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ORIGINAL ARTICLE

2,3,7,8-Tetrachlorodibenzo-p-dioxin influences bovine herpesvirus 1 replication through upregulation of SIRT3 and cytoskeletal reorganization

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Abstract Infection of kidney cells (MDBK) with Bovine Herpesvirus 1 (BoHV-1) is affected by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which accelerates BoHV-1-induced apoptosis and increases virus replication. Herein, to elucidate the mechanism through TCDD modifies BoHV-1 infection, we analyzed the modulation of a members of Sirtuin proteins family in MDBK cells. We found that mitochondrial SIRT3 was upregulated during infection. This change was accompanied by cytoskeletal rearrangements and cell extensions. All these trends were drastically modified by TCDD. We hypothesize that, taken together, these results might further clarify the processes responsible for the action of TCDD on the BoHV-1 replication, resulting in enhanced virus production.

Keywords TCDD · BoHV-1 · SIRT3 · Cytoskeletal reorganization

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Abbreviations

TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
BoHV-1	Bovine herpesvirus 1
MDBK cells	Madin-Darby bovine kidney cells

Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), known as dioxin, is a toxic and persistent environmental contaminant. TCDD may provoke a broad range of toxic effect, both in humans and in animals, including chloracne, hepatotoxicity, thymic atrophy, reproductive toxicity, teratogenesis, and the International Agency for Research on Cancer has been classified TCDD as carcinogen since 1997. In addition, dioxin has the potential to disrupt multiple endocrine pathways and to provoke immunosuppression and increased susceptibility to infectious agents (Mandal 2005; Fiorito et al. 2017a). Indeed, it has been described that dioxin decreases host resistance to several viruses, both in vivo and in vitro. In particular, dioxin enhances mortality in mice infected respectively with herpes simplex II virus (Clark et al. 1983), human coxsackievirus B3 (Funseth and Ilbäck 1994; Funseth et al. 2000, 2002), and with different subtypes of influenza A viruses (House et al. 1990; Burleson et al. 1996; Warren et al. 2000; Vorderstrasse et al. 2003). Furthermore, TCDD increases virus replication in cells infected with human immunodeficiency virus-1 (HIV-1) (in MT-4 or U1cells) (Pokrovsky et al. 1991; Gollapudi et al. 1996) and cytomegalovirus (CMV) (in MRC-5 cells) (Murayama et al. 2002).

Bovine Herpesvirus 1 (BoHV-1), a member of the alpha-herpesvirinae subfamily, is an important cattle pathogen which may provoke infectious bovine rhinotracheitis (IBR), genital disorders, conjunctivitis and abortions. Moreover, the virus induces immunosuppression

that could render the animals more susceptible to secondary bacterial infections, as pneumonia, and occasionally to death (Jones 2003).

In experimental conditions, such as in permissive Madin-Darby bovine kidney (MDBK), BoHV-1 induces cell death apoptosis (Devireddy and Jones 1999; Fiorito et al. 2008b), which is significantly anticipated by TCDD (Fiorito et al. 2008b), through a down-regulation of telomerase activity (Fiorito et al. 2014a). All changes above reported may contribute to determine the increase in virus replication due to TCDD exposure (Fiorito et al. 2008b, 2014a, 2017a).

Recently, high levels of TCDD were detected in dairy products from farms in Campania region (Italy) (Diletti et al. 2003; Santelli et al. 2006), where BoHV-1 is widespread (2004/558/CE; Ackermann and Engels 2006; Raaperi et al. 2014). An extraordinary plan of official control was carried out to monitor dioxins levels in cow's and buffalo's milk from farms in Campania region, and the geo-referencing analysis allowed to individuate a restricted area of the region where was located the majority of the non-compliant farms (Esposito et al. 2009, 2010). To verify the possibilities of the use of data obtained in vitro to in vivo conditions, we performed an epidemiological analysis on the distribution of virus in the above monitored farms. We collected serum and plasma samples to detect antibodies for IBR from cattle raised on those farms, by using IBR-gB and IBR-gE E.L.I.S.A. kit, which represents the test procedure of choice in many European IBR programs. And we found a significant prevalence of IBR on samples collected from farms in contaminated areas, compared to samples collected in uncontaminated areas (Fiorito et al. 2015).

