Effects of supplemental β-carotene with whey on IgA transfer from maternal milk and mucosal IgA induction in neonatal mice and calves

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ABSTRACT: Data from 17 pregnant mice and 33 Japanese Black calves were collected to clarify the effect of supplemental β-carotene with whey on IgA transfer from maternal milk and mucosal IgA induction in neonatal mice and calves. Dietary treatments in milk replacers were 1) 26% CP as in skim milk (control), 2) 26% CP as whey and 3) 26% CP as whey and 30 mg/kg β-carotene. Diets were offered from 6.5 days postcoitus to 14 days postpartum in pregnant mice and from 3 to 63 days postpartum in calves. Supplemental β-carotene with whey increased the numbers of IgA antibody secreting cells (ASC) in the mammary gland in maternal mice and IgA concentration in stomach contents in neonatal mice at 14 days

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postpartum, which was the consequence of the higher IgA transfer from maternal milk to neonates. The number of IgA ASC in the mammary gland in maternal mice fed whey was higher than that of control mice, but intestinal IgA concentration of neonatal mice was not affected by treatments. Supplemental β-carotene with whey drastically increased serum β-carotene concentration in calves at 14 and 42 days postpartum. Supplemental β-carotene with whey had no effects on fecal IgA concentration and fecal water in calves. These results suggest that β-carotene supplementation with whey to maternal mice during pregnancy and lactation enhances IgA transfer from maternal milk to neonates, but supplemental β-carotene has little effect on mucosal IgA induction in neonatal mice and calves.

Key Words: IgA, β-carotene supplementation, neonatal mice, calves, whey

1. Introduction

Mortality and morbidity of neonates continue to be major problems in humans and animals, and their most common disease is diarrhea, which can cause growth retardation and death of young animals. Successful neonatal health depends on many factors related to management and nutrition, but the improvement of the immune system is required for preventing diarrhea. Supplemental vitamin A and β-carotene enhance the immune system in neonates, and β-carotene has pro-vitamin A activity (Bendich, 1989; Chew and Park, 2004; NRC, 2001; Rühl, 2007). It is well known that vitamin A deficiency is associated with an increased risk of death from common childhood infections (Mora and von Andrian, 2009), and β-carotene deficient calves were found to have a higher incidence of diarrhea and mortality in the first week of life (Kume and Toharmat, 2001; Lotthammer, 1979).

Passive immunity is critical to the survival and health of neonates, and colostrum or milk is a source of nutrients and immune components for neonates (Blum, 2006). IgA is the most

abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces (Fagarasan and Honjo, 2003; Mora and von Andrian, 2009). However, the mucosal immune induction is also needed in neonatal calves, because the disease resistance acquired from colostrum Ig is only temporary (Quigley and Drewry, 1998). Peyer's patches in the gut-associated lymphoid tissue are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cells home preferentially to the gut lamina propria (Fagarasan and Honjo, 2003; Mora and von Andrian, 2009). Recent studies (Iwata et al., 2004; Mora et al., 2006) showed that the vitamin A metabolite all-trans retinoic acid (RA) plays important roles in gut immunity and that RA is necessary for the imprinting of gut-homing specificity on T cells and the induction of gut-homing receptors on B cells and IgA ASC. In the previous study (Nishiyama et al., 2010), supplemental β-carotene (50mg/kg in the diet) to maternal mice during pregnancy and lactation increased the number of maternal IgA antibody secreting cells (ASC) in the mammary gland and guts during lactation and IgA transfer from milk to neonatal mice. However, it is not clear whether β-carotene enhances mucosal IgA induction in neonates, although β-carotene supplementation has been expected to enhance RA-mediated immune response in neonates (Rühl, 2007).

Whey protein concentrate has an adequate amino acid profile than that of dried skim milk and casein, and higher proportion of whey protein concentrate in milk replacers improved calf performance when only milk replacer was fed (Lammers et al., 1998). Additionally, whey protein concentrate contains antiviral and immunomodulatory components, and supplemental whey protein concentrate reduces rotavirus-induced disease symptoms in suckling mice (Wolber et al., 2005) and enhances mucosal innate immunity during early life in suckling rats (Perez-Cano et al., 2007).

