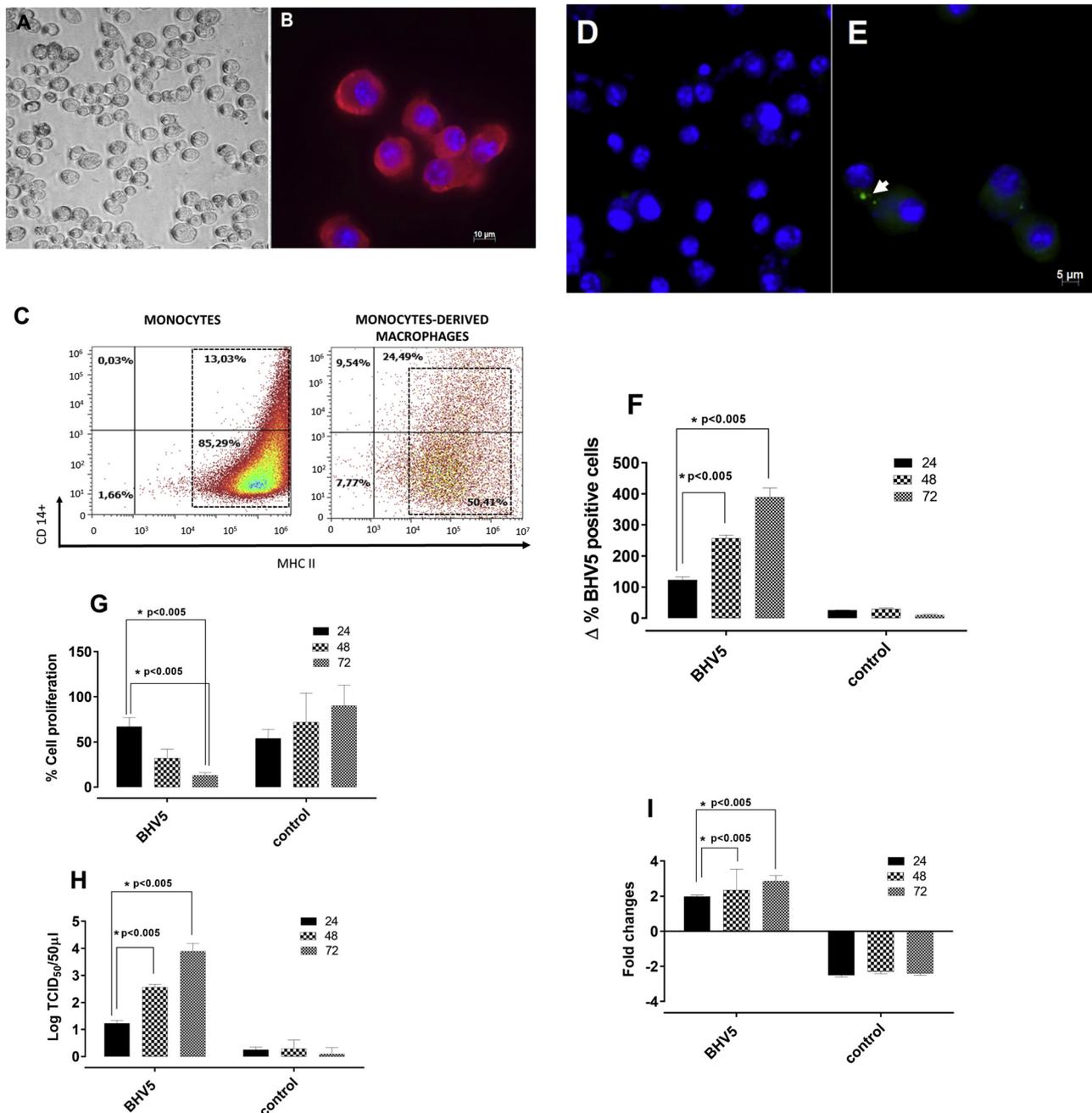
Fig. 1. A) Bovine monocyte derived-macrophage culture observed under phase contrast microscopy; B) Immunofluorescence assay illustrating positivity corresponding to CD14-Cy3.3 in monocyte derived-macrophage C) Flow cytometer analysis revealing 74.90% of positive monocyte derived-macrophages after culture under differentiation media in the early stage of adherence considered non-adherent cells), x-axis: MHC II + cells (major histocompatibility complex type II) and y-axis: CD 14 + cells; data were calculated as variation mean of positive fluorescent signals (Δ); D and E) Control and BHV5 infected cells under immunofluorescence assay to detect viral *g*C antigen, respectively; F) Percentage of BHV5 infected cells showing an increase during times after infection obtained in flow cytometer analysis; G, H and I) Loss of cell proliferation, increase of BHV 5 titres and genome transcription during infection, respectively.

