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The β-catenin signaling pathway stimulates bovine herpesvirus 1 productive infection.

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Abstract

Bovine herpes virus 1 (BoHV-1), an important bovine pathogen, causes conjunctivitis and disorders in the upper respiratory tract. Following acute infection, BoHV1 establishes life-long latency in sensory neurons. Recent studies demonstrated that viral gene products expressed in trigeminal ganglionic neurons during latency stabilize β-catenin levels, an important signaling molecule that interacts with a family of DNA binding proteins (T-cell factors) and subsequently stimulates transcription. In this study, we provide new evidence demonstrating that BoHV-1 transiently increased β-catenin protein levels in bovine kidney (CRIB) cells, but not in rabbit skin cells. β-catenin dependent transcription was also stimulated by infection of CRIB cells. The β-catenin small molecule inhibitor (iCRT14) significantly reduced the levels of BoHV-1 virus during productive infection of CRIB cells and rabbit skin cells. In summary, these studies suggested the ability of β-catenin to stimulate cell survival and cell cycle regulatory factors enhances productive infection in non-neuronal cells.

Keywords

bovine herpesvirus 1 (BoHV-1); productive infection; β-catenin; β-catenin inhibitor (iCRT14)

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1), a widespread bovine pathogen, is an important cofactor for the most important disease in cattle, bovine respiratory disease complex (BRDC), reviewed in (1–3). BRDC is initiated by stress and/or viral infection(s) resulting in immune-

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suppression and life-threatening pneumonia. Mannheimia haemolytica, a commensal bacterium in the upper respiratory tract of cattle, is the cause of pneumonia in most BRDC cases. Bovine herpesvirus 1 (BoHV-1) infection can be a significant risk factor for BRDC because it causes clinical disease in the upper respiratory tract (4, 5), suppresses immune responses (2, 3), and cooperates with Mannheimia haemolytica to induce pneumonia in calves (6). Like many Alphaherpesvirinae subfamily members, BoHV-1 impairs immune responses during acute infection, which enhances replication (2). Following acute infection of mucosal linings in the oral, nasal or ocular cavity, BoHV-1 establishes latency in sensory neurons that reside within trigeminal ganglia, reviewed in (1–3). The ability to establish, maintain, and reactivate from latency is crucial for virus transmission.

We recently discovered that BoHV-1 regulates the Wnt/β-catenin signaling pathway in latently infected sensory neurons (7). These studies suggested that the ability of BoHV-1 to regulate the β-catenin signaling pathway was important during latency because this pathway stimulates axonal growth and navigation of axons to their synaptic targets, reviewed in (8–12). Wnt family members are secreted glycoproteins that interact with Frizzled receptors and a co-receptor LRP5/LRP6, reviewed in (13). Upon Wnt binding, the transcription factor β-catenin, is stabilized and enters the nucleus where it interacts with TCF (T-cell factor) family members specifically bound to a consensus DNA motif (5'-T/A-T/A-CAAAG-3'), reviewed in (13, 14). Binding of β-catenin to TCF displaces bound co-repressors and recruits co-activators to activate Wnt target genes (15). The Wnt/β-catenin signaling pathway is required for embryogenesis and adult homeostasis, reviewed in (13, 16).

In this study, we demonstrate that β-catenin steady state protein levels and β-catenin dependent transcription were increased following infection of bovine kidney (CRIB) cells. A specific inhibitor of the Wnt/β-catenin signaling pathway (iCRT14) reduced the plaque forming efficiency in CRIB and rabbit skin cells. These observations suggest that β-catenin has the potential to stimulate productive infection.

RESULTS

Analysis of β-catenin protein expression during productive infection of bovine kidney or rabbit skin cells

To understand whether the β-catenin signaling pathway influences productive infection, we initially examined β-catenin protein levels following infection of cultured cells. For these studies, bovine kidney (CRIB) and rabbit skin (RS) cells were infected with BoHV-1 because both cell lines support BoHV-1 replication. Relative to uninfected cells, β-catenin levels increased at four and eight hours after CRIB cells were infected but hen decreased to levels that were similar to mock-infected cells (Figure 1A). In contrast,β-catenin protein levels in RS cells were similar in mock-infected cells and four hours after infection (Figure 1B). At eight, 16, and 24 hours after infection, β-catenin levels were consistently reduced relative to mock-infected RS cells or cells infected for four hours. bICP0 protein expression was readily detected in CRIB, but not RS cells, at 4 hours after infection. In general, bICP0 expression was lower in RS cells compared to CRIB cells. Although β-catenin protein levels were transiently induced in CRIB cells, they were reduced at 16 and 24 hours after infection in CRIB and RS cells, which was likely a result of the virus host shutoff activity.

