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The Effect of Dietary Intake of the Acidic Protein Fraction of Bovine Colostrum on Influenza A (H1N1) Virus Infection[§]

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Acidic protein levels in the milk decrease markedly as lactation progresses, suggesting that it is an important part of the colostrum. However, little attention has been paid to their biological function. In this study, we isolated the acidic protein fraction of bovine colostrum (AFC, isoelectric point <5) by anion-exchange chromatography, and investigated the effect of its dietary intake on influenza A (H1N1) virus infection. 100% of mice infected with 1 LD_{50} of the virus survived when administered AFC for 14 days prior to infection, compared with 33% survival when administered phosphate buffered saline (PBS). Moreover, consumption of AFC reduced the weight loss associated with infection. We propose that dietary intake of AFC has a prophylactic effect on influenza A virus infection.

Keywords: bovine colostrums, acidic protein, dietary intake, influenza A virus

Introduction

Breast milk delivers not only immunologic components that protect the infant against pathogen invasion, but also nutritional factors that promote normal organ development (Wang and Brand-Miller, 2003). Colostrum is the natural food produced by female mammals during the first 24–72 h after giving birth (Robison *et al*., 1988). It contains concentrated nutrients, antibodies, cytokines, and growth factors (Playford *et al*., 2000; Jouan *et al*., 2006), providing immediate immune protection to the newborns (Asakuma *et al*., 2007; Benson *et al*., 2012).

Influenza is a single-stranded RNA orthomyxovirus, subdivided into influenza A, B and C (Gatherer, 2009). The H1N1 and H3N2 subtypes of influenza A and influenza B are widely recognized as causes of seasonal flu (Goldstein *et al*., 2011). The virus can evade humoral immunity by antigenic drift, i.e. changes in the amino acid sequence of the surface hemagglutinin and/or neuraminidase (Webster *et al*., 1982; Sandbulte *et al*., 2011), which is why influenza can cause epidemics annually. Although in most cases symptoms resolve within a week without the need for treatment, in high-risk groups influenza can lead to severe illness or death (World Health Organization, 2009). In the United States, the influenza-attributable mortality rate is estimated at 1.4–16.7 deaths per 100,000 cases, which translates to between 3,339 and 48,614 deaths annually (Centers for Disease Control and Prevention, 2010). Influenza is a serious public health and economic issue. In developed countries especially, epidemics can result in high levels of worker absenteeism and productivity losses (World Health Organization, 2009). Therefore, much effort has been put into preventing infections.

It is known that the oligosaccharide contents of bovine colostrum are different from those of human colostrum. 6-sialyllactosamine and 3-sialyllactose are the most abundant in bovine colostrum while 3-sialyl-3-fucosyllactose and sialyllacto-N-tetraoses are in human colostrum (Martin-Sosa *et al*., 2003). Although there are differences in the oligosaccharide contents, both colostrums have been regarded to have biological functions to modulate diseases. Previous reports have shown that human colostrum can modulate the immune system and enhance protection against pathogens (Claud *et al*., 2003; Wang and Brand-Miller, 2003). Dietary intake of bovine colostrum has also been suggested to prevent upper respiratory tract (URT) infections (Brinkworth and Buckley, 2003). However, there has been no direct evidence that dietary bovine colostrum enhances protection against influenza infection.

The amount of acidic proteins and sialic acid in milk is known to decrease significantly as lactation progresses (Bezkorovainy, 1965; Wang and Brand-Miller, 2003), therefore, the acidic protein fraction is thought to have an important function in colostrum. In this study we isolated the acidic protein fraction of bovine colostrum (AFC) and investigated how dietary intake of AFC modulates the symptoms of influenza infection.

Materials and Methods

Materials and apparatus

Bovine colostrum powder was provided by Ildong Foodis (Korea). DEAE-sepharose CL-6B was purchased from GE Healthcare (USA). A Power Pac 3000 power supply and electrophoresis kit (both from Bio-Rad, USA) were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Mini Protean® II, Bio-Rad). The protein fraction separated by SDS-PAGE was visualized by silver staining (GE Healthcare).

