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The effect of human interferon alpha on replication of different bovine viral diarrhea virus strains

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Summary. – Bovine viral diarrhea virus (BVDV) exists in two main biotypes: cytopathic (cp) and noncytopathic (ncp). Although some studies were done on the effect of interferon alpha (IFN-α) on BVDV, the effect of exogenous IFN against BVDV biotypes remains unclear. In the present study, we evaluated the comparative effect of exogenous human IFN-α (HuIFN-α) on different BVDV biotypes and genotypes. The results showed that exogenous HuIFN-α greatly inhibited the growth of different BVDV biotypes and genotypes. However, HuINF-α has a significant inhibitory effect on cp biotype compared to ncp one without significant variation between different genotypes. The effect of HuIFN-α on BVDV reached the maximum level at early stages of infection (0–20 h post infection) and increased in a dose-dependent manner (10–500 U/ml). Quantitative real-time RT-PCR was used to evaluate the effect of exogenous HuIFN-α on RNA synthesis of both BVDV biotypes. HuIFN-α reduced RNA production of cp by 4 logs compared to only 2 logs for ncp strains. Additionally, the antiviral effect of IFN-α against both BVDV biotypes seems to be independent of the RNA-dependent protein kinase (PKR) activation as assayed by direct analysis of *in vivo* phosphorylation of eIF2-α and by 2-aminopurine (2-AP) treatment. Collectively, these results indicated that the exogenous HuIFN-α treatment has an inhibitory effect not only on cp BVDV biotype but also on the ncp BVDV. The antiviral effect of exogenous HuIFN-α was biotype, time, dose but not genotype dependent. PKR has no role in the inhibitory effect suggesting that other IFN-antiviral pathways were involved.

Keywords: BVDV biotypes; HuIFN-α; RNA synthesis; PKR-independent

Introduction

Bovine viral diarrhea virus (BVDV) infections result in major economic losses in cattle. The virus belongs to the genus *Pestivirus* in the family *Flaviviridae* (Meyers and Thiel, 1996). BVDV is classified into two genotypes (type 1 and type 2) with each genotype subdivided into subtypes (1a-1t and 2a-c). The prevalence of genotype type 1 or type 2 varies by region. Within each genotype, pathogenicity varies by strain (Ridpath *et al.,* 1994; Vilcek *et al.,* 2001; Ularamu *et al.,* 2013). BVDV is also categorized into two biotypes, cytopathic (cp) and noncytopathic (ncp), with distinct pathologic characteristics (Lee and Gillespie, 1957; Gillespie *et al.,* 1960; Donis and Dubovi, 1987). Ncp BVDV is the most commonly isolated BVDV biotype from the field and induces persistent infections. In contrast, cp BVDV is the most frequently used virus in BVDV vaccines and is isolated from cattle with

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Abbreviations: 2-AP = 2-aminopurine; BoIFN-α = bovine IFN-α; BVDV = bovine viral diarrhea virus; cp = cytopathic biotype; eIF2α = alpha subunit of eukaryotic initiation factor 2; HuIFN-α = human IFN-α; IFN-α = interferon alpha; ncp = non-cytopathic biotype; PKR = RNA-dependent protein kinase; YRA = yield reduction assay; VSV = vesicular stomatitis virus

mucosal disease (Brownlie *et al.,* 1984; Sentsui, *et al.,* 2001; Fulton *et al.,* 2003; Reid and Charleston, 2014).

Interferons (IFNs) are cytokines having broad-spectrum of antiviral effects and they are divided into three major subtypes: type 1-alpha (α)/beta (β), type 2-gamma (γ) and type 3-lambda (λ). IFNs have direct or indirect antiviral activities via stimulating a large number of cellular genes (Schoggins and Rice, 2011). One of the best-characterized IFN-inducible cellular components is double-stranded RNA-dependent protein kinase (PKR). The IFN-inducible PKR is a serine/threonine kinase with multiple functions that control transcription and translation. The viral infection activates PKR, which in turn phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α). This phosphorylation of eIF2α inhibits the translation of the cellular and viral proteins. Other cellular factors especially molecules that limit the virus replication, may be activated by IFN (Thomis *et al.,* 1992).

IFN-α is considered to be an effective antiviral agent against BVDV (Bielefeldt Ohmann and Babiuk, 1988; Sentsui *et al.,* 1998; Woodhouse *et al.,* 2008; Zhang *et al.,* 2010; Wang *et al.,* 2011). Bovine IFN-α has decreased BVDV replication in bovine fibroblast cells persistently infected with BVDV (Bielefeldt Ohmann and Babiuk, 1988). Human IFN-α suppressed BVDV replication in peripheral mononuclear cells from persistently infected animals (Sentsui *et al.,* 1998). IFN-α in combination with antiviral agents decreased BVDV growth and RNA production (Peek *et al.,* 2004; Woodhouse *et al.,* 2008; Zhang *et al.,* 2010). PKR is implicated in mediating the IFN-induced antiviral state against BVDV and consequently controls the virus replication (Gale and Katze, 1998; Gil *et al.*, 2006).