Sirtuin proteins (SIRTs) are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases identified as regulatory molecules which modulate the organism life span in many species. Particularly, in mammals, there are seven homologues of Situins, SIRT1-7, that are involved in the control of critical cellular processes such as differentiation, proliferation, metabolism and apoptosis (Saunders and Verdin 2007). And, in a recent study, we showed that sirtuins like SIRT3 and SIRT4 are involved in canine coronavirus type II-induced apoptosis (Marfè et al. 2011).

Viruses have developed a multiplicity of interactions with host cell to facilitate phases as viral replication, persistence and spread. The actin cytoskeleton of the host cell can be involved in several of these interactions. Following uptake in host cells, herpesviruses interact with actin when they enter into the cytoplasm, during replication and assembly in the nucleus, maturation and egress (Favoreel et al. 2007). In addition, cytoskeletal rearrangements and cell extensions, induced by the US3 kinase, a conserved viral protein among the Alphaherpesvirinae, are associated with enhanced spread of the pseudorabies virus, an alphaherpesvirus (Favoreel et al. 2005).

Herein, to better characterize the influence of TCDD on BoHV-1 infection, we analyzed the regulation of SIRT3, as well as the cytoskeleton reorganization of bovine kidney cells, when virus induced-apoptosis occurred in cells exposed to TCDD.

Materials and methods

Cell cultures, virus infection and TCDD exposure

MDBK cells (American Type Culture Collection, CCL22) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 2% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin, 0.2% sodium pyruvate and 0.1% tylosin, a macrolide-class antibiotic. Cells were maintained in an incubator at 37 °C (in 5% CO_2/95% air). This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus by real time PCR analysis (Cordero Camacho et al. 2011). The BoHV-1 Cooper strain was used throughout the study. Virus stocks were routinely grown on MDBK cells and were also used for determination of virus titers (Fiorito et al. 2008a, 2013). We used 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), 10 µg/mL in toluene (Supelco, 48,599). TCDD was initially diluted to give a 10,000 pg/mL stock solution by mixing with DMEM. This stock solution was then diluted to give working solutions of 0.01, 1 and 100 pg/mL in DMEM (from $3{,}1 \times 10^{-14}$ to $3{,}1 \times 10^{-10}$ M), which were added to cultures, according to Fiorito et al. (2008a,b,2010, 2011, 2013, 2014a, 2014b, Santamaria et al. 2011). In preliminary studies, we treated control cells (MDBK) with a toluene/DMEM mix at the same concentrations used to dilute the TCDD stock solution in toluene at working solutions of 0.01, 1 and 100 pg/mL in DMEM. In our experimental model, no significant differences were detected in cell viability, as well as in cellular morphological features between MDBK treated with DMEM or toluene in DMEM at 0.01, 1 and 100 pg/mL (data not shown). All other chemicals were of the highest commercially available purity.

MDBK cells, at confluency, were washed with DMEM and then infected or not with BoHV-1, at multiplicity of infection (MOI) of 5, at the same time, in the presence or not of different concentrations of TCDD (0.01, 1 and 100 pg/mL). After 1 h of adsorption at 37 °C, the cells were incubated for 4, 8, 12, 24, 36 and 48 h post infection (p.i.) and then processed. The virus was present in the culture media throughout the course of the experiment.

Cell viability

Cell viability was evaluated by MTT test, as previously described by us (Fiorito et al. 2008b, 2011, 2013, 2014a).

Data are calculated as a percentage of the control, and results are the mean ± SEM of four independent experiments performed in duplicate.

