Bovine Complex Milk Lipid Containing Gangliosides for Prevention of Rotavirus Infection and Diarrhoea in Northern Indian Infants

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ABSTRACT

Rotavirus (RV) is a leading cause of morbidity and mortality in children younger than 5 years of age, presenting commonly with diarrhoeal symptoms. In a prospective 12-week double-blind randomised controlled trial we assessed acceptability and efficacy of a high-ganglioside complex milk lipid (CML) for prevention of RV infection in 450 infants, ages 8 to 24 months, at 3 sites in northern India. Prevalence of diarrhoea and RV was unseasonably low at baseline (all-cause diarrhoea [ACD], n = 16; RV diarrhoea [RVD], n = 2; RV infection, RV positive [RV^+], n = 20) and throughout the trial, with only 110 total episodes of ACD for 12 weeks (CML, n = 62; control, n = 48) of which 10 were RVD (CML, n = 4; control, n = 6). Mean duration that RVD persisted was lower in the CML group (2.3 ± 0.5 days) than that in the control group (3.8 ± 1.3 days, $P = 0.03$), but only 3 of 450 end of trial stool samples were identified as RV^+ (<1%; CML, n = 2; control, n = 1). This hampered the assessment of efficacy of CML, despite the large a priori determined sample size. During the trial similar numbers of infants reported adverse events (AEs: CML 41%, control 46%), with the majority of events classified as mild and not related to the intervention. In conclusion, further clinical trials against a higher background of seasonal prevalence are necessary to assess efficacy of this nutritional intervention to prevent RVD. More important, however, high-ganglioside CML was acceptable for long-term consumption in infants ages 8 to 24 months.

Key Words: complex milk lipid, diarrhoea, infant formula, randomised controlled trial, rotavirus

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Rotavirus (RV) is a major cause of gastroenteritis-induced mortality in children younger than 5 years of age, with an estimated 2 million hospitalisations and 450,000 to 700,000 deaths globally per year (1,2). RV infects mature enterocytes of the small intestine that induces gastroenteritis and diarrhoea (3,4). Rotavirus diarrhoea (RVD) is a year-round concern for countries with temperate climates (5), and young infants are particularly susceptible. Therefore, the development of interventions to prevent RV during infancy is needed (6). The fortification of paediatric nutritional products with bioactive nutrients may help reduce RV burden, with several reported to suppress RV infection in cell and animal models, but these have yet to be clinically tested (7). Complex milk lipid (CML) present in cow's milk provides a rich source of polar phospholipids (PLs) and glycosphingolipids such as gangliosides, which form the essential component of cell membranes (8). The development of the intestinal mucosa barrier plays an important role in the defence against foreign antigens (9). PLs such as phosphatidylcholine may improve intestinal barrier function (10) and protect mature enterocytes against infection. Dietary gangliosides play a role in gastrointestinal maturation and have been shown to improve immune function including reducing the risk of infection (11). Our preclinical studies have shown that high-ganglioside CML decreases diarrhoeal occurrence in rat pups infected with human RV (M.A. McConnell, unpublished data). Hence, we undertook a prospective trial to evaluate acceptability and efficacy of CML in infants ages 8 to 24 months in an RVD-prone area in northern India.

METHODS

Design

A prospective double-blind randomised controlled trial was conducted to assess the acceptability, safety, and efficacy of high-ganglioside CML in the prevention of RV infection in infants ages 8 to 24 months. Infants visited the research clinics on 2 occasions, at screening/enrollment/baseline (week 0) and at 12-week follow-up (F/U, week 12). During the trial, fieldworkers visited the infants at home twice each week to dispense the dietary supplements, review diarrhoeal incidence, collect faecal samples, and review general health. The primary outcome measure was total number of days with RVD during the 12-week intervention. The secondary measures were total number of days with diarrhoea of any type (all-cause diarrhoea [ACD]); number of episodes, duration, and severity of RVD and ACD; and RV load in stool samples collected in the clinic at baseline and 12-week F/U. For safety evaluation, adverse events (AEs) were recorded and compared between infants randomised to CML and those to control treatment. The sample size was determined a priori based on the primary endpoint being total

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number of days with ACD. The main assumption based on published data for diarrhoeal episodes in the northern Indian region was 1 episode per child during the 12-week intervention. Power calculations showed that a total of 300 infants, 150 in each of the CML and control groups, would achieve a power of 90% and a significance of 5% for the comparison between the 2 supplemented groups. A meaningful difference between CML and control group was considered to be 38% of the within-group standard deviation (SD), for example, a difference of 1 day with an SD of 2.65 days or a difference of 2 days with an SD of 5.3 days. To allow for a lower than predicted incidence of diarrhoea, noncompliance, or dropout, a total of 450 infants were randomised into the 12-week trial.

