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Full Length Research Paper

Bovine colostrum as immunomodulator for prevention of *Escherichia coli* diarrhea in weaned rabbits

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The study was conducted on weaned New Zealand White rabbits aged 4 to 5 months. First milking liquid bovine colostrum (BC) was lyophilized to make powder. Three different doses of BC (200, 400 and 600 mg/kg b.wt. per os for 15 days) were evaluated for immunomodulatory potential against cow milk control. The best immunomodulatory dose was then evaluated for assessing the potential of BC for the prevention of *Escherichia coli* diarrhea. It was observed that lyophilization decreased IgG non-significantly by 10.52%. Further, BC supplementation enhanced the cell mediated immune response with significant protective potential on causation of *E. coli* diarrhea, as long as it was given with residual effect as well.

Key words: Bovine colostrum, immunomodulation, *Escherichia coli* diarrhea, rabbit.

INTRODUCTION

Bovine colostrum is used as a nutraceutical owing to its immunomodulatory, antioxidant and health promoting properties (Pandey et al., 2011). It plays a protective role with respect to neonatal gastrointestinal immunity due to its direct effect on stimulation and development of infantile gut associated lymphoid tissues (Korhonen et al., 2000). It is highly effective in the prophylactic treatment of recurrent diarrhea in reducing not only the episodes but also the hospitalization (Patel and Rana, 2006). It goes down the digestive tract unchanged due to trypsin inhibitors and helps in maintaining the health of epithelial lining and immune system. The colostral oligosaccharides and glycoconjugate sugars attract and bind the pathogenic bacteria, thereby preventing their entry in the mucosal lining (Ogra and Ogra, 1978). Feeding of colostrum is reported to be beneficial even after the absorptive phase because of the local activity of immunoglobulins in the intestine against *Escherichia coli* (Okabe, 1983), rotavirus (Davidson et al., 1989), Candida and *Helicobacter pylori* (Marnila et al., 2003). Colostrum could be effectively used as protective and therapeutic agent across the species (Anderson et al., 1989). Its supplementation increased weight gain in rabbits without affecting biochemical parameters (Dar et al., 2010). Similarly, calves supplemented with colostrum developed less diarrhea and received fewer antimicrobial treatments than placebo and control calves (Berge et al., 2009). The present study was aimed to evaluate the immmuno-modulatory and protective potential of bovine colostrum in rabbits, so that large amounts of it could be collected and processed for further use in a wide range of species against a variety of ailments.

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Abbreviations: BC, Bovine colostrums; **IgG,** immunoglobulin G; **PBS,** phosphate buffer saline; **NZW,** New Zealand white; **SRID,** Single radial immunodiffusion; **SRBCs,** sheep red blood cells; **HA,** haemagglutination; **DTH,** delayed type hypersensitivity; **PHA-p,** phytohemagglutinin-p; **LTT,** lymphocyte transformation test; **MTT,** (3-(4, 5-dimethyl thiazole-2-yl) 2, 5-diphenyl tetrazolium bromide); **FS,** fecal score; **DpS,** depression score; **DS,** dehydration score; **Con-A,** concanavalin-A; **RPMI-GM,** RPMI growth medium; **ANOVA,** analysis of variance; **EGF,** epidermal growth factor; **IGF,** insulin like growth factor; **TGF,** transforming growth factor.

MATERIALS AND METHODS

Animals

The study was carried out using New Zealand White (NZW) weaned rabbits of similar (4 to 5 months) age group. They were

housed in individual cages, fed *ad libitum* with same diet and had free access to water. The managemental conditions in terms of temperature, humidity etc were ideal for the experimental animals.

Collection and preservation of BC

The first milking colostrum was collected immediately after calving under proper sanitation to avoid contamination, frozen at -20°C and lyophilized to form powder. Before use in the experiment it was thawed and warmed to room temperature.

Immunological assessment of BC

Both frozen and lyophilized BC were analyzed for IgG content using standard method of single radial immunodiffusion (SRID) (VMRD Inc.). The study was undertaken in two stages.

