Short communication

Lactoferrin from bovine milk inhibits bovine herpesvirus 1 in cell culture but suppresses development of in vitro-produced bovine embryos

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Received 16 January 2008; received in revised form 30 April 2008; accepted 22 May 2008

Abstract

Bovine herpesvirus 1 (BoHV-1) is widely distributed among cattle populations and has been associated with cells, fluids, and tissues collected from donor animals for use in reproductive technologies. The purpose of this study was to determine if lactoferrin would inhibit BoHV-1 in cell culture and to evaluate if embryos could develop normally when cultured in vitro with lactoferrin. In Experiment 1, lactoferrin (10 mg/mL) inhibited up to 25,000 plaque forming units (PFU)/mL of BoHV-1 in Madin Darby bovine kidney (MDBK) cell culture. In Experiment 2, lactoferrin (10 mg/mL) combined with cidofovir (62.5 μg/mL) inhibited up to 100,200 PFU/mL of virus in cell culture. In Experiment 3, following fertilization, presumptive zygotes were cultured in media containing lactoferrin (10, 5, and 2.5 mg/mL). Embryonic development and quality were assessed, and embryonic viability was determined by counting the nucleated cells of developed blastocysts. While lactoferrin did not affect the nucleated cell count of the treated embryos, it did significantly decrease blastocyst development. In conclusion, lactoferrin from bovine milk can inhibit BoHV-1 in cell culture. However, supplementation of in vitro culture medium with lactoferrin inhibits blastocyst development of in vitro-produced embryos.

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Keywords: Cattle; Embryo; IVF; BoHV-1; Lactoferrin

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1. Introduction

Bovine herpesvirus 1 (BoHV-1) is an alphaherpesvirus that is responsible for abortion, infertility, genital disease, and respiratory infection in cattle (Radostits et al., 2000). Bovine herpesvirus 1 is associated with gametes, serum, and co-culture cells which are used for in vitro embryo production, and transferable embryos can be produced from infected cumulus oocyte complexes (Bielanski and Dubuc, 1994; Weiblem et al., 1992). Further, while standard trypsin treatment is effective at rendering in vivo-derived embryos free of virus (Singh et al., 1982), it is not effective for in vitro-produced embryos (Bielanski et al., 1997) making it theoretically possible to transmit embryo-associated virus to the recipient. Thus, the identification of effective and non-cytotoxic anti-herpesvirus agents could provide disease control options for use in bovine in vitro embryo production.

Lactoferrin, a glycoprotein that is produced by mucosal epithelial cells, has antiviral effects in cell culture against herpes simplex virus, cytomegalovirus, canine herpesvirus, and feline herpesvirus (Beaumont et al., 2003; Hasegawa et al., 1994; Tanaka et al., 2003). Because resistance to antiviral agents can arise and long-term treatment can result in undesirable side-effects associated with cytotoxicity, combination therapy has been used to overcome these negative effects (van der Strate et al., 2003). van der Strate et al. (2003) demonstrated that bovine lactoferrin works synergistically with cidofovir to inhibit cytomegalovirus in fetal lung fibroblasts. In addition, cidofovir (4 µg/mL) is effective against BoHV-1 in tissue culture (Gilliam and Field, 1992) as well as in vivo (Gilliam and Field, 1993).

Therefore, the aim of this study was to determine if lactoferrin individually and in combination with cidofovir would be effective against BoHV-1 in cell culture and determine the impact on developmental efficiency of bovine embryos cultured with lactoferrin.

2. Materials and methods

2.1. Antiviral compounds

Lactoferrin from bovine milk was purchased from Sigma (St. Louis, MO, USA). It was supplied as a dry powder, dissolved in minimum essential medium (MEM), and filter sterilized (0.2 µm) before use. Cidofovir was purchased from Gilead Sciences (Foster City, CA, USA).

2.2. Cytotoxicity assay

The cytotoxic effects on MDBK cells, as determined by the number of viable cells, of four concentrations of lactoferrin (10, 5, 2.5, and 1.25 mg/mL) combined with each of three concentrations of cidofovir (62.5, 31.3, and 15.6 µg/mL) were quantified using Cell Counting Kit-8 (Dojindo, Gaithersburg, MD, USA).

2.3. Embryo production

Embryo production was performed as previously described (Stringfellow et al., 2000). On Day 7.5 of in vitro culture, embryonic development and quality were assessed as previously described (Stringfellow, 1998). The equine serum, BSA, and FBS used in media were determined to be free of BoHV-1 and anti-BoHV-1 antibodies by virus isolation and virus neutralization, respectively.
2.4. Experimental design

After performing cytotoxicity assays, the project was divided into three experiments. The first experiment determined the non-toxic concentration of lactoferrin that was effective against BoHV-1 in MDBK cells. The second experiment evaluated the inhibition of BoHV-1 in MDBK cells resulting from the combination of cidofovir and lactoferrin. The third experiment assessed the development of cattle embryos cultured in the presence of lactoferrin.