To further analyze the inhibitory effect of IFN-α on BVDV, this study was aimed to (i) investigate the comparative antiviral effects of exogenous HuIFN-α on different BVDV biotypes and genotypes in MDBK cells, (ii) test the effect of HuIFN-α on RNA synthesis of both BVDV biotypes, and (iii) test the PKR activity in IFN-induced BVDV inhibition.

Materials and Methods

Cells and viruses. Madin Darby bovine kidney (MDBK) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, USA). The media was supplemented with 10% horse serum (Hyclone, USA) and antibiotics (streptomycin 100 μg/ml, penicillin 100 IU/ml and amphotericin B 250 μg/ml) at 37°C in 5% CO_2. The cell cultures were tested by the immunoperoxidase staining protocol (Afshar *et al.,* 1991) using anti-p80/p125 monoclonal antibodies and RT-PCR and were BVDV-free. Four laboratory BVDV strains; NADL (cp type 1a), NY-1 (ncp type 1b), A125 (cp type 2a), 890 (ncp type 2a), and vesicular stomatitis virus (VSV-Indiana) (ATCC, USA) were used in this study. Additional one pair of type 2a BVDV isolates, 296-cp and 296-ncp, kindly supplied by Dr. Julia Ridpath (National Animal Disease Center, USA), were used. The viruses were grown in MDBK cells and harvested after two freeze-thaw cycles. The titers were determined by $TCID_{50}$ through recording the CPE for the cp BVDV and the reddish-brown color (the positive result of immunoperoxidase test) induced by ncp BVDV, respectively.

Reagents. Human recombinant IFN-α (Sigma, USA) was prepared according to manufacturer's instruction and stored at -80°C till use. A 200 mM solution of the nitrate salt of 2-aminopurine (2-AP) (Sigma, USA) was prepared in MEM and the pH was adjusted by adding 1M Tris until the cell culture medium turned to pink as described by Rowland (Rowland *et al.,* 2001).

BVDV cytopathic effect (CPE) inhibition assay. Confluent monolayers of MDBK cells, grown in 24-well tissue culture plates, were infected with NADL, A125, or VSV at a multiplicity of infection (MOI) of 1 for 1 h at 37°C followed by removal of medium. The infected cell monolayers were overlaid with DMEM containing 0, 10, 100, 500, or 1000 U/ml of IFN-α. Interferon-free medium was added to control wells. The cells were incubated for 72 h at 37°C in humidified CO_2 incubator. To observe the CPE, the cells were fixed with 80% acetone for 20 min, dried and stained with 1% crystal violet. The CPE was observed by phase contrast microscopy (Nikon TMS, Japan).

BVDV yield reduction assay (YRA). Confluent MDBK cells, grown in 24-well tissue culture plates, were infected with NADL, A125, NY-1 or 890 strains as well as 296-cp and 296-ncp strains (MOI = 1). After the viruses were allowed to be adsorbed to the cells for 1 h at 37°C, the excess inoculum was removed and the infected monolayers were washed twice with DMEM. The infected cells were treated with DMEM containing 0, 10, 100, 500, or 1000 U/ml of IFN-α at 0, 10, 20 or 30 h post-infection. After incubation for 72 h at 37°C in 5% CO_2 incubator, the cells were freeze/thawed three times and the amount of infectious virus was titrated on MDBK cells according to the method of Reed and Muench (Reed and Muench, 1938). The titers were determined by $TCID_{50}$. In case of ncp strains, the immunoperoxidase staining protocol was performed according to Afshar (Afshar *et al.,* 1991) using anti p80/p125 monoclonal antibodies (Mab 20.10.6, kindly supplied by Dr. Edward Dubovi, Cornell University, USA).

Effect of IFN on RNA synthesis of BVDV. In 24-well tissue culture plates, MDBK cells were infected with A125 or 890 (MOI = 1) for 1 h at 37°C and then treated with IFN-α 100 U/ml or 500 U/ml. The cells were incubated at 37°C for 16 h and the RNA level was determined by qRT-PCR as previously described by Bhudevi (Bhudevi and Weinstock, 2001). Total RNA was extracted from BVDV-infected cells using the QIAamp viral RNA mini kit (QIAGEN, USA) following manufacturer's instruction. In addition, the total RNA was extracted from IFN- and BVDV- free MDBK cells as a negative control. Pestivirus specific primers to NS3 region were; SPTL 5'-CAAGGAGGGATCAGCTCGGTAGAC-3' and SPTU 5'-CTTCGGACACCTGAGTCGG-3'. A fluorogenic molecular