Productive infection stimulates β-catenin dependent transcription

To test whether infection regulated β-catenin dependent transcription, CRIB cells were transfected with Super 8x TOPFlash and then promoter activity measured after infection. When β-catenin is localized to the nucleus, it can interact with a TCF (T-cell factor) family member bound to a consensus site (5'-T/A-T/A-CAAAG-3'), reviewed in (13, 17). β-catenin binding to TCF displaces bound corepressors and recruits transcriptional coactivators, thus stimulating transcription of promoters containing TCF binding sites (14). The plasmid Super 8x TOPFlash contains 8 TCF binding sites upstream of a minimal promoter that drives firefly luciferase reporter expression and thus accurately measures β-catenin dependent transcription. β-catenin dependent transcription increased as a function of the moi used for infection at 8 hours after infection (Figure 2A).

To test whether the effect on Super 8x TOPFlash was due to β-catenin dependent transcription or merely the effect of viral infection stimulating transcription, we analyzed promoter activity in the presence of a β-catenin specific inhibitor (iCRT14). This small molecule was chosen because it specifically interferes with interactions between β-catenin and TCF family members (18). Super 8x TOPFlash promoter activity was similar to uninfected cells when infected cultures were treated with 10 uM iCRT14 (Figure 2B). 1uM iCRT14 also reduced β-catenin dependent transcription but not significantly different compared to infected cells. In summary, these studies revealed that BoHV-1 infection stimulated β-catenin dependent transcription in CRIB cells at 8 hours after infection.

The β-catenin inhibitor, iCRT14, inhibits BoHV-1 productive infection

The effect that the β-catenin signaling pathway has on productive infection was examined using the small molecule inhibitor iCRT14. Initial studies tested whether iCRT14 was toxic for CRIB cells. Ten uM iCRT14 had no obvious toxic effects on CRIB cells, as judged by measuring intracellular levels of ATP (Figure 3A). At higher iCRT14 concentrations (25, 50, and 100 uM), toxicity increased in a dose dependent manner. Furthermore, 10 uM iCRT4 had little effect on steady state levels of β-catenin protein levels in CRIB cells (data not shown). We then examined the effect of increasing concentrations of iCRT14 on BoHV-1 replication in CRIB cells. Relative to the DMSO control, 1 uM iCRT14 had no obvious effect on virus replication, conversely 10 or 25 uM iCRT14 reduced the levels of infectious virus by approximately 3 logs (Figure 3B). When cell morphology was examined, 10 uM iCRT14 reduced the effect of virus induced cytopathology (Figure 3C). Additional studies were performed to examine the effects that ten uM iCRT14 had on virus infection at different times after infection. Ten uM iCRT14 consistently inhibited virus production in CRIB cells by 2–3 logs at 16 or 24 hours after infection (Figure 3D).

The effect of iCRT14 on BoHV-1 infection was then examined in RS cells. In contrast to CRIB cells, 10 uM iCRT14 exhibited low levels of toxicity in RS cells 24 hours after treatment, however 2.5 uM iCRT14 was not toxic (Figure 3A). Consequently, studies were performed using 2.5 uM iCRT14. BoHV-1 replication was inhibited approximately 2 logs at 24 hours after infection when treated with 2.5 uM iCRT14 (Figure 4). In summary, the β-catenin small molecule inhibitor, iCRT14, significantly reduced BoHV-1 replication in two permissive cell lines, CRIB and RS cells.

DISCUSSION

In this study, we provide evidence that a β-catenin specific inhibitor reduced virus replication at least 100 fold. CRIB cells, but not RS cells, contained higher β-catenin protein levels during early stages of productive infection suggesting viral infection induced cell-type or species-specific effects. Regardless, inhibiting interactions between β-catenin and TCF family members with iCRT14 significantly reduced BoHV-1 replication in CRIB and RS cells. An active Wnt/β-catenin signaling pathway also stimulates human cytomegalovirus and herpes simplex virus 1 (HSV-1) replication (19, 20) suggesting β-catenin provides important functions that can directly or indirectly stimulate replication of more than one herpesvirus.