Ethical approval was obtained in northern India from the Society for the Promotion of Ethical Clinical Trials (New Delhi sites) and the institutional ethics committee (Lucknow site). Written consent to participate was obtained from each parent or primary caregiver. The trial was registered with the Clinical Trial Registry—India (no. CTRI/2009/091/000873).

Participant Flow and Recruitment

A total of 470 infants at 3 clinical sites in northern India were assessed for eligibility (Fig. 1). The inclusion criteria were as follows: boy or girl, ages 8 to 24 months, living at home within a community setting, with or without a history of diarrhoea, and located near the designated study sites. The exclusion criteria were as follows: prior vaccination for RV, allergy to milk or dairy products, or any serious medical condition including prolonged hospitalisation for diarrhoea. All 470 infants met the inclusion criteria, of which 20 declined to participate. A total of 450 infants (284 boys; 166 girls) were randomised to receive CML (n = 225) or control intervention (n = 225). The present study was a community-wide prevention trial, with no requirement for a positive diarrhoeal test at entry. A stool sample was collected at baseline to determine presence or absence of RVD using commercial rapid screen immunochromatographic dipsticks (QuickStripe Rotavirus, Savyon Diagnostics Ltd, Ashdod, Israel). This was repeated whenever diarrhoea

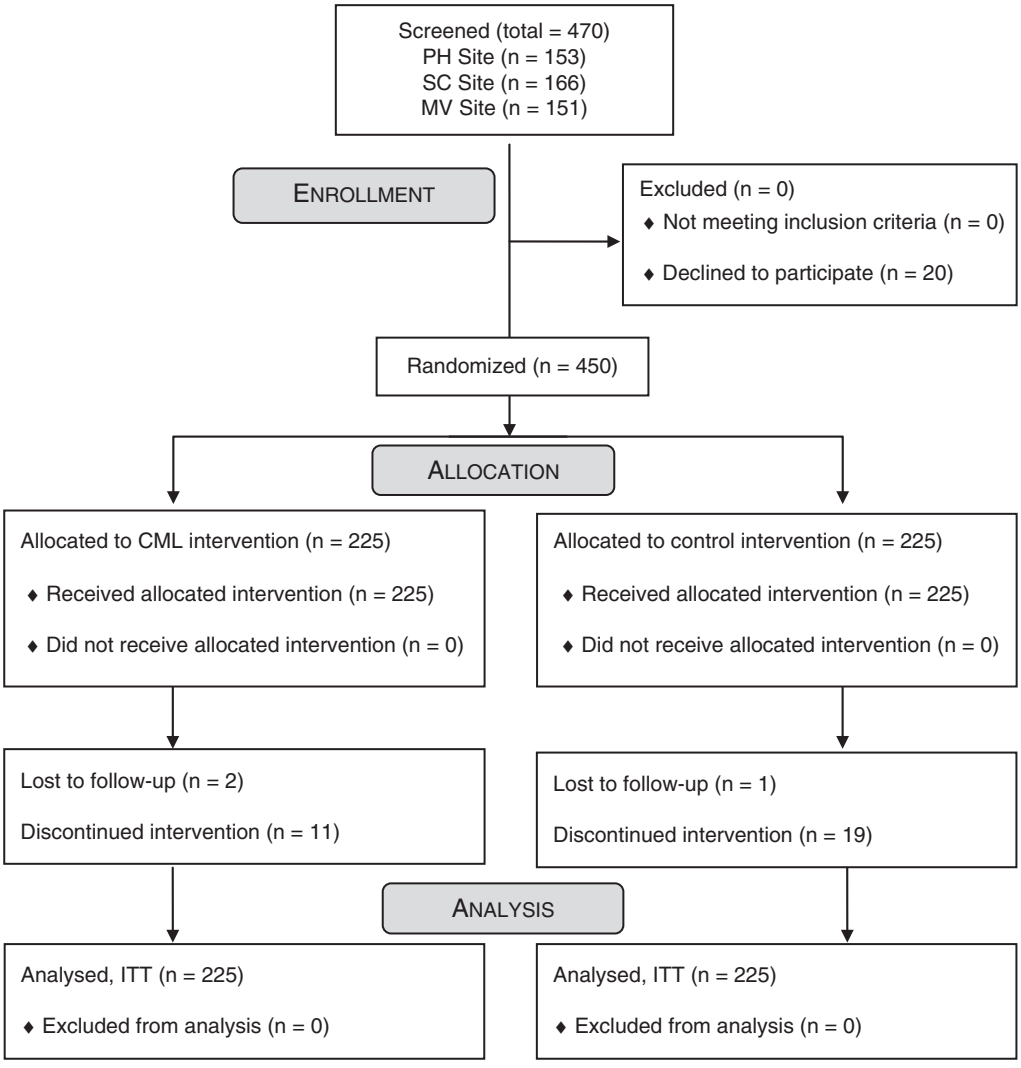

FIGURE 1. Participant flow and recruitment. Infants were randomised at 3 clinical sites, 2 based in New Delhi (PH, Panchsheel Hospital; SC, Sarvodaya Clinic) and 1 based in Lucknow (MV, MV Hospital), northern India. All sites enrolled and randomised 150 infants into the 12-week study. Complex milk lipid (CML; 2 g CML + 3 g whole-milk powder) and control (5 g whole-milk powder) supplements were given daily stirred into a glass of fresh milk; ITT = intention to treat.

TABLE 1. Characteristics of infants at baseline

	CML	Control
n	225	225
Age, mo	15.4 ± 4.6	15.6 ± 4.5
Sex	135 boys, 90 girls	149 boys, 76 girls