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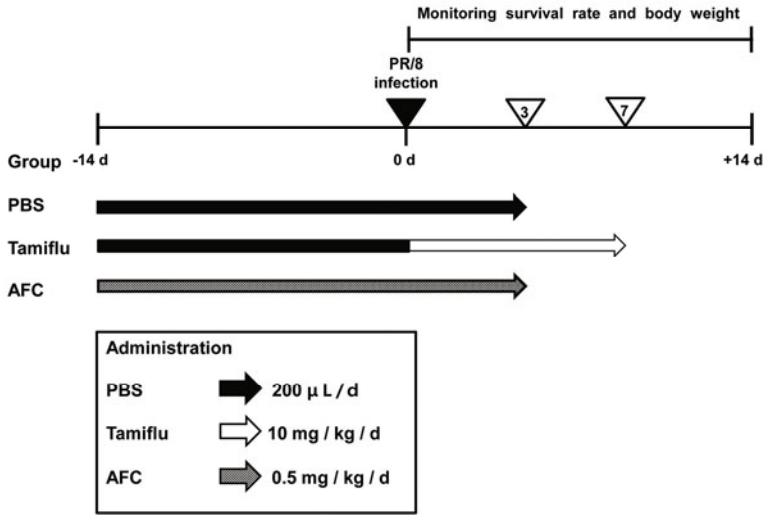

Fig. 1. Diagram of the animal experiments. The three mouse groups were given oseltamivir, PBS and AFC orally for 14 days prior to infection with influenza A virus. After the virus challenge, the mice continued to receive oseltamivir, PBS and AFC for 7, 3 and 3 days, respectively. They were then infected with 1 LD_{50} or 0.2 LD_{50} of the virus. Survival rates and body weight changes were monitored for 14 days after infection.

Influenza A/PR/8/34 virus (H1N1) was kindly donated by Prof. Man-Seong Park (Hallym University, Korea). Zoletil 50 (Virbac, France) and Rompun (Bayer Animal Health, Germany) were used to anesthetize the mice. Phosphate buffered saline (PBS) and oseltamivir (Roche, Switzerland) were used as negative and positive controls in mouse experiments, respectively.

AFC purification by anion-exchange chromatography

A 40 g sample of bovine colostrum powder was dissolved in binding buffer (2,000 ml of PBS containing 0.025% Tween 80, pH 7.0, final NaCl concentration was adjusted to 0.13 M). This starting sample was dialyzed against 10 L of binding buffer at 4°C for 4 h. The dialysate was centrifuged for 10 min at 14,000×*g*, to obtain the supernatant (i.e. the loading sample). Anion exchange chromatography was performed in batch mode. The loading sample was mixed with 40 ml DEAE-sepharose CL-6B resin equilibrated with binding buffer in a 2 L bottle, and the mixture was left at 4°C for 72 h. The unbound fraction was removed by centrifugation at 84×*g* for 5 min. The DEAE resin was centrifuged at 84×*g* for 5 min with 200 ml of the binding buffer. The AFC fraction was isolated with elution buffer (to make elution buffer NaCl was added to the binding buffer, final NaCl concentration of elution buffer was adjusted to 0.652 M). The protein content of the starting sample, the loading sample and AFC were analyzed as described below.

Proteomic analysis

The proteins in each sample were separated on 12% SDS-PAGE gels as described by Laemmli (1970) and visualized by silver staining. Protein concentration was determined by the Bradford protein concentration assay (Bio-Rad). Two-dimensional electrophoresis (2DE) of 800 μg protein samples was performed by Genomine Inc. (Korea), and the separated proteins were visualized by Coomassie blue staining.