Our previous studies demonstrated that BoHV-1 latent gene products stabilize β-catenin expression during latency suggesting the β-catenin pathway plays a role during the establishment and/or maintenance of latency (7), which seems at odds with the results from this study. However, the Wnt/β-catenin signaling pathway has tissue and cell-type specific functions that may enhance latency in one cell type but stimulate productive infection in other cell types. For example the Wnt/β-catenin signaling pathway in neurons maintains differentiation specific functions of neurons by stimulating axon growth and targeting axons to specific targets (8–12), as well as inhibiting neuro-degeneration (21–24). In non-neuronal cells, the β-catenin signaling pathway stimulates cell growth and inhibits cell death, as exemplified by the fact that β-catenin enhances oncogenesis (13, 16, 25).

The core Wnt/β-catenin response element (5’-T/A-T/A-CAAAG-3’) (26), which includes a TCF binding site, is only present in the BoHV-1 genome twice (data not shown). Thus, β-catenin does not appear to stimulate productive infection by directly trans-activating many, if any viral promoters. Consequently, we suggest β-catenin promotes productive infection by enhancing expression of factors that mediate certain aspects of cell proliferation and cell survival. For example, β-catenin trans-activates the cyclin D1 promoter (27) and it is generally accepted that cyclin D1, Rb, and E2F cooperate to promote G1 cell cycle progression, reviewed by (28, 29). These observations correlate with the finding that BoHV-1 replicates more efficiently in actively dividing cells, as demonstrated by the finding that silencing E2F1 or E2F2 inhibits BoHV-1 replication and E2F1 trans-activates the bICP0 early promoter (30, 31).

In conclusion, BoHV-1 productive infection is stimulated by the ability of β-catenin to maintain the growth potential of non-neuronal cells. In contrast, neuronal-specific functions of the Wnt/β-catenin signaling pathway are proposed to cooperate with BoHV-1 latent gene products to establish and maintain latency in sensory neurons.

MATERIALS AND METHODS

Cells and cell viability assay

Bovine kidney cells (CRIB) and rabbit skin (RS) cells were grown in Eagle’s minimal essential medium (EMEM) supplemented with 10% FCS, penicillin (10 U/ml), and streptomycin (100 ug/ml).

CRIB or RS cells were seeded at 3.5 $\times 10^6$ cells/ml in 24-well plates and then incubated overnight at 37^o C. Cells were treated with various concentrations of iCRT14 (Sigma-Aldrich) for 24 hours. To assess cell viability, ATP levels in cultures were then measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega; G7572) according to the manufacturer's instruction.

Viruses & plasmids

The Cooper strain of BoHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, IA. BoHV-1 stocks were prepared in CRIB cells.

Super 8x TOPFlash contains a simple promoter that is stimulated by β-catenin and was a gift from Randall Moon (Addgene plasmid # 12456) (32).

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (Thermo-Scientific). The respective samples were boiled in Laemmli sample buffer for 5 minutes and all samples were separated on an 10% SDS–polyacrylamide gel. Western blots were performed as previously described (7). The anti-β-catenin antibody (Abcam, ab32572) or a peptide specific bICP0 antibody was used for Western blot studies.

Dual-luciferase reporter assay

CRIB cells were cotransfected with the Super 8x TOPFlash plasmid and a plasmid encoding Renilla luciferase under the control of a minimal herpesvirus promoter (Promega) using Lipofectamine® 2000 Transfection Reagent (11668019, Invitrogen). At the designated times after infection, cells were harvested and protein extracts were subjected to a dual-luciferase assay using a commercially available kit (E1910; Promega) according to the manufacturer's instructions. Luminescence was measured with a GloMax 20/20 luminometer (E5331; Promega).

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Highlights

- BoHV-1 transiently increased β-catenin protein levels in bovine kidney cells
- β -catenin dependent transcription was stimulated by BoHV-1 infection
- A β -catenin inhibitor (iCRT14) reduced BoHV-1 productive infection