Stage 1: Evaluation of immunomodulatory potential of BC

NZW rabbits (30) were randomly divided into five groups of 6 each. Group A comprised of healthy rabbits maintained on normal diet (without treatment with either cow milk or BC) to serve as healthy control (control 1). Group B rabbits received cow milk a 3ml/kg b.wt, instead of BC to serve as control 2. Groups C, D and E rabbits were given BC orally at dose 1 (200 mg/kg b.wt.), dose 2 (400 mg/kg b.wt.) and dose 3 (600 mg/kg b.wt.) respectively for 15 days, by dissolving BC in sterilized phosphate buffer saline (PBS- 0.1 M, pH 7.2). The orally administered cow milk / BC to different groups were in addition to the normal diet to rabbits.

Humoral immune response

Determination of serum IgG: Serum samples were used in a SRID kit (VMRD Inc.,) for the quantitative determination of IgG.

Haemagglutination test: The rabbits from different groups were immunized with sheep red blood cells (SRBCs) by intravenous (ear vein) injection of 0.5 ml of SRBCs suspended in PBS (0.1 M, pH 7.2) a 4.75×10^{6} cells per animal on day 1. Serum was collected on 11^{th} and 21^{st} days of immunization**.** Haemagglutination (HA) was carried out by microtitration techniques according to the procedure described by Beard (1980).

Cell mediated immune response

Delayed type hypersensitivity test (DTH): Cutaneous DTH response was measured after 15 days of colostrum feeding as described by Kim et al. (2000). All rabbits were injected with 100 µl PBS (0.1 M, pH 7.2) as a negative control on right neck region and phytohaemagglutinin – p (PHA-p) 50 µg in 100 µl PBS (0.1 M, pH 7.2) intra-dermally in left neck region. The DTH response was assessed by measuring skin induration using vernier calipers at 0, 24, 48 and 72 h post- injection, and expressed as increase in skin thickness (mm) compared with 0 h.

Lymphocyte proliferation assay (LTT): LTT using 3-(4, 5-dimethyl thiazole-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) was performed in 2 groups (Control-1 and Group E) on 15^{th} day of colostrum feeding as described by Bounous et al. (1992).

Stage 2: Evaluation of protective role of BC in *E. coli* induced diarrhea in rabbits

The study was conducted under standard feeding and managemental conditions in 30 healthy weaned NZW rabbits of 3 to 5 months age.

E. coli challenge

Rabbits were randomly divided into five groups of six each. Group A comprised of healthy rabbits maintained on normal diet (without treatment with either cow milk or BC) to serve as healthy control (control 1). Group B rabbits received cow milk (3 ml) instead of BC to serve as control 2. Groups C, D and E rabbits were given BC orally at 600 mg/kg b.wt for 20 days. The rabbits of Groups A,B and C were fasted overnight on day 21 and then challenged orally with 2 ml of the bacterial suspension (2×10^{8} cfu/ml) preceded by per os 5 ml of 10% sodium bicarbonate (Blake and Cantey, 1977). Groups D and E rabbits were challenged with same culture on day 30 and 40 of the study respectively. The rabbits were then allowed food and water *ad libitum*. Fecal shedding was recorded as positive when lactose fermenting Gram –ve bacteria were observed on MacConkey plates after overnight incubation at 37°C. Diarrhea was recorded as present when either perineal soiling was seen or soft and unformed stool was recovered from rectal swabs.

Clinical profile

The fecal consistency (FS), depression (DpS) and dehydration (DS) scores (0 to 3) were noted on day 20, day of onset of diarrhea and 5 days post diarrhea as per the method of Walker et al.(1998).

Immunological studies

Serum IgG: Serum samples were used for the quantitative determination of IgG by SRID kit on day 20, day of onset of diarrhea and 5 days post diarrhea.

DTH: Cutaneous DTH response was measured after 20 days in 3 groups – Control-1, Control-2 and BC (600 mg/kg b.wt.) group using PHA-p and Con-A as mitogens as described by Kim et al. (2000).