In this study, our first objective was to clarify the effect of supplemental β-carotene with whey to maternal mice during pregnancy and lactation on IgA transfer from maternal milk

and on mucosal IgA induction in neonatal mice. The second objective was to evaluate the effect of supplemental β-carotene with whey on growth rate and levels of fecal IgA in newborn calves.

2. Materials and methods

2.1. Experimental design in neonatal mice

Pregnant ICR mice (n=17) were purchased from Clea Japan (Tokyo, Japan). They were housed in polycarbonate cages and maintained in an air-conditioned room (24±2℃) under controlled lighting conditions (light:dark cycle, 14:10 h). All mice were allowed free access to water and treated in accordance with ”Regulation on Animal Experimentation at Kyoto University” (Animal Research Committee. Kyoto University, revised 2007).

Dietary treatments were 1) commercial milk replacer which contained 26% CP as in skim milk (control group, n=5), 2) experimental milk replacer which contained 26% CP as whey (whey group, n=6) and 3) experimental milk replacer which contained 26% CP as whey and 30 mg/kg β-carotene (β-carotene group, n=6). These milk replacers were provided by Chubu Shiryo Co. Ltd (Ohbu, Japan), and dietary ratio of protein source and chemical composition in milk replacers are shown in Table 1. Diets were offered from 6.5 days postcoitus to 14 days postpartum (d pp) in pregnant mice. Maternal mice from each group were dissected at 14 d pp. At birth, the litter sizes (Mean±SD) of mice born from control, whey and β-carotene groups were 15.0±1.7, 15.0±2.5 and 13.0±3.3, respectively. All neonatal mice were alive by 7 d pp, and subsets of neonatal mice in each group, except 5 female and 5 male neonatal mice born to each mother, were dissected at 7 d pp. Then, 5 female and 5 male neonatal mice born to each mother were dissected at 14 d pp.

Body weights of maternal mice before birth and feed intake of maternal mice throughout the experiment were measured at 10.00 hours every day. Body weights of neonatal mice were measured at 10.00 hours every day. Blood samples from maternal mice at 14 d pp were obtained by cardiac puncture under anesthesia with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich Chemical, MO, USA), and then mammary gland was removed and immediately frozen in dry ice-cooled isopentane (2-methylbutane, Wako Pure Chemicals, Osaka, Japan) for immunohistochemical analysis. Blood samples from neonatal mice at 7 and 14 d pp were obtained by incising their hearts and collecting with hematocrit tubes under anesthesia with Avertin, and then small intestine and stomach contents were rapidly removed. At 7 d pp, samples of blood, small intestine and stomach contents of neonatal mice born to each mother were pooled, and samples were separately pooled for female or male neonatal mice born to each mother at 14 dpp. The samples of small intestine were frozen in liquid nitrogen and stored at -80℃, and the samples of stomach contents were stored at -20℃. Blood samples from maternal or neonatal mice were left to stand at room temperature for 1 hour or 30 min and then centrifuged at 3,000 × g for 15 min or 10,000 × g for 5 min, respectively. Serum was fractionated for IgA analysis.

IgA immunoassay of serum, stomach contents and intestine and immunohistochemical analysis of mammary glands were determined as previously described (Nishiyama et al, 2010). IgA concentration was measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instructions. The sections obtained by immunohistochemical analysis were examined under a confocal laser scanning microscope (FV300, Olympus, Tokyo, Japan), and the resulting images were analyzed by ImageJ software (National Institute of Health, Bethesda, MD, USA). The IgA-positive cells in mammary gland were counted in eight randomised fields from each mouse and represented as

IgA ASC/field of view (field = 700 μm × 700 μm).

2.2. Experimental design in neonatal calves

This research was approved by the guide for the care and use of animals in Northern Center of Agricultural Technology (Asago, Japan) and Nara Prefectural Livestock Technology Center (Mitsue, Japan). Thirty three Japanese Black calves born in their centers were used, and calves consisted of 24 males and 9 females.

