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EFFECT OF BOVINE VIRAL DIARRHEA VIRUS INFECTION ON DNA INTEGRITY AND INTERFERON-GAMMA (IFN-Γ) RELEASE IN BUFFALO MACROPHAGE

(With 2 Tables and 5 Figures)

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(Received at 8/3/2009)

تأثير العدوى بفيروس الإسهال البقرى على سلامة الحمض النووى مع إفراز الإنترفيرون جاما على الخلايا البلعومية للجاموس

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تهدف هدة الدراسة إلى دراسة تأثير العدوى بالعترة المرجعية العالمية لفيروس الإسهال البقرى على سلامة الحمض النووى للخلايا البلعومية المستخلصة من دم الجاموس والمنماة معملياً فى طبق زرع نسيجى ذات ٢٤ عين مع قياس نوعى لمستوى الإنترفيرون جاما المفرز من تلك الخلايا. تم حقن الخلايا بالفيروس دات العيارية $١٠^{٦.٤}$ مع إعتبار يوم الحقن هو اليوم صفر. تم تجميع الميديا يوميا من عمود من طبق الزرع لقياس مستوى الإنترفيرون جاما وتجميع الخلايا لدراسة سلامة الحمض النووى. أيضا تم تجميع الميديا والخلايا غير المحقونة كعينة ضابطة حيادية يوميا. بدراسة سلامة الحمض النووى للخلايا البلعومية المحقونة على مدار الأربعة أيام وجد أن هناك تكسير فى الحمض النووى بنسب تصاعدية حيث يبدأ بعد ٢٤ ساعة من الحقن ويصل إلى أعلى مستوى عند اليوم الثالث والرابع من الحقن مقارنة بالعينة الضابطة الحيادية. بقياس مستوى الإنترفيرون جاما قياسا نوعيا وجد أن هناك زيادة تدريجية خلال الأربعة أيام حيث تبدأ من اليوم الثانى للحقن مع الوصول إلى أعلى مستوى فى اليوم الثالث و الرابع. تخلص تلك الدراسة إلى أن فيروس الإسهال البقرى له تأثير سلبى ومدمر على الخلايا المناعية.

SUMMARY

The present study aimed to investigate the effectiveness of a national animal disease laboratory (NADL) strain as cytopathic bovine viral diarrhea virus (cp- BVDV) infection on DNA integrity of blood derived buffalo macrophages (MΦ) and Interferon-Gamma (IFN-γ) release *in vitro*. The virus ($10^{6.4}$ $TCID_{50}$) infect the matured MΦ, cells in one column of inoculated MΦ as well as non infected MΦ were scraped daily and collected for DNA damage using Comet assay. The level of IFN-γ was determined too. Comet microphotograph of inoculated MΦ along the 4 days tested period showed, balloon shaped tail with different percent of DNA damage. The DNA damage is beginning at 24 h postinoculation (PI) and reached to the maximum at 72-96 h PI as compared to the negative control. A qualitative measurement of IFN-γ in the supernatant of inoculated MΦ as compared with the negative control revealed gradually increased of IFN-γ secretion through out tested period that beginning at the 2nd day PI and reach to the plateau out through the 3rd and 4th day PI. In conclusion, BVDV has suppressive effects on immune cells as demonstrated *in vitro* that damage of macrophage.

Key words: *BVDV, Macrophage, Comet assay, IFN-γ.*

INTRODUCTION

Bovine viral diarrhea virus (BVDV) could be a key component in multiple-etiology diseases and immunosupression which still a matter of debate (Piccinini *et al.*, 2006). Bovine viral diarrhea virus is a heterogeneous group of viruses of the family *Flaviviridae* grouped in the genus *Pestivirus* together with closely related classical swine fever virus (CFSV) of pigs and border disease virus (BDV) of sheep (Thiel *et al.*, 2005). There are two genetically distinct types of BVDV, BVDV-1 and BVDV-2, which can be differentiated from each other and from other pestiviruses (Ridpath *et al.*, 1994). These 2 genotypes are further characterized into two biotypes, cytopathic (cp) and non cytopathic (ncp), defined by their effect on cultured cells (Paton, 1995). Three additional genotypes have been proposed (Becher *et al.*, 1997; Ridpath, 2003).

