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RESEARCH ARTICLE

Anti-rotaviral activity of whey proteins derived from milk of different animal species

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Abstract

Rota viruses are the leading pathogens causing diarrhea in children and animals. Whey protein was shown to contain bioactive ingredients that may activate immune cells and/or prevent infection. The current study was designed to assess whether whey proteins prepared from cow's, goat's and camel's milk could protect against rotavirus. Four groups of Swiss albino mice were treated once daily from the age of 9 to 20 day with whey proteins prepared from cow's milk (group I), goat's milk (group II), farm camel's milk (group III) and dessert camel's milk (group IV) and the fifth group was untreated. All groups were infected with bovine Rota virus (BRV) at the age of 11 day. Disease symptoms, viral shedding in fecal samples and serum levels of anti-BRV antibodies was measured during the post-infection period. Severe diarrhea occurred in 90% of control mice; there was a significantly reduced to 10%, 30%, 40% and 60% in group IV, III, II and group I, respectively. Severe diarrhea occurred for a 4-day period in the control group but only for a one day period in group II, III, IV and for 2 day in group I. Rota virus-specific antibody levels in serum did not differ between groups. Thus, cow's, goat's and camel's whey proteins could be used as a preventive measure for rotaviral disease. Furthermore, whey proteins prepared from dessert's camel milk have the best anti-rotaviral efficacy for treatment of infected mice followed by those prepared from goat's milk..

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Introduction

Diarrhea is one of the most important health problems globally and a leading cause of morbidity and mortality in children especially in developing countries (WHO, 1996). This is a result of absence of potable water, proper sanitary habits, good fecal disposal systems, and good hygienic practices by impoverished citizens and over crowding (Cheeshbrough, 1994).

Although diarrhea is self-limiting, at times it may require antibiotic therapy (Prescott et al., 2002). However, because of the growing resistance of microorganisms to conventional antibiotics most of the commonly employed antibiotics are becoming ineffective especially for viral infection. Among etiological agents, group A Rota virus, members of the genus rotavirus within the family reoviridae, are the major cause of acute diarrhea among newborn animals and humans leading to death (Saif et al., 1994).

Rota virus is transmitted by the fecal-oral route, via contact with contaminated hands, surfaces and objects (Hunter, 2009) and possibly by the respiratory route (Dennehy, 2000). Furthermore, rotavirus can spread in food and water by infected handlers. In the same context, milk can be a vehicle for rota-viral transmission through the same route and this leads to Rota-viral infection through consumption of raw milk and/ or dairy products

manufactured from contaminated raw milk. Several outbreaks were reported among students on a college campus in Washington D.C. during March and April, 2000 due to consumption of rota-viral infected foods (Hunter, 2009).

Although development of Rota virus vaccine candidates has been reported (Glass et al., 1996), passive protection could have advantage over vaccine owing largely to the following reasons: the immune system in young infants is immature and they are incapable of active production of adequate amounts of intestinal antibodies. In addition, the elderly and immunocompromised may be particularly prone to more severe symptoms when infected with Rota virus (Roitt et al., 1996). Passive oral prophylaxis is thus of practical importance for the prevention of diarrhea due to infectious Rota virus.

Food supplementation with several compounds (i.e. probiotics, prebiotics, or protein concentrates) in RV-infected animals has been shown to provide a significant reduction in the incidence and severity of diarrhea as well as changes in laboratory parameters, such as viral shedding or RV concentration in intestinal segments (Wolber et al., 2005). Many of studies involving passive protection against Rota virus diarrhea have been done using bovine colostrums or immunoglobulin concentrates prepared from bovine milk in which whey is an important source for immunoglobulin in its whey protein (Davidson, 1996).

Whey is the solution left during the cheese-making process. It contains a variety of factors and compounds which have been reported CDRF (2006) to have health promoting effects and prevent diseases. Some of the factors are immunoglobulin, lactoferrin, lactoperoxidase, glycomacropeptide, bovine serum albumin, α -lactalbumin and β -lactoglobulin. In addition to the health promoting effect of whey, Olorunfemi et al (2007) also found that it has growth inhibitory activity against common bacteria that cause diarrhea. So, it could be exploited in treating viral diarrhea that not responded to antibiotic treatment.