Dietary treatments in milk replacers were similar to the mice (Table 1), and 33 calves were assigned to control (7 males and 4 females), whey (9 males and 2 females) and β-carotene (8 males and 3 females) groups. Calves lived with their dams after birth and received only their dam's colostrum. At 3 days of age, calves were separated from their dams and housed in individual pens. From 3 to 63 days of age, calves received appropriate amounts of milk replacers and calf starter pellets to meet recommendations (Agriculture, Forestry, and Fisheries Research Council Secretariat, 2000) for TDN, protein and minerals of calves. The amounts of milk replacers offered to calves were increased from 0.5 to 0.9 kg/d during 3 to 15 days of age, maintained at 1.0 to 1.1 kg/d during 16 to 55 days of age and decreased by 0.5 kg/d during 56 to 63 days of age. Milk replacers were diluted with warm water at 40℃ and offered twice a day throughout the experiment. Calf starter pellets (TDN, 75%; CP, 20%) were offered from 7 days after birth, and the amounts of calf starter were gradually increased by 63 days of age, according to the pellet refusals of calves. Additionally, the calves were given free access to timothy hay from 20 days after birth.

Fecal consistency of calves was observed every day throughout the experiment. Fecal scores were measures on a scale of 1 to 3 (1= firm, normal; 2=soft, 3=watery), and their data were averaged by each week. Intake of milk replacers and calf starter pellets were measured

every day. Body weights of calves were measured on day 0, 7, 14, 21, 28, 42, 56 and 63 after birth. Blood and fecal grab samples were collected at 13:00 hour on day 2, 14 and 42 after birth. Blood samples were left to stand at room temperature for 1 hour and then centrifuged at 3,000 × g for 15 min. Serum β-carotene was determined by HPLC (Shimadzu LC-10AT, Kyoto, Japan).

Fecal samples were oven-dried for 24 hours at 60℃ and then DM contents of feces were determined by oven drying at 135℃ for 2 hours. Subsets of feces were strongly vortexed in cold PBS containing bovine fetal serum (GIBCO, CA, USA), centrifuged at 3,000 × g for 15 min at 4℃ and stored at -20℃ until IgA analysis. Fecal IgA concentration was measured using the Bovine IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instructions. Plates obtained from the procedures were read at 450 nm with a Microplate Reader (BIO RAD Model 550, CA, USA).

2.3. Statistics

Data from body weight and feed intake of mice were analyzed by least squares ANOVA using the general linear model procedure of SAS (1997). The model was as follows:

$$Y_{ijk}=\mu + T_i + M_{(i)j} + D_k + TD_{ik} + e_{ijk}$$

where μ is the overall mean, T_i is the effect of treatment, $M_{(i)j}$ is the random variable of mice nested in treatment, D_k is the effect of sampling day, TD_{ik} is the interaction between treatment and sampling day, and e_{ijk} is the residuals. The general linear model procedure of SAS (1997) was used to analyze the effects of treatment on variables in maternal mice or neonatal mice at 7 d pp, the effects of treatment, sex and their interaction on variables in neonatal mice at 14 d pp and the effect of age on variables in neonatal mice.

Data of calves were analyzed by least squares ANOVA using the general linear model procedure of SAS (1997). The model was as follows;

$$Yijk = u + T_i + S_j + E_k + C_{(ijk)l} + D_m + TS_{ij} + TD_{im} + e_{ijklm}$$

where u is the overall mean, T_i is the effect of treatment, S_j is the effect of sex of calves, E_k is the effect of the experimental center, $C_{(ijk)l}$ is the random variable of calves nested in treatment, sex of calves and experimental center, D_m is the effect of sampling day, TS_{ij} and TD_{im} are the interactions, and e_{ijklm} is the residuals.

An ANOVA was performed, and the differences were tested by least significant difference. Significance was declared at $P < 0.05$.

3. Results

3.1. IgA in serum and tissues in mice

Feed intake of whey group was higher ($P<0.05$) than that of β-carotene group, although feed intake increased ($P<0.001$) rapidly after birth (Fig. 1). Body weight of maternal mice increased more in controls than in whey and β-carotene groups, but differences were not significant. Body weights of neonatal mice born from control and β-carotene groups were higher ($P<0.05$) than those of neonatal mice born from whey group.

