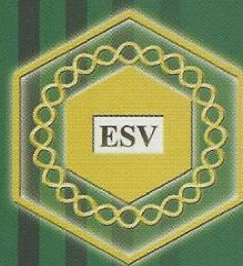


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INHIBITORY EFFECT OF CEFOTAXIME ON BOVINE ROTAVIRUS

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ABSTRACT

Bovine Rotavirus (BRV) is a major cause of neonatal calf diarrhea throughout the world. The successful effects of cefotaxime sodium (100 µg/ ml) on bovine rotavirus were studied. MDBK cells were treated with cefotaxime (CTAX) pre- or post-infection and the virus cytopathic effect was observed and virus proteins were analyzed by western blot technique. Also, the diarrheas were scored in BRV infected mice treated with CTAX. Results showed that the CTAX inhibited virus cytopathic effect on MDBK cells, and prevented virus induced diarrhea in experimentally infected mice. As revealed by western blot analysis of viral proteins expressed in CTAX pretreated MDBK cells, the effect of CTAX on BRV was related to the inhibition of synthesis of the VP3 and NSP2 rotavirus proteins, which are major components of the early replication intermediate of rotavirus. In conclusion, CTAX could be used as inhibitor of BRV replication.

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INTRODUCTION

Rotaviruses, members of the family *Reoviridae*, are the leading etiologic agent of viral gastroenteritis in infants and young animals (Parashar *et al.*, 2006).

Significant economic losses induced by group A rotaviruses in the dairy and beef industry due to increased morbidity and mortality, treatment costs, and reduced growth rates, were reported (Maes *et al.*, 2003). These non-enveloped

viruses are formed from three concentric layers of protein that surround a viral genome, formed of 11 segments of double-stranded RNA. The outermost layer of the virion is formed by two proteins, VP4 and VP7, which are involved in the early interactions of the virus with its host cell (Lopez and Arias, 2004). VP4 is involved in receptor binding and cell penetration. The role of VP7 is less clear, although it has been shown that it interacts with the cell surface molecules at a post-attachment step (Graham *et al.*, 2003). After binding to the cell surface, the virus penetrates the plasma membrane to infect the cell. This penetration depends on the trypsin treatment of the virus, which results in the specific cleavage of VP4 to polypeptides VP8 and VP5. This cleavage promotes VP4 rearrangements in the viral particles that rigidify the spikes (Dormitzer *et al.*, 2004). The genome, also, encodes six non-structural proteins, NSP1–NSP6, shown to function in transcription, dsRNA replication, translation of viral mRNA, cellular pathology and virus particle maturation (Estes, 2007). Endogenous transcription of rotavirus dsRNA occurs in double layer virus particles (DLP) formed

after virus entry to the cells and loss of the outer VP4/VP7 capsid layer. DLP are formed from the middle layer which consists of 260 trimers of VP6 and the inner layer which made of 60 dimers of the capsid protein VP2. Incorporation of the RNA-dependent RNA polymerase VP1 and guanylyltransferase methylase VP3 into the core of the virion occurs through the amino terminus of VP2 (Lawton *et al.*, 1997) and the non-specific single-stranded RNA and dsRNA binding activities of VP2 (Labbe *et al.*, 1994) made the double layer particles (DLP) transcriptionally competent structures (Estes *et al.*, 2001) present in cellular cytoplasm. Transcription of mRNA from dsRNA genome occurs in the DLP through VP1, the RNA-dependent-RNA polymerase (Valenzuela *et al.*, 1991), and VP3 for 5' end capping of mRNA to facilitate translation by the cellular translation machinery (Chen *et al.*, 1999).

Cefotaxime (CTAX), a member of Betalactam antibiotics (BLA) is the most widely used antibacterial drugs in practical medicine. The antibacterial mechanism of BLA centers around a specific and covalent binding of

the drug to bacterial enzymes, the so called penicillin binding proteins located at the outer side of the plasma membrane (Spratt, 1983). In addition, BLA have inhibitory effect on DNA polymerases of herpes simplex and vaccinia viruses (Nefel and Hubscher, 1987). In the present study we have demonstrated the inhibitory effect of CTAX on bovine rotavirus (BRV) replication *in vitro* (MDBK cells) and *in vivo* (mice).

MATERIAL & METHODS

1. Virus

Cell culture adapted strain of BRV of 10^6 TCID₅₀/ml titer was supplied by the department of Rinderpest-like diseases; Veterinary Serum and Vaccines Research Institute (VSVRI), Abbassia, Cairo.