2.4.1. Experiment 1

Four trials were performed. Twofold dilutions of lactoferrin (10, 5, 2.5, 1.25, and 0.625 mg/mL) were tested. The media over monolayers of MDBK cells in a 96-well cell culture plate (0.32 cm²) were supplemented with concentrations of each compound to be tested. After 15 min, each well was inoculated with 10³–⁵ PFU/mL of BoHV-1 (Colorado strain), and culture plates were incubated for 5 days. Virus isolation procedures, previously described (Marley et al., 2006), were used to determine the effect of the antiviral agent on the presence of BoHV-1 in MDBK cells. The cells were examined for evidence of cytopathic effect (CPE). In addition, the inhibition of BoHV-1 in incubating cells was quantified via plaque assay (Stringfellow et al., 1990). Controls included the following: (1) MDBK cells with antiviral agent (to evaluate toxicity of agent to cells). (2) MDBK cells with BoHV-1 (positive control for viral CPE). (3) MDBK cells only (negative control). The percent viral inhibition for each treatment was determined.

2.4.2. Experiment 2

Four trials were performed. The four concentrations of lactoferrin (10, 5, 2.5, and 1.25 mg/mL) were combined with each of three concentrations of cidofovir (62.5, 31.3, and 15.6 μg/mL). The experiment was performed as described in Experiment 1.

2.4.3. Experiment 3

Six trials were performed. During each trial, oocytes were distributed as follows: (1) untreated control and (2) treated with lactoferrin (10, 5, or 2.5 mg/mL) in IVC media. After embryonic development was assessed on Day 7.5, embryonic quality and viability were evaluated. Embryonic quality was assessed by grading the embryos as described in the Manual of the International Embryo Transfer Society (Stringfellow, 1998). Embryonic viability was assessed by counting the nucleated cells of developed blastocysts (Pursel et al., 1985).

2.5. Statistical analysis

The mean values of OD for control and antiviral samples were analyzed by one-way ANOVA, followed by Tukey–Kramer HSD multiple comparison test (JMP IN software version 3.2.1, SAS Institute, Cary, NC, USA). For all analyses, \( P \leq 0.05 \) was considered significant. As well, the toxicity of each antiviral combination was calculated as a percentage of the cell control. First, the average OD of the medium control was subtracted from the OD of the cell control and antiviral samples. Next, the OD of the antiviral samples was compared to the cell control \[\text{antiviral values/average cell control value} \times 100 = \text{percentage of cell control}\]. These results were analyzed by one-way ANOVA, followed by Tukey–Kramer HSD.

For Experiments 1 and 2, the amount of PFU/mL virus inhibited by each antiviral combination was calculated \[\text{quantity of virus in the positive control/quantity of virus in the antiviral sample}\]. These results were analyzed by one-way ANOVA, followed by Tukey–Kramer HSD. As well, the
percent of virus inhibited by each antiviral agent was determined as a percentage of the positive control cultures [percentage of virus inhibited = (quantity of virus in the positive control – quantity of virus in the antiviral sample)/quantity of virus in the positive control × 100].

In Experiment 3, comparison of embryos that developed to blastocysts was made between the control and treatments using two-tail Fisher’s exact test (GraphPad Software, San Diego, CA, USA). Additionally, the mean values of embryo nucleated cell count and embryo grade for control and treatments was analyzed by Kruskal–Wallis one-way ANOVA.

3. Results

3.1. Cytotoxicity assay

None of the OD values of the combinations of lactoferrin (10, 5, 2.5, and 1.25 mg/mL) with each of three cidofovir concentrations (62.5, 31.3, and 15.6 μg/mL) were significantly different from the control samples. As well, the antiviral OD values evaluated as a percentage of the control were not significantly different from the control samples. Thus, the antiviral compounds were not cytotoxic to MDBK cells as demonstrated by the number of living MDBK cells.

3.2. Experiment 1

In MDBK cells, lactoferrin (10 mg/mL) inhibited 100–25,000 plaque forming units (PFU)/mL of virus (99% viral inhibition) (Fig. 1). At a concentration of 5 and 2.5 mg/mL of lactoferrin, 10–1000 PFU/mL of virus (90–99% viral inhibition) were inhibited, and a concentration of 1.25 and 0.625 mg/mL inhibited 1–100 PFU/mL of virus (80–99% viral inhibition). The amount of PFU/mL virus inhibited by each antiviral was not significantly different from the control or one another.