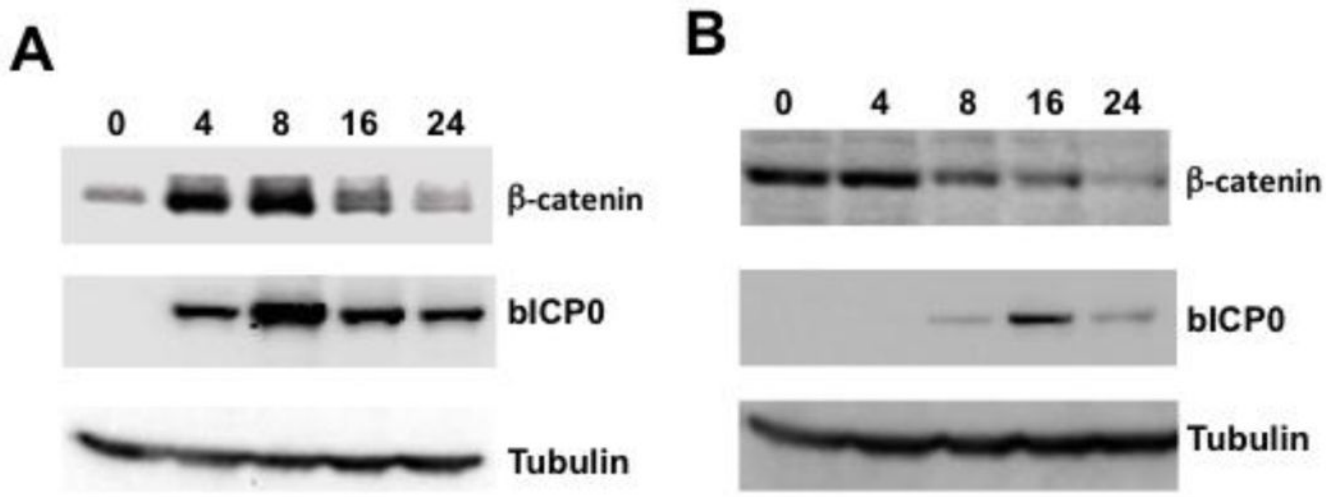

Fig 1. Expression of β-catenin in cultured bovine kidney and rabbit skin cells during productive infection.

Bovine kidney (CRIB; Panel A) or rabbit skin (RS; Panel B) cells were infected with BoHV-1 (1 pfu/cell) for the designated times (hours after infection). Mock-infected cells were denoted as 0. At the indicated times after infection, total cell lysate was prepared and Western blots performed using specific antibodies that recognize the denoted proteins. For each lane, 60 ug protein was loaded. These results are representative of five independent experiments.

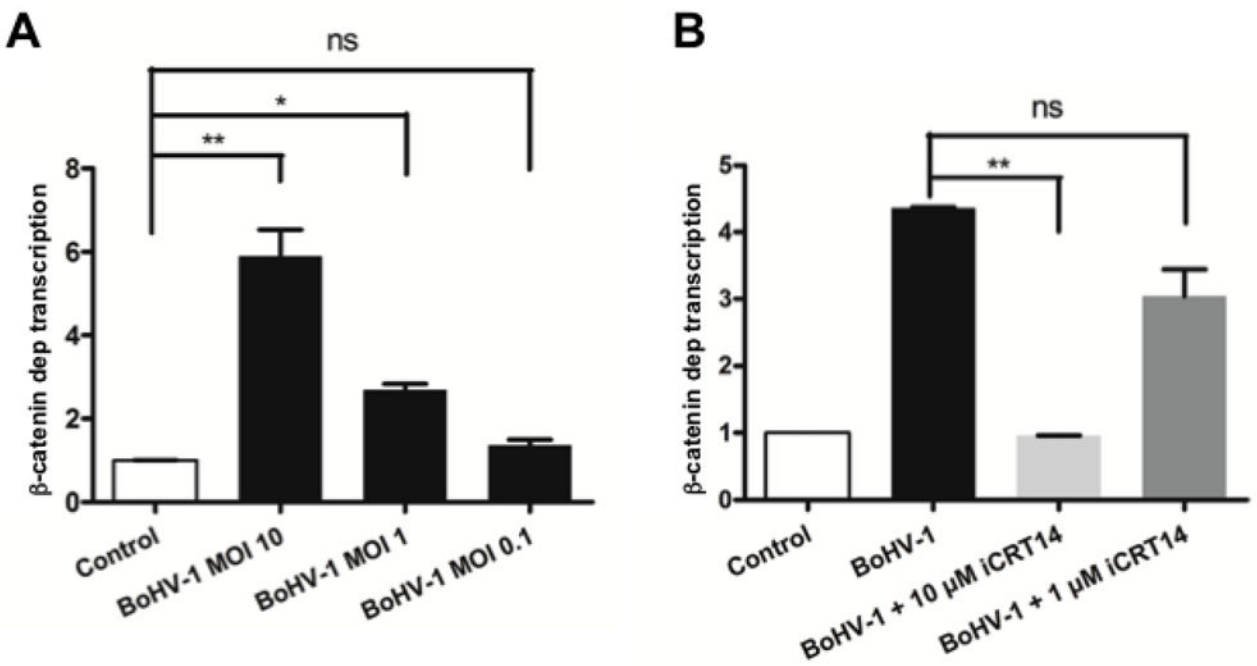

Fig 2. BoHV-1 infection of CRIB cells stimulated β-catenin dependent transcription.