In vitro, BVDVs infection of monocytes or macrophages (MΦ) causes the synthesis of cytokines, reduction of the phagocytic activity and Fc and C3 receptors expression on alveolar MΦ (Welsh *et al.*, 1995),

inhibition of basic metabolic activities of peripheral blood mononuclear cells (PBMCs) such as DNA synthesis (Hou *et al.*, 1998).

Viruses have evolved strategies to modulate (either induce or prevent) apoptosis in their host cells. These strategies include modulation of the Bcl-2/Bax pathway, interference with Caspases or inhibition of the PKR/RNase L pathway. Some of these apoptosis-modulating activities have been mapped to specific viral genes. The cp-BVDV-infected cells undergo apoptosis and may promote apoptosis in uninfected cells (Razvi and Welsh, 1995; Lambot *et al.*, 1998; Schweizer and Peterhans, 1999).

There are several techniques employed for evaluating defects in DNA integrity or chromatin structure. These techniques are, single cell gel electrophoresis (Comet assay), terminal transferase dUTP Nick End Labeling (TUNEL), sperm chromatin structure assay (SCSA), in situ nick translation (ISNT) and acridine orange test (Shamsi *et al.*, 2008).

The present study aimed to investigate the effectiveness of a national animal disease laboratory (NADL) strain, as cp-BVDV, infection on DNA integrity of blood derived buffalo Mϕ and IFN-γ release *in vitro*.

MATERIALS and METHODS

1. Preparation of macrophage (Mϕ):

Blood-derived Mϕ was isolated and cultured from buffalo's calves as described briefly by Jungi *et al.* (1997). Peripheral blood mononuclear cells (PBMC) were isolated from heparinzed blood using Ficoll-Hypaque and centrifuged at 1600 *xg* for 30 minutes. The interface layer (lymphocytes and monocytes) was collected and washed 3 times by HEPES balance salt solution (HBSS) and centrifuged at 1800 *xg* for 10 minutes per a time. Macrophages were permitted to differentiate by culturing the suspended cells in growth RPMT-1640 [G-RPMI; RPMI-1640 with 10% fetal bovine serum (FBS)] medium in 24 well tissue culture (TC) plate as 4 x 10^6 cells/ml for 3 h under the standard culture conditions (37 °C, 5% CO_2, and 90% humidity). The non adherent cells as well as the culture medium were removed and a new G-RPMI was added and re-cultured the plate under the standard culture conditions for 5 days.

2. Virus titration:

A reference international cp-BVDV-NADL strain was cultured and titrated in Madin-Darby bovine kidney (MDBK) cell cultures (CC) and the titer of the virus was calculated according to Reed and Muench (1938) method.

3. Virus inoculation of cultured Mϕ :

cp-BVDV-NADL strain at a titer $10^{6.4}$ was used to infect the matured Mϕ in the CC plate as 20 µl/well and a maintenance RPMI-1640 (M-RPMI; RPMI-1640 with 2% FBS) medium was added. The plate was incubated for 5 days under the standard culture conditions. The day of inoculation was considered as a zero day. Daily, cells in one column of inoculated Mϕ as well as non infected Mϕ (a negative control) were scraped and collected for DNA damage examination over the tested period. The supernatant M-RPMI of inoculated as well as non infected Mϕ was collected daily for the released IFN-γ measurement.

4. A single cell gel electrophoresis (Comet assay):

As described by Bock *et al.* (1999), a layer of 1% (200 µl) normal melting agarose (Sigma, Ames Iowa, USA) was prepared on special full forsted microscopical slide (Labcroft, Great Britain) precoated with 30 µl of 1% normal melting agarose. The scraped cellular Mϕ was mixed with 0.8% low melting agarose (Sigma, Ames Iowa, USA) to obtain a final concentration 1.5 X 10^5 cells/ml. Fifty µl of Mϕ /agarose mixture was pipette onto the precoated slide and covered with a coverslip (22 X 40 mm). The agar was allowed to cool down for 5 minutes on cold metal plate. The coverslip was removed and the microscopical slide was immersed in cold lyses solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM tris pH 10, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) at pH 10 and left at 4°C for 3 h. The slide was washed by cold distilled water, placed in alkaline electrophoresis buffer at pH 13 (0.3 M NaOH and 1 mM Na_2 EDTA) and left at 4°C for 1 h. Subsequently, the slide was transferred to an electrophoresis tank in a freshly prepared electrophoresis buffer and run at 70 volts for 50 minutes. The slide was then neutralized with 0.4 M tris at pH 7.5 for 5 minutes before stained with 25 µM ethidium bromide (Sigma, Ames Iowa, USA). Finally, the slide was covered with a coverslip and incubated in the dark at the room temperature for 5 minutes.