beacon probe (BVDV type 2, A125): FAM-5'-cgcacgGAGACT GAGTATGGCATCAAGcgtgcg-3'-DABCYL was synthesized by Integrated DNA Technologies (IDT, USA). The RNA extracts were quantified using the spectrophotometer. The standard reaction mix (50 µl final volume) consisted of RNA sample, 0.75 U AmpliTaq™ DNA polymerase (PE Biosystems, USA), 2 mM $MgCl_2$, 250 µM of dNTPs, 2 µM of each PCR primers, 2.5 U of the MuLV reverse transcriptase (RT) and 1 U RNase inhibitor and 50 ng BVDV type 2-FAM beacon. Nuclease-free water was used as a non-template negative control in each run. The TaqMan PCR amplification was performed as follows: 50°C for 5 min, 42°C for 20 min, and 95°C for 5 min. This was followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1min and elongation at 72°C for 1 min and then a final extension step at 72°C for 10 min. Real-time fluorescence measurements were taken. The threshold cycle (C_T) value for each sample was calculated by determining the end point at which the fluorescence exceeds a threshold limit. As a positive control, the pcBVDV80 plasmid was constructed using pcDNA 3.1 plasmid (Clonetech, USA) and the full length NS3 gene of BVDV type 2 (A125).

Western blot analysis of PKR induction. Monolayers of MDBK cells were infected with NADL or NY-1 (MOI = 1). Following virus adsorption for 1 h, the cells were treated with DMEM containing 500 U of IFN-α. As a positive control for eIF2-α phosphorylation, MDBK cells were treated with 1mM hydrogen peroxide (Sigma, USA). Additional controls of BVDV-infected non IFN-treated cells and non-infected non-treated cells were included. After incubation at 37°C for 16 h, the cells were lysed in 1 ml of a lysate buffer containing 50 mM Tris-HCl, pH 7.5, 1% Nonidet P40, 150 mM NaCl, 0.5% sodium deoxycholate and protease inhibitor cocktail (EDTA 2 mM, aprotinin 2 µg/ml, pepstatin 5 mM, and leupeptin 2 µg/ml) and the DNA was sheared by 5–10 passages through a 27-gauge needle. The lysate was centrifuged at 12,500 x g for 15 min at 4°C, the supernatant was obtained and protein amount was measured by spectrophotometer. Approximately 50 µg of the whole cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels. The proteins were immunologically detected after electro-transfer onto nitrocellulose membranes (Schleicher and Schuell, USA). The transferred nitrocellulose membranes were incubated with rabbit polyclonal antibodies against eIF2-α (Cell Signaling Technology, USA). Alkaline phosphatase-linked goat anti-rabbit immunoglobulin G was used to detect the antigen-antibody complexes. The sizes of the proteins on SDS-PAGE were estimated by using molecular weight marker (Amersham Biosciences, USA).

Treatment with 2-aminopurine (2-AP). Monolayers of MDBK cells, grown in 24 wells plates, were infected with NADL, A125, NY-1 or 890 strains (MOI = 1). The BVDV-infected MDBK cells were treated with 2-AP according to the method described in the literature (Rowland *et al.,* 2001). Briefly, IFN-α and 2-AP were added to MDBK cells at approximately 15 h and 2 h, respectively, prior to BVDV infection. After 1 h of infection, the medium was removed and the cells were replenished with fresh medium containing freshly prepared IFN-α and 2-AP. The cells were incubated for 72 h and the virus yield was determined as $TCID_{50}$ on MDBK cells according to the method of Reed and Muench (Reed and Muench, 1938).

Statistical analysis. The analysis of variance (ANOVA) was done with PROC GLM of SAS (SAS Institute, Inc., USA). All variables were tested in triplicate for each experiment and the results were expressed as mean ± standard error. The significance of the differences between the RNA levels of both BVDV biotypes at different times was done by repeated measure design. The differences between the effects of IFN on BVDV replication was assessed by the 2-way factorial design. The P value of <0.05 was considered statistically significant.