RNA isolation and northern blot analysis

Total RNAs from infected cells, exposed or not to TCDD, were obtained by using Tri Reagent (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany), as previously described (Marfè et al. 2011). Aliquots of RNA were electrophoresed on 1% agarose formaldehyde gels and subsequently blotted onto nylon membranes (Hybond N, Amersham, Braunschweig, Germany). The membrane was then UV crosslinked, and hybridized to ^{32}P-labeled probe. The relative amount of mRNA level was quantified throughout Gel-Doc Phosphorimanger and Quantity One software (Bio-Rad) and normalized by the band intensity of β-actin.

Protein extraction and Western blot analysis

At 4, 8, 12, 24, 36 or 48 h post infection, cells, exposed or not to TCDD, were washed twice with PBS and removed from the flask by treatment with trypsin–EDTA solution. Then cells were mixed with cells previously collected by centrifugation in supernatant from the same flask and re-suspended at an adequate concentration in PBS. The pellets, obtained by centrifugation, were stored at -20 °C. Cells were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 1,4000 rpm x 10 min. Protein concentrations were estimated by an assay (Bio-Rad) and boiled in Laemmli buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue] for 5 min before electrophoresis. Proteins were subjected to SDS–PAGE (12.5% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA) (Fiorito et al. 2017b; Liguori et al. 2017); complete transfer was assessed using pre-stained protein standards (Bio-Rad, Hercules, CA). After blocking with Tris-buffered saline-BSA [25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA], the membrane was incubated with the primary antibodies. The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: anti-SIRT3 (dilution 1:1,000) (Cell Signalling Technology, Inc) and anti-ß-actin (Sigma) (1:7,500); (β-tubulin was also used as loading control for western blot in experiments on the influence of TCDD on cellular and viral proteins during BoHV-1 infection. The levels of β-actin and/or β-tubulin were unchanged between control cells and TCDD treated cells over time). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000) (at room temperature), and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK). The relative amount of protein expression was quantified using Gel-Doc phosphorimanger and Quantity One software (Bio-Rad) and normalized by the band intensity of β-actin.

Staining of cell cytoskeleton by fluorescent phalloidin solution

At 4, 8, 12, 24, 36 and 48 h p.i., actin filaments were stained by incubating the cells with Phalloidin, Fluorescein Isothiocyanate labeled (Sigma, Milan, Italy), as previously described by Wulf et al. (1979). Briefly: cells grown on glass coverslips were rinsed in phosphate-buffered saline (PBS) (pH 7.4), fixed for 5 min in 3.7% formaldehyde in PBS at room temperature and washed extensively in PBS. Cells were dehydrated with acetone, permeabilized with 0.1% TRITON X-100 in PBS, and washed again in PBS. Then, cells were stained with a 50 mg/mL fluorescent phalloidin conjugate solution in PBS for 40 min at room temperature and washed with PBS and observed under UV with a fluorescence microscope.

Virus production assay

MDBK cells, at confluence, were infected with BoHV-1 at MOI 5, in the presence or not of different concentrations of TCDD (0.01, 1 or 100 pg/mL). For the virus production assay, we analyzed both viral cytopathic effects (CPE) and virus titration. At 4, 8, 12, 24, 36 or 48 h p.i., all groups were observed under light microscope to evaluate CPE, represented by ample syncytia formation along with elimination of the cellular sheets.

For virus titration, at 4, 24 or 48 h p.i., cell extracts, obtained by three cycles of freezing and thawing, were collected and stored in aliquots at -80 °C. Virus titers were assayed by $TCID_{50}$ method according to Reed and Muench (1938).

Statistical analysis

Results Data are presented as mean ± S.E.M. One-way ANOVA with Tukey's post-test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). P value < 0.05 was considered statistically significant.

