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ORIGINAL CONTRIBUTION

Single oral dose of micellar β-carotene containing phospholipids improves β-carotene metabolism and plasma lipids in vitamin A-deficient rats

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Abstract
Background Vitamin A (VA) deficiency is still a major health problem in the developing world. It affects various cellular functions and causes hypolipidemic effects in the body. β-Carotene (BC)-rich foods are promising sources of VA. Phospholipids are reported to improve BC bioefficacy in normal rats, but whether they show similar effects during VA deficiency is unknown.
Aim To compare the BC metabolism and plasma lipid responses in VA-sufficient (+VA) and VA-deficient (−VA) rats after a single oral dose of micellar BC containing phospholipids.
Methods Groups of rats were fed with a VA-free diet and when they attained the weight-plateau stage of deficiency, both +VA and −VA rats were divided into 2 groups (phosphatidylcholine, PC and lysophosphatidylcholine, LPC). Each group was further divided into 4 subgroups (1, 2, 3, and 6 h; $n = 5$ rats/time point) and determined the BC metabolism and plasma lipid responses to a post-dose of micellar BC with phospholipids.
Results Maximal plasma BC (pmol/mL) levels were observed at 2 h in PC (1330 ± 124) and at 1 h in LPC (1576 ± 144) groups of +VA rats, and at 3 h in the PC (1621 ± 158) and LPC (2248 ± 675) groups of −VA rats. Liver BC (pmol/g) was maximum at 1 h in the PC (218 ± 32) and LPC (249 ± 24) groups of +VA rats, and at 2 h in PC (228 ± 23) and at 3 h in LPC (277 ± 18) groups of −VA rats. Plasma and liver BC levels were significantly ($P < 0.05$) higher in −VA rats than +VA rats. Plasma retinyl palmitate (pmol/mL) was maximum at 3 h in PC (97 ± 18) and at 2 h in LPC (126 ± 14) groups of +VA rats, and at 2 h in the PC (92 ± 13) and LPC (134 ± 27) groups of −VA rats. The higher ($P < 0.05$) BC monoxygenase activity in −VA rats compared to +VA rats supports the BC bioefficacy. Plasma retinol level was improved in the PC and LPC groups, but the effect of LPC was higher ($P < 0.05$) than PC. Micellar phospholipids mitigate the VA deficiency–induced hypolipidemic effects.
Conclusions Micellar phospholipids improved BC metabolism and reinstated the hypolipidemic effects, perhaps by modifying the fat-metabolizing enzymes and repairing the altered intestinal membrane structure.

Keywords Bioavailability · β-Carotene · Mixed micelles · Phospholipids · Vitamin A deficiency

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Introduction

Vitamin A deficiency (VAD) continues to be a major public health problem, particularly in developing countries, including India [1], much of which is attributed to the vitamin A (VA)-deficient diets [2]. Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene (BC), the VA

precursor. VA supplementation programs have often been effective for many years, but difficulties are found in reaching higher risk population and its cost effectiveness, and therefore may not be a permanent solution [3]. It is reported that between 1.3 and 2.5 million deaths of the children under the age of 5 years could be prevented every year by proper VA nutrition. Long-term solution proposed to combat VAD is implementing the dietary diversity, and emphasis has been on the increased intake of green leafy vegetables, yellow-orange fruits and vegetables to improve VA intake [4].

Several human intervention studies have shown the efficacy of BC-rich foods as a source of VA. Bioefficacy of BC dissolved in oil was determined in Indonesian children using isotopic enrichment of serum BC and retinol [5]. Serum retinol concentrations were improved in young children who consumed orange-fleshed sweet potatoes [6]. However, BC from green leafy vegetable is less absorbed compared to yellow-orange fruits and roots or tubers, although the presence of dietary lipids modulates the bioefficacy of dietary BC [7, 8]. Dietary fat improves the carotenoids absorption by stimulating bile salts secretion and pancreatic lipases required for the release of carotenoids from food matrix, micelle formation, and chylomicron synthesis [9]. In addition, nutritional status of an individual is the primary determinant to regulate the intestinal bioconversion of absorbed carotene to VA [10]. Altered intestinal carotene cleavage activity has been reported in VA- and protein-deficient rats [11]. Reduced BC uptake was observed in the brush border membrane vesicles of VA-deficient rats [12].