Results

Effect of IFN-α on CPE and replication of BVDV in MDBK cells

Addition of IFN-α to the MDBK monolayers infected with cytopathic strains of BVDV (NADL and A125) resulted in a clear inhibition of the CPE intensity and progression in a dose-dependent manner (Fig. 1a). IFN-α inhibited the CPE induced by the cp BVDV strains NADL and A125 at a concentration as low as 10 U/ml. This effect was similar to that seen in the VSV infected MDBK cells (Fig. 1a). Further evaluation of CPE using phase-contrast microscopy was performed prior to crystal violet staining. No CPE was detected at 500 or 1000 U of IFN-α (data not shown). To evaluate the inhibitory effect of IFN-α on BVDV replication, we used the yield reduction assay (YRA). The MDBK cells were infected with one of the four BVDV laboratory strains; NADL (cp/type 1a), NY-1 (ncp/type 1b), A125 (cp/type 2a), or 890 (ncp/type 2a) at MOI of 1. The virus yield was calculated at 72 h post-infection. The treatment of BVDV infected cells with IFN-α dramatically reduced BVDV yields in both, dose- and time-dependent manners. IFN-α inhibited cp BVDV replication up to 5 logs, while the maximum inhibition of ncp was 2–3 logs (Fig. 1b, c). Low doses of IFN-α (10 U/ml) resulted in little to no inhibition to both BVDV biotypes (1 log or less) while the higher dose (500 U/ml) reduced cp BVDV replication by 4 or 5 logs and ncp BVDV by 2–3 logs (Fig. 1b). With both BVDV biotypes, the maximum inhibition of viral replication was observed when IFN-α treatment was at the time of infection (0 h post-infection) and decreased over time to only slight inhibition at 20 h after infection (Fig. 1b, c). At 30 h post-infection, IFN-α had no effect on the replication of cp BVDV while it inhibited ncp BVDV replication by 1–2 logs (Fig. 1c). Similar results were obtained with 296-cp and 296-ncp strains (data not shown).