Examination of the tested slide was performed with an inverted epifluorescence phase-contrast trinuclear microscope (Nikon ECLIPSE-TS100, Japan) with 40 X plan a chromatic lens and a digital camera

DS-U2 with software NIS elements. Comet was analyzed using computerized image analysis system (Kinetic imaging opticals, MÜnchen, Germany). The concept of tail moment = tail length (µm; micro meter) X percentage of damaged DNA in the tail (Olive *et al.*, 1990).

5. Interferon-Gamma (IFN-γ) production assay:

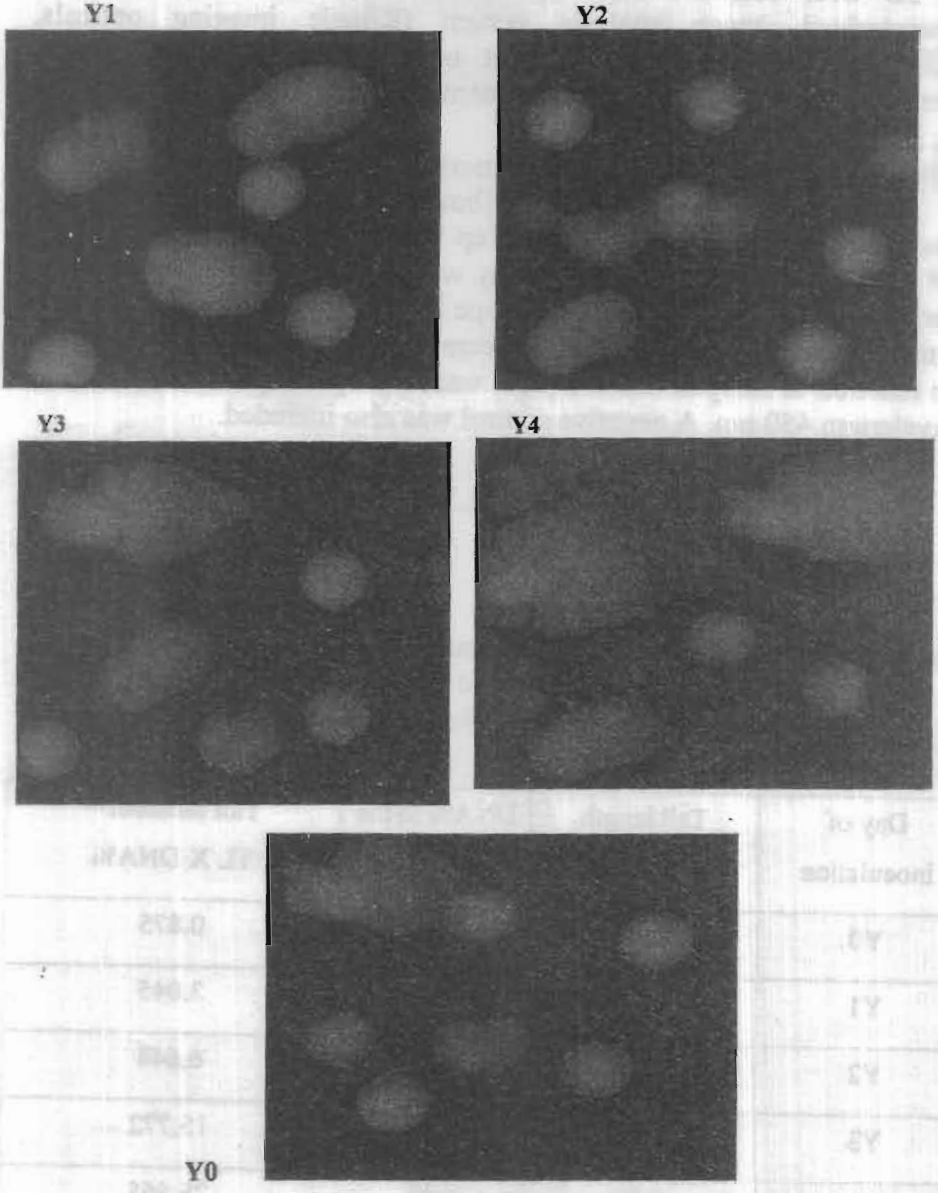
A qualitative assessment of bovine interferon-gamma (IFN-γ) production in cell culture medium of cp-BVDV-NADL strain-inoculated Mϕ using an enzymatic immunoassay was assessed as described by the manufacturer guide (BioSource, Europe S.A.). The assay was performed in microplate and is designed to measure the IFN-γ in two steps. Finally, the reaction is stopped and the plate was read spectrophotometrically at wavelength 450 nm. A negative control was also included.