Results

We confirmed that TCDD (0.01, 1 or 100 pg/mL) induces a significant time and dose dependent viability decrease of BoHV-1 infected cells (Fig. 1a). In particular, after 8 h p.i., we observed a significant ($p < 0.05$) reduction in cell viability in the presence of the highest dose of dioxin (100 pg/mL). From 12 h to the end of infection, TCDD drastically and significantly ($p < 0.001$) increased cell death of infected groups in a dose-dependent manner (Devireddy and Jones 1999; Fiorito et al. 2008a, b, 2014a).

Following infection, SIRT3 transcript levels considerably augmented from 36 h p.i. onwards (Fig. 2a). In the presence of TCDD, a significant ($p < 0.001$ and $p < 0.05$) dose-dependent increase in SIRT3 transcript levels from 8 h p.i. to the end of infection was detected (Fig. 2a).

When SIRT3 protein expression was evaluated, the levels were found increased at the end of infection (Fig. 2b-c). Whereas, in TCDD exposed groups, SIRT3 significantly ($p < 0.001$, $p < 0.01$ and $p < 0.05$) enhanced, from 4 h p.i. onwards (Fig. 2b-c).

We evaluated the effects of TCDD on cytoskeletal organization of MDBK cells, during BoHV-1 infection, in the presence or not of TCDD. Herein, we only showed the results at 12 h p.i., in the presence of the medium dose of TCDD (1 pg/mL). The cytoskeletal actin in control cells, at all times, was arranged in many thin and short fibers surrounding the nucleus and, from circular filaments, fibers diverge radially as spokes toward the cell cortex, like a net of concentric fibers, as previously demonstrated (Wulf et al. 1979). Both actin fibers and cortical actin remained unaffected in, exposed or not, infected cells until 4 h p.i.; starting from 8 h to the end of infection, a complete reorganization of the actin based cytoskeleton it has been occurred (data not shown). In particular, at 12 h p.i. in TCDD uninfected group, in a shape similar to control, only an enhancement of radial fibers was detected (Fig. 3), according with previous data showing that dioxin did not alter actin filaments in human uterine RL95-2 cells (McGarry et al. 2002). In infected group, more stress fibers were arranged in spherical structures and cortical actin increased (Fig. 3). Moreover, filopodia were intercellular networks (Fig. 3). At the same time, in the presence of TCDD, a similar pattern of actin was detected in BoHV-1 infected cells, which loosed spherical shape and appeared polyhedral (Fig. 3). Cortical actin accumulated, thin stress fibers were shorter, were arranged in a confused manner and did not reach the cell periphery, in contrast to those observed at 4 h p.i. (data not shown).

These results that BoHV-1 infection modifies the cytoskeletal actin in MDBK cells. TCDD further modifies these structures.

Then, we explored virus production, analyzing both viral cytopathic effects and virus titration in MDBK cell cultures infected with BoHV-1 and exposed or not to all doses of TCDD. The CPE, represented by ample syncytia formation along with elimination of the cellular sheet, was always more evident in TCDD exposed cells after 12 h p.i., when virus-induced apoptosis occurred (Fig. 4a). Virus titers, assayed by $TCID_{50}$ method, confirmed the above data. In fact we evidenced a statistically significant ($p < 0.01$ and $p < 0.001$) increase of viral production in all TCDD treated groups after 48 h p.i. (Fig. 4b).

Discussion

This study suggests that BoHV-1 modifies the cytoskeleton reorganization of MDBK cells, through the regulation