The numbers of IgA ASC in the mammary gland were highest ($P<0.05$) in β-carotene group and those of whey group were higher ($P<0.05$) than in control group. Serum IgA concentrations of maternal mice at 14 d pp were not affected by treatment (Table 2). IgA concentrations in stomach contents of neonatal mice born from β-carotene group were higher ($P<0.001$) than those of control and whey groups at 14 d pp. IgA concentrations in serum and small intestine were not affected by treatment. Compared with IgA concentrations of neonatal

mice at 7 d pp, IgA concentrations in serum, stomach contents and small intestine increased (P<0.001) drastically at 14 d pp, but IgA concentrations were not affected by sex.

3.2. Body weight gains, health status and fecal IgA in calves

Body weights and daily gains of calves were not affected by treatment and sex (not shown) (Fig. 2). Weight gains (Mean±SD) in control, whey and β-carotene groups were 0.72±0.31 , 0.72±0.21 and 0.73±0.29 kg/d, respectively. Intake of milk replacers and calf starter pellets did not differ between treatment and sex. Calves were fed almost all the milk replacers. Calf starter intake was increased from 66g/d at 2 weeks of age to 257g/d at 6 weeks of age and reached at 705g/d at 9 weeks of age. Fecal scores of calves were not affected by treatment and sex, and fecal scores (Mean±SD) were 1.27±0.30, 1.26±0.27 and 1.24±0.26 in control, whey and β-carotene groups, respectively.

Serum β-carotene concentration was higher (P<0.001) in β-carotene group at 14 and 42 d pp than in the other groups, but fecal water and fecal IgA concentration were not affected by treatment and sex (Fig. 2). Compared with 2 d pp, fecal water increased (P<0.001) at 14 and 42 d pp, while fecal IgA concentration decreased (P<0.01) at 14 d pp. Fecal IgA concentration (Mean±SD) at 2 d pp was 3.95±5.45 mg/g, range 0.003 to 17.8 mg/g, and fecal IgA concentrations (Mean±SD) at 14 and 42 d pp were 1.03±1.03 (0.10 to 4.91) and 2.76±2.15 (0.18 to 8.06) mg/g, respectively.

4. Discussion

4.1. Effects of supplemental β-carotene with whey on passive immunity and mucosal immune induction in neonatal mice

The importance of adequate consumption of high quality colostrum or milk for acquisition of optimal nutrition and passive immunity is widely recognized in neonates of many species (Quigley and Drewry, 1998). Most IgA in neonates after birth is derived from milk IgA, and IgA antibodies in milk are specific for antigens of the intestinal microflora and act to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium (Harris et al., 2006; Roux et al., 1977). Passive immune protection of the newborn gastrointestinal tract is dependent on an active production and storage of IgA in ASC in the lactating mammary gland of the mother (Morteau et al., 2008). However, vitamin A-depleted mice show impaired IgA secretion and protection in mucosal tissues (Mora et al., 2006). Most IgA ASC express chemokine receptor CCR10, but IgA ASC from CCR10-deficient mice do not efficiently accumulate in the lactating mammary gland and lead to a significant decrease in milk IgA and fecal IgA of neonatal mice (Morteau et al. 2008). In the previous study (Nishiyama et al., 2010), supplemental β-carotene (50 mg/kg in the diet) increased the number of maternal IgA ASC in the mammary gland and IgA transfer from milk to neonatal mice, and these effects may be mainly due to the RA-mediated immune responses because mice efficiently convert β-carotene to vitamin A (Lee et al., 1999).

In the present study, we have shown that supplemental β-carotene (30 mg/kg in the diet) with whey to maternal mice during pregnancy and lactation increased the number of maternal IgA ASC in the mammary gland during lactation and IgA concentration in stomach contents in neonatal mice, which indicated as the higher IgA transfer from maternal milk to neonates (Jiang et al., 2001). Additionally, compared with the skim milk feeding, feeding whey protein increased IgA ASC in the mammary gland of maternal mice. The globulin fraction of whey was shown to contain a nondialyzable factor that is chemotactic for IgA-positive lymphocytes when these are obtained from mesenteric lymph nodes as a source of mucosal-associated

lymphoid tissue (Czinn and Lamm, 1986). Whey protein concentrate promoted the expansion of cell subsets involved in innate and mucosal immune response in suckling rats (Perez-Cano *et al.* 2007). Thus, our data imply that feeding β-carotene with whey protein may be useful to increase IgA transfer from maternal milk to neonatal mice.