LTT: LTT using MTT was performed on 20^{th} day of colostrum feeding in control-1 and BC supplemented group as described by Bounous et al. (1992). A total of 5×10^{6}/ml mononuclear cells from blood of rabbits of control group and the group fed with 600 mg of BC for 20 days were placed in phenol red free RPMI-GM. 100 µl of the cell suspension was added to 3 sets of triplicate wells of a RPMI medium including 20 % foetal calf serum, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. The second and third sets received 100 µl of RPMI-GM containing 100 µg/ml of PHA-p and100 µl of RPMI-GM containing 100 µg/ml of ConA.

Statistical analysis

The data were analyzed statistically using ANOVA and 't' test, to find out the significance of difference in mean values.

RESULTS AND DISCUSSION

Immunoglobulin status of lyophilized colostrum

The IgG content of BC and its freeze dried powder was

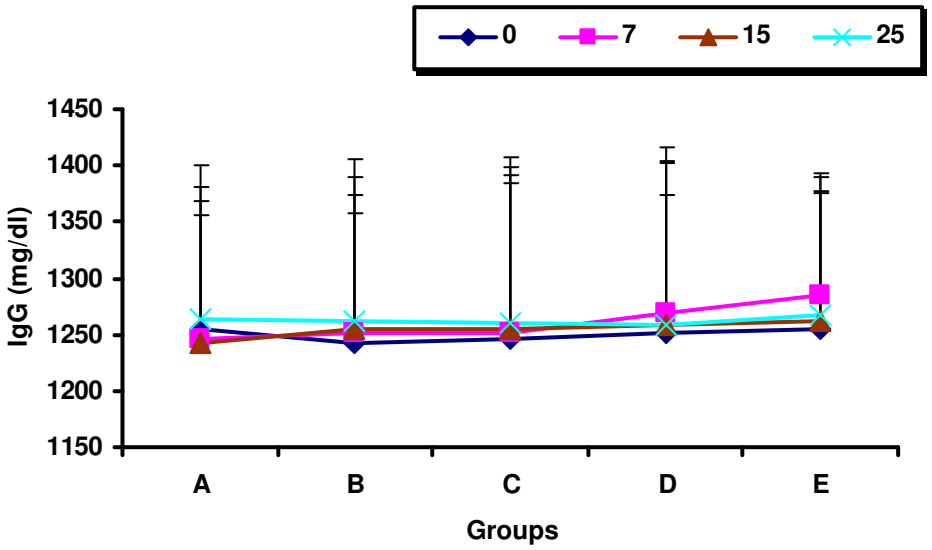

Figure 1. Serum IgG levels (mg/dl) in rabbits supplemented with different doses of BC.

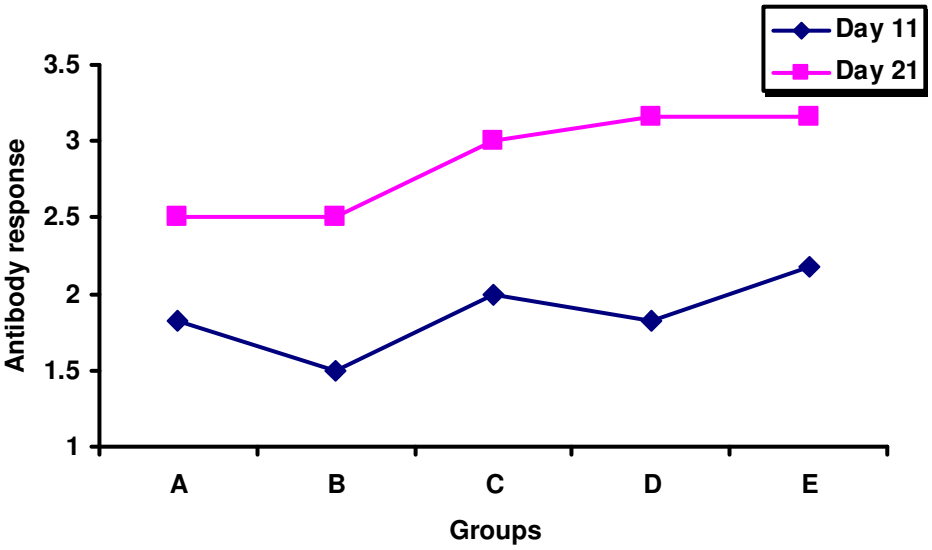

Figure 2. Antibody response (log_2 titre) against SRBCs by HA test.