Aldrich). Brief, fifty microliters of supernatant was mixed with 200 μL of Griess reagent (1% sulfanilamide, 0.1% napthylethylenedamine dihydrochloride, and 2.5% H_3PO_4) and incubated at 25 °C for 10 min. Absorbance was determined at 540 nm, converted to μM concentrations and results compared to a standard curve.

To evaluate apoptosis, the same macrophage culture conditions were used. Flow cytometric analysis of apoptosis/necroptosis and ROS production were acquired using an Attune acoustic focusing cytometer system at 24, 48, and 72 h after BHV1 and BHV5 infection (Applied Biosystems). Apoptosis/necroptosis was measured using a double staining method APO-BrdU TUNEL Assay Kit (Molecular Probes,

Eugene, OR, USA) according to manufacturer's instructions. The differentiation of early apoptotic, secondary necrotic, necroptotic, and viable cells was made according to their phenotype: BrdU +/PI − cells were considered early apoptotic, BrdU −/PI + were considered necroptotic, BrdU +/PI + were considered secondary necrotic, and BrdU −/PI − were considered viable cells. The results are expressed as Δ % of each cell phenotype.

2.6. Mitochondrial membrane potential, complex I and ROS evaluation

Cell mitochondria were isolated by procedure based on

Table 1
Proportion of BrdU/PI labeled monocytes derived-macrophage cells after 24, 48 and 72 h of BHV5 infection.

	Dead (% ± sd)	Secondary necrotic (% ± sd)	Early apoptotic (% ± sd)	Viable (% ± sd)
	(BrdU-/PI+)	(BrdU+/PI+)	(BrdU++/PI-)	(BrdU-/PI-)
24 h	1.7 ± 3.8	3 ± 1.0	6.6 ± 6.0	88.7 ± 1.3
48 h	1.2 ± 1.5	1.3 ± 2.9	5.8 ± 4.7	91.7 ± 3.6
72 h	6.8 ± 4.9	9 ± 0.9	7.4 ± 3.9	76.8 ± 7.9
control	5.6 ± 1.2	0.5 ± 0.1	5.0 ± 0.6	88.9 ± 7.8
staurosporine	70 ± 9.7	16 ± 2.3	10.7 ± 2.7	3.3 ± 1.7

mitochondria isolation kit for profiling culture cells MITOISO2 (Sigma-Aldrich).

To perform the mitochondrial membrane potential assay, 2×10^6 infected and mock infected cells were incubated with 10 μg/ml of JC-1 (5,5`,6,6``-tetrachloro-1,1`,3,3`-tetrathylbenzimidazolyl-carbocyamine iodide; Molecular Probes,) for 10 min at 37 °C and analyzed by acoustic flow cytometry performed in the dark as described by a previous study (Ferrarezi et al., 2013). The staurosporine (Sigma-Aldrich) was used as positive control to mitochondrial membrane depolarization as described previously (Cardoso et al., 2015). Mitochondrial complex I (NADH dehydrogenase) activity production by macrophages infected by BHV5 at 24, 48 and 72 h was measured as previously described (Da Silva et al., 2018). Briefly, complex I activity was determined in BHV5 infected-macrophage lysates in 24-well plates. In the presence of NADH, electrons are transferred from complex III to *cytochrome c* resulting in an increase in absorbance at 550 nm. This reaction was blocked by rotenone, a specific inhibitor of complex I activity. The activity of complex I was determined by comparison of the curve's slope before and after the addition of rotenone.