Height/length, cm	76.2 ± 6.9	76.9 ± 6.3
Weight, kg	9.0 ± 1.9	9.3 ± 1.9
Breast-feeding, %		
No breast-feeding	13	15
Some breast-feeding	86.5	84.5
Full breast-feeding	0.5	0.5
Prior rotavirus vaccination, %	0	0
Healthy at baseline, %	91	86
Major illness in last 6 mo, %	8	7
Diarrhoeal episode in last 6 mo, %	43	40
Prescription medications at baseline, %	8	15
All-cause diarrhoea at baseline (ACD), n	7	9
Rotavirus diarrhoea at baseline (RVD), n	1	1
Rotavirus infection in stool at baseline, RV^+, n	12	8

Age, height/length, and weight shown as mean ± SD. % = percentage of the 225 infants randomised into each treatment group; CML = complex milk lipid; RV^+ = stool sample positive for rotavirus infection: of the 20 stool samples that tested positive only 2 children presented with symptomatic diarrhoea.

was reported during the intervention period, and also from a stool sample collected at the end-of-trial 12-week F/U. In the CML group, 2 infants were lost to F/U and 11 infants discontinued the intervention. In the control group, 1 infant was lost to F/U and 19 infants discontinued the intervention. The baseline characteristics of the infants at week 0 are shown in Table 1. Mean age at baseline was 15 months in both treatment groups, and >85% of infants were receiving some breast-feeding. A total of >40% of infants in both groups had a diarrhoeal event in the last 6 months, but >85% were reported as healthy at the time of entry into the intervention.

Intervention

The trial was conducted between December 2009 and September 2010. Infants received 2 g CML + 3 g whole-milk powder (CML supplement) or 5 g whole-milk powder (control supplement) in powdered form, to be consumed at home with fresh milk, once daily for 12 weeks. Both supplements were provided in individual, daily, sealed sachets. The CML was a spray-dried ganglioside concentrate, commercially available as a speciality lipid product (Fonterra Co-Operative Ltd, Auckland, New Zealand), and a component of commercial infant formula. The control whole-milk powder contained neutral lipid triglyceride.