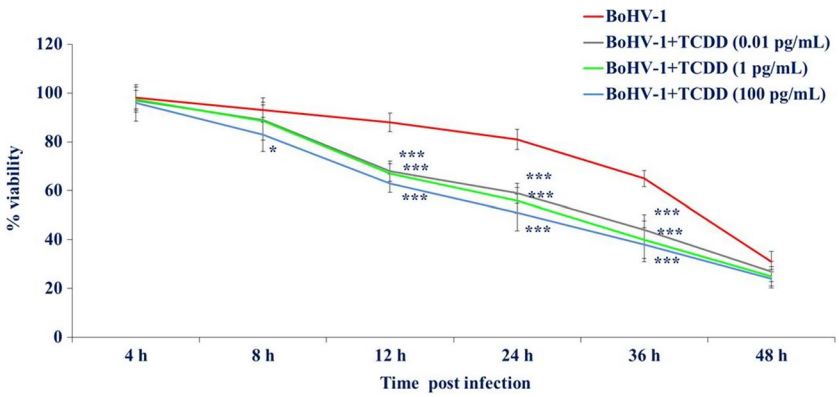

Fig. 1 TCDD increases cytotoxicity of MDBK cells during BoHV-1 infection. Dose response curve of MDBK cells treated with different concentrations of TCDD and observed at different times on cell viability. Viable, adherent cells were stained with MTT at different times of incubation and the absorbance assayed as described in Materials and Methods section. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