Supplementation of vitamin A and carotenoids affects the immune-cell function during ontogenesis (Garcia et al., 2003). However, supplemental β-carotene had no effect on IgA concentrations in serum, small intestine and feces in neonatal mice at 7 and 14 d pp in a previous (Nishiyama et al., 2010) and the present study. Thus, supplemental β-carotene may have little effect on mucosal IgA induction in neonatal mice before weaning. However, further study is needed to clarify the effect of β-carotene on mucosal IgA induction in neonatal mice, because the intestinal secretions of IgA in mice could hardly be found at weaning and increased drastically after weaning (Tanneau et al., 1999).

4.2. Effects of supplemental β-carotene with whey on passive immunity and mucosal immune induction in neonatal calves

Severe diarrhetic feces of calves contain more than 85 % moisture, while feces that contain less than 80% moisture are considered as normal (Abe et al., 1999). The increased supplementation of vitamin A improved fecal consistency of calves at 3 and 4 weeks of age (Eicher et al., 1994), but additional vitamin A could be detrimental to calves that are already receiving vitamin A supplementation (Franklin et al., 1998). Krüger et al. (2005) reported that colostrum feeding had several selective effects on expression of nuclear receptors and target genes in neonatal calves, but the effects of vitamin A feeding were limited. Supplemental β-carotene (30 mg/kg in the diet) with whey had no effect on fecal IgA concentration and fecal water in calves, although supplemental β-carotene with whey drastically increased

serum β-carotene concentration in the present study.

The absorbed and retained N in dairy calves fed 16.1, 18.5, 22.9 and 25.8% of CP from whey protein sources increased linearly as dietary CP increased (Blome et al., 2003). The lean tissue gain of calves continued to increase with dietary CP up to 26% when calves were fed at 1.75% of BW daily (Bartlett et al., 2006). In the present study, 40,000 IU/kg vitamin A and 26% CP were offered by milk replacers to the neonatal calves, and the growth rates and average fecal scores of calves were very similar in the 3 groups. These results suggest that supplemental β-carotene has little effect on mucosal IgA induction in neonatal calves, but the appropriate supply of vitamin A and CP probably maintains the health status of calves.

Kume and Toharmat (2001) reported that colostral β-carotene was a primary source for newborn calves and that diarrhetic calves had decreased plasma β-carotene concentrations at 7 days of age. Fecal IgA in calves at 2 d pp was likely derived from milk IgA and varied from 0.003 to 17.8 mg/g in the present study. Thus, the occurrence of diarrhea in the β-carotene deficient calves may be partly due to the low IgA concentration in milk, because milk IgA concentrations in maternal cows as well as maternal mice may be enhanced by supplemental β-carotene. However, further studies are needed to evaluate the role of β-carotene on the immune system in neonatal calves.

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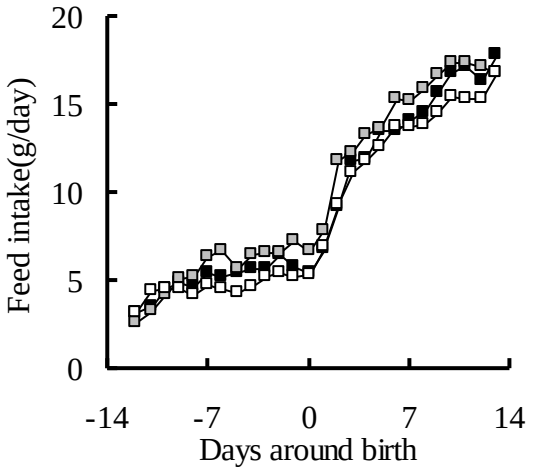
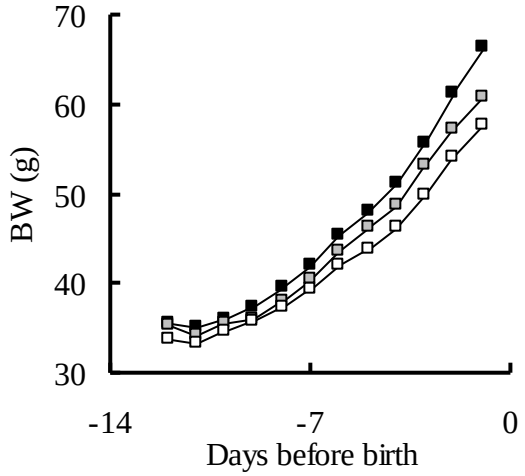
Figure 1. Body weight (SE=0.7) and feed intake (SE=0.4) of maternal mice and body weight (SE=0.12) of their neonatal mice in control (■), whey (■) and β-carotene (□) groups.