2. Cells

Madin derby bovine kidney cell line (MDBK) established by (Mackpherson and Stocker, 1962) were supplied by the same department. It was used for virus propagation.

3. Cefotaxime sodium (CTAX)

It was purchased from Epico pharmaceutical company as vials of

Cefotax[®], containing 500 mg cefotaxime sodium, used to study its effect on BRV infection in tissue culture and mice.

4. Estimation of CTAX cytotoxic effect

MDBK cells were treated with 10, 50, 70, 90, 100, 110, 150, and 1000µg/ml of CTAX in macroplates. The mono-layers were incubated at 37°C for 1 hour before washing twice with PBS and flooding with the overlay medium. Controls using PBS instead of CTAX were prepared, inoculated, and assayed in similar manner.

5. Observations on the nature of the CTAX-virus cell interaction

Effect of CTAX pretreatment to infection of cells by BRV

Monolayer cultures of MDBK cells were inoculated with 1ml of PBS containing 100µg of CTAX and incubated at 37°C for 60 min. Thereafter, 100 TCID₅₀ of BRV were added, and the cultures were incubated at 37°C for 1 hour before washing twice with PBS to remove un-adsorbed viruses. Afterwards, washed cultures were flooded with the overlay maintenance medium. 24 hours post virus inoculation cells monolayer was observed for cytopathic effect (CPE). Controls

using PBS instead of CTAX were prepared and assayed in similar manner.

Effect of CTAX post-treatment to infection of cells by BRV

CTAX (100µg/ml) in PBS was added to the monolayer cultures of MDBK cells, after inoculation with 100 TCID₅₀ of BRV, at various times (0, 15, 30, 45 and 60 minutes). After 1 hour of virus inoculation, cultures were washed twice with PBS and flooded with the overlay maintenance medium. 24 hours post virus inoculation, cells monolayer was observed for CPE. Controls using PBS instead of CTAX were prepared and assayed in similar manner.

6. SDS-PAGE and Western blot assay

To determine the effect of CTAX on BRV replication, detection of viral proteins synthesized in CTAX pre-treated MDBK cells was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Briefly, 48 hours after CTAX treatment, cells were washed twice with PBS (pH 7.4) and mono-layers were harvested by

adding SDS-PAGE sample buffer [12 mM Tris/HCl (pH 6.8), 5% glycerol, 0.4% SDS, 10% β-mercaptoethanol and 0.02% bromophenol blue] followed by boiling for 10 min for complete lyses of cells. SDS-PAGE of 20µl of cell lysates and 10µl SeeBlue[®] Plus2 molecular weight protein standard (Life Technologies), loaded in parallel, was performed in a 10% separating gels and Tris-Glycine electrophoresis buffer. Thereafter, separated proteins were electro-blotted onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk with 0.1% Tween-20 in PBS before incubation with rabbit anti-BRV serum diluted 1:4000. Bound antibodies were detected by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). Molecular weight of detected proteins was compared with the protein standard.

7. Effect of CTAX on BRV infection in mice.

A total of 15 mice were divided into three groups. Group-1 included 5 mice treated with CTAX for 24 hours prior to inoculation with 100µl of BRV (10⁵ TCID₅₀). Group-2 included 5 mice

inoculated with 100µl of BRV (10^5 TCID₅₀) for 24 hours, thereafter, treated with CTAX. Group-3 included 5 mice inoculated with 100µl of BRV (10^5 TCID₅₀). Therapeutic doses of CTAX were calculated according to **Paget and Barnes (1964)**. All groups were examined for diarrhea till the 10th day post virus inoculation

RESULTS

1. Estimation of CTAX cytotoxic effect

Per estimations, 100 µg/ml was the maximum concentration of CTAX, which did not produce CPE in cultures of MDBK cells (Table 1).

Table 1: Effect of cefotaxime concentration on MDBK cell.

cefotaxime conc. µg/ml	CPE
10	-
50	-
100	-
110	-to +
150	+
1000	+

-: no CPE

+: CPE

2. Effect of CTAX pretreatment on the BRV-infected cells

CPE was completely inhibited with CTAX for 48hr post virus inoculation. These results suggest that the CTAX effect occurs very early in the viral infectious cycle, through its effect on cell receptor before virus entry or viral replication component. Notably, this inhibitory effect was reversible on the cells where CPE consisted of cytoplasmic vacuolation and degeneration and

detachment of cells from the monolayer, started to appear on BRV infected MDBK cells 72 hours post CTAX treatment.

3. Effect of CTAX post-treatment on BRV-infected cells

The time of appearance and extent of virus induced CPE were similar in test and control. These results suggest that no inhibitory effect of CTAX on BRV infected cells could be detected in case of post-treatment table (2).