2.7. ROS evaluation, ATP levels and oxygen consumption

ROS levels were determined using the Amplex Red Hydrogen peroxide/Peroxidase kit (Invitrogen®). In the presence of horseradish peroxidase, Amplex red reagent reacts with hydrogen peroxide released by the BHV5 infected cells. The product of this reaction, resofurin, was detected by measuring absorbance at 560 nm after 2 h of incubation of the 96-well plate at 37 °C. Levels of ATP were determined using the firefly luciferin– luciferase assay system according to ATP determination Kit (Molecular Probes™) following the manufacturer's recommendation. Bioluminescence was measured using Lucetta™ Luminometer (Lonza Inc, Alpharetta, GA, USA). Oxygen consumption was measured using 5 mM succinate (+ 5 nM rotenone) or 5 mM pyruvate + 5 mM malate as respiratory substrates in the absence (state-4 respiration) or presence (state-3 respiration) of 400 nmol ADP. CCCP (m-chlorophenyldrazone) was used at 1 μM of as an uncoupler.

2.8. 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).

3. Results

3.1. Cell survival and virus replication

The morphology of monocyte derived-macrophage cell culture visualized under inverted phase-contrast microscopy revealed rounded cells as a unique cell type after microbeads isolation (Fig. 1A). Immunocytochemistry labelling CD14-Cy3.3 on fixed cells showed the monocytes derived-macrophages phenotype in of the cells undergoing the first passage (Fig. 1B). In addition, flow cytometer analysis

demonstrated that 98.32% of the cells were considered positive for CD14 + at the first passage *in vitro* (Fig. 1C). After induction, 74.90% of the cells were considered monocyte derived-macrophage phenotype and express CD14 + and MHCII (Fig. 1C). After BHV5 infection, fluorescence in the cytoplasm of infected cells, in comparison to uninfected cells, was observed at 72 h (Fig. 1D and E, respectively). The percentage of infected cells measured by flow cytometer demonstrated an increase of positive fluorescent signals according to subsequent periods after infection (Fig. 1F). However, the viability of infected cells decreased at the same times p.i. (Fig. 1G), contrasting with BHV5 titres and partial genome quantification, that increased progressively reflecting production of progeny virus (Fig. 1H and I, respectively). These results indicated productive replication of BHV5 in bovine macrophages *in vitro*, leading to loss of cell viability. Decrease of cell viability, however, seemed not to interfere with the virus replicative cycle.

3.2. Macrophage apoptosis, NO production and mitochondrial dysfunction

The APO-BrdU TUNEL Assay was applied in attempts to characterize the effects of BHV5 infection in macrophage survival and death. The results in Table 1 shows that the number of early apoptotic cells did not differ statistically at different post-infection periods. On the other hand, at 72 h p.i, the percentage of dead and secondary necrotic cell increased in comparison with uninfected cells used as controls, but lower than monocytes derived-macrophages exposed to staurosporine used as positive control (Table 1). Therefore, the set of experiments described next were performed to evaluate alterations on mitochondrial function.

The mitochondrial membrane depolarization measured by detection of JC-1 complex revealed an increase, according to the progression of infection, similar to the results observed by the use of staurosporine (Fig. 2A). Complex I activity was intense at all three post-infection points (Fig. 2B). The same phenomenon was observed when ROS and ATP content were quantified (Fig. 2C and D), contrasting with oxygen consumption, which decreased from 24 to 72 h p.i. (Fig. 2E). Macrophage activity was measured by NO production, which showed an increase according to the time post-infection (Fig. 2F). These experiments confirm that BHV5 replication induces macrophage early, secondary and necrotic apoptosis damage at 72 h p.i. followed by NO production, affecting probably mitochondrial membrane depolarization, activation of complex 1 from respiratory chain and consumption of ATP followed by ROS release.