7600±352.2 and 6800±400 mg/dl respectively which indicated that lyophilization decreased IgG non significantly (P<0.05) by 10.52%. Freeze-drying of colostral whey decreased the content of Igs by 25% (Lindmark-Mansson et al., 2002). No significant decrease in immunoglobulin concentration after lyophilization was observed by Dar (2009).

Serum IgG

There was no significant change observed in the serum IgG values of rabbits of all the five groups throughout the study (Figure 1). IgG being a macromolecule, cannot be absorbed beyond the absorptive phase. This indicated that short term BC supplementation does not influence the endogenous antibody production. No significant increase in serum IgG consequent to BC supplementation for 8 days was found by Mero et al. (1997).

Haemagglutination test

The HA titres showed no significant difference (P<0.05) on day 11 and 21 (Figure 2). No differences in antibody production against SRBC, between pigs fed 20% and13.9% whey protein, was reported by Crenshaw et al. (1986).

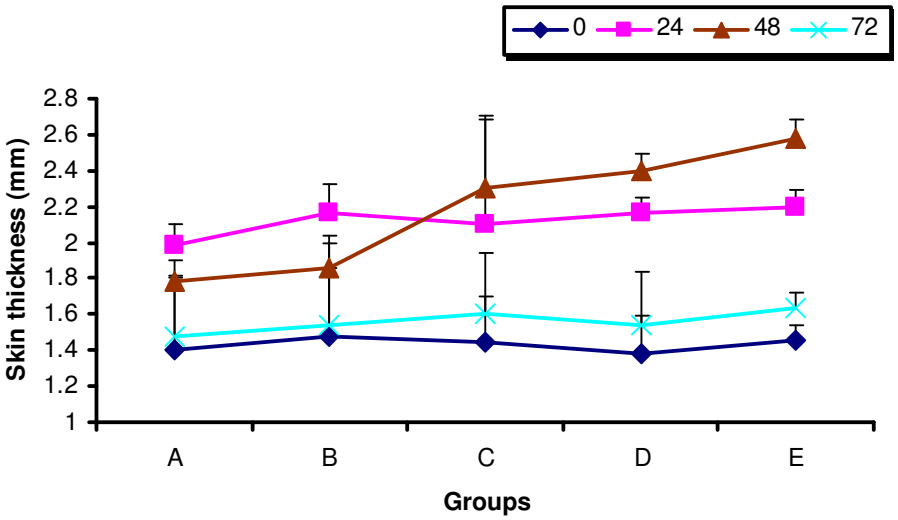

Figure 3. DTH response to PHA-p in rabbits.

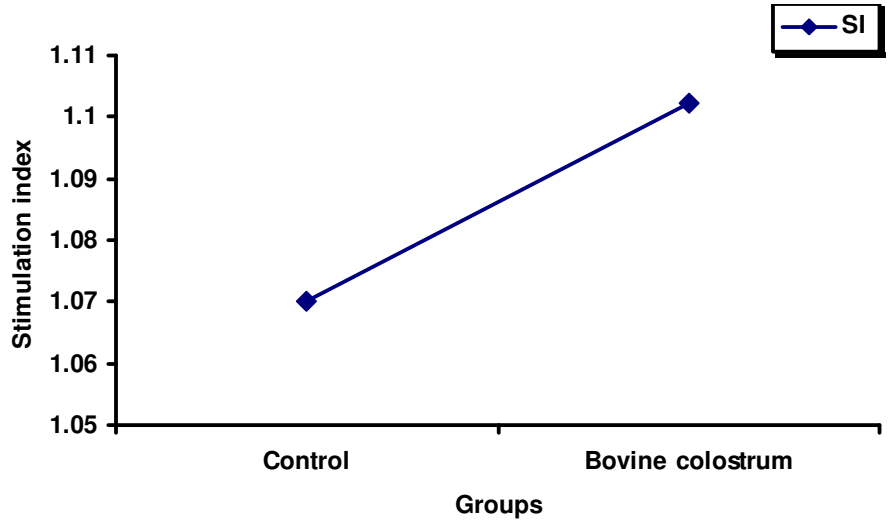

Figure 4. Stimulation index (mean±SE) of mononuclear cells from rabbits.