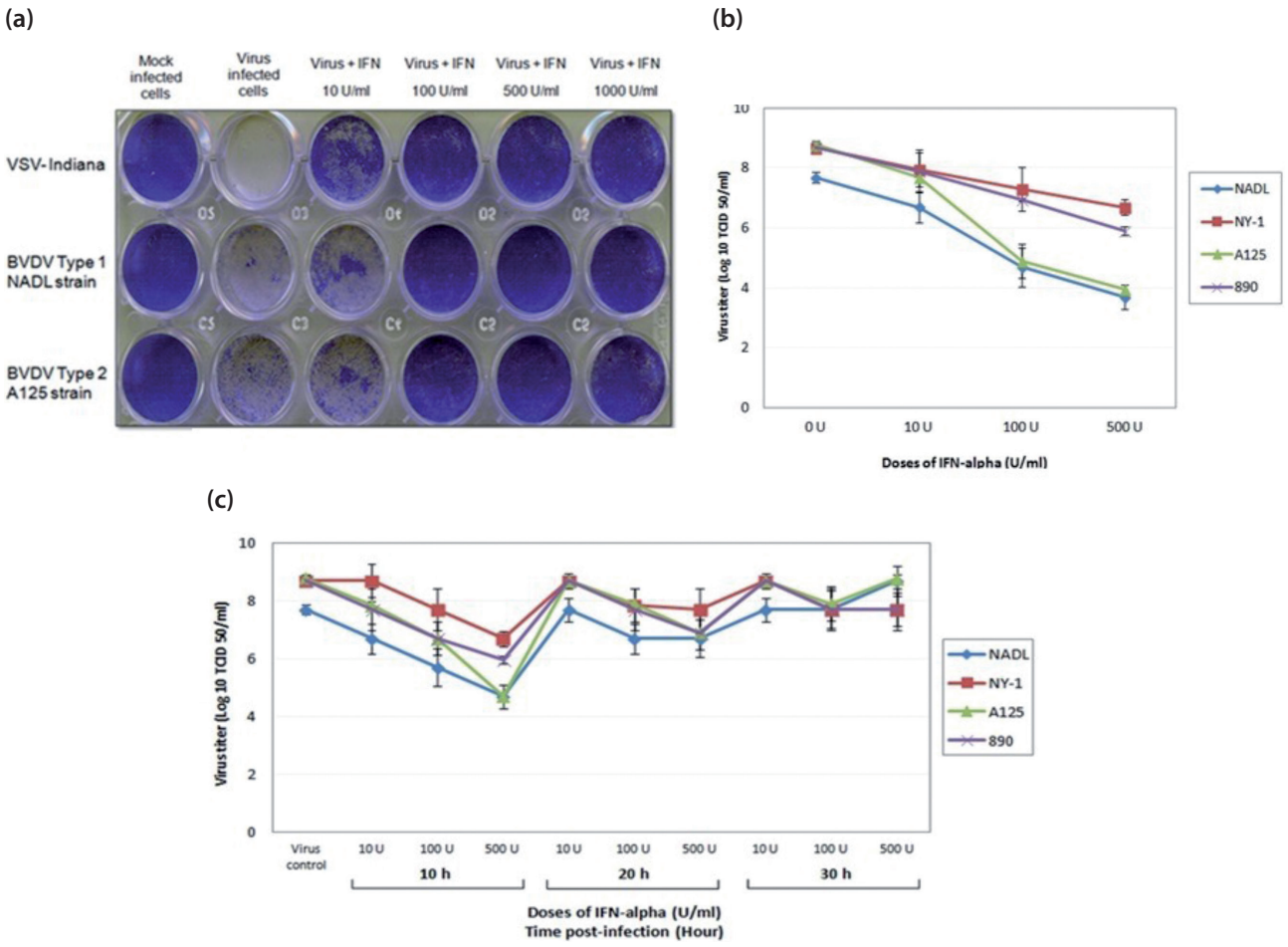

Fig. 1

Inhibitory effect of IFN-α on CPE and replication of BVDV

Different doses of IFN-α (10, 100, 500, or 1000 U/ml) were added to MDBK cells infected with different cp and ncp strains of BVDV. IFN-free medium was added to mock-infected cells. **(a)** The intensity and progression of CPE of cp BVDV strains and VSV were analysed 72 h post-infection after staining with 1% crystal violet. **(b)** Dose-response curve of the reduction in the titers of both cp (NADL, A125) and ncp (NY-1, 890) BVDV strains after IFN-α addition (YRA) at time of infection. The virus yield was determined by $TCID_{50}$. The difference between IFN effect on the replication of cp and ncp BVDV strains and the effect of IFN dose on both biotypes were highly significant (P value was <0.0001). **(c)** The effect of IFN-α on the replication of cp (NADL, A125) and ncp (NY-1, 890) BVDV strains (YRA) at different times post-infection. The time effect of IFN-α on both BVDV biotypes was highly significant (*P* value was < 0.0001).

Inhibition of RNA synthesis of cp and ncp BVDV strains by IFN-α

The standard curve was obtained by linear regression analysis of a plot of the CT values against the log10 initial copy number of three replicates of the pcBVDV80 positive control plasmid. The cp BVDV RNA was detected in infected cells at 8 h compared to 10 h for ncp. The RNA level of cp strains was 2 logs higher than the ncp strains at 12 h to 20 h post-infection but decreased to less than 1 log at 24 h post-infection (Fig. 2a). Similar results were obtained after analyzing RNA accumulation levels of the type 2 BVDV, 296 (cp and ncp). The cp BVDV RNA was detected earlier and the RNA level was 2 logs higher then ncp RNA indicating that the differences in RNA level are maintained (Fig. 2a).

To test the hypothesis that IFN inhibition of BVDV replication occurs through the direct inhibition of viral RNA production, the effect of IFN-α on viral RNA levels of infected cells with either BVDV biotypes was analysed by qRT-PCR. MDBK cells were infected with A125 (cp) or 890 (ncp) at MOI of 1 and then treated at different times of infection with IFN-α (100 U or 500 U/ml). The cells were incubated at 37°C for 16 h post-infection and the RNA level was determined by qRT-PCR. IFN-α greatly decreased RNA production and its effect was dose- and biotype-dependent