Virus preparation

Influenza A virus was propagated in 11-day old fertilized chicken eggs for 48 h at 37°C. Egg allantoic fluid was clarified by centrifugation at 682×*g* for 10 min and filtered using a 0.22 μm syringe filter. The 50% lethal dose (LD_{50}) of the virus was determined as described (Reed and Muench, 1938).

Oral administrations of AFC and infection with influenza A (H1N1) virus

Five-week-old female Balb/c mice purchased from Orient Bio (Korea) were divided into three groups (PBS, oseltamivir and AFC) of five to six mice each. The dosing schedule for each group is shown in Fig. 1. The mice received PBS (200 μl/day) or AFC (0.5 mg/kg/day, 0.5 mg indicates protein amount) orally for 14 days before infection. The protein concentration of AFC was determined by Bradford protein assay using commercial protein assay kit (Bio-Rad). They were anesthetized intraperitoneally with a 4:1 mixture of Zoletil 50 and Rompun and infected intranasally with 1 LD_{50} or 0.2 LD_{50} of the influenza A (H1N1) virus. Following infection, the PBS and AFC groups continued to receive PBS (200 μl/day) and AFC (0.5 mg/kg/day), respectively, for 3 days, and the oseltamivir group continued to receive oseltamivir (10 mg/kg/day) for 7 days. Survival rates and changes in body weight were monitored for 14 days after infection.

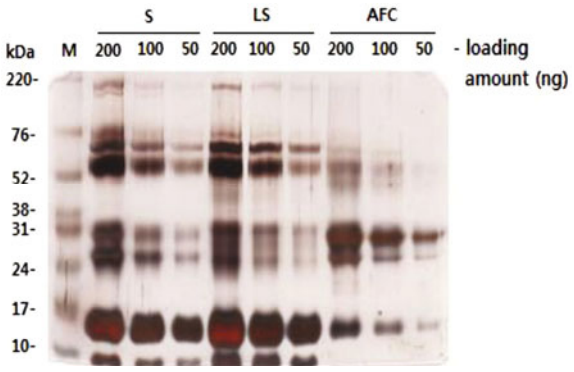

Fig. 2. SDS-PAGE analysis of starting sample (S), loading sample (LS), and AFC.

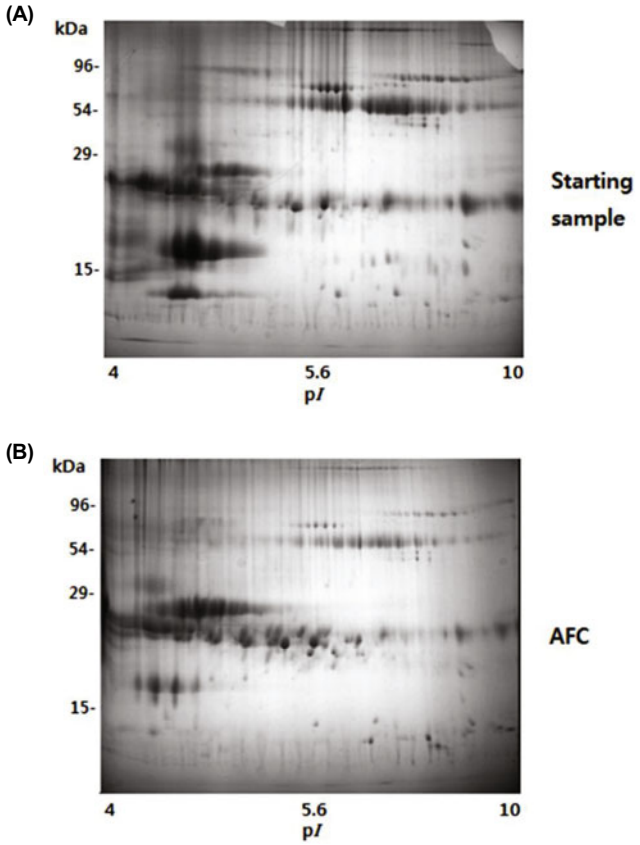

Fig. 3. 2-DE analysis of starting sample and AFC. 800 μg protein samples were analyzed, and the results are shown in (A) the starting sample and (B) AFC. The separated proteins were visualized by Coomassie blue staining.