3.3. Experiment 2

Lactoferrin (10 and 5 mg/mL) inhibited 500–100,000 PFU/mL of virus (99.8–99.9% viral inhibition) when combined with each cidofovir concentration (Fig. 2). At a concentration of 2.5 mg/mL lactoferrin, 100–10,000 PFU/mL of virus (99–99.8% viral inhibition) were inhibited when combined with each cidofovir concentration, and a concentration of 1.25 mg/mL lactoferrin inhibited 10–5000 PFU/mL of virus (90–99.9% viral inhibition) when combined with each

Fig. 2. Results of Experiment 2—the mean values and standard error of virus inhibited in MDBK cells following treatment with lactoferrin and cidofovir is given. PFU, plaque forming unit; MDBK, Madin Darby bovine kidney.

Table 1
Embryonic development and viability, as demonstrated by nucleated cell number, of control embryos and those cultured with lactoferrin from bovine milk (10, 5, or 2.5 mg/mL)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 3.5 development (&gt;4 cell embryos/oocytes fertilized)</th>
<th>Day 7.5 development (blastocysts/oocytes fertilized)</th>
<th>Nucleated cell number (mean of Day 7.5 blastocysts)</th>
<th>Embryo grade (mean of Day 7.5 blastocysts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>191/470 (41%)</td>
<td>89/470 (19%)</td>
<td>90 ± 5</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>10 mg/mL lactoferrin</td>
<td>69/491 (14%)a</td>
<td>3/491 (1%)a</td>
<td>65 ± 25</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>5 mg/mL lactoferrin</td>
<td>101/461 (22%)a</td>
<td>9/461 (2%)a</td>
<td>82 ± 15</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>2.5 mg/mL lactoferrin</td>
<td>65/435 (15%)a</td>
<td>16/435 (4%)a</td>
<td>91 ± 11</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean differs from control (*P* ≤ 0.05).

cidofovir concentration. The amount of PFU/mL virus inhibited by each antiviral combination was not significantly different from the control or one another.

3.4. Experiment 3

Embryos were able to develop in the presence of lactoferrin (Table 1). Lactoferrin did not significantly affect the nucleated cell count or embryo grade of the treated embryos. However, lactoferrin did adversely affect blastocyst development. This decrease in blastocyst development of treated embryos was statistically different from the untreated controls.

4. Discussion

Lactoferrin has been shown to inhibit the in vitro replication of herpes simplex virus, cytomegalovirus, canine herpesvirus, feline herpesvirus, human immunodeficiency virus, respiratory syncytial virus, and hepatitis virus (Beaumont et al., 2003; Tanaka et al., 2003). Tanaka et al. (2003) demonstrated that bovine lactoferrin at concentrations ranging from 0.125 to 1 mg/mL was effective at reducing canine herpesvirus in Madin Darby canine kidney cells by $10^2$ cell culture infectious dose (CCID$_{50}$)/0.1 mL. As well, Beaumont et al. (2003) showed that lactoferrin from bovine colostrum at concentrations of 0.5–10 mg/mL inhibited 87–96% feline herpesvirus infection of Crandell–Reese feline kidney cells. Both these studies also demonstrated...
that lactoferrin was not cytotoxic to the cells. The present study revealed similar antiviral results against BoHV-1. While the present results were not statistically significant, further replicates might have detected a difference. Lactoferrin (10 mg/mL) reduced BoHV-1 in MDBK cells by an average of 7400 PFU/mL with a viral inhibition of 99.9% while the combination of lactoferrin (10 mg/mL) and cidofovir (62.5 µg/mL) reduced BoHV-1 in MDBK cells by an average of 32,000 PFU/mL with a viral inhibition of 99.9%.

However, while these results seem promising, if this antiviral is to be used in media for in vitro embryo production, total inhibition of viral replication is needed to confirm embryos as virus-free. In addition, while the quality of embryos cultured with lactoferrin was comparable to the control embryos, the number of embryos developing to blastocysts was severely diminished. Thus, while the cytotoxicity assay demonstrated that the combination of cidofovir and lactoferrin was not toxic to MDBK cells and in vivo studies have not shown appreciable side-effects (Sato et al., 1996), the assessment of embryo development is obviously a much more sensitive test of antiviral toxicity. Therefore, additional research is needed to identify an antiviral agent or combination of agents that will be effective against BoHV-1 associated with in vitro-produced embryos while not interfering with normal embryonic development.

5. Conclusion

Bovine herpesvirus 1 is a likely contaminant of bovine in vitro embryo production. A non-toxic concentration of an antiviral agent might prevent replication of BoHV-1 within in vitro production. This research did confirm that lactoferrin, similar to its effect on human, canine, and feline herpesviruses, also has antiviral effects against bovine herpesvirus. However, lactoferrin severely inhibited embryonic development. Therefore, the use of lactoferrin at the concentrations tested in the present study cannot be recommended as an antiviral supplement during in vitro culture of developing bovine embryos.

References