CRIB cells in 12-wells plates (60% confluent) were transfected with 0.4 μg of the Super 8x TOPFlash luciferase reporter construct and 0.05 μg of the Renilla reporter construct that was used as an internal control to allow normalization of promoter activity. **Panel A:** At 48 hours after transfection, cells were infected with BoHV-1 at the designated MOI. At 12 hours after infection, dual luciferase activity was measured. **Panel B:** CRIB cells that were transfected as described in Panel A were infected with BoHV-1 at an MOI of 10. Certain cultures were treated iCRT14 at the designated concentrations. Dual luciferase assays were performed at 12 hours after infection. Mock-infected cells were normalized to a value of 1 and the fold activation for other samples calculated. The results are the average of three independent experiments and error bars denote standard error. An asterisk denotes significant differences ($P < 0.05$) in promoter activity compared to the indicated control by the one-way ANOVA and Fisher's LSD multiple means comparison tests.

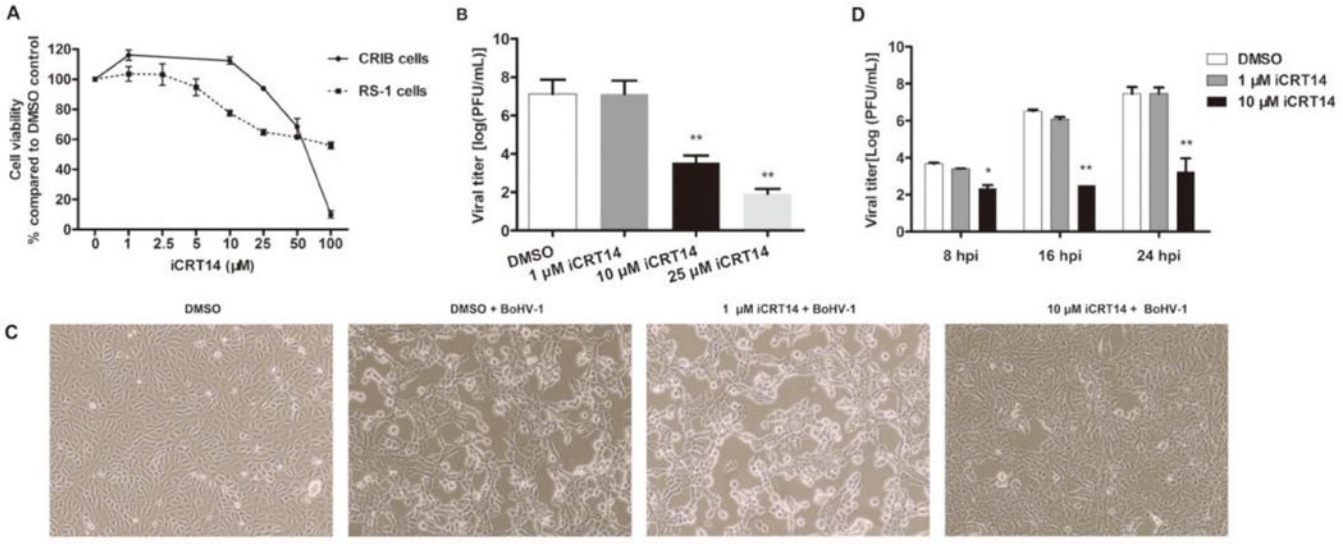

Fig 3. The β-catenin inhibitor iCRT14 reduced BoHV-1 replication in Bovine kidney (CRIB) cells.