RESULTS

1. Comet assay analysis:

Table 1: Comet assay analysis of inoculated Mϕ with cp-BVDV-NADL strain along the 4 days tested period (Y1-Y4), Y0 represented the viability of Mϕ-DNA before the virus inoculation (negative control).

Day of inoculation	Tail length (TL; µm)	DNA% in the tail	Tail moment = TL X DNA%
Y0	1.51	0.58	0.875
Y1	2.16	1.41	3.045
Y2	3.21	2.07	6.644
Y3	5.42	2.91	15.772
Y4	6.71	3.81	25.565



Slides (Y1-Y4) showed DNA damage in cp-BVDV-NADL strain inoculated MΦ along the 4 days with balloon shaped tail of the nuclei and different percent of DNA damage (different density of fluorescence). **Slide** (Y0) represented the viability of MΦ- DNA before the virus inoculation (negative control).

2. IFN-γ production:

Table 2: The IFN-γ induction in macrophage inoculated with cp-BVDV-NADL strain.

Day of MΦ inoculation	Y0	Y1	Y2	Y3	Y4
OD at 450 nm wavelength	0.089	0.123	0.150	0.165	0.169

DISCUSSION

Overview on the DNA damage of cultured MΦ that is inoculated with the NADL strain in this study revealed the damage of DNA is beginning at 24 h PI and reached to the maximum at 72-96 h PI as compared to the negative control. It is noteworthy that the cleaved NS23 protein into NS2 and NS3 in the cp-biotype clearly correlates with its cytopathogenicity on infected cells (Meyers and Thiel, 1996). That, the NS3 (specially its N-terminal part) has nucleotide triphosphatase (NTPase), RNA helicase, and protease activities (Grassmann *et al.*, 1999 and Gu *et al.*, 2000). The cp-BVDV-infected cells undergo apoptosis that is associated with the activation of ICE/CED-3 cysteine protease results in cleavage of poly (ADP-ribose) polymerase (PARP) either direct participation of the viral protease or indirect induction of an endogenous signal (Hoff and Donis, 1997 and Schweizer and Peterhans, 1999). Cleavage of PARP disrupts its ability to coordinate genome maintenance activity (Kaufmann *et al.*, 1993). As the comet assay is a sensitive technique for detecting DNA damage, it is used in a large scale in the diagnosis. The assay is based on the principle of faster rate migration of smaller fragmented DNA towards anode in an electrophoretic field as compared to larger non fragmented DNA (Shamsi *et al.*, 2008 and Smart *et al.*, 2008), and the 'Tail moment' as simple way to quantify the amount of DNA damage using the digital comet image analysis (Frazer, 2005).

A qualitative measurement of IFN-γ in the supernatant of cultured MΦ that is inoculated with the NADL strain in comparison to the negative control revealed, gradually increased the IFN-γ secretion over the tested period that beginning at the 2nd day and reached to the plateau at the 3rd and 4th day PI. The replication of flaviviruses, including BVDV, is known to involve replicative intermediates and replicative forms of viral RNA. Therefore, BVDV-dsRNA can be

expected to be produced and is an important trigger for IFN synthesis (Lee and Esteban, 1994 and Castelli *et al.*, 1998). IFNs have been shown to prime the cellular apoptosis via increased synthesis of 2', 5'-oligoadenylate (2-5A) and its activation of RNase L (Diaz-Guerra *et al.*, 1997 and Castelli *et al.*, 1998), or via inhibition of protein synthesis by protein kinase (PKR) (Jagus *et al.*, 1999).

In conclusion, BVDV has suppressive effects on immune cells as demonstrated *in vitro* that damage of macrophage.

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