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Role of Bovine Colostrum and its Biofunctional Fraction PRP in Oral Treatment of Enterogenic Endotoxaemia in Rats

SHERIF S. RAGAB AND MOHAMED K. IBRAHIM†
Department of Nutrition and Food Science, Faculty of Home Economics, Minufiya University, Minufiya, Egypt
†Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt
†Corresponding author's e-mail: kkkmohamed1@hotmail.com

ABSTRACT

Bacterial endotoxins in the intestines can traverse the mucosal barrier by translocation and enter the blood and lymphatic system and hence affect the general immune responses. Colostrum biofunctional constituents like immunoglobulins, lysozymes and lactoferrins have been reported to neutralize endotoxins and control many of viral and bacterial infections in humans. This study was done on a total of 60 male Wistar rats that were randomly assigned to four groups, each comprising 15 animals: groups I, II and III in addition to a control group. Endotoxaemia was caused by administrating an oral suspension of enterotoxigenic *Escherichia coli* ($1.0x10^6$ CFU/mL) directly into the duodenum of anaesthetized rats after giving intraperitoneal carrageenan. At the same time, bovine colostrum and/or proline-rich polypeptide (PRP) were given. Therapeutic effects were studied by examining both plasma endotoxin activity and bacterial load of both mesenterial lymph nodes and peritoneal lavages. Results were compared with those obtained with the milk whey which was used in a control group. Value of plasma endotoxin activity of groups I, II and were 68.1±4.3, 112.3±3.5 and 56.0±3.5 EU/dl, respectively after 6 h of the infection incidence compared with the control group value 152.8±8.8 EU/dl. The reduction in bacterial infection of lymph nodes and peritoneal lavages was also evident with different degrees of efficiency. The incidence of bacterial infection in lavage was 42.1, 46.8, 36.3 and 64.8% in the experimental groups I, II, III and the control one respectively, whereas, the incidence of lymph nodes infection was 40.2, 44.4, 33.9 and 66.7% in the experimental groups I, II, III and the control one, respectively. Whole bovine colostrum and/or PRP may help to eliminate and control *Escherichia coli* endotoxaemia in rats when administered into the gut in conditions of septic shock.

Key Words: Colostrum; PRP; Endotoxaemia; Experimental septic shock

INTRODUCTION

Clinical data underscores the fact that subsequent high mortality rates occur in-patients who survive acute septic episodes. It is generally acknowledged that severe sepsis/septic shock is a major problem in clinical medicine, yet the extent of the problem and its basic immunology remain poorly defined (Benjamim *et al*., 2003). Possible translocation of bacteria and endotoxins renders the gastrointestinal tract a crucial factor in this condition (Saadia *et al*., 1990).

The problem of sepsis is further complicated by the remarkably diverse spectrum of illness encompassed under the term 'sepsis'. Sepsis may range in severity from mild systemic inflammation without significant clinical consequences to multisystem failure in septic shock with an exceedingly high mortality rate (Opal, 2003). The innate immune response is the first line of defence against infection. Toll-like receptors (TLRs) recognize bacterial lipopolysaccharides and other pathogen-associated molecular patterns (PAMPs) (Carrillo-Esper, 2003). The protective function of the gastrointestinal tract breaks down and bacteria from the gut enter the blood and lymph system (Brunser *et al*., 1992). Immunological approaches have been considered as an alternative therapeutic option for the treatment of enteric infections over the past few years. Per-oral administration of bovine milk immunoglobulin has proven to be effective in the treatment of intestinal *Escherichia coli* infection (Kawasaki *et al*., 2000; Ashraf *et al*., 2001). The importance of colostrum for the growth and health of newborn offspring is well known. In bovine colostrum, the antibody (immunoglobulin) complement system provides a major antimicrobial effect against a wide range of microbes and confers passive immunity (Korhonen *et al*., 2000). Proline-rich polypeptide was reported to have multifunctional importance for human health as an immuno-modulator to correct immunological disorders, improve mood and cognitive activities, psycho-enhancing, interleukin-inducing (Inglot *et al*., 1996; Leszek *et al*., 2002). The present study was conducted to determine whether those findings could be confirmed *in vivo* by using bovine colostrum and/or its multifunctional PRP fraction in animal experiments.