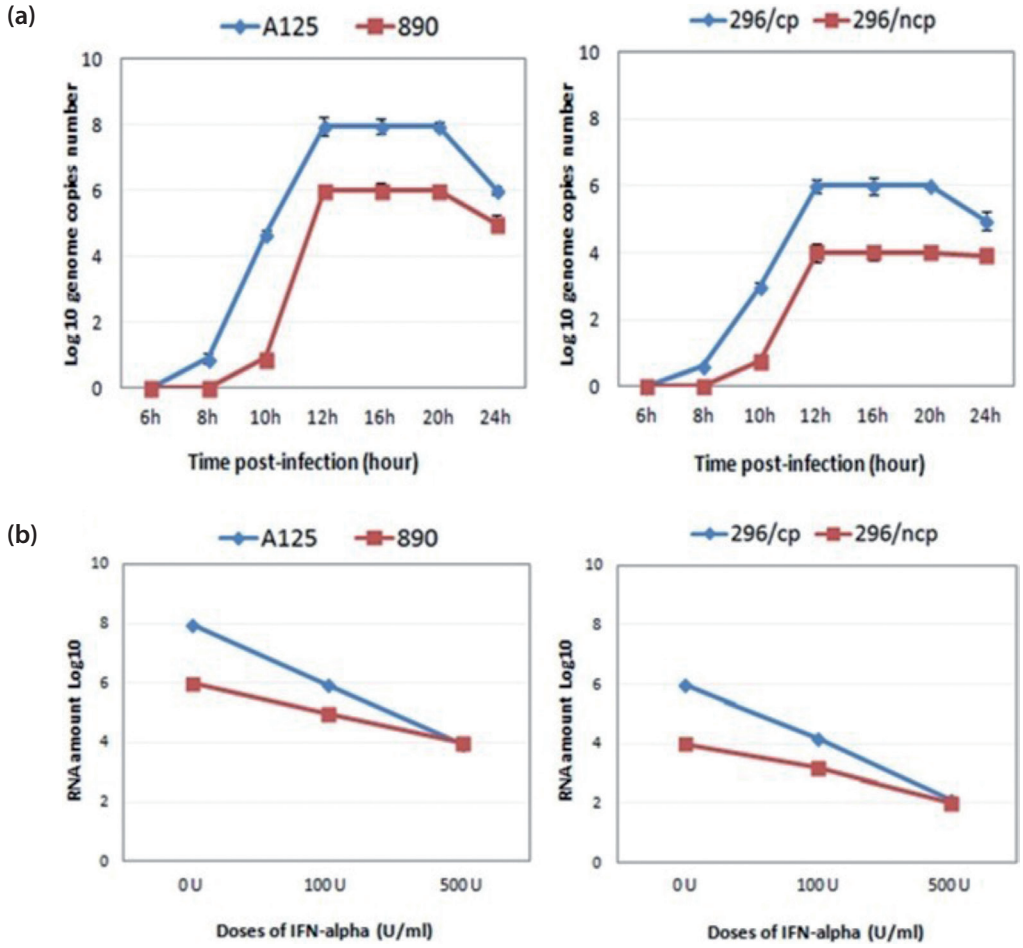

Fig. 2

RNA level of cp (A125 and 296/cp) and ncp (890 and 296/ncp) biotypes of BVDV in infected MDBK cells without (a) and with (b) exogenous IFN-α treatment

Viral RNA levels in the culture harvests were measured by qRT-PCR, using Taqman. **(a)** The kinetics of RNA production by the laboratory BVDV strains (cp, A125 and ncp, 890 strains,) (left chart) and by BVDV strains isolated from infected animals (296/cp and 296/ncp) (right chart) at different time points of 6 h, 8 h, 10 h, 12 h, 16 h, 20 h, and 24 h post-infection. The experiment was done in triplicate and the mean values are shown. **(b)** Quantitative analysis of the effect of two different concentrations of IFN-α (100 or 500 U/ml) at time of virus infection on RNA level of the laboratory BVDV strains (cp, A125 and ncp, 890 strains) (left chart) and of the BVDV strains isolated from infected animals (296/cp and 296/ncp) (right chart). The experiment was done in triplicate and the mean values are shown.

(Fig. 2). In cp BVDV, IFN-α decreased the viral RNA production by nearly 4 logs, while ncp BVDV RNA production was only reduced by 2 logs (Fig. 2b). Similar results were also obtained after using the 296 pair (cp and ncp) of type 2 BVDV. IFN-α decreased the viral RNA production of cp 296 BVDV by almost 4 logs, which was 2 logs more than that of ncp 296 BVDV (Fig. 2b).

PKR activity in IFN-induced BVDV inhibition

Since PKR plays a role in the antiviral action of IFN, we tested its activity in BVDV-infected and IFN-treated cells. The role of PKR in IFN-inhibition of BVDV replication was tested by monitoring the recovery of virus replication after the addition of 2-AP, an inhibitor of PKR phosphorylation. Although the phosphorylation state of PKR was not evaluated, the assumption was made that the reversal of IFN inhibition of virus replication following addition of 2-AP could only occur by preventing PKR phosphorylation. Three different concentrations of 2-AP (250, 500 or 1000 μM) were added 2 h prior to infection with BVDV strains (NADL, NY-1, A125, or 890) or 15 h after incubation with 100 U/ml IFN-α. The 2-AP did not rescue virus replication of either BVDV biotypes (Fig. 3a). Further, increasing the 2-AP to higher doses (up to 5 mM) had no effect on virus recovery and was toxic for MDBK cells (data not shown).