On the sight of these facts, the aim of the current study was to study and compare the effect of cow's, goat's and camel's whey proteins on bovine rotavirus infection and how to supplement the whey proteins in milk and its products to control their contamination with rotavirus.

MATERIAL AND METHODS

Virus strain

Cell culture adapted strain of BRV of a titer 10^6 TCID₅₀/mL was supplied by the Department of Rinderpest-like diseases; Veterinary Serum and Vaccines Research Institute (VSVRI), Abbassia, Cairo. It was used in mice inoculation as previously described (Pe' rez-Cano et al., 2007).

Preparation of whey protein

Acid precipitation of milk from different species (cow, camel and goat):

Whey protein obtained by acid precipitation for the separation of casein and whey proteins, 10% (v/v) acetic acid and 1M Sodium acetate was added, to the point where the pH of the milk was lowered close to the isoelectric point (4.6) and once the desired pH of 4.6 was obtained, milk sample was centrifuged at 5000g for 30 minutes at 15°C. Once the centrifugation is concluded, separation of casein and whey proteins is quite vivid, such that the white casein pellet rests at the bottom of the centrifugation tube, whereas the milky whey (supernatant) floats at the top (Bordin et al. 2001). The resultant whey, which contained the undenatured whey proteins (WPs), was saturated with ammonium sulfate to a final saturation of 80% to precipitate the WPs

Chemical analysis of whey:

Whey samples were analyzed chemically. Protein content was determined by Kjeldahl method and fat content by Gerber butyrometer according to Ling (1963). While lactose determination was according to the method described by AOAC (1990). Ash content was determined according to IDF (1975). Protein concentration of WPs was measured spectrophotometrically according to AOAC (1990).

Mice and experimental design

Fifty Albino Swiss mice were obtained from Department of Pet Animal vaccine Research; Veterinary Serum and Vaccine Research Institute; Abassia, Cairo. Mice were distributed in 5 experimental groups (10 mice each) and were orally supplemented beginning day 9 of life with whey protein of cow (Group I), goat (Group II), farm camel (Group III) and dessert camel (Group IV). RV was inoculated at day 11 of life to suckling rats from the 4 supplemented groups. The fifth group (Group V) was used as an un-supplemented infected comparison group

Clinical indexes and fecal specimen collection

BRV infection was evaluated from 1 to 14 day post-inoculation (DPI) by growth rate and clinical indexes such as severity of diarrhea, and incidence of diarrhea (%). Collection of individual fecal samples was performed once daily by gently pressing and massaging the abdomen. Severity of diarrhea was assessed by fecal material examination and by scoring stools from 1 to 4 (diarrhea index [DI]) based on color, texture, and amount. Scoring

was as follows: normal feces (score of 1), loose yellow-green feces (2), totally loose yellow-green feces (3), high amount of watery feces (4). Incidence of diarrhea corresponded to the percentage calculated by dividing the number of diarrheic samples by the number of total samples collected each day. Collected fecal samples were immediately frozen at -20°C for further BRV particle quantification.

ELISA for Rota virus load in feces

Fecal samples were diluted in PBS (20 mg/mL) and homogenized by a Polytron. Homogenates were centrifuged, and supernatants were frozen at -20°C until use. BRV particles in fecal samples were detected by Bovine Rotavirus ELISA kit BIO K 343 according to manufacture instructions.

ELISA for sera anti-BRV Ig quantification

Serum samples were obtained for anti-BRV Ab detection by ELISA. Ninety-six-well plates were incubated with BRV proteins (10 $\mu\text{g}/\text{mL}$) from sonicated virus suspension. After blocking with PBS-1% BSA (1 h, RT), appropriate diluted sera in PBS-Tween-1% BSA were added (3 h, RT). Abs were detected by adding rabbit anti-rat Ig conjugated to peroxidase (Sigma Chemical Co., 3 h, RT) followed by enzyme substrate and stopping solutions as above. Pooled sera from RV inoculated animals were used as a standard in each plate.