Table 2: Effect of CTAX on viral infectious cycle

Time CTAX added in min	appearance of CPE
0	+
15	+
30	+
45	+
60	+
Positive virus control (no CTAX)	+
Negative Control (CTAX)	0

0: no CPE

+: CPE

3. The effect of different concentrations of CTAX on different dilutions of BRV

Virus induced CPE was demonstrable in MDBK cells in the higher dilutions of the inoculums, even with high CTAX

concentrations 70 and 90 µg/ml. CPE was absent in mixtures containing 100 µg of CTAX. The data suggest that, if a CTAX-virus complex is formed, it is readily dissociated on dilution of CTAX (Table 3).

Table 3: Lack of inactivation of BRV virus by CTAX

Dilution of Virus	Saline control	CTAX conc. (µg/ml) in CTAX-virus mixture		
		70	90	100
Undiluted	+	+	+	-
10 ⁻¹	+	+	+	-
10 ⁻²	+	+	+	-
10 ⁻³	+	+	+	-
10 ⁻⁴	+	+	+	-

- = no CPE; + = CPE

4. SDS-PAGE and Western blot assay

Comparative analysis of proteins expressed in uninfected MDBK cells (negative control), cells pretreated with CTAX then infected with BRV, and MDBK

cells infected with BRV (positive control) suggest that, CTAX inhibits the synthesis of 88 and 36 kDa molecular weight polypeptides that are related to rotavirus proteins VP3 and NSP2, respectively, as demonstrated in Figure (1).

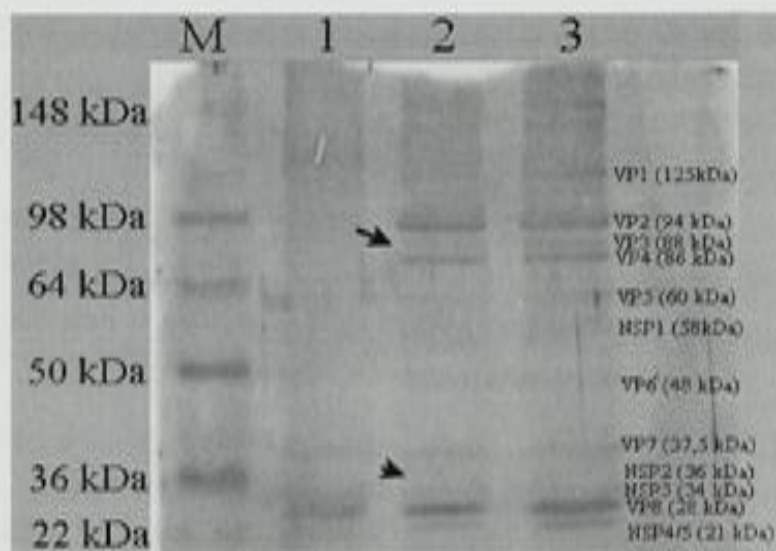


Figure 1: Analysis rotavirus proteins expressed in CTAX pretreated MDBK cell using western blot. (M); is the SeeBlue[®]Plus2 molecular weight protein standard with the corresponding apparent molecular weights in Tris-Glycine buffer system. (Lane1); MDBK cells treated with CTAX (negative control). (lane 2); MDBK cells pretreated with CTAX before infection with BRV. (Lane3); MDBK cells infected with BRV (virus positive control). Molecular weight of rotavirus structural (VP) and non-structural proteins (NSP) detected in western blot of the positive control lane was indicated at right side. Position of the rotavirus structural proteins VP3 and NSP2, which are inhibited by CTAX treatment (Lane 2), was indicated by arrows.

5. Clinical score of diarrhea in BRV infected mice pre- and post-CTAX treatment

The signs of diarrhea were stopped in the treated groups of mice (pre- or post- treatment with CTAX). The clinical scores of diarrhea in BRV infected mice pre- and post-treatment with CTAX and BRV infected mice without any treatment of CTAX (positive virus

control group) are illustrated in table 4. Both CTAX pre- and post-treated groups did not show any signs of infection with BRV as fecal pellets were observed. In contrast positive virus control mice group infected with BRV but not treated with CTAX, showed clear signs of infection appeared as pasty fecal consistency and diarrhea 5-7 days post virus infection.

Table 4: Clinical score of diarrhea in mice treated with CTAX in different times post BRV inoculation.