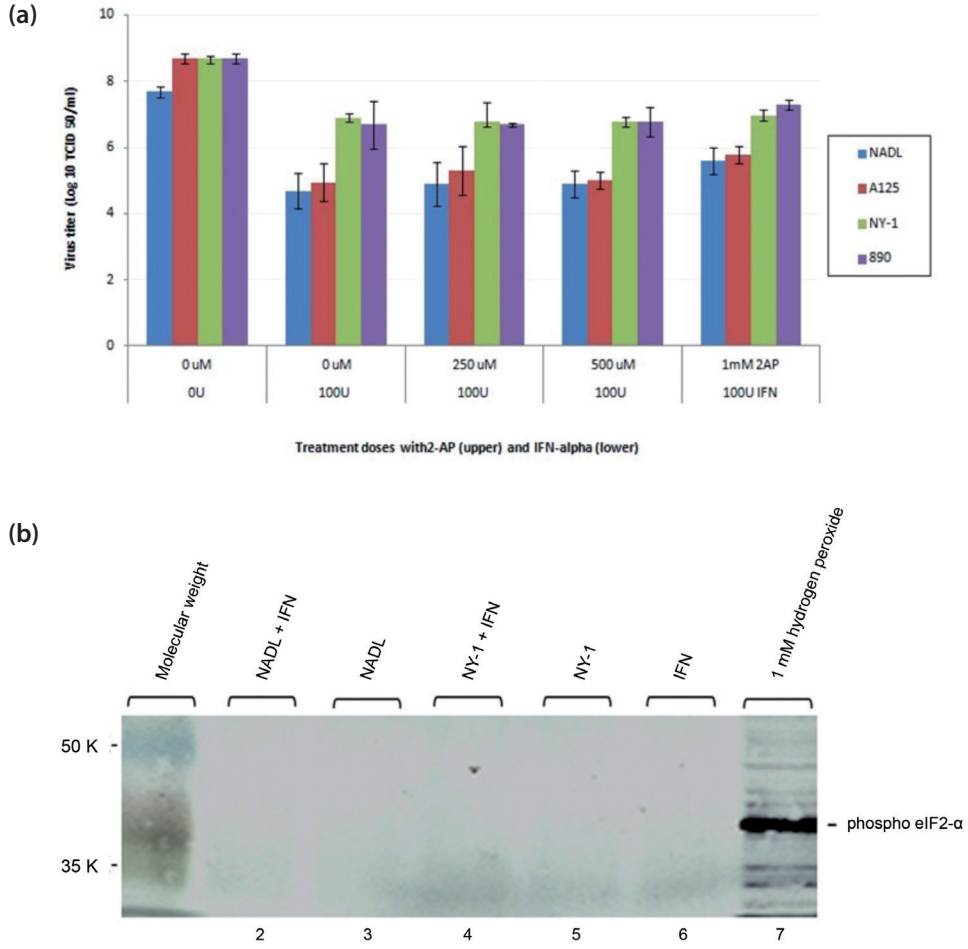

Fig. 3

The role of PKR activity in IFN-induced BVDV inhibition in MDBK cells

(a) Recovery of BVDV replication in IFN pretreated MDBK cells using 2-AP. Different concentrations of 2-AP (250, 500 or 1000 μM) were added on IFNα pretreated (15 h) MDBK cells at approximately 2 h prior to infection with NADL, A125, NY-1 or 890 BVDV strains. The virus yield was calculated by $TCID_{50}$ on MDBK cells. The experiment was done in triplicate and the mean values are shown. **(b)** Western blot detection of phosphorylated eIF2-α in IFN (500 U) pretreated MDBK cells that were simultaneously infected with NADL or NY-1 BVDV strains. As controls, cells treated with 1 mM hydrogen peroxide and infected non-treated cells were included.

To further determine the activity of PKR in IFN antiviral mechanism, we measured the phosphorylation of eIF2-α, a PKR target and phosphorylated by the activated PKR, using western blot assay. The phosphorylated eIF2-α was not detected in either BVDV biotypes and there were no differences between IFN-α treated or non-treated cells (Fig. 3b).

Discussion

There have been several previous reports of IFN-α and its effect on BVDV (Bielefeldt Ohmann and Babiuk, 1988; Sentsui *et al.,* 1998; Woodhouse *et al.,* 2008; Zhang *et al.,* 2010). However, the comparative effect of exogenous IFN-α on both BVDV biotypes remains unclear. To widen the study of IFN-α effect on BVDV, we have tested the effect of HuIFN-α on replication of six different BVDV strains *in vitro*. Evaluation of antiviral activities of bovine IFN-α (BoIFN-α) against BVDV *in vitro* would have been ideal; however, these recombinant products were unavailable at the time these experiments were performed. Recombinant HuIFN-α -2a was used in our study because it had profound antiviral activity against BVDV *in vitro* (Peek *et al.*, 2004) and because bovine cells, compared with cells of other species, are the most sensitive to the antiviral effect of recombinant human interferons *in vitro* (Cutrone and Langer, 2001; Bridgman *et al.*, 1988). HuIFN-α had higher antiviral activity in MDBK

cells than recombinant BoIFN-α by a factor of 5–10 based on vesicular stomatitis virus (VSV) plaque reduction assay (Capon *et al*., 1985).

First, to determine whether the inhibitory effect of IFN-α on replication of BVDV biotypes was due to inhibition of the initiation of BVDV replication, the infected cells were treated with different concentration of HuIFN-α at different time points post-infection. Significant inhibition ($P<0.0001$) of replication was observed with both BVDV biotypes and the degree of inhibition was higher (up to 3 log) in cp than ncp. IFN had the maximum inhibition in the early stage of infection, while in the late stage it had no effect on the cp strains but inhibited replication of some ncp strains. The differences in BVDV biotype inhibition could be attributed to 1) the difference in replication rate of both biotypes, where the cp biotype replication rate is higher and reaches its maximum state of infection earlier than ncp when the IFN is no longer effective; 2) the cell condition, where the cell machinery is destroyed in cp infected cells while ncp does not induce apoptosis and the cellular response to IFN is still intact. The inhibitory effect of HuIFN-α on both biotypes of BVDV started at a dose up to 10 U/ml and increased proportionally reaching its maximum effect at 500 U/ml. Our results were in agreement with previous work indicating that HuIFN-α led to a decrease in BVDV replication and this reduction was dose dependent (Sen, 2001; Durantel *et al.,* 2004). However, only doses above 1000 U/ml inhibited BVDV replication *in vitro* (Sentsui *et al.,* 1998).