RESULTS

Chemical analysis of whey

There was variation in chemical compositions of whey among milk from different species especially the protein %. The highest protein % was reported in whey derived from dessert's camel milk (1.1 ± 0.2) followed by that derived from farm's camel milk, while the lowest protein % was reported in whey prepared from cow's milk (0.68 ± 0.2) (**Table 1**). These results reflected the concentration of protein in the final undenatured whey protein. It ranged from 34.4% in cow's milk to 55% in dessert's camel milk (**Fig.1**).

Infection of Albino Swiss mice groups with BRV and clinical evaluation

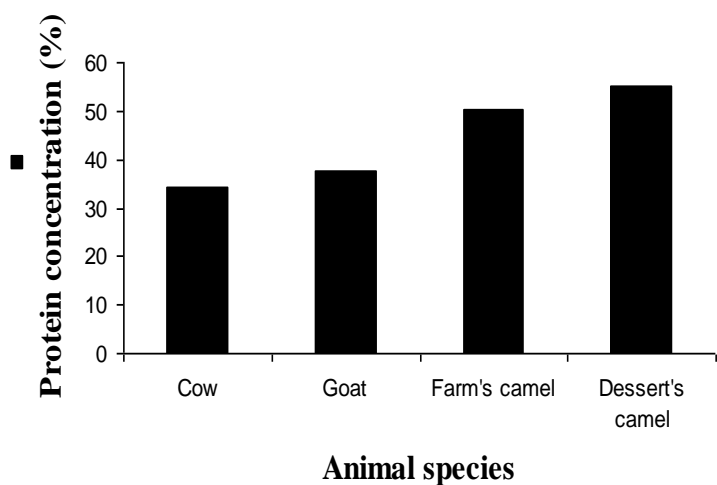
All Albino Swiss mice inoculated groups developed diarrhea as was observed by incidence results (**Fig. 2**) and the DI (**Fig. 3**). Severe diarrhea occurred in 90% of control mice; there was a significantly reduced to 10%, 30%, 40% and 60% in group IV, III, II and group I, respectively. Severe diarrhea occurred for a 4-day period in the control group but only for a one day period in group IV, III, II and for 2 day in group I. Most of Group II, Group III, Group IV developed mild diarrhea (DI 2) during one DPI, while Group I showed loose yellow-green feces resembling that in Group V.

Bovine rotaviral shedding

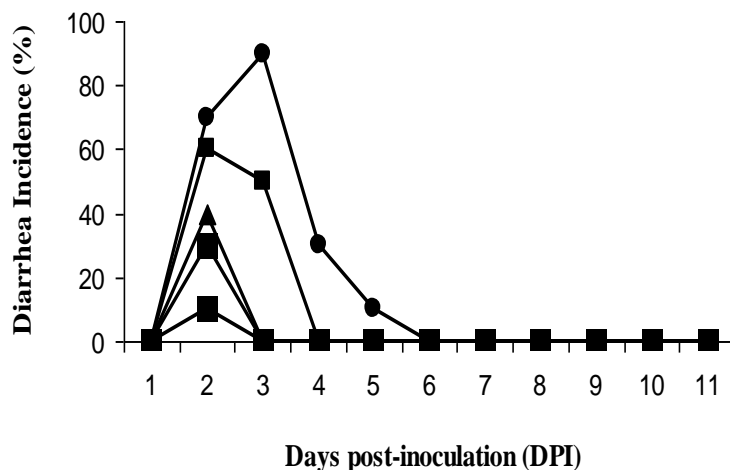
Viral load was analyzed in All Albino Swiss mice inoculated groups during the post-infection period with maximum virus shedding immediately after inoculation in Group V (**Fig 4**). Whereas the load of fecal BRV particles was almost undetectable by ELISA after 5 DPI in Group V. Viral shedding in fecal samples were absent in group II, III and IV except for one DPI for Group II, Group III, Group IV and 2 DPI for group I.