Assessment of Bowel Habits and General Health

Parents/caregivers were given 14-day diaries to record occurrence, duration, and severity of diarrhoeal episodes, and change in general health. On 2 occasions per week (24 visits) fieldworkers reviewed the infants at home, recorded bowel and general health, dispensed supplement packs and collected empty supplement sachets, completed diarrhoeal report forms, and collected stool samples (~48 hours after onset) for measurement of RV incidence. Severity of diarrhoeal symptoms was recorded using a modified Vesikari score (12) designed for community assessment.

Adverse Events

AEs (serious and nonserious) were recorded by fieldworkers during home visits, and defined as any symptom, disease, syndrome, intercurrent illness, and/or abnormal laboratory finding that emerged or worsened during the intervention, relative to baseline.

Data Analysis

Statistical analysis of primary and secondary outcomes was performed in R 2.14.2 (R Foundation for Statistical Computing, Vienna, Austria). Baseline characteristics are presented as mean ± SD. Data were analysed on the basis of intention to treat; hence, all infants randomised to treatment were included in the analysis. Missing data were assumed missing at random. Primary outcomes and secondary outcomes including RV and ACD are presented as proportions. Differences in the number of days and severity of ACD between treatment and control groups were assessed with 2-sample *t* test. Confidence intervals (CIs) were

TABLE 2. Diarrhoeal incidence during the intervention and at 12-week follow-up

	CML	Control
During intervention		
All-cause diarrhoea (ACD), total duration, days	196	163
Rotavirus diarrhoea (RVD), total duration, days	9	23
ACD, total episodes, n	62	48
RVD, total episodes, n	4	6
End of intervention: 12-week F/U		
ACD, n	0	0
RVD, n	0	0
Rotavirus infection: RV^+ in stool at 12-week F/U, n	2	1

CML = complex milk lipid; F/U = follow-up; RV^+ = stool sample positive for rotavirus infection.

TABLE 3. Severity, relation, and frequency of adverse events

	CML	Control
Mild, n	157	163
Not related to treatment	*154*	*163*
Abdominal pain	0	1
Acute gastroenteritis	0	3
Bronchitis	3	0
Chest infection, respiratory tract infection	0	1
Common cold	11	11
Conjunctivitis	0	1
Constipation	1	3
Cough	27	28
Dermatitis	0	1
Diarrhoea	54	39
Fever	36	36
Loss of appetite	0	1
Malaria	0	1
Pain in legs from fall from stool	1	0
Pneumonia	0	4
Pustular lesions	5	2
Rectum prolapse	0	1
Rhinorrhoea	0	1
Respiratory tract infection	1	6
Sneezing	0	1
Stool with mucous	0	1
Upper respiratory tract infection	2	9
Viral fever	1	0
Vomiting	10	10
Watery stool with blood	1	0
Whitish papular lesion	0	1
Worm infestation	1	1
Possibly related to treatment	*3*	*0*
Diarrhoea	2	0
Vomiting	1	0
Moderate, n	32	50
Not related to treatment	*26*	*45*
Acute gastroenteritis	1	1
Bronchitis	1	1
Common cold	3	1
Constipation	0	2
Cough	4	6
Diarrhoea	1	3
Eye infection	1	0
Fever	10	17
Impetigo	1	1
Lower respiratory tract infection	1	0
Malaria	0	1
Nasal blockage	1	0
Pneumonia	0	3
Rickets	0	1
Rashes	0	1
Upper respiratory tract infection	1	6
Vomiting	1	0
Wheeze	0	1
Possibly related to treatment	*3*	*5*
Acute gastroenteritis	1	0
Diarrhoea	2	3
Vomiting	0	2
Probably related to treatment	*3*	*0*
Diarrhoea	1	0
Fever	1	0
Vomiting	1	0
Severe, n	0	3
Not related to treatment	*0*	*3*
Convulsion	0	1
Febrile seizure	0	1
Fever	0	1
Total	189	216

CML = complex milk lipid.

determined. Differences in general health at 12 weeks were assessed using Fisher exact test. Significance was declared at $P < 0.05$. AEs were classified based on severity and relation to treatment.