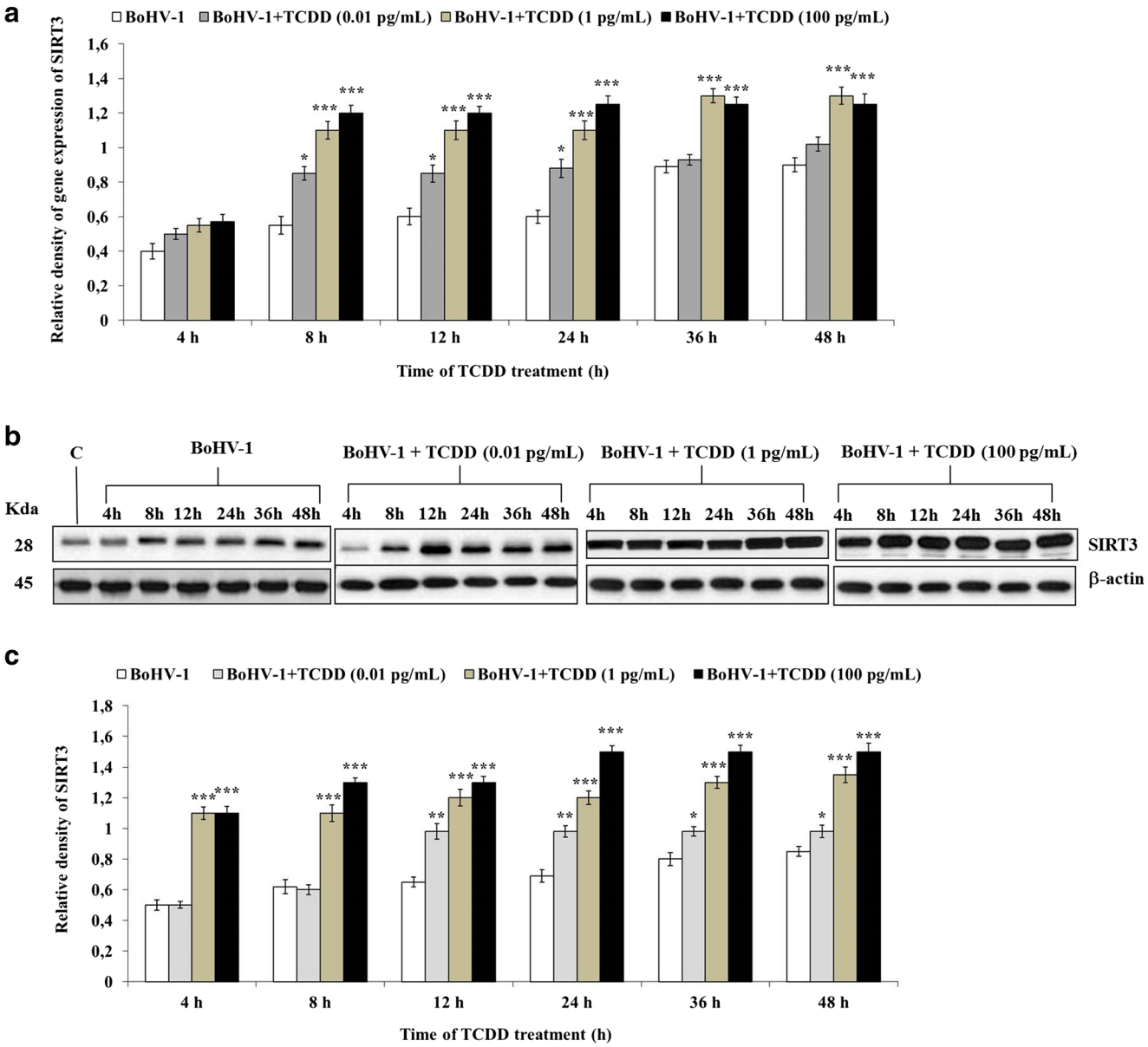

Fig. 2 TCDD induces an increase of SIRT3 during BoHV-1 infection. A) To perform Northern blot assay, RNA was extracted from untreated cells, infected cells, or infected and exposed to different concentrations of TCDD (0.01 pg/ml), (TCDD 1 pg/ml) or (TCDD 100 pg/ml) cells at the indicated times, electrophoresed and hybridized with a labelled probe as described under Material and Methods. β-actin was used as loading control. Densitometry analysis of the blot (B) Whole-cell lysate was prepared from untreated cells, infected cells, or infected and exposed to different concentrations of TCDD (0.01 pg/ml), (TCDD 1 pg/ml) or (TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h p.i., Western blot analysis was performed with an antibody which specifically recognized SIRT3 or β-actin. β-actin was used as an internal loading control. (C) Densitometry analysis of the relative blots shown in (A). Results are the mean ± S.E.M. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $*p<0.05$, $**p<0.01$ and $***p<0.001$

of a member of SIRT family, and these results are further modified by TCDD exposure. Our previous studies (Fiorito et al. 2008b, 2014a) indicate that TCDD in infected cells accelerates BoHV-1 induced apoptosis. The sirtuin protein family has also been proposed to be involved in cellular stress response pathways including DNA damage, cell cycle arrest and apoptosis (Saunders and Verdin 2007). SIRT3 is located in the mitochondria, dynamic organelles that play crucial roles in intracellular signaling and apoptosis (Verdin et al. 2010). Herein, following infection in MDBK cells, we detected an increase of SIRT3 both in transcript and in protein levels (Fig. 2). Moreover, SIRT3 had a similar trend previously observed in canine coronavirus type II-induced apoptosis (Marfè et al. 2011). In particular at 12 h p.i. when,