MATERIALS AND METHODS

A total of 60 male Wistar rats (200-230 g) were anaesthetized with ketamine. In order to achieve a degree of

immunosuppression (i.e. an inflammatory state) in the gut and a higher initial level of plasma endotoxin activity (Lissner *et al*., 1996; Pricolo *et al*., 1996), 80 mg of type IV carrageenan/kg (Sigma-Aldrich Corp., St Louis, MO, USA) were injected into the peritoneal cavity. Animals were randomly assigned to four groups, each comprising 15 in number (Table I): groups I, II and III received 200 mg bovine colostrum/kg, 25 mg PRP/kg, and a mixture of 200 mg bovine colostrum with 25 mg PRP/kg, respectively. Group 4 received 400 mg milk whey/kg (control substance). All animals used in this experiment were adminstrated wuth 10 mg neomycin.

Bovine colostrum was delivered as a scientific grant from Immuno-Dynamic Inc., Perry – Iowa , USA in purified powder package of 250 g. PRP was prepared according to Janusaz *et al.* (1981).

At the beginning of the 6 h period of observation, the first blood samples were taken from the exposed external jugular vein. Neomycin is clear bactericidal agent and stimulates the release of lipopolysaccharide from *E. coli*, and their administration results in increasing plasma endotoxin levels. Therefore, a combination of 1.0×10^6 colony-forming units of *E. coli*/kg (strain O:NT H16 clinical isolate; American Type Culture Collection); 10 mg neomycin/kg; and the group-specific colostrum, PRP or milk whey were administered through a per-oral tube (diameter 2 mm). The tube was fixed in the duodenum by laparotomy (L.N.), carefully avoiding damage to the efferent billiary tract.

Preliminary investigations without carrageenan have shown that the maximum endotoxin level in plasma (50±2 EU/dl) was reached 6 h after administration of the bacteria/neomycin suspension. Therefore, the plasma endotoxin activity was measured hourly for 6 h. At the same time points, 1 mL blood was taken from the jugular vein for culture. Laparotomy (L.N.) and peritoneal lavages with 10 mL endotoxin-free 0.9% saline solution followed the last assessment.

Furthermore, mesenteric lymph nodes from two different areas of the mesenterium (duodenum & colon) were resected and homogenized. Bacterial load of the lymph nodes and the hourly blood cultures were examined using smears on sheep blood agar plates incubated for 48 h at 37°C. Each lymph node area was examined on one agar plate and the peritoneal lavage on three agar plates. Quantity and specification of the bacterial contamination were not assessed and no anaerobic cultures were performed.

In order to measure the biological endotoxin activity in serum, we used the modified Limulus amoebocyte/lysate test with a chromogenic substrate (Harris *et al*., 1983; van Saena *et al*., 1998). First, 100 L of a 1:40 diluted (0.9% NaCl) plasma sample were heated for 5 min at 80°C. Then 50 µL of Limulus lysate (Pyroquant 50, ChB: 42-109-551; Pyroquant Diagnostik GmbH, Mörfelden-Walldorf, Germany) were added and incubated for 45 min at 37°C. inally 100 L of chromogenic substrate (S-2423; Chromogenic Company, Mölndal, Sweden) were added and incubated for 4 min. at 37°C. The reaction was stopped with 50 L acetic acid (100%) and the extinction was measured by a photometer at a wavelength of 405 nm.

Statistical analysis of plasma endotoxin activity was based on the arithmetic means and standard deviations. Statistical evaluation of analytical data was done by student's 't' test (Snedecor & Cochran, 1976).

RESULTS

Data presented in Table I indicate the endotoxin activity in plasma of rat groups as affected by ingestion of bovine colostrum and/or PRP. There was an approximately linear rise in plasma endotoxin up to 6 h in control group with endotoxin activity value of 145±18 EU/dl. In groups I and II, the increase in the value of plasma endotoxin activity was observed up to 5 h with values of 78.3±4.4 and 115.1±2.2 EU/dl, respectively. Group IV shows a decrease in the measured value after 4 h with value of 66.2±5.4 EU/dl.

The most effective suppression of biological activity was observed in group III which received a mixture of both bovine colostrum together with PRP, in which the maximum plasma endotoxin value was 67.4±6.2 EU/dl after 4 h. At the end of the observation period the value was just 56.0±3.5 EU/dl. This amounts to a reduction of 63.3% after 6 h. In groups I and II endotoxin activity was reduced to 68.1±4.3 and 112.3±3.5 EU/dl, respectively (i.e. a maximum reduction was 55.4 and 26.5%, respectively after 6 h) as shown in Table II.