4. Discussion

Tonsils are lympho-epithelial immunocompetent tissues strategically located in areas surrounding the main local of penetration and primary replication of BHV1 and 5 in natural infections (Pérez et al., 2005; Favier et al., 2014). However, little information is available about which immune cells are targets for virus infection. Macrophages are among the most important antigen presenting cells and participate in the early, primary response against virus infection. Bomac cells, a bovine transformed macrophage lineage have been used as an *in vitro* model to study host-pathogen interactions (Bürgi et al., 2018). However, primary bovine macrophages present an obvious advantage for
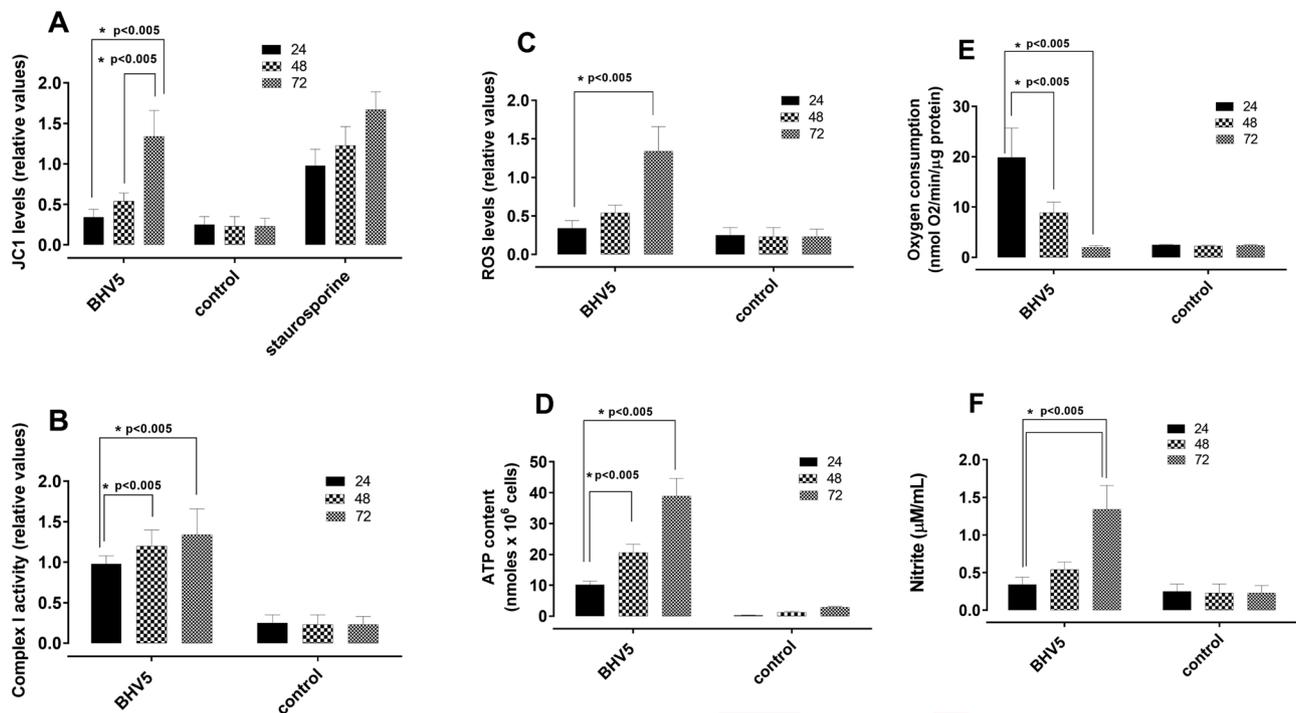
Fig. 2. Mitochondrial bioenergy and function analysis of monocytes derived-macrophages infected by BHV5. A) Depolarization of mitochondrial membrane after virus infection; B) Activation of mitochondrial complex I; C and D) Increase of ROS levels and ATP content during viral infection; E and F) Oxygen consumption increase in contrast to NO after BHV5 infection.

such studies since they probably better reproduce *in vitro* situation, are more convenient to work with, display less variability and are not genetically manipulated (Bürgi et al., 2018). Thus, primary bovine monocytes derived-macrophages were chosen to evaluate BHV5 replication and interference on cell metabolism.