Statistical analysis

Student's *t*-test was used to evaluate differences between the groups. *P*-values <0.05, or <0.01 in two-tailed tests, were considered statistically significant.

Results

Separation and characterization of AFC

As shown in Fig. 2, the starting sample and the loading sample were both found to contain three major protein bands with molecular masses of 52–76, 24–31, and 5–17 kDa. There was no significant difference between the two samples in terms of protein composition. It has been suggested that bovine colostrum is composed of 50–75, 25–35, and 5–20 kDa proteins (Senda *et al*., 2011), and our results were consistent with this finding. The 24–31 kDa protein band was the most prominent in AFC, the largest proportion of which was the 28 kDa fraction (Fig. 2). We also compared the isoelectric points (pIs) of the proteins in the starting sample and in AFC (Fig. 3). Most of the proteins in AFC had pIs <5 (Fig. 3B), while the pIs of the proteins in the starting sample ranged from 4 to 10 (Fig. 3A), suggesting that AFC contains mainly acidic proteins.

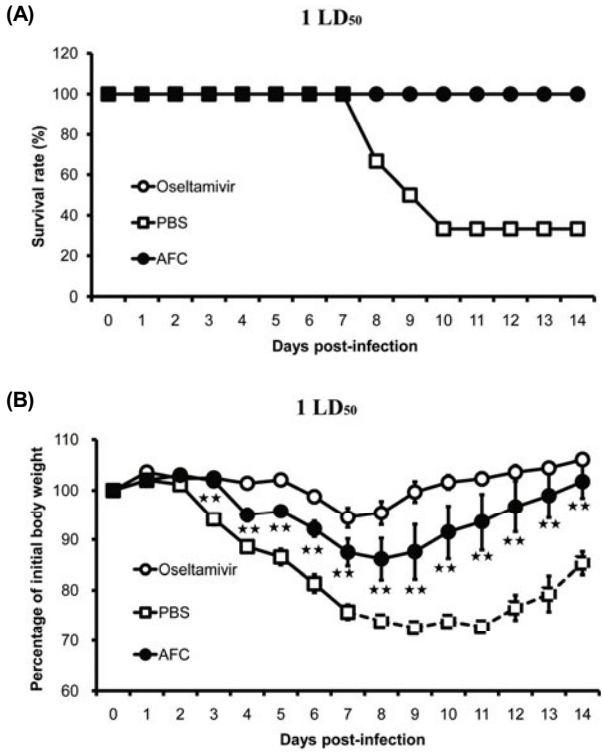

Fig. 4. The effect of dietary intake of AFC on infection with 1 LD_{50} of influenza A virus. Survival rates (A) and changes in body weight (B) were monitored for 14 days after infection. Body weight measured 1 day prior to virus challenge was set at 100%. In panel (B), the values are presented as mean±SEM of 5–6 mice. The dotted line represents the mean body weight of the PBS group of mice that survived. ** *P*<0.01 indicates a difference in body weight between the AFC and the PBS group.

The effect of dietary intake of AFC on influenza A virus infection in mice

Whereas the survival rates in both the oseltamivir and AFC groups were 100%, in the PBS group, only 33% of the animals challenged with 1 LD_{50} of the virus survived (Fig. 4A). At the same time, oral administration of AFC significantly