Figure 2. Body weight (SE=1.0), serum β-carotene concentration (SE=1.2), fecal water content (SE=1.7) and fecal IgA concentration (SE=1.2) of neonatal calves in control (■), whey (■) and β-carotene (□) groups after birth. Fecal IgA concentration was expressed on a fresh matter basis. Serum and fecal samples were obtained at 2, 14 and 42 days after parturition.

a) Mother





b) Neonate

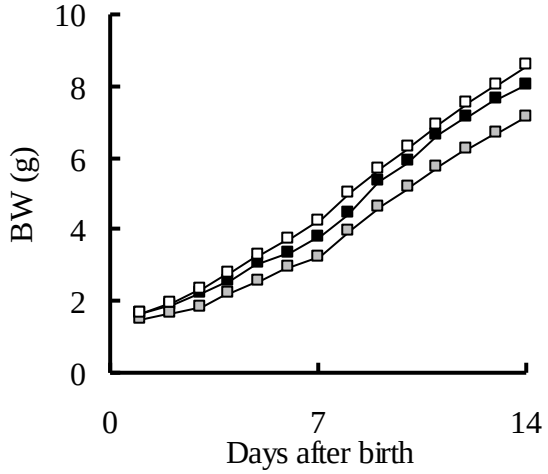


Figure 1.

