Note

Effect of Beta-Carotene Supplementation on the Peripheral Blood Leukocyte Population in Japanese Black Calves

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Summary The purpose of this study was to determine the effect of beta-carotene supplementation to Japanese Black calves on the peripheral blood leukocyte population. Twenty-two Japanese Black calves were alternately assigned to two groups. Eleven calves received 20 mg/d of beta-carotene orally from 2 to 8 wk of age (BC group), and the other 11 calves did not receive (control group). The serum beta-carotene concentration in the BC group at 4, 8 and 12 wk of age were significantly higher than those in the control group ($p < 0.05$). The numbers of CD4⁺ cells in the BC group were significantly higher than those in the control group at 4 wk of age ($p < 0.05$). These results confirmed that beta-carotene supplementation to Japanese Black calves affected the peripheral blood leukocyte population.

Key Words beta-carotene, calves, Japanese Black, peripheral blood leukocyte population, supplementation

Young calves have immature immune systems compared with adult cows, due to their lower numbers of peripheral blood lymphocytes, which are responsible for cell-mediated and humoral immunity (1). Japanese Black is a breed of beef cattle which has different metabolism (2) with lower numbers of peripheral blood lymphocytes compared with Holstein calves (3), which makes Japanese Black calves more prone to infectious diseases.

Beta-carotene plays an important role as an antioxidant inside the body for maintaining the stability of biological membranes (4). Many researches of human and animal supplementation with beta-carotene have reported reduction of oxidative stress and improvements of immunity (5–10). In particular, many human and animal researches of oral beta-carotene supplementation reported improvement of the peripheral blood leukocyte population (7–10). Therefore, supplementation of beta carotene to Japanese Black calves was expected to improve the peripheral blood leukocyte population.

The purpose of this study was to evaluate the effect of beta-carotene supplementation on the peripheral blood leukocyte population in Japanese Black calves in order to keep calves healthier.

Materials and Methods

Animals. We used 22 Japanese Black calves kept at one farm in Kagoshima Prefecture, Japan. All samples were collected from December 2017 to April 2018. All the calves stayed with their mothers for 3 d after birth and were housed indoors. Starting at 4 d after birth, they were fed with milk replacer and were raised individually in calf hutches, and all calves were weaned at

11 wk of age. The amount and nutrients composition of feed are shown in Table 1.

Beta-carotene administration. The calves were randomly assigned to two groups. Eleven calves (average weight $\pm$ standard deviation; 47.2 ± 7.1 kg) were orally supplemented with 20 mg of beta-carotene (Rovimix beta-carotene, DSM Nutrition Products, Basal, Switzerland) (The dose was based on the previous studies (11–13)) once daily from 2 to 8 wk of age (BC group), and 11 calves (48.4 ± 6.9 kg) were not supplemented with beta-carotene (control group). All calves were managed in the same manner and fed to meet their nutritional requirements according to the Japanese beef cattle feeding standard (14). Peripheral blood samples were collected from all calves at 2, 4, 8 and 12 wk of age via the jugular vein using plain Vacutainer tubes and Vacutainer tubes containing dipotassium ethylenediamine tetraacetic acid (EDTA-2AK). Serum was separated from blood samples collected in plain tubes by centrifugation and stored at -30°C until analysis. Blood collected in tubes containing EDTA-2AK was used for white blood cell (WBC) analysis within 4 h after collection. All calves were weighed at start of the experiment (2 wk of age) and at 42 d after experiment (8 wk of age). No calves developed disease during the study period. The calves were raised according to guidelines of animal care of the Joint Faculty of Veterinary Medicine at Kagoshima University. The protocol was reviewed and approved by the Kagoshima University Laboratory Animal Committee, Japan (study number: VM17019).

Beta-carotene, retinol and white blood cell analysis. The serum beta-carotene was measured using a Labospect 7020 autoanalyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). The serum retinol concentration was measured using a high performance liquid chro-

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Table 1. Amount and nutrient composition of feed without supplement.