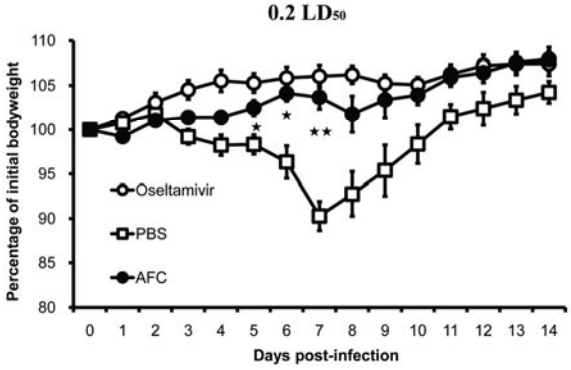

Fig. 5. The effect of dietary intake of AFC on infection with 0.2 LD_{50} of influenza A virus. Body weight measured 1 day prior to virus challenge was set at 100%. The values are means±SEM of six mice. * *P*<0.05 and ** *P*<0.01 indicate differences in body weight between the AFC and the PBS group.

reduced body weight loss in the animals receiving 1 LD_{50} of the virus (Fig. 4B). We also compared the changes in body weight in the three groups infected with 0.2 LD_{50}. As shown in Fig. 5, the mice in the oseltamivir and AFC groups experienced no change in body weight, while significant weight loss was observed in the PBS group in days 4 to 7 after infection. These results demonstrate that dietary intake of AFC can ameliorate the symptoms associated with influenza.

Discussion

Picornaviruses, coronaviruses, adenoviruses, parainfluenza viruses and influenza viruses are the causal pathogens in the vast majority of URT infections (Fendrick *et al*., 2003). Dietary supplementation with concentrated bovine colostrum proteins has been shown to reduce the incidence of symptoms of URT infection in adult men (Brinkworth and Buckley, 2003). Recently, Patiroglu and Kondolot also reported that oral administration of bovine colostrum lessened the severity of viral URT infections in IgA-deficient children (Patiroglu and Kondolot, 2011). Based on these findings, we hypothesized that bovine colostrum would improve the symptoms associated with influenza virus. We performed a large number of animal experiments investigating the effect of total bovine colostrum, and found that while some experiments yielded significant reductions in influenza-related morbidity and mortality, the symptom-ameliorating effects of the same bovine colostrum products varied significantly (Supplementary data Fig. S1). Therefore, the effect of total bovine colostrums on influenza virus infection in mice was significantly different from our expectation. On the other hand, AFC showed promising effect for ameliorating symptoms caused by influenza infections (Figs. 4 and 5). Previous studies implied that the intestinal conditions, i.e. bacterial flora and digestive enzymes involved in the absorption of colostrum components differed between human and animal subjects (Petschow and Talbott, 1994; Johnson *et al*., 2007; Kobayashi *et al*., 2012). In other words, some components contained in bovine colostrum decrease the ameliorating effect or hinder the functions of novel compenents. We suggest that separations of novel components such as AFC from the hindering components are thought to be critical in mouse experiment.

We found that AFC contains significantly higher level of proteins harboring α2,3-linked sialic acids than total bovine colostrums and acid protein fraction of mature bovine milk does (Supplementary data Fig. S2), indicating that the anion-exchange chromatography separates sialic acid rich fraction from the bovine colostrum. The sialic acid is known to inhibit influenza virus-mediated haemagglutination and infection (Matrosovich and Klenk, 2003). In addition, α2,3-linked sialic acid is a cell receptor for influenza A virus infection (Glaser *et al*., 2005). Therefore, it is assumed that other components of AFC such as free sialyl oligosaccharides may be beneficial on ameliorating the symptom caused by influenza virus infection. The composition and quality of pooled colostrum is known to depend on a number of factors, such as the health of the cows, the timing of colostrum collection, and the manufacturing process (Kelly, 2003). However, it is uncertain what amount of each component is required for its biological activity, and what specific components are biologically active in specific diseases, and this has hindered the standardization of bovine colostrums (Struff and Sprotte, 2007). Therefore, we believe that separating novel factors from colostrum, and collecting more data on the composition of colostrum and the activity of its components are critical for obtaining high-quality colostrum products.

In this study, we provide the first evidence that orally supplemented AFC is effective in reducing the symptoms associated with influenza A virus infection. Further study of the mechanisms through which AFC modulates these symptoms will provide new insight into why colostrum components are necessary for newborns.

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