The incidence level of the bacterial infection in-group III which was fed on a mixture of both bovine colostrum and PRP was the lowest as 33.9 and 36.3% in lymph node and lavage tissues, respectively (Table III).

DISCUSSION

The gastrointestinal tract is of great importance for the development and prognosis of septicemia (Prins *et al*., 1994). However, such bactericidal preparations can liberate lipid A fragments from the bacterial cell wall and thus increase the translocation of endotoxin (Prins *et al*., 1994; Dean-Nystrom *et al*., 2000). It therefore appears rational to combine antibiotic with a substance that inactivates both bacteria and endotoxins (Burke *et al*., 1989; Warny *et al*., 1999). An oral dietetic would be of particular importance in this regard because plain parenteral nutrition lowers the concentration of secreted IgA in bile. This weakens immunological resistance and thus diminishes the barrier function of the intestinal mucosa (Feist *et al*., 2000). We attempted to demonstrate that bovine colostrum is better able to inactivate lipopolysaccharide than albumin. Lactoferrin is well documented to have multifunctional activity as the most active principal of bovine colostrum (Roberts *et al*., 1992; Antonius *et al*., 2000). Several

Table I. Plasma endotoxin activity in rats fed bovine colostrum and/or PRP

Time (hours)	Group I (BC[1])	Group II (PRP[2])	Group III (BC+PRP)	Group IV (Control)
0	2.7 ± 2.4^{a}	2.7 ± 4.2^{a}	2.7 ± 1.2^{a}	2.8 ± 1.2^{a}
1	25.6 ± 1.5^{a}	27.1 ± 5.1^{b}	24.9 ± 3.5^{a}	29.6 ± 3.4^{c}
2	44.1 ± 3.2^{a}	48.6 ± 3.3^{b}	41.4 ± 4.2^{c}	54.8 ± 5.3^{d}
3	64.6 ± 3.6^{a}	75.4 ± 4.7^{b}	56.8 ± 4.4^{c}	88.3 ± 2.1^{d}
4	74.4 ± 1.1^{a}	98.9 ± 3.7^{b}	67.4 ± 6.2^{c}	121.1 ± 3.4^{d}
5	78.3 ± 4.4^{a}	115.1 ± 2.2^{b}	66.2 ± 5.4^{c}	146.5 ± 6.5^{d}
6	68.1 ± 4.3^{a}	112.3 ± 3.5^{b}	56.0 ± 3.5^{c}	152.8 ± 8.8^{d}

BC= [1]± SE. P<0.05, versus control; Values are expressed as the mean PRP= proline-rich polypeptide;Values with different [2] ,bovine colostrum letter symbols are shown significant difference

Table II. Inhibition percentage of plasma endotoxin activity in rats fed bovine colostrum and/or PRP

Time (hours)	Group I (BC[1])	Group II (PRP[2])	Group III (BC+PRP)
0	1.8 %	1.8 %	1.8 %
1	13.4 %	8.3 %	15.6 %
2	19.4 %	11.2 %	24.3 %
3	26.8 %	14.5 %	35.6 %
4	38.8 %	18.6 %	44.5 %
5	46.5 %	21.4 %	54.8 %
6	55.4 %	26.5 %	63.3 %

Table III. Bacterial contamination after 48 h of incubation of peritoneal lavage and lymph node specimen in rats fed bovine colostrum and/or PRP

Tissue	Group I (BC[1])	Group II (PRP[2])	Group III (BC+PRP)	Group IV (Control)
+ve (%) Lavage	40.1^{a} %	46.8^{b} %	36.3^{c} %	64.8^{d} %
+ve (%) Lymph node	40.2^{a} %	44.4^{a} %	33.9^{b} %	66.7^{c} %