Item		Weeks of age			
		2	4	8	12
Amount (dry matter)					
Milk replacer	(kg)	0.72	0.92	0.92	0.00
Concentrate	(kg)	0.05	0.10	0.50	0.85
Hey (oats)	(kg)	0.01	0.01	0.05	0.10
Composition (dry basis)					
Crude protein	(%)	30.6	30.1	26.8	18.6
Crude fat	(%)	15.7	15.2	11.6	2.6
Total digestible nutrients	(%)	101.1	100.1	92.9	74.8
Retinol	(mg/d)	18.9	18.3	14.1	3.5
Beta-carotene	(mg/d)	0.0	0.0	0.0	0.1

Table 2. Primary antibodies used to identify peripheral blood leukocytes.

Angiten	MAb clone	Isotype	Specificity	Source
CD3	MM1A	IgG1	Pan T cell	WSUMAC
CD4	CACT138A	IgG1	Helper T cell	WSUMAC
CD8	BAQ111A	IgM	Cytotoxic T cell	WSUMAC
CD14	CAM66A	IgM	Monocyte	WSUMAC
CD21	GB25A	IgG1	B cell	WSUMAC
CD335	AKS1	IgG1	Natural killer cell	BIO-RAD

WSUMAC: WSUMAC, WA, USA. BIO-RAD: BIO-RAD, CA, USA.

matography (Prominence, Shimazu, Kyoto, Japan) as previously reported (15). The total WBCs were determined with a blood cell counter. Peripheral blood mononuclear cells (PBMCs) and granulocytes in WBCs were analyzed by WBC cytogram, and then the numbers of PBMCs and granulocytes were calculated using their percentages and total WBCs counts. The peripheral blood leukocyte population was assessed using a FACSscan flow cytometer (Becton, Dickinson and Company, Mountain View, CA, USA) as previously reported (3). The primary antibodies used and a description of the working solutions are listed in Table 2.

Statistical analysis. Data were expressed as the mean $\pm$ SD. The statistical analysis was conducted to determine the differences between the two groups within the same weeks of age using Student's *t* test with SPSS Statistics 24 software (IBM, Tokyo, Japan). *p* values less than 0.05 were considered statistically significant.

Results and Discussion

The serum beta-carotene concentration in the BC group at 4, 8 and 12 wk of age were significantly higher than those in the control group ($p < 0.05$) (Fig. 1). The serum retinol in both groups increased gradually from 2 to 12 wk of age without significant difference between the groups (Fig. 2). The numbers of WBCs, PBMCs, granulocytes, CD3⁺, CD8⁺, CD14⁺, CD21⁺ and CD335⁺

cells did not show a significant difference between groups (Table 3) and within the same group. The numbers of CD4⁺ cells in the BC group were significantly higher than those in the control group at 4 wk of age ($p < 0.05$).

The results of the present study suggested that the increase in the serum beta-carotene concentration caused by oral supplementation in the Japanese Black calves changed the numbers of immune component cells in the peripheral blood. The higher population of CD4-positive cells by oral beta-carotene supplementation in this study was in agreement with other studies in human (7, 8).

Regarding daily oral supplementation of beta-carotene to calves, increased blood beta-carotene level was reported with 20 mg/d by Bierer et al. (11) and Poor et al. (12), and with 6–96 mg/d/50 kg body weight by Hoppe et al. (13). Therefore, in this study, calves were supplemented daily with 20 mg beta-carotene.

As a result, in the present study, the serum retinol concentration in both groups gradually increased and showed similar patterns from 2 to 12 wk of age. However, the serum beta-carotene concentration in the BC group was significantly higher than that in the control group at 4, 8 and 12 wk of age. These results indicate that supplemental 20 mg/d of beta-carotene in Japanese Black calves is effective in increasing serum beta-carotene concentration with minor influence on serum retinol concentration. Previous researches have also shown that supplementation of beta-carotene to calves did not cause major influences on blood retinol concentration (16, 17).

Most CD4-positive cells are helper T cells, which appear to be necessary for regulation of the immune response and play a critical role in the overall immunological competence of the host (18). It has been reported that increased oxidative stress in peripheral blood decreased T cell proliferation (19). In addition, oxidative stress promoted apoptosis of CD4-positive cells (20), and decrease in the number of CD4-positive cells in peripheral blood suppressed cell-mediated as well as humoral immunity (21, 22). In human studies, oral beta-carotene

Fig. 1. Changes in serum concentrations of beta-carotene in the beta-carotene group (dark square) and the control group (empty square). Data are expressed as mean $\pm$ SD. Asterisks indicate significant difference between groups at the same age (* $p < 0.05$).