RESULTS

Baseline Visit

At baseline, the prevalence of ACD was low (3.6%) and there was surprisingly little correlation between diarrhoea and RV infection. The characteristics of the infants are shown in Table 1. Of the 450 infants tested, only 16 had ACD, of which 2 were RVD (12.5% of ACD). These community data were far lower than recent reports showing that RV contributes up to 40% of diarrhoea-related hospitalisations in India (13). When the 450 stool samples were analysed for presence or absence of RV at baseline, only 20 stool samples were RV positive (RV^+, 4.4% of all infants; CML, n = 12; control, n = 8), of which 18 were from healthy infants without diarrhoea.

Reported Incidence of Diarrhoea During the 12-Week Intervention

Table 2 presents diarrhoeal incidence through the 12-week intervention. The total number of days with ACD was 359 (CML, n = 196 days; control, n = 163 days), an extremely low incidence equivalent to <1% of monitored trial days. Of this, only 32 days were RVD (8.9% of ACD; CML, n = 9 days; control, n = 23 days). The total number of episodes of ACD was 110 (CML, n = 62; control, n = 48) with only 80 infants (18% of those randomised) reporting 1 or more diarrhoeal events, of which 10 episodes were identified to be RVD (9.1% of ACD; CML, n = 4; control, n = 6). In the 80 children in whom diarrhoeal events occurred, mean (±SD) number of days of ACD was not significantly different between groups (CML, 3.1 ± 1.5 days; control, 3.4 ± 1.6 days, 95% CI −0.3 to 0.8 days, $P > 0.05$); however, mean duration that RVD persisted was lower in the CML group (CML, 2.3 ± 0.5 days; control 3.8 ± 1.3 days, 95% CI 0.16–3.0, $P = 0.03$). There was no difference in severity based on the Vesikari scale for either ACD (CML, 4.4 ± 1.8; control, 4.5 ± 1.9, 95% CI −0.5 to 0.9, $P > 0.05$) or RVD (CML, 4.8 ± 2.1; control 4.0 ± 0.6, 95% CI −3.9 to 2.4, $P > 0.05$).

Twelve-Week Follow-Up Visit

At 12-week F/U, body weight had increased in both the CML and control groups by 0.7 kg, as expected in these growing infants (baseline: CML, 9.0 kg, control, 9.3 kg; 12 weeks: CML, 9.7 kg; control, 10.0 kg). None of 415 infants who attended the clinic visit were reported to have diarrhoea (0%; Table 2). Prevalence of RV infection was again low, with only 3 (CML, n = 2; control, n = 1; <1%) samples testing positive for RV^+, a decrease of >80% in both groups compared with baseline. There was no difference in general

health at 12-week F/U, and the reported prevalence of minor illness was not significantly different. The reported prevalence of major illness during the prior 12 weeks was lower in the CML group (OR 3.5, 95% CI 0.9–20.4, $P = 0.05$).

Adverse Events

During the course of the trial, 405 AEs were recorded of varying severity from mild to severe affecting 41% infants in the CML and 46% infants in the control group (Table 3). The majority of events were classified as mild and not related to the intervention. The proportion of AEs was similar regardless of treatment; however, no severe AEs occurred in the CML group.

DISCUSSION

In the present prospective trial, the infants received 2 g CML that contained 150 to 300 mg per day PL, which was 10- to 20-fold higher than typical commercial infant formula or follow-on formula. Seasonal hospitalisations in New Delhi for severe RVD have been reported to peak between October and December (14). The unexpectedly low incidence of RV throughout the present trial hampered the main objective of the trial to assess the efficacy of CML to prevent RVD, despite a large a priori determined sample size. Compliance with 12-week supplementation in the 450 infants and young children who entered the trial was good, with 94% of daily sachets consumed. More important, the present trial indicated CML to be acceptable and safe for long-term consumption in infants ages 8 to 24 months without known milk allergy or intolerance. The development and maturation of the immune system is important in infants to protect the host against pathogenic organisms, and introduction of weaning foods by this age exposes infants to novel antigens and immune maturation (15). A large prospective community clinical trial in infants at risk from RVD was shown to be feasible in northern India. Nevertheless, further clinical trials against a higher background of seasonal prevalence are necessary to assess the efficacy of nutritional interventions such as CML as an alternative preventive measure for infants in whom RV vaccination is not accessible.

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