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Note

In Vitro and *in Vivo* Evaluation of the Efficacy of Bovine Colostrum against Human Rotavirus Infection

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We found that skimmed and concentrated bovine late colostrum (SCBLC) obtained from normal cows at 6–7 d after parturition exhibited high potency in inhibiting replication of human rotavirus (HRV) *in vitro*. Furthermore, prophylactic oral administration of SCBLC once before inoculation of HRV prevented the development of diarrhea in suckling mice *in vivo*. SCBLC from normal cows might be useful in the prevention of HRV-induced severe gastroenteritis in immunocompromised hosts.

Key words: bovine colostrum; human rotavirus; infection; diarrhea; suckling mice

Human rotavirus (HRV) is one of the major causes of severe dehydrating gastroenteritis in infants and young children worldwide. It causes more than 527,000 deaths per year.[1)] Two rotavirus vaccines have been developed and approved in certain countries,[1)] but the development of alternative prophylactic approaches is warranted, because most patients with rotavirus diarrhea are immunocompromised, especially infants and young children.

Bovine colostrum is the early milk produced by cows during the first several days post-parturition. The importance of colostrum for the growth and health of newborn offspring is well known, as is a high concentration of immunoglobulins. Over the past two decades, it has been proposed that passive protection against rotavirus diarrhea can be achieved by using cow's milk containing a high level of specific anti-rotavirus antibodies.[2,3)] These are commonly produced by hyper-immunization of pregnant cows with certain rotavirus strains,[2,3)] while bovine lacteal secretions in the normal state contain antibodies against rotavirus.[4)] There is a review regarding the clinical effects of supplementation with bovine colostrum,[5)] but the clinical use of bovine colostrum from hyper-immunized cows has been limited, due to difficulties in large-scale production. In addition, shipping early colostrum from cows within 5 d of parturition is illegal as a food in Japan.

To our knowledge, there has been no study on the efficacy of colostrum from normal healthy cows against HRV infection in an experimental animal model. The aim of the present study was therefore to evaluate the protective efficacy of skimmed and concentrated bovine milk from healthy lactating cows at 6–7 d after parturition, referred to as skimmed and concentrated bovine late colostrum (SCBLC), against HRV infection *in vitro* and *in vivo*. It would be of great potential if SCBLC from normal cows shows potent inhibitory activity against HRV, comparable to that exhibited by early colostrum from hyper-immunized cows.

SCBLC from normal cows was prepared at an industrial level in the facility of Kobayashi Pharmaceutical (Osaka). Briefly, pooled late colostrum from healthy cows at 6–7 d after delivery was defatted by centrifugation, pasteurized by HTST condition (heating at 73 °C for 15 s), and then concentrated by ultrafiltration, followed by spraydrying.

We compared the protective efficacy against the HRV gastroenteritis between SCBLC from normal cows and the colostrum whey within the first 3 d after parturition from cows hyper-immunized with HRV (rota whey). As previously reported,[2)] rota whey contained high titers of neutralizing antibodies against HRV.

Replication inhibition (neutralization) assay for HRV MO strain (serotype G3P[8]) was performed using MA104 cells (African rhesus monkey kidney cell line) following a procedure described previously.[6)] Briefly, a suspension containing infectious virus at a titer of 10^5–10^6 FCFU/ml was treated with 10 μg/ml trypsin (Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C. After appropriate dilution with Eagle's minimum essential medium (MEM) containing 10% fetal calf serum to give a titer of approximately 4×10^3 FCFU per 100 μl, aliquots were mixed with an equal volume (100 μl) of one-half serially diluted samples in microtubes for 1 h at 37 °C. The diluted mixtures were then used to inoculate MA104 cells (2×10^5 cells/ml, 200 μl), and 20-μl aliquots of each were placed into the wells of a 24-well heavy teflon (HT)-coated slide (AR Brown, Tokyo). The control gave about 100 infected foci/well without test samples. The cells were further cultured for 22 h at 37 °C under an atmosphere of 5% CO_2, and then

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Abbreviations: SCBLC, skimmed and concentrated bovine late colosturm; HRV, human rotavirus; HTST, high-temperature short-time method sterilization

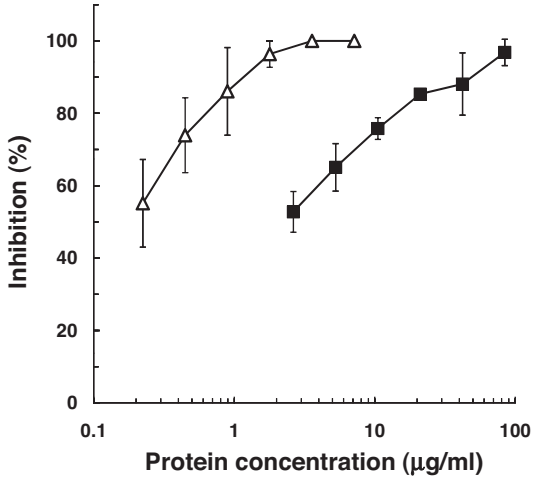

Fig. 1. Inhibitory Effect of Bovine Colostrums on Infection by the Human Rotavirus MO Strain on MA104 Cells.