Fig. 2. Changes in serum concentrations of retinol in the beta-carotene group (dark square) and the control group (empty square). Data are expressed as mean $\pm$ SD.

tene supplementation reduced oxidative stress in peripheral blood (5, 6), and stimulated proliferation of lymphocytes (7, 8). Decrease in serum beta-carotene concentration was associated with the development of respiratory and digestive diseases (23). We previously reported that the supplementation of Japanese Black calves with beta-carotene could increase the serum antioxidative stress and decreased the serum oxidative stress (24). Therefore, increase in the number of CD4-positive cells in the BC group compared with the control group in the present study might have been associated with decreased oxidative stress in blood and/or increased the serum beta-carotene concentration. The number of T and B cells in the peripheral blood of young calves has been reported to increase with their growth (1, 3, 25). In particular, it has been reported that the number of CD4-positive cells increased up to 9–12 wk of age (1, 3). In the present study, although the beta-carotene concentration in the BC group was significantly higher than those in the control group at 4, 8, and 12 wk of age,

significant difference in the number of CD4-positive cells in the BC group was observed only at 4 wk of age. Natural increase in the number of CD4-positive cells associated with growth might have made the differences between groups obscure at 8 and 12 wk of age. Otomaru et al. (26, 27) reported that vitamin E supplementation to calves increased the number of CD4-positive cells in peripheral blood and enhanced antibody response to vaccination. Therefore, increased number of CD4-positive cells in the peripheral blood in the present study might have enhanced the immunity in calves.

These results confirmed that oral beta-carotene supplementation of Japanese Black calves increased blood beta-carotene concentration, ameliorated the numbers of immune cells in the peripheral blood. In order to enhance health condition in calves, further research is needed to clarify whether beta-carotene supplementation improve the peripheral blood leukocyte function in Japanese Black calves.

Table 3. Changes in the number of leukocyte subsets by age.

Cell type	2 wk		4 wk		8 wk		12 wk	
	BC group	Control group	BC group	Control group	BC group	Control group	BC group	Control group
WBC ($\times 10^2/\mu\text{L}$)	78.9 $\pm$ 8.5	81.2 $\pm$ 26.3	86.5 $\pm$ 19.5	90.9 $\pm$ 20.3	84.0 $\pm$ 15.1	87.4 $\pm$ 21.7	84.1 $\pm$ 17.5	89.5 $\pm$ 19.2
PBMC ($\times 10^2/\mu\text{L}$)	46.2 $\pm$ 9.3	47.7 $\pm$ 8.0	46.2 $\pm$ 6.8	49.3 $\pm$ 6.6	51.9 $\pm$ 4.7	51.7 $\pm$ 8.5	52.2 $\pm$ 9.5	50.6 $\pm$ 5.8
Gran ($\times 10^2/\mu\text{L}$)	32.7 $\pm$ 10.5	33.5 $\pm$ 22.4	40.3 $\pm$ 16.3	41.6 $\pm$ 17.3	32.1 $\pm$ 13.1	35.7 $\pm$ 19.3	31.9 $\pm$ 18.3	39.0 $\pm$ 17.0
CD3 ⁺ ($\times 10^2/\mu\text{L}$)	29.1 $\pm$ 4.8	28.5 $\pm$ 7.3	28.7 $\pm$ 5.0	30.2 $\pm$ 7.3	31.6 $\pm$ 6.0	35.6 $\pm$ 7.4	37.4 $\pm$ 7.6	38.5 $\pm$ 6.6
CD4 ⁺ ($\times 10^2/\mu\text{L}$)	8.5 $\pm$ 1.0	8.2 $\pm$ 1.8	9.7 $\pm$ 1.3*	8.2 $\pm$ 1.9	9.5 $\pm$ 1.6	8.8 $\pm$ 1.3	9.6 $\pm$ 1.8	9.5 $\pm$ 2.5
CD8 ⁺ ($\times 10^2/\mu\text{L}$)	4.7 $\pm$ 2.0	4.3 $\pm$ 0.8	4.8 $\pm$ 2.5	4.2 $\pm$ 1.0	5.0 $\pm$ 1.7	4.6 $\pm$ 1.3	4.8 $\pm$ 0.7	5.0 $\pm$ 1.3
CD14 ⁺ ($\times 10^2/\mu\text{L}$)	4.6 $\pm$ 1.7	4.3 $\pm$ 1.7	4.8 $\pm$ 1.5	4.3 $\pm$ 1.4	4.0 $\pm$ 0.5	4.4 $\pm$ 1.1	4.7 $\pm$ 1.1	4.1 $\pm$ 1.3
CD21 ⁺ ($\times 10^2/\mu\text{L}$)	6.7 $\pm$ 3.2	8.2 $\pm$ 3.5	11.1 $\pm$ 4.5	12.0 $\pm$ 4.6	14.3 $\pm$ 2.1	14.0 $\pm$ 6.1	14.4 $\pm$ 5.0	14.1 $\pm$ 4.1
CD335 ⁺ ($\times 10^2/\mu\text{L}$)	3.5 $\pm$ 1.6	2.6 $\pm$ 0.9	2.7 $\pm$ 0.5	2.7 $\pm$ 0.8	3.6 $\pm$ 2.3	3.3 $\pm$ 1.1	3.8 $\pm$ 1.4	3.0 $\pm$ 0.6