Values are expressed as the mean ± SE; P<0.05, versus control; [1]BC= bovine colostrum[2] ,PRP= proline-rich polypeptide; Values with different letter symbols are shown significant difference

physiological functions have been attributed to bovine lactoferrin, including the pronounced protective effects against many of enteric and gastrointestinal infectious agents in both newborn and adult humans through its oral intake (Reiter, 1983; Dial & Lichtanberger, 2002). It could be stated that oral administration of colostrum lactoferrin is involved in the retardation of the growth of wide range of both Gram+ve and Gram–ve infectious bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Kelbsiella pneumoniae* and *Streptococcus mutans* through either bacteriostatic or bactericidal mechanisms (Ajello *et al*., 2002). In addition to the pronounced antibacterial potency of bovine lactoferrin, it has potent anti-viral activity against many viruses associated with human diseases including hepatic virus C infections and its possible hepatic carcinoma, AIDS, influenza, herpes, cytomegalo-viral infections and others (Harmsen *et al*., 1995; Swart, 1998). Many of fungal and parasitic human infections are completely prevented or at least cured and recovered by regular oral ingestion of bovine lactoferrin (Samaranayake *et al*., 2001; Nibbering *et al*., 2002).

In addition, administration of bovine colostrum has already proven effective in treating bacterial and viral enteritis in babies and infants (Raqib *et al*., 2000; Marshall *et al*., 2002). Enteral administration of hyperimmunized bovine colostrum has been found to reduce peri-operative translocation of endotoxin from the gastrointestinal tract (Appelmelk *et al*., 1994). However, it is still unclear which constituents of bovine colostrum are the crucial biological factors in this therapeutic effect. Neutralization of endotoxins and bacteria has been reported for immunoglobulins and lactoferrin (Nebermann *et al*., 1994; Pricolo *et al*., 1996). On the other hand, Guillen *et al*. (2002) and Kimber *et al*. (2002) reported that bovine lactoferrin could mediate both antimicrobial and immunomodulation activities with downstream effects on the outcome of microbial and other inflammatory diseases.

Yu and Schrijvers (2002) stated that bovine lactoferrin molecules have the ability to interact with the bacterial cell wall through specific binding sites. There are some other suggested mechanisms involved in the expression of the antibacterial potency of bovine lactoferrin. Semenov *et al*. (1998) and Welling *et al*. (2000) reported that bovine lactoferrin could inhibit the non-specific bacterial esterase without affecting both cell wall and cell membrane permeability. This indicates that lactoferrin molecules affect and interact with the bacterial ATP system. These interaction mechanisms could offer some of the critical disturbance in most of the bacterial physiology then damage and inactivate the whole bacterial cell. Several other studies indicated that lactoferrin has its pronounced bactericidal properties through the ability to release the cellular lipopolysaccharides of the bacterial cell by the action of the highly cationic bactericidal domain of lactoferrin at N-terminus of the molecule (Ballamy *et al*., 1992; Yamauchi *et al*., 1993). This domain is involved directly interacting with the bacterial cell wall and lipopolysaccharide content, therefore acting as strong bactericidal agent. This mechanism highly agree with that reported by Ellison *et al*. (1990) and Rossi *et al*. (2002). Other mechanisms could be considered also to explain the antibacterial activity of bovine lactoferrin as that its ability to block bacterial carbohydrate metabolism (Arnold *et al*., 1980).

In this study, group III which received a mixture of both bovine colostrum and PRP, exhibited the greatest suppression of plasma endotoxin level. This may be due to the high immunoglobulin content received in addition to immunomodulator activity of ingested PRP. The reduction in endotoxin activity was also significant in the other two experimental rat groups more than control group. Because that colostrum contains only half as much immunoglobulins as the colostra, a distinct effect of lactoferrin in-groups I and III is possible. The iron-saturation of the preparations rules out the possibility that the bacteriostatic effect of lactoferrin derives from removal of iron from the bacterial cell wall. Therefore, lactoferrin also confers a specific defence mechanism. Intensified elimination of the endotoxin by

'natural killer cells' is conceivable because iron-saturated lactoferrin can activate these cells. The positive therapeutic effect of combining colostrum with PRP supports this concept.

In a large cohort of patients with microbiologically confirmed severe sepsis, appropriate initial antimicrobial therapy was an important determinant of survival. New approaches aimed at improving detection and treatment of early sepsis are needed (Harbarth *et al*., 2003). The synergistic antitoxigenic effect of PRP and bovine colostrum is unclear and needs to be clarified and to unveil its possible mechanisms. It is highly suggested to incorporate PRP in the daily therapeutic program in case of endotoxingenic conditions in order to ensure the perfect and pronounced recovery.

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