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Original article

Bovine herpes virus type 4 alters TNF-α and IL-8 profiles and impairs the survival of bovine endometrial epithelial cells

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ABSTRACT

Bovine herpes virus type 4 (BoHV-4) can be transmitted by contaminated semen to cows at the time of breeding and may cause uterine disease. The aim of this study was to characterize the susceptibility of bovine endometrial epithelial cells (bEEC) to BoHV-4 by using an *in vitro* model. When bEEC were challenged with different multiplicity of infection (MOI; from 0.001 to 10) of BoHV-4 for 6 days, a significant decrease in cell survival with increasing MOI was observed. The bEEC were subsequently challenged with BoHV-4 MOI 0.1 for 7 days. During the first 4 days, numbers increased in a similar way in controls and infected group ($p < 0.01$ when compared to Day 0). After Day 4, numbers of live cells in infected samples decreased when compared to controls and were lower than control at Day 7 ($p < 0.01$). From titration and qPCR, increasing number of viral particles was observed from Day 1, and reached a plateau at Day 5. Concentrations of IL-8 increased with time and were higher in supernatants from infected cells than in controls ($p < 0.0001$). TNF-α concentrations presented similar profile as cell survival ones. In conclusion, the survival of bEEC was strongly impaired by BoHV-4 infection in a time and dose dependent manner and supernatant cytokine profiles were altered. This information supports BoHV-4 implication in clinical cases of uterine diseases and the existence of a risk of BoHV-4 transmission from infected males through animal breeding.

1. Introduction

Modern dairy cows are at very high risk of suffering from uterine disease and the number of exposed cows in Europe has reached several million per year [1]. Cows diagnosed with metritis have low fertility, extended unproductive periods and high culling rates [1,2] thus affecting herd profitability and animal welfare. These negative impacts are probably stronger due to undiagnosed forms of uterine dysfunction such as asymptomatic persistent inflammation of the endometrium (endometritis) which can perturb embryo-maternal interactions at the time of implantation and impair fertility [1,2]. So far, several specific pathogens have been identified as metritis and endometritis inducing agents [1,3–6]. *Escherichia coli* (*E. coli*) and *Trueperella pyogenes (T. pyogenes)* are the most prevalent bacteria isolated from diseased cows. In addition to their direct effects, these pathogens pave the way for subsequent infection by other bacteria or viruses such as bovine herpesvirus 4 (BoHV-4) [7–9]. BoHV-4, a double stranded DNA virus and member of the *Herpesviridae* family has been first isolated from a variety of diseases such as

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respiratory and ocular disease in calves [10]. Moreover, an association between BoHV-4 seropositivity, postpartum metritis, abortion and chronic infertility has been reported in many studies [11–13] and BoHV-4 infection is considered as a risk factor in uterine diseases [14–16]. BoHV-4 causes cytopathic effects (CPE) and replicates in culture in a wide range of cell lines and primary cultures of various animal species [17,18]. However, different cell types can be more or less susceptible to BoHV-4 [19,20]. In bovines, BoHV-4 has a striking tropism for bovine endometrial cells and these cells are highly susceptible to BoHV-4 replication as evidenced by the strong CPE reported for bovine endometrial cell cultures [21,22]. In stromal cell cultures, viral replication and CPE have been shown by immunofluorescence [20]. Virus-infected cells synthesise and secrete type I interferons (IFN α/β) which are a major players in the antiviral defence response against all kinds of viruses [23]. Immune cells or infected cells secrete more cytokines such as IL-1α, IL-1β, IL-6, and TNF-α, which kill virus-infected cells and act as a bridge between innate and adaptive response [24]. Mechanisms by which BoHV-4 alters cell function have been documented mainly in immune and stromal cells whereas responses to BoHV-4 have not been so well documented in endometrial epithelial cells. It is important to define the responses of these cells to BoHV-4 infection because they are the first to be exposed to the virus especially if the contamination occurs at the time of reproduction through natural or artificial insemination. These routes of infection have not been investigated specifically to date, but the presence of BoHV-4 DNA has been reported in cases of oedematous orchitis and also in the sperm of healthy bulls indicating that sperms represent a potential vector for BoHV-4 transmission to cows [25,26]. Therefore, we have developed an *in vitro* model to investigate interactions between endometrial epithelial cells and BoHV-4. This present study investigated the impact of changes in the number of live endometrial epithelial cells in connection with viral replication while taking into account individual host cow variation. The impact of infection on the patterns of a selected set of cytokines was also investigated.