Data are expressed as mean $\pm$ SD. Asterisk indicates a significant difference between two groups at the same age ($p < 0.05$).

Authorship

Research conception and design: KO and YI; experiments: KO, RO, SO, YI, SI and KN; statistical analysis of the data: KO, RO and YU; interpretation of the data: KO and RO; writing of the manuscript: KO.

Disclosure of state of COI

No conflicts of interest.

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REFERENCES

- 1) Kampen AH, Olsen I, Tollersrud T, Storset AK, Lund A. 2006. Lymphocyte subpopulations and neutrophil function in calves during the first 6 months of life. *Vet Immunol Immunopathol* **113**: 53–63.
- 2) Otomaru K, Wataya K, Uto T, Kasai K. 2016. Blood biochemical values in Japanese Black calves in Kagoshima Prefecture, Japan. *J Vet Med Sci* **78**: 301–303.
- 3) Ohtsuka H, Ono M, Saruyama Y, Mukai M, Kohiruimaki M, Kawamura S. 2011. Comparison of the peripheral blood leukocyte population between Japanese Black and Holstein calves. *Anim Sci J* **82**: 93–98.
- 4) Chew BP, Park JS. 2004. Carotenoid action on the immune response. *J Nutr* **134**: 257S–261S.
- 5) Dixon ZR, Shie FS, Warden BA, Burri BJ, Neidlinger TR. 1998. The effect of a low carotenoid diet on malondialdehyde-thiobarbituric acid (MDA-TBA) concentrations in women: a placebo-controlled double-blind study. *J Am Coll Nutr* **17**: 54–58.
- 6) Ma'rifah B, Roosita K, Sinaga T. 2019. The galohgor nutraceutical cookies effects on β -carotene serum and oxidative stress of postpartum mothers. *Prev Nutr Food Sci* **24**: 381–386.
- 7) Prabhala RH, Maxey V, Hicks MJ, Watson RR. 1989. Enhancement of the expression of activation markers on human peripheral blood mononuclear cells by in vitro culture with retinoids and carotenoids. *J Leukoc Biol* **45**: 249–254.
- 8) Alexander M, Newmark H, Miller RG. 1985. Oral beta-carotene can increase the number of OKT4⁺ cells in human blood. *Immunol Lett* **9**: 221–224.
- 9) Chew BP, Park JS, Weng BC, Wong TS, Hayek MG, Reinhart GA. 2000. Dietary beta-carotene is taken up by blood plasma and leukocytes in dogs. *J Nutr* **130**: 1788–1791.
- 10) Bendich A. 1991. Beta-carotene and the immune response. *Proc Nutr Soc* **50**: 263–274.
- 11) Bierer TL, Merchen NR, Nelson DR, Erdman JW. 1993. Transport of newly-absorbed beta-carotene by the pre-ruminant calf. *Ann NY Acad Sci* **691**: 226–228.
- 12) Poor CL, Bierer TL, Merchen NR, Fahey GC Jr, Erdman JW Jr. 1993. The accumulation of alpha- and beta-carotene in serum and tissues of preruminant calves fed raw and steamed carrot slurries. *J Nutr* **123**: 1296–1304.
- 13) Hoppe PP, Chew BP, Safer A, Stegemann I, Biesalski HK. 1996. Dietary beta-carotene elevates plasma steady-state and tissue concentrations of beta-carotene and enhances vitamin A balance in preruminant calves. *J Nutr* **126**: 202–208.
- 14) Agriculture, Factory and Fisheries Research Council Secretariat, ed. 2008. Japanese Feeding Standard for