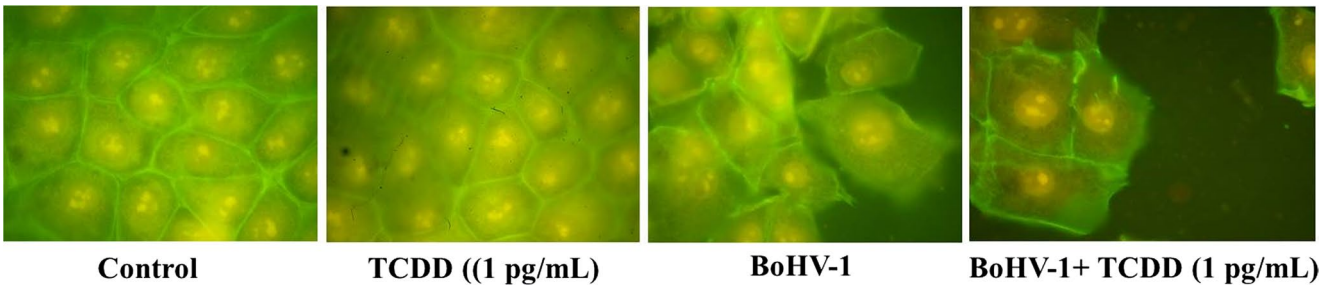

Fig. 3 TCDD modifies cytoskeletal organization of MDBK cells during BoHV-1 infection Photomicrographs showing cytoskeletal structure of MDBK cells infected or not with BoHV-1, at MOI of 5, in the presence or in the absence of different concentrations of TCDD (1 pg/mL). After 12 h p.i., fixed cells were stained with Phalloidin, Fluorescein Isothiocyanate labeled and then observed under fluorescent microscope (magnification, ×1000). In TCDD uninfected group, similarly to control, an enhancement of fibers which diverge radially was detected. In infected group, more stress fibers were arranged in spherical structures and cortical actin increased and filopodia were intercellular networks. In the presence of TCDD, a similar pattern of actin was detected in infected cells, which loosed spherical shape and appeared polyhedral. Cortical actin accumulated, thin stress fibers were shorter, were arranged in a confused manner and did not reach the cell periphery