Cell mediated immune response

DTH

The skin thickness, measured at 0 h in all the five groups showed no significant ($P<0.05$) difference. In comparison to 0 h, the DTH response showed a significant ($P<0.05$) increase in all the five groups at 24 h. A significant ($P<0.05$) increase in DTH response was also observed at 48 h of post-inoculation in Groups C, D and E, indicating that BC supplementation enhanced the CMIR (Figure 3). This could be due to the presence of cytokines like IL-1, IL-2, IL-6, TNF-α and IFN-γ in the colostrum which enhance B and T cell maturation (Watkins et al., 1995). An increase in CD4+ T lymphocytes in lutein-supplemented cats was correlated with the enhanced DTH response (Kim et al., 2000). Similarly, increased DTH response to SRBCs in mice supplemented with BC was reported by Bounous and Kongshavn (1985).

LTT

It was observed that peripheral blood mononuclear cells (PBMC) from rabbits supplemented with BC showed a significantly higher ($P<0.05$) proliferative response to PHA-p on day 15, as compared to PBMC from rabbits of control group (Figure 4). Improvement in cellular immunity of mice with increasing levels of BC in the diet, as measured by a variety of immunological assays, lymphocyte blastogenesis, and resistance to viral infections was observed by Cooper et al. (1974).

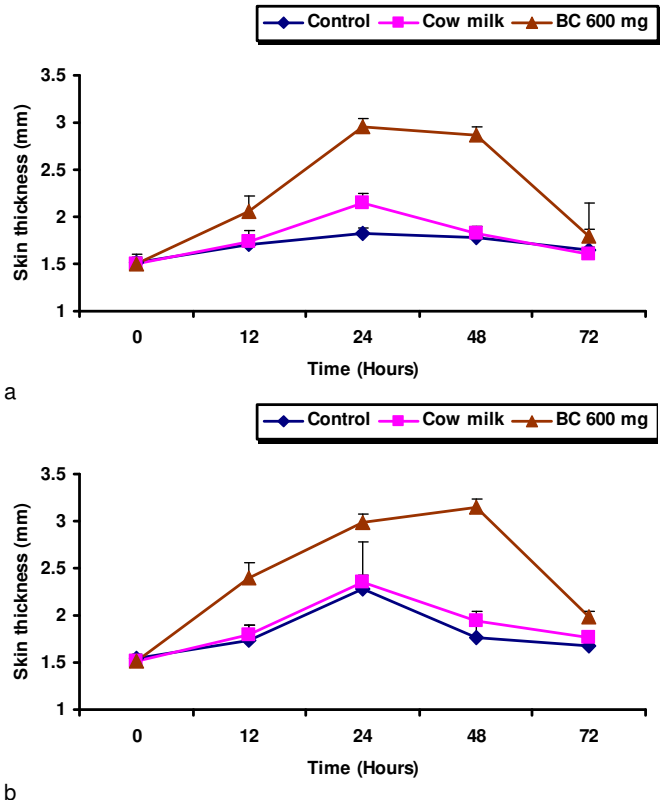

Figure 5. DTH response to (a) PHA-P in rabbits. b) Con-A in rabbits.

Evaluation of protective/prophylactic role of BC

E. coli challenge

The rabbits of Groups A and B developed diarrhea by 96±12 h post challenge with *E. coli*. However, Group C rabbits did not develop diarrhea during the study period. The rabbits of Groups D and E developed diarrhea by 102±6 and 108±8 h post challenge respectively. After the onset of diarrhea the rabbits were found dull, depressed and dehydrated. *E. coli* 015 (RDEC-1) at dose ranging from 1.5×10^{2} to 4×10^{10} cfu/ml produced diarrhea (onset 5.8±3.1 days and mean duration of 4.1±2.8 days) in rabbits without invading the mucosa or without synthesizing enterotoxin (Blake and Cantey, 1977). *E. coli* (RDEC-1) required 24 h to attach to the Peyer's patch lymphoid follicles after inoculation (Cantey and Inman, 1981).