Apoptosis is a major way of programmed cell death following virus infection and may be elicited by virus infection with negative and positive influence on replication (Zhou et al., 2017). Previously, it has been demonstrated that BHV5 can induce different levels of apoptosis depending on the cell type (Brenner et al., 2012; Silva-Frade et al., 2014; Cardoso et al., 2015, 2016b). The present study demonstrates that BHV5 is able to productively infect *in vitro* cultured bovine monocyte derived-macrophages. In addition, the percentage of dead, secondary necrotic and early apoptotic macrophages were less than ≈ 10%in comparison with 76.8% of viable cells. These findings suggest that BHV5 infection leads to a block or delay in apoptosis in bovine monocyte derived-macrophages, probably as a means to produce sufficient progeny and to establish successful viral replication. Similar results were found when *in vitro* produced bovine embryos were exposed to BHV5 (Brenner et al., 2012; Silva-Frade et al., 2014). However, when neuron-like cells were infected by BHV5, apoptosis was evident during infection (Cardoso et al., 2015).

Regarding to mitochondrial function, our results showed that membrane depolarization was synchronous with BHV5 replication and NO and ROS production. In addition, ROS is mainly originated from NADPH oxidases and mitochondria. Overproduced cellular ROS or oxidative stress are factors that influence disease outcome. In this respect, BHV1 replication in MDBK (Madin-Darby bovine kidney) cells resulted in an increased in ROS production, which seems to be crucial for virus replication (Zhu et al., 2016). Previously, high ROS production was also observed in neuronal and glial-derived tumor cells infected by BHV5 (Cardoso et al., 2016b).

It is conceivable that mitochondria are potential targets for some viruses that are able to induce damage to complex I (NADH dehydrogenase). To our knowledge, our data are the first to demonstrate that BHV5 infection of immune cells activates mitochondrial membrane depolarization, complex I activity, ROS levels and ATP content. One of the main functions of mitochondria is to synthetize ATP from dietary sugar or amino acids by ATP synthase in the electron transport chains (ETC). The ATP produced relies upon an intact and efficient ETC, the activity of four key proteins that make up the redox carrier, named complex I-IV, and the surface area of cristae (El-Bacha and Poian, 2013). Membrane potential ($\Delta\Psi$m) is also critical for the generation of ATP and loss of $\Delta\Psi$m leads to ATP depletion within the cell thereby contributing to cell death.

The results obtained herein using mitochondria energized with pyruvate (electron donors to complex I) showed that BHV5 replication inhibits state-3 respiration, which involves mitochondria, ADP, and a respiratory substrate; and the speed of ADP phosphorylation is the limiting factor of the process. The activation observed in the complex I indicates that BHV5 replication has a direct action on the electron transport chain in infected macrophage cells; however, it cannot be ruled out that virus replication events interacts with the oxidative phosphorylation system (F_0F_1 ATPase and/or ADP/ATP translocator). Complex I (NADH ubiquinone oxidoreductase) accepts electrons from reduced nicotinamide adenine dinucleotide (NADH), while complex II (succinate dehydrogenase) accepts electrons from reduced Flavin adenine dinucleotide ($FADH_2$). Complex III (ubiquinol-cytochrome c oxidoreductase) is responsible for electron transfer between ubiquinone to *cytochrome c*. Finally, complex IV (cytochrome oxidase) promotes oxidation of *cytochrome c* and reduction of molecular oxygen to water. The energy derived from the electrons transfer is related to complex I, II and IV that produce cell energy. Based on this, it may be speculated that BHV5 infection may impair the electron transfer by high activating complex I, producing reactive oxygen species in excess but not activating apoptosis and/or necrosis at high levels. Further studies should be performed to investigate the interaction by macrophage mitochondria regulation and innate and inflammatory network during BHV 5 infection.

5. Conclusion

In summary, our results demonstrate that BHV5 replication in cultured bovine macrophages apparently does not alter the tested parameters in comparison to untreated cell with staurosporine. The percentage of viable cells was close to the controls in spite of high levels of mitochondrial damage, especially activation of complex I. Indeed, the results obtained in this *in vitro* study support the implication of BHV5 infection on mitochondrial metabolism and the ability of the virus to maintain productive replication during the process.

Conflict of interest statement

All authors declare no conflicts of interests.

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