Systemic humoral and cell immune response

Specific humoral immune response was evaluated by quantification of serum anti-BRV antibodies in inoculated groups; all BRV-inoculated mice produced high titers of a specific antibody against BRV (2.4 ± 0.07 Absorbance Units, determined by ELISA).

Figure 1. Protein concentration (%) of whey protein derived from milk of different animals' species**Figure 2. Diarrhea incidence (%) in Albino Swiss mice different groups inoculated with**

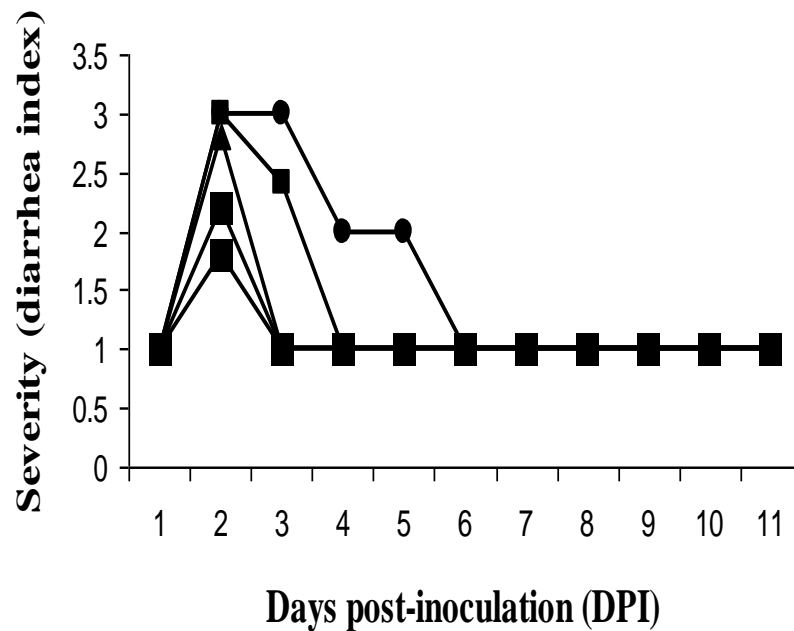
BRV: Group I (■), Group II (▲), Group III (×), Group IV (*) and Group V (●).



Group I: Group supplemented with whey protein derived from cow's milk
 Group II: Group supplemented with whey protein derived from goat's milk
 Group III: Group supplemented with whey protein derived from farm camel's milk
 Group IV: Group supplemented with whey protein derived from dessert camel's milk
 Group V: Un-supplemented infected group (control)

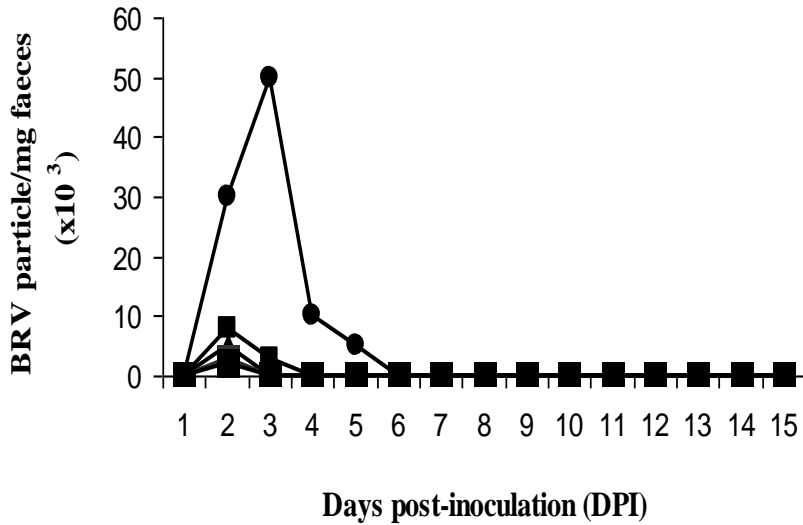
Figure 3. Severity of diarrhea (DI) in Albino Swiss mice different groups inoculated with

BRV: Group I (■),Group II (▲),Group III (×),Group IV(*),and Group V(●).