One of remarkable differences between the two BVDV biotypes is viral RNA accumulation in infected cells (Gil *et al.,* 2006). The kinetics of RNA production in both BVDV biotypes was studied by qRT-PCR. Cp BVDV RNA was detected earlier and viral RNA levels from cp BVDV infected cells were 2 logs higher than ncp. Interestingly, the difference in RNA levels between the two biotypes was decreased to less than 1 log at 24 h post-infection. Using Northern blot technique, similar results were obtained by Mendez and Vassilev (Mendez *et al.,* 1998; Vassilev and Donis, 2000). The reason for the increased RNA production in cp BVDV infected cells could be attributed to the expression of NS3 that increases the replication rate (Lackner *et al.,* 2004). While the later reduction in RNA level could result from the increased IFN production in cp BVDV infected cells. Previous results indicated that cp but not ncp strains of BVDV induce the synthesis of α/β interferons (Perler *et al.,* 2000; Charleston *et al.,* 2001; Schweizer and Peterhans, 2001; Baigent *et al.,* 2002; Peterhans *et al.,* 2003; Reid *et al.,* 2016). In contrast, other works indicated that also ncp BVDV can induce IFN (Charleston *et al.,* 2002; Smirnova *et al.,* 2008). To test the hypothesis that HuIFN-α inhibition of BVDV replication occurs through inhibition of viral RNA production, the effect of HuIFN-α on RNA levels of both BVDV biotypes was measured. Our results showed that HuIFN-α treatment of BVDV decreased RNA levels of both biotypes and the effect on cp exceeded ncp about 2 logs. Why the ncp biotype viral growth and RNA accumulation was less affected by IFN-α treatment than cp biotype? It seems likely that a state of equilibrium exists between IFN and ncp biotype, with lower induction or possibly inhibition of IFN activity. Due to expression of NS3 in cp biotype, this equilibrium is shifted by increasing the viral replication rate, with subsequent over-expression of IFN. The increased IFN response in cp BVDV infected cells results in increased antiviral effect on the cp biotype.

PKR is an essential protein mediator of the antiviral effects induced by interferon. It is still unclear to which extent the inhibitory effect induced by the exogenous IFN on both BVDV biotypes relies on PKR pathway. PKR activation in HuIFN-α-treated-BVDV-infected MDBK cells was examined. Although western blot analyses of PKR activation in BVDV-infected bovine testicle cells had significant PKR activation in the cp-BVDV infected cells (Gil *et al.,* 2006), in our study with MDBK cells, neither cp- nor ncp-BVDV viruses activated PKR. The difference in cell culture systems used in both studies could be the reason for such different results. For further assessment of PKR activation in the BVDV-infected MDBK cells, an additional experiment with 2-AP treatment was done. After binding to viral RNA, PKR is auto-phosphorylated and then it phosphorylates the alpha subunit of eIF2 which inhibits the viral RNA translation. The PKR phosphorylation is inhibited by 2-AP, an ATP analog. In our results, the addition of 2-AP to infected cells pre-incubated with 100 U/ml of IFN-α did not rescue viral growth of either BVDV biotypes indicating that IFN-α did not activate the PKR pathway. However, other works showed that cp BVDV but not ncp biotype was a PKR inducer (Baigent *et al.,* 2002; Gil *et al.,* 2006). Other IFN antiviral effects such as the 2'5'A/RNase L or Mx proteins may be utilized. For most viruses, IFN impairs more than one step of the viral life cycle, so there is a cumulative effect on virus yields (Vilcek, 2003). Most recently, TRIM56, the IFN inducible E3 ubiquitin ligase was shown as novel antiviral host factor that restricts pestivirus infection (Wang *et al.,* 2011).

In conclusion, the results of this study demonstrated that the exogenous HuIFN-α inhibited the replication and RNA synthesis of both BVDV biotypes and the inhibitory effect was time and dose dependent. The IFN-inhibitory effect was more potent in cp BVD biotype with no differences among different genotypes. Further studies such as the level of endogenous IFN produced by the cells and other IFN pathways in the BVDV infections need to be explored.

There may have been some difference in responses if bovine IFN-α had been used and would be a consideration in further studies. Administration of naturally infected animals with IFN-α could be a new strategy for the treatment of the disease.

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