2. Materials and methods

2.1. Sample collection and selection

All animal samples used were obtained from cows which were not slaughtered for the purposes of this study and therefore no ethical permission was required. Genital tracts were collected from the slaughter house and brought back on ice to the laboratory within 1 h of collection. The left uterine horn of 9 cows was dissected and used for cell culture and challenges with virus. Tissues from the right uterine horn were fixed in 4% (v/v) paraformaldehyde (PFA) and used for uterine health characterization. Uterine health was appraised from the number of CD11b positive cells (primary antibody, ab75476 Abcam, UK) following immunohistochemistry [27]. CD11b is expressed on the surface of many leukocytes including monocytes, neutrophils, natural killer cells, granulocytes and macrophages. All cell cultures used in this work originated from cow tissue presenting low numbers of CD11b positive cells (<5 cells/mm^2 cross section of endometrium). Moreover, the materials used in the study originated from cows in the dioestrus stage [27].

2.2. Cell culture

Bovine endometrial epithelial cells (bEEC) were prepared from left uterine horn tissue as previously described [27]. The uterine tissue was cut into 5–6 cm long and 4–5 mm deep pieces which were digested with collagenase IV (C5138, Sigma, USA) and hyaluronidase (250 U/mL) (H3506, Sigma, USA) diluted in PBS (phosphate-buffered saline) plus 20 mg/mL bovine serum albumin (BSA) under stirring for 2 h at 39 °C. The suspension was then filtered through 250 μm gauze to remove mucus and undigested tissue. The filtrate was passed through a 40 μm nylon sieve, which allowed fibroblast and blood cells to pass through while epithelial cells were retained. Epithelial cells were collected from the filter by backwashing with 30 mL PBS. Cells were centrifuged at 170 × *g* for 6 min and the pellet was re-suspended in 3 mL of PBS. To disperse the pellet into a single cell suspension, cells were disrupted by passing through a fine gauge needle. The cells were then seeded into a 25 cm^2 ventilation flask with F-12 medium (Dulbecco 's modified Eagle 's medium, D6434, Sigma, USA) containing 10% (v/v) foetal bovine serum (FBS), 1% Penstrep® (5000 unit/mL penicillin/streptomycin), 2 mM L- glutamine, 0.5% Liquid Media Supplement (ITS), 5 μg/mL gentamycin and 100 unit/mL nystatin. All cell cultures were kept at 39 °C in 5% CO_2 atmosphere and the medium was changed every 1-2 days. Sub-cultivations were performed at 5–7 days when epithelial cells attained 80–90% confluence. bEEC from 9 cows were prepared for use in experiments. The purity of the epithelial cell culture was checked by flow-cytometry labelling cytokeratin (primary Anti-cytokeratin 18 Ab, Abcam, UK, cat. no. ab668 and secondary Anti-alex 488 Ab, Abcam, UK, cat. no. ab175473 used following the manufacturer's instructions). From passage 2 and thereafter, more than 98% of cells expressed cytokeratin, confirming the very high purity of the cell culture system [27].

2.3. BoHV-4 strain and inoculum preparation

The BoHV-4 strain was originally isolated from a postpartum metritis cow (titre 10^6 tissue culture infectious dose, $TCID_{50}$/mL) [28]. A virus stock was prepared by propagating the virus in Madin-Darby Bovine Kidney cells (MDBK CCL-22, American Type Culture Collection, USA). The MDBK cells were cultured in Eagle's Minimal essential Medium (EMEM, Hatunalab AB, Sweden) containing 10% (v/v) horse serum, 2 mM L-glutamine (Sigma, USA), 200 IU/mL penicillin G sodium (Sigma, USA) and 100 μg/mL streptomycin sulphate (Sigma, USA). One millilitre of virus (titre 10^6) was diluted to 1:10 (v/v) in the tissue culture medium and virus (in solution) was inoculated on MDBK cells in a 25 cm^2 flask. After 2 h of incubation at 37 °C under 5% CO_2, the virus solution was discarded. The monolayer was then disrupted by trypsin treatment and re-suspended in EMEM with 10% (v/v) horse serum. After three days of incubation at 37 °C under 5% CO_2, when the cells showed > 50% CPE the incubation was stopped by freezing the flask at −70 °C. After thawing, the flask contents was aliquoted into 1 mL tubes and then transferred to a −70 °C freezer. The titre of the stock of amplified virus used for the experiment was estimated to be $10^{7.7}$ per mL.

2.4. BoHV-4 challenges

First challenges were performed *in vitro* to define viral multiplicity of infection (MOI) and lethal dose of virus (LD_{50}) to bEEC. bEEC from passage 5 were challenged by different viral MOIs (MOI 0.001, 0.01, 0.1, 1 and 10). The bEEC were cultured in the flasks until confluence and then detached by trypsin (Tryple™ Express (1 ×), ref.12605-010, Gibco®, USA). The sample materials were then transferred to 15 mL tubes and centrifuged at 215 × *g* for 5 min. Supernatant was discarded and 7 mL of 2% (v/v) FBS medium were added to suspend the cell pellet. The material was aliquoted into 6 centrifuge tubes. In each tube serial viral MOI; 0.001, 0.01, 0.1, 1 and 10 was added to five these tubes, while one was used as a control, to which 2% (v/v) FBS medium was added instead of virus.