Group I: Group supplemented with whey protein derived from cow's milk
Group II: Group supplemented with whey protein derived from goat's milk
Group III: Group supplemented with whey protein derived from farm camel's milk
Group IV: Group supplemented with whey protein derived from dessert camel's milk
Group V: Un-supplemented infected group (control)

Figure 4. Viral antigen–shedding curves of fecal samples in Albino Swiss mice different groups inoculated with BRV: Group I (■),Group II (▲),Group III (×),Group IV(✱) and Group V(●).



- Group I: Group supplemented with whey protein derived from cow's milk
Group II: Group supplemented with whey protein derived from goat's milk
Group III: Group supplemented with whey protein derived from farm camel's milk
Group IV: Group supplemented with whey protein derived from dessert camel's milk
Group V: Un-supplemented infected group (control)

Table 1. Chemical analysis of whey derived from milk of different animal species

	<i>Cow</i>	<i>Camel</i>		<i>Goat</i>
		<i>Dessert</i>	<i>Farm</i>	
Fat	0.25 ± 0.06	0.13 ± 0.06	0.30 ± 0.1	0.70 ± 0.1
Lactose	4.00 ± 0.08	4.15 ± 0.2	4.30 ± 0.05	3.27 ± 0.1
Protein (whey protein)	0.68 ± 0.2	1.1 ± 0.2	0.83 ± 0.1	0.71 ± 0.07
Ash	0.4418 ± 0.01	0.5063 ± 0.09	0.4299 ± 0.03	0.9162 ± 0.1

The values indicated was the mean of triplicates ± standard deviation

DISCUSSION

Rota viruses are implicated in severe dehydrating gastroenteritis in children and young animals; hence, considerable efforts are being made to develop effective preventive and therapeutic strategies for this condition. Today, whey is a popular dietary protein supplement purported to provide antimicrobial activity, immune modulation, improved muscle strength and body composition, and to prevent cardiovascular disease and osteoporosis. Advances in processing technology, including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange, have resulted in development of concentrated whey protein that has better biological and antimicrobial activities than whey itself (Marshall, 2004).

The experimental results in this study indicate diarrhea symptoms significantly improved with daily supplementation of mice's diet with cow's, goat's and camel's whey proteins. Specifically, supplementation with these dairy compounds decreased the incidence and severity of diarrhea caused by BRV and modulated viral shedding with the highest effect for whey protein prepared from dessert's camel milk (Fig. 2-4). These data provide further support for the idea that cow's, goat's and camel's whey proteins are effective for ameliorating the clinical course of BRV infectious diarrhea and are consistent with the finding of Wolber et al (2005), who describe a reduction in RV-induced disease symptoms in suckling mice infected with Epizootic Diarrhea Infant Mice virus RV.

Recent studies suggest that whey protein concentrates, fractions, or individual peptides present in milk and colostrum might be useful for the treatment of a wide variety of gastrointestinal disorders, such as inflammatory bowel disease, necrotizing enterocolitis, and infective diarrhea (Playford et al., 2000). This is came in accordance with the results of current study in which the whey proteins derived from cow's, goat's and camel's milk showed antiviral activity against Rota virus with the highest efficacy to dessert's camel whey protein. It contained the highest protein % in its whey in comparison to that in other milk species (Table 1) and also in the final undenatured whey protein (55%) (Fig. 1). The difference in efficacy and composition of whey derived from both dessert's and farm's camel may be attributed to feeding's and diet's nature. Generally, camel's whey protein include a heterogeneous group of proteins, including serum albumin, α -lactalbumin, immunoglobulin, lactophorin, peptidoglycan recognition protein, lysozyme and lactoferrin more than those in cow's and goat's milk in reverse to β -lactoglobulin. In the same context, goat's whey protein contain double amount of lysozyme than that of cow's whey protein (Kappeler et al., 2004). Lysozyme may adsorb on viruses by electrostatic interactions between its positive charges and negative charges of virus which alter viral capsid structure and viral nucleic acids that reflect a loss viral infectivity (Ly-Chatain et al., 2013).