Clinical profile

Clinical profile as measured in terms of FS, DS and DpS revealed significantly increased scores on the day of onset of diarrhea in all the groups except Group C rabbits which did not suffer from diarrhea indicating that colostrum supplementation prevented *E. coli* organisms from setting up the infection by offering immediate protection against the challenge. The protective potential of bovine colostrum could be attributed to factors other than systemically absorbed IgG which include lactoferrin, oligosaccharides, proline rich polypeptides, cytokines, lactalbumin, EGF, IGF-I, IGF-II, TGF-α and TGF-β etc (Playford et al., 2000). Similar clinical findings were observed in *E. coli* diarrhea of various species of animals (Chanjkija, 2002). Human colostrum IgA antibodies seem to prevent *E. coli* induced diarrhea in breast infants (Carbonare et al., 1997). Supplementation of BC at higher doses significantly improved clinical scores in dogs suffering from gastroenteritis (Harish, 2008). Pooled colostrum given to infants infected with diarrheagenic *E. coli,* shiga toxin producing *E. coli* or enterohaemorrhagic *E. coli* experienced diminished frequency of loose stools compared to placebo (Huppertz et al., 1999).

Gay et al. (1965) reported the protective role of colostral immunoglobulins against colibacillosis. Cantey (1978) observed that oral feeding of surface-IgA to rabbits prevented the colonization and diarrhea after challenge with *E. coli RDEC-I* strain. Kawasaki et al. (2000) observed that bovine immunoglobulin administration proved to be effective in the treatment of intestinal *E. coli* infection. Palmer et al. (2001) reported that immune factors in bovine colostrum, when taken orally, were effective against disease causing organisms in the intestinal tract. Bogstedt et al. (1996) demonstrated sufficient prophylactic activity of a daily dose of 500 mg bovine immunoglobulins against rotavirus diarrhea.

Serum IgG

No significant ($P<0.05$) change in serum IgG was observed irrespective of the groups:

DTH: The DTH response to intra-dermal inoculation of PHA-p and Con-A was assessed on day 20 of the study. The dermal induration showed a significant ($P<0.05$) increase in all the three groups at 24 h post-inoculation (PI), before finally subsiding to basal levels at 72 h PI. There was a significant ($P<0.05$) increase in skin thickness in BC supplemented group at 12, 24, 48 h PI (Figure 5a). A similar trend was observed in all the three groups in response to Con-A inoculation (Figure 5b). The DTH response to PBS as a control was low and did not differ significantly among the treatment groups.

LTT: After stimulating rabbit mononuclear cells of control-1 and BC supplemented (600 mg/kg b.wt.) group with PHA-P and Con A; it was observed that BC supplemented group showed a significant ($P<0.05$) increase in stimulation index of mononuclear cells at day 20 compared to control group cells (Figures 6a, b, 7a, and b).