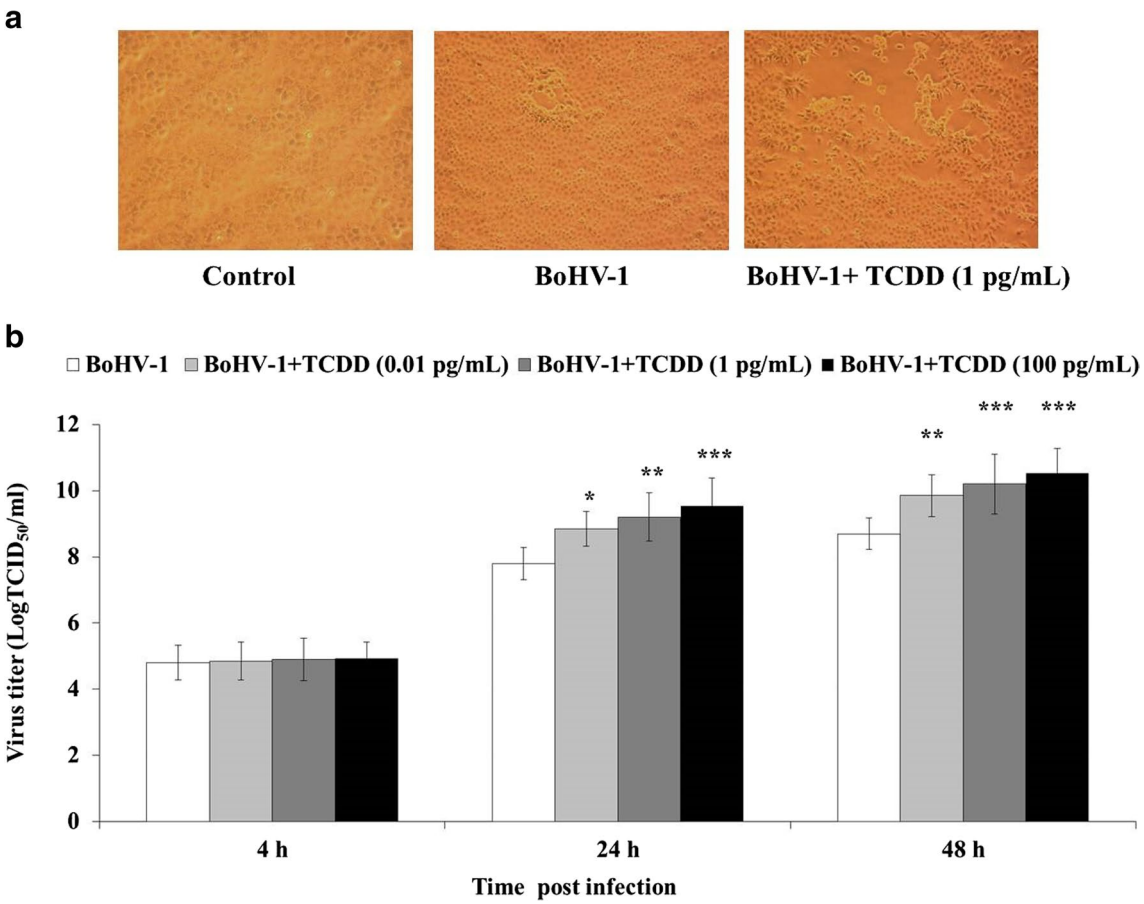

Fig. 4 TCDD increases virus production. **a** Representative microphotographs by phase-contrast light microscopy of MDBK cells infected with BoHV-1, exposed or not to 1 pg/mL of TCDD at 12 h post infection, showing the cytopathic effects and the morphological changes on cellular monolayers. **b** Virus titers, assayed by $TCID_{50}$ method and reported as Log $TCID_{50}$/mL, in MDBK cells infected with BoHV-1, exposed or not to 0.01, 1, 100 pg/mL of TCDD, as indicated in the legends. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

in the same experimental conditions, in the presence of TCDD, BoHV-1 induced apoptosis occurred (Fiorito et al. 2008b, 2014a), we detected a significantly increase in SIRT3 at all doses studied (Fig. 3).

Herein, following infection in MDBK cells, in BoHV-1 infected groups, we detected high differences in cytoskeletal organization, which from 12 h p.i. (Fig. 3) increased until the end of infection. While, in all infected groups, TCDD drastically and significantly modified cytoskeletal organization starting from 12 h p.i. (Fig. 3) when, in the same experimental conditions, accelerated virus-induced apoptosis occurred (Fiorito et al. 2008b, 2014a), to the end of infection. In addition, caspase-3 induces, by inactivating PARP, cytoskeletal reorganization (Elmore 2007; Green et al. 2014) and TCDD induced the cleavage of PARP earlier than control groups during the early stages of infection (Fiorito et al. 2008b, 2014a). As reported above, viruses have developed several types of interactions with host cells to produce viral replication, persistence and spread. The actin cytoskeleton is involved in many crucial cellular processes, including providing cell integrity, mobility and shape, driving cell division and contraction, and the uptake of extracellular molecules. We already described morphological changes, such as rounding and loosening from basic structures, in MDBK during BoHV-1 infection, in the presence or not of TCDD, by Acridine Orange staining (Fiorito et al. 2008b), Giemsa staining (Fiorito et al. 2010), or by ample syncytia formation along with elimination of the cellular sheets, as in CPE (Fiorito et al. 2008a). In general, changes in cell shape are related to alterations in the architecture of the cellular cytoskeleton. The actin cytoskeleton is also implicated in herpesvirus capsid assembly and nuclear egress. Later stages of the herpesvirus replication cycle have been associated with disassembly of actin stress fibers, retraction and rounding of the cells and the formation of actin-containing cell projections (Favoreel et al. 2007). Herein, we evidenced that BoHV-1 infection of MDBK cells provokes a disassembly of cytoskeletal actin, modified by TCDD. Concomitantly, we detected a significant increase in virus replication (Fig. 4). Indeed, following infection in MDBK cells, in BoHV-1 infected groups, at MOI of 5, exposed to different concentrations of TCDD, we detected an increase of virus titer which significantly increased at the end of infection (Fig. 4a), as we previously showed at MOI of 0.1 as well as at MOI of 1 (Fiorito et al. 2008a, 2013). On the basis of the overall results obtained by investigating the effects of BoHV-1 infection and TCDD exposure, alone or in combination, we can state that the main factor that influences virus replication is represented by dioxin.

Until now, there are no reports in the literature describing cytoskeletal reorganization induced by TCDD in virus-infected cell cultures. This is the first evidence that an epithelial line of mammalian cells infected with BoHV-1, an alphaherpesvirus, induces cytoskeletal rearrangements and cell extensions, that are further modified by the administration of TCDD, which enhanced SIRT3.

We hypothesize that these modifications might further explain the mechanisms as to how TCDD exerts its effects on the BoHV-1 replication, resulting in anticipated cell death apoptosis and increased virus replication.

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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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