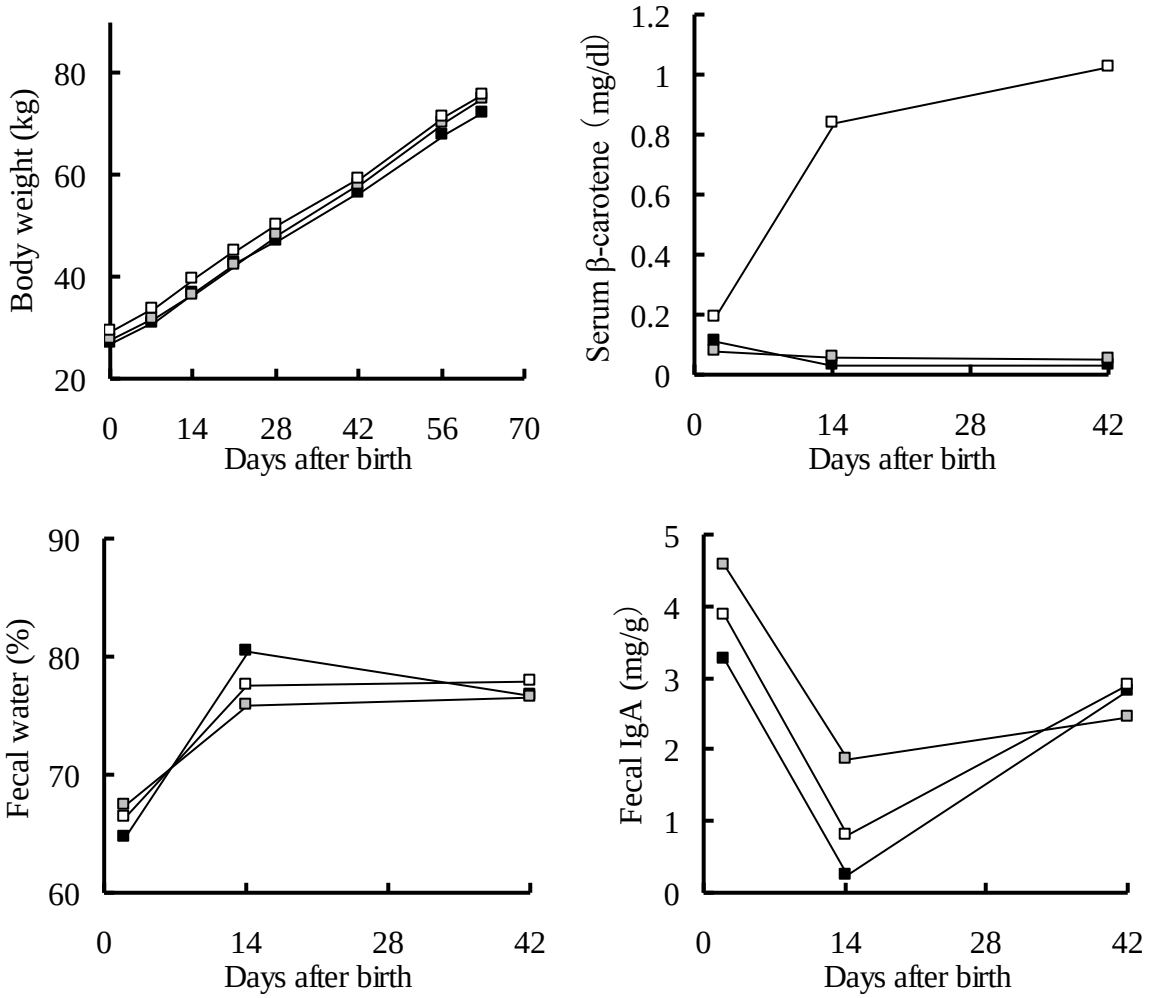

Body weight (kg)
80
60
40
20
0
14
28
42
56
70
Days after birth
Serum β-carotene（mg/dl）
1.2
1
0.8
0.6
0.4
0.2
0
0
14
28
42
Days after birth
Fecal water (%)
90
80
70
60
0
14
28
42
Days after birth
Fecal IgA (mg/g)
5
4
3
2
1
0
0
14
28
42
Days after birth

Figure 2

412

413

414

415 **Table 1** Dietary ratio of protein source and chemical composition in milk replacers for

416 control, whey and β-carotene groups of mice and calves.

	Control	Whey	β-carotene
Ingredient (%)			
Skim milk	66.3	0	0
Dried whey	3.1	17.5	17.5
Whey protein concentrate	7.4	56.5	56.5
Soybean protein concentrate	2.0	0	0
Composition (as-fed basis)			
CP, %	26.3	26.1	26.1
Crude fat, %	17.2	17.3	17.3
TDN, %	105.5	105.1	105.1
β-carotene[1)], mg/kg	0	0	30
Vitamin A[1)], IU/kg	40,000	40,000	40,000

417 [1)]Supplemented amounts.

418

Table 2. The numbers of IgA ASC in mammary gland and IgA concentration in serum of maternal mice at 14 days postpartum and IgA concentration in serum, stomach contents and small intestine of neonatal mice at 7 and 14 days postpartum in groups fed milk replacer (control), whey and whey plus β-carotene.

		Diets		
	Days	Control	Whey	β-carotene
The numbers of IgA ASC				
Mother				
Mammary gland	14	7.6 ± 0.7[c]	9.8 ± 0.7[b]	12.0 ± 0.7[a]
IgA, μg/g				
Mother				
Serum	14	376.3 ± 72.0	482.0 ± 65.7	273.8 ± 65.7
Neonate				
Serum	7	0.09 ± 0.01	0.14 ± 0.01	0.09 ± 0.01
	14	1.45 ± 0.20	1.16 ± 0.18	0.98 ± 0.18
Stomach	7	24.0 ± 6.1	37.6 ± 5.4	29.0 ± 5.4
	14	46.8 ± 5.5[e]	50.0 ± 5.0[e]	89.1 ± 5.0[d]
Intestine	7	22.9 ± 11.1	34.9 ± 11.1	64.7 ± 11.1
	14	274.2 ± 83.3	426.1 ± 75.2	488.8 ± 71.7

[a,b,c] $P<0.05$. [d,e] $P<0.001$.

Each value represents mean ±SE, and the numbers of IgA ASC in mammary gland were counted in eight randomized fields from each mouse.