Pe' rez-Cano et al (2008) evaluated the immunomodulatory action of WPs during RV disease in which they stimulate gut immune response in early life to confer protection against pathogens in suckling rats. It has been described that serum IgA titers correlate with protection from RV disease and high titers correlate with protection from reinfection (Matson, 1996).

Milk whey is reported to contain components having antimicrobial and immune modulatory activity (Kelleher and Lonnerdal, 2001), although the precise mechanisms by which bovine's, ovine's and camel's whey can help in gastrointestinal disorders are not fully clarified. Nonetheless, several factors, such as the large amounts

of Ig present in these compounds, challenge by neutrophils and NK cells, blockage of pathogens, and the specific biological activities of some molecules present in whey protein, such as lactoferrin, have been proposed to contribute to the beneficial action of milk whey against pathogens. In addition to controlling diarrhea, camel's whey proteins showed the superiority among other used whey proteins and this may be attributed to their constituent from lactoferrin. Lactoferrin is able to inhibit the replication of Rota virus in a dose-dependent manner, lactoferrin being the most active (Beaulieu et al., 2006). It was shown that lactoferrin hinders virus attachment to cell receptors since it is able to bind the viral particles and to prevent both rotavirus haemagglutination and viral binding to susceptible cells. Moreover, this protein markedly inhibited rotavirus antigen synthesis and yield in HT-29 cells when added during the viral adsorption step or when it was present in the first hours of infection, suggesting that this protein interferes with the early phases of Rota virus infection (Superti et al., 1997).

The protection against infection conferred by consumption of camel's milk is observed in experiments performed in mice infected with *Schistosoma mansoni* (Magharaby et al., 2005), and in humans suffering from hepatitis B. The creation of a better immunological situation probably occur through a better adjustment of the expression T1/T2-type cytokines which could strengthen the cellular immune response, inhibiting the replication of the virus DNA and promoting recovery in chronic hepatitis B patients (Saltanat et al., 2009).

The difference in the antiviral activity against Rota virus of the different whey proteins in the current experiment could be explained as cow's milk and camel's milk show different antioxidant and antimicrobial activities, higher for camel's milk (Salami et al., 2010). The protein concentration of WPs (Fig. 1) and structure of the milk whey from different animals' species are not the same (Table 1). Camel milk's lactoferrin has very high levels of bactericidal and bacteriostatic properties against Gram-positive and Gram-negative bacteria (Conesa et al., 2008), more than cow's and human's lactoferrin. The action is similar against viruses; in this case, for example, it prevents the penetration of hepatitis C virus in leukocytes (Redwan and Tabll, 2007).

Recent studies have indicated that camel's whey proteins have many therapeutic properties. It increases the antioxidant activity in the body, combats fatigue and inflammation, hastens healing, improves stamina and may discourage related infections due to the immune system-enhancing and natural antibiotic properties of its components (Weinberg, 2007). Recently, Badr (2013) investigated that camel's whey proteins enhance diabetic wound healing

CONCLUSION

The findings of this study suggest that daily supplementation of whey proteins prepared from cow's, goat's and camel's milk in early life considerably reduce Rota-viral disease by decreasing the prevalence of severe diarrhea and by decreasing the time period during which severe symptoms and high viral shedding occur so can be used in prophylactic treatment. In addition the various compounds present in whey protein of camel's milk have the most potent antiviral activity against rotaviral infection. Moreover, we recommend the application of camel's whey protein (concentrated form) as food additives especially for milk and dairy products.

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