- Beef Cattle. Japan Livestock Industry Association, Tokyo (in Japanese).
- 15) Adachi K, Katsura N, Nomura Y, Arikawa A, Hidaka M, Onimaru T. 1996. Serum vitamin A and vitamin E in Japanese black fattening cattle in Miyazaki prefecture as determined by automatic column-switching high performance liquid chromatography. *J Vet Med Sci* **58**: 461–464.
 - 16) Chew BP, Wong TS, Michal JJ. 1993. Uptake of orally administered beta-carotene by blood plasma, leukocytes, and lipoproteins in calves. *J Anim Sci* **71**: 730–739.
 - 17) Jin Q, Cheng H, Wan F, Bi Y, Liu G, Liu X, Zhao H, You W, Liu Y, Tan X. 2015. Effects of feeding β -carotene on levels of β -carotene and vitamin A in blood and tissues of beef cattle and the effects on beef quality. *Meat Sci* **110**: 293–301.
 - 18) Ma CS, Deenick EK, Batten M, Tangye SG. 2012. The origins, function, and regulation of T follicular helper cells. *J Exp Med* **209**: 1241–1253.
 - 19) Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, Kappler J, Marrack PC. 1999. Reactive oxygen species regulate activation-induced T cell apoptosis. *Immunity* **10**: 735–744.
 - 20) Gollapudi S, Gupta S. 2016. Reversal of oxidative stress-induced apoptosis in T and B lymphocytes by coenzyme Q10 (CoQ10). *Am J Clin Exp Immunol* **5**: 41–47.
 - 21) Howard CJ, Sopp P, Parsons KR, Finch J. 1989. In vivo depletion of BoT4 (CD4) and of non-T4/T8 lymphocyte subsets in cattle with monoclonal antibodies. *Eur J Immunol* **19**: 757–764.
 - 22) Gill HS, Watson DL, Brandon MR. 1992. In vivo inhibition by a monoclonal antibody to CD4+ T cells of humoral and cellular immunity in sheep. *Immunology* **77**: 38–42.
 - 23) Kuçukbay H, Yakinci C, Kuçukbay FZ, Turgut M. 1997. Serum vitamin A and beta-carotene levels in children with recurrent acute respiratory infections and diarrhoea in Malatya. *J Trop Pediatr* **43**: 337–340.
 - 24) Otomaru K, Ogawa R, Oishi S, Iwamoto Y, Hong H, Nagai K, Hyakutake K, Kubota C, Kaneshige T. 2018. Effect of beta-carotene supplementation on the serum oxidative stress biomarker and antibody titer against live bovine respiratory syncytial virus. *Vet Sci* **5**: E102.
 - 25) Fossum C, Larsson B, Matsson P. 1986. Development of mononuclear cell subpopulations and their function during calffhood. *J Vet Med B* **33**: 518–527.
 - 26) Otomaru K, Saito S, Endo K, Kohiruimaki M, Ohtsuka H. 2015. Effect of supplemental vitamin E on the peripheral blood leukocyte population in Japanese Black calves. *J Vet Med Sci* **77**: 985–988.
 - 27) Otomaru K, Saito S, Endo K, Kohiruimaki M, Fukuyama S, Ohtsuka H. 2013. Effect of supplemental vitamin E on antibody titer in Japanese black calves vaccinated against bovine herpesvirus-1. *J Vet Med Sci* **75**: 1671–1673.