The human rotavirus (HRV) MO strain was incubated with each of the samples for 60 min prior to inoculation. The virus-sample mixture was then added to MA104 cells, followed by incubation under a 5% CO_2 atmosphere at 37 °C for a further 22 h. Control cells were prepared in the same way, except for preincubation without sample. Concentrations of the samples are given in a logarithmic scale: the whey from the coloctrum of cows hyper-immunized with HRV (rota whey, open triangles); skimmed and concentrated bovine late colostrum (SCBLC, solid squares). The inhibitory activity of each sample is expressed as percent of infected cells as compared to control cells (100%). The experiments were carried out in triplicate at least 3 times, and representative results for each sample are given as means ± SD.

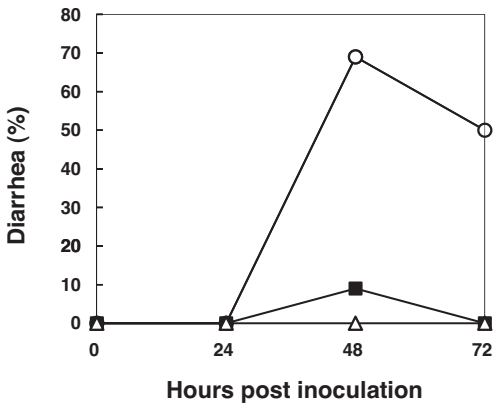

Fig. 2. Protective Efficacy of Bovine Colostrum on Human Rotavirus MO Strain-Induced Diarrhea in Suckling Mice.

Litters of 5-d-old mice were given orally by either PBS (n = 13) (open circles) or 0.25 mg of the whey from the colostrum of cows hyper-immunized with human rotavirus (HRV) (rota whey) (n = 8, open triangles) or 2.5 mg of skimmed and concentrated bovine late colostrum (SCBLC) (n = 11, solid squares) for 60 min before virus inoculation with 1.7×10^5 FCFU of HRV MO strain/mouse. Stools were examined daily to assess diarrhea 3 d after inoculation.

fixed with cold acetone for 20 min. The foci numbers of infected cells were measured by indirect immunofluorescence assay using PO-13 monoclonal antibody against pigeon rotavirus[7] and FITC-conjugated goat anti-mouse IgG antiserum (American Qualex, San Clemente, CA). Neutralizing activity was expressed as percentage reduction in the foci number of infected cells observed by fluorescence microscopy. The minimum inhibitory concentration (MIC), the finally diluted protein concentration showing 50% reduction of infected cells, was calculated for each sample from logarithmic regression of the concentration-dependent percentage focus reduction. The protein concentration was determined with a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

As expected, rota whey was remarkably potent in inhibiting the replication of HRV, with a MIC of 0.15 μg/ml (Fig. 1). SCBLC exhibited weaker but still high inhibitory activity against HRV, with a MIC of 2.1 μg/ml, while the MIC of normal milk was approximately 200 μg/ml. These data suggest that SCBLC is a promising candidate for prophylactic treatments against HRV infection.

Next, we investigated to determine whether SCBLC exhibits a protective efficacy against HRV-induced diarrhea *in vivo*. We used a previously developed mouse model of HRV gastroenteritis to study protective efficacy in the development of diarrhea.[6] Pregnant BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Litters of 5-d-old mice were orally inoculated with a single dose of 1.7×10^5 FCFU of the MO strain in 50 μl by gavage. Stools were examined daily for the development of diarrhea by gentle abdominal palpation beginning at 1 d after inoculation for 3 d. Observation of muddy-mucous or liquid-mucous yellow stool yielded a judgment of diarrhea. To assess the effects of the colostrums in the prevention of HRV-induced diarrhea, test samples were given orally by gavage every 60 min before virus inoculation. Control mice were given phosphate buffered saline, pH 7.2 (PBS). The care and experimental procedures were approved by the Animal Care and Use Committee of Gifu University.

In the case of rota whey, the suckling mice were given orally a single dose of 0.25 mg in 50 μl of PBS before inoculation of the HRV MO strain. In the same experimental model, we used a single dose of 2.5 mg in 50 μl of PBS for SCBLC. This dosage was adopted based on an experimental observation *in vitro* (Fig. 1), showing 10-times less effective activity against the HRV MO strain than that of rota whey. As shown in Fig. 2, 2 d after inoculation of HRV, nine of the 13 mice developed diarrhea in the PBS group, and half of the mice still suffered from diarrhea even 3 d after inoculation. In contrast, all eight mice did not develop diarrhea at all in the rota whey group, and only one of the 11 mice developed diarrhea in the SCBLC group throughout the experimental period.

The effect of a single administration of a sample before inoculation was examined in this study, and a 10-times higher dose was thus required for SCBLC to be effective against HRV gastroenteritis in comparison with rota whey. A considerable reduction in the immunoglobulin level would be expected in cow's milk as lactation progress. In SCBLC preparation, concentration through ultrafiltration was therefore applied to keep the level of antibodies high. However, for practical purposes, the smaller the quantity is ingested in a single dose the better for the immunocompromized hosts to accept it orally. Multiple administrations per d can reduce the quantity in a single dosage, as was found for a synthesized sulfated sugar compound.[8]

In conclusion, the present study indicates promising efficacy in immunocompromised hosts of SCBLC,

skimmed and concentrated late bovine colostrum, for inhibition of infection and prevention of the development of diarrhea caused by rotaviruses. Further studies might delineate the potential of SCBLC as a prophylactic food additive against HRV infection in more detail.

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