Panel A: The designated concentrations of iCRT14 were incubated with CRIB or RS cells for 24 hours. ATP levels in the respective cultures were measured to assess cell viability using CellTiter-Glo Luminescent Cell Viability Assay (Promega; G7572), according to the manufacturer's instruction. **Panel B:** Confluent CRIB cells in 60 mm dishes were pretreated with the designated concentration of iCRT14 at two hours prior to infection. Cultures were infected with BoHV-1 at MOI of 1 for 1 h in the presence of iCRT14 or just DMSO. After extensive washing with PBS, fresh medium containing inhibitors or DMSO control were replaced. At 24 h post infection, the amount of total virus in the culture was measured by plaque assay. These studies were repeated 3 times and asterisks denote significant differences between the DMSO control and cultures treated with iCRT14 (**: $P < 0.005$) as determined by the Student t test. **Panel C:** Before collecting the virus in the study described in Panel B, cells were photographed to compare the effect of viral induced cytotoxicity to uninfected cells (magnification is 40x). **Panel D:** As in panel B, CRIB cells were pretreated with 10 uM iCRT14 two hours prior to infection. Cultures were then infected with BoHV-1 at an MOI of 1 for 1 h in the presence of iCRT14 or just DMSO. After extensive washing with PBS, fresh medium containing inhibitors or DMSO control were replaced. At 8, 16, or 24 h post infection, the amount of total virus in the culture was measured by plaque assay. These studies were repeated 3 times and an asterisk denotes significant differences between the DMSO control and cultures treated with iCRT14 (*: $P < 0.05$ and **: $P < 0.005$) as determined by the Student t test.

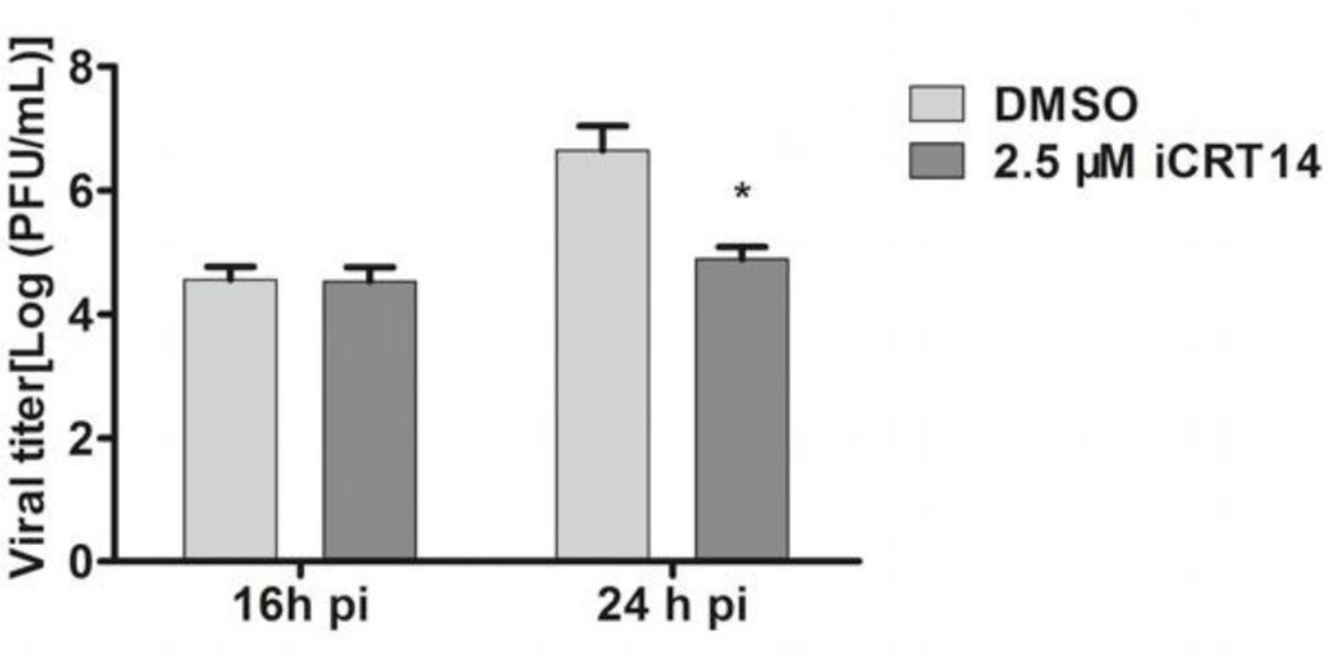

Fig 4. The β-catenin inhibitor iCRT14 reduced BoHV-1 replication in rabbit skin cells.

RS cells were incubated in 10% fetal calf serum and then sub-cultured 24 hours prior to infection. Two hours prior to infection, RS cells were pretreated with the designated concentration of iCRT14 and then infected with BoHV-1 at an MOI of 1. Cells were treated with iCRT14 throughout infection. Infectious virus was collected at 24 hours after infection by three rounds of freeze-thawing and then plaque assays performed. These studies were repeated 3 times and an asterisk denotes significant differences between the DMSO control and cultures treated with iCRT14 ($P < 0.05$) as determined by the Student t test.