Group	Days post virus inoculation				
	1 st	3 rd	5 th	7 th	10 th
1	pellet*	pellet	pellet	pellet	Pellet
2	pellet	pellet	pellet	pellet	Pellet
3 (positive control group)	pellet	pasty	diarrhea	diarrhea	Pellet

* faeces consistency

DISCUSSION

Although rotavirus vaccines are often administered to pregnant domestic animals to increase levels of rotavirus antibodies in colostrum, neonates still frequently exhibit rotaviral diarrhea that may be attributed to failure of passive transfer of antibodies from dam to calf due to low levels of immunoglobulins in the dams milk (Hassan *et al.*, 2007). Accordingly, other therapeutics is needed to control rotavirus infection.

Our results demonstrated that cell viability was not significantly affected by 48 hours exposure to 100µg of CTAX (table 1), which come in agreement with Prokesch and Hand (1982) whereas betalactam antibiotics did not accumulate intra-cellular and the ratio of cellular-to-extracellular concentration of BLA, in different cell types, has been found to be less

than 1.0. Inhibitory effect of CTAX on BRV replication was detected when cells treated with CTAX then inoculated with BRV indicating their effect on cell receptor or viral replication component.

Comparative analysis of viral proteins expressed in BRV infected cells pretreated with CTAX indicated that CTAX inhibited the formation of a two polypeptides with molecular weights of 88 and 36 kDa corresponding to VP3 and NSP2 rotavirus proteins (Figure 1). VP3 is essential for transcription of rotavirus mRNA from dsRNA genome in the DLP where VP3 enzyme activity guarantees that the cap structure at the 5' end of each mRNA transcript, synthesized by VP1, is properly guanylated and methylated to facilitate translation by the cellular translation machinery (Valenzuela *et al.*, 1991; Chen *et al.*, 1999).

Inhibitory effect of Cefotaxime on bovine rotavirus

In addition to the inhibition of VP3 synthesis by CTAX treatment, the non-structural protein NSP2 was also inhibited as demonstrated by western blot analysis (Fig. 1). Non-structural proteins, except NSP1, are essential for rotavirus replication. NSP1 binds to the cellular transcription factor of the interferon regulatory factor 3 (IRF3) (Graff *et al.*, 2002) and targets it for degradation by the proteasome early post-infection (Barro and Patton, 2007). The loss of NSP1 does not seem to negatively affect rotavirus replication in cultured cells (Silvestri *et al.*, 2004). On the other hand, NSP2 along with NSP5 and VP1 are co-localized in the viroplasm acting as the main constituents of the replication intermediate (Taraporewala and Patton, 2001). In addition, NSP2 is crucial for virus packaging where it readily forms an octamer and has NTPase (nucleotide triphosphatase), ssRNA-binding activity, and helix destabilizing activities, promoting its function as a molecular motor using the energy derived from NTP hydrolysis to facilitate genome packaging.

Consequently, down regulation of VP3 and NSP2

synthesis by CTAX treatment correlates with the inhibition of BRV replication in MDBK cells and loss of CPE induced by the cell culture adapted BRV strain for 48 hours after virus inoculation. In addition, detection of other BRV proteins in the BRV infected cells and pre-treated with CTAX, as revealed by western blot, indicates that no interference with virus entry into MDBK cells occurred; supporting the notion that CTAX inhibited virus replication components rather than blocking of cell receptors necessary for virus entry.

It was not surprising that the inhibitory effect of CTAX on BRV lasts only for 48 hours before appearance of CPE, as instability of CTAX under conditions of *in vitro* microbiological testing was reported before and attributed to the metabolism or degradation of CTAX to less active or inactive metabolites by serum esterases, elevated temperatures or a pH outside of its stability range (Marchbanks *et al.*, 1987). We speculate that late appearance of the CPE, in BRV infected MDBK cells at 72 hours post-CTAX treatment, might be attributed to a reversible inhibitory effect of

CTAX on BRV replication due to the degradation of CTAX to its primary metabolites as mentioned above.

In addition to the down-regulation of rotavirus VP3 and NSP2 expression, BLA could also modify the immune functions by their proliferative effects including generation of virus-specific cytotoxic T cells and proliferation of lymphocytes (Bessler *et al.*, 2000). This may explain the absence of diarrhea in mice treated with CTAX pre- and post-virus inoculation.

To the best of our knowledge, this work is the first research evaluating the *in-vitro* effect of CTAX on BRV replication on cell culture as well as the *in-vivo* effect in mice.

In conclusion, cefotaxime was indicated in rotavirus infected cases till vaccination. Besides, it can be added to tissue culture media to prevent contamination with rotavirus and we recommend further research to confirm this application.

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