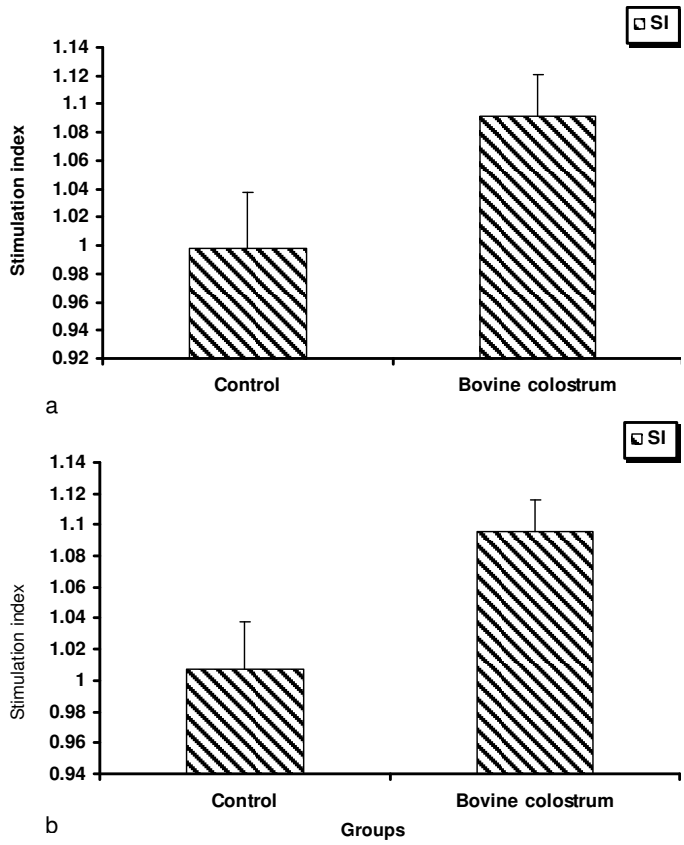

Figure 6. Stimulation index (mean±SE) of mononuclear cells a) (with PHA-p) from rabbits. b) (with Con-A) from rabbits.

Conclusions

The results of the present study suggest that keeping quality of bovine colostrum is enhanced excessively by lyophilization, without any significant loss in IgG concentration, paving way for establishment of colostral bank for use in orphaned newborn animals and commercial utilization.

Further, bovine colostrum could be of significant use in other than bovine species, for increasing the cell mediated immune response for maintenance of health, and its supplementation in newborn animals irrespective of the species during the first 2 months of critical period of stress, and gastrointestinal infections has a potential to protect them from *E. coli* diarrhea and stress of various reasons.

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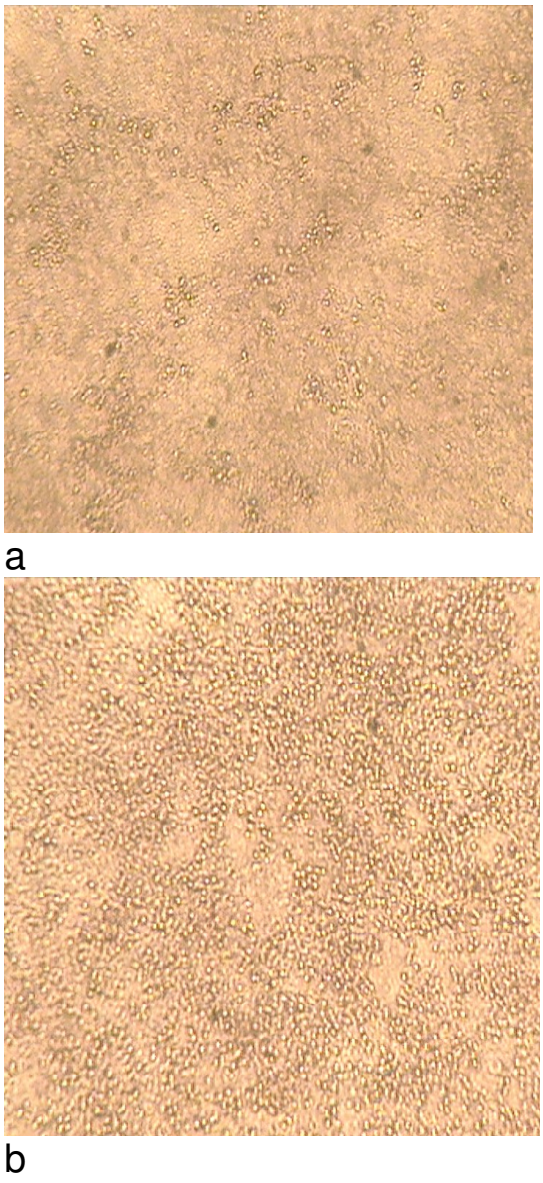

Figure 7. LTT- (a) Control stimulated cells with Con A. (b) Colostrum stimulated cells with Con A.

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