Earlier, we have demonstrated that micellar phospholipids improve the BC uptake and its cleavage enzyme activity in mice and rats [13–15]. Phospholipids derived from bile and foods influence the cellular uptake of carotenoids in the intestinal tract. However, whether they tend to show similar effects during VAD is unknown. Despite the reported enhancement of the carotene cleavage enzyme activity, VAD may negatively impact animal's ability to absorb carotenoids and/or preformed VA via impaired intestinal structure. There are reports on the improved gastrointestinal integrity in retinol-deficient children, who consumed BC-rich mango or received VA supplementation [16]. We hypothesize that micellar phospholipids may positively influence the restitution of the VAD-induced structural and functional changes in the small intestine and thereby modulate BC uptake and metabolism. Therefore, in this study we compared the role of micellar phospholipids on BC metabolism in VA-deficient and VA-sufficient rats. Furthermore, plasma lipid responses were determined to the post-dose of micellar phospholipids to support the hypothesis.

Materials and methods

Chemicals

Vitamin-free casein, vitamins, minerals, cellulose, dextrose, and methionine were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India) and were of high purity food grade. Choline chloride was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Cornstarch and refined groundnut oil were purchased from local market. Dithiothreitol (DTT), N-[Tris (hydroxymethyl)-methyl] glycine (tricine), N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), high-performance liquid chromatography (HPLC) grade acetonitrile, hexane, methanol, and dichloromethane were purchased from Sisco Research Laboratories (Mumbai, India). Sephadex G-25 was obtained from Pharmacia Biotech (Uppsala, Sweden). Standard all-*trans* β-carotene (98%), DL-α-tocopherol, all-*trans* retinol (95%), retinyl palmitate (RP), monooleoylglycerol, sodium taurocholate, linoleic acid, oleic acid ($\sim$99%), egg-yolk phosphatidylcholine (PC, 99%), lysophosphatidylcholine (LPC, 99%), methyl pentadecanoate ($\sim$99%) and trans β-8′-apocarotenal (96%) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animals and diet

Weanling male albino rats [OUTB- Wistar, IND-cft (2c)] were bred at our animal house facilities (Central Food Technological Research Institute, Mysore, India), housed in steel cages at room temperature (28 $\pm$ 2 °C) with 12-h dark: light cycles. Animal Ethics Committee approved all procedures for the use and care of animals, and necessary steps were taken for the humane treatment of the experimental animals. Vitamin A-free diet [17] was prepared freshly every week by mixing all the ingredients (Table 1) and stored in airtight containers at 4 °C. Rats were fed ad libitum with freshly prepared diet and had free access to tap water. Diet intake was monitored every day and gain in body weight was measured every week.

Experimental plan: induction of vitamin A deficiency

The experimental plan is given in the Fig. 1. Weanling male rats ($n = 90$) weighing 32 $\pm$ 3 g were randomly divided into 3 groups; VA deficient (−VA, $n = 45$), restricted diet control (+VA, $n = 40$) and unrestricted diet control (++VA, $n = 5$) and put on VA-free diet**.** The control rats (+VA and ++VA) received an oral dose of RP (7 μg/100 g body wt) in groundnut oil (0.2 mL/rat) every week. The ++VA control group was included to determine the unrestricted diet–induced changes on the growth and

Table 1 Composition of vitamin A-free diet

Ingredients	g/Kg
Vitamin-free casein	200
DL-Methionine	3
Cellulose	50
Fat (groundnut oil)	50
Corn starch	325
Glucose	324
AIN vitamin mixture (excluding vitamin A)	10
AIN mineral mixture	35
Choline chloride	2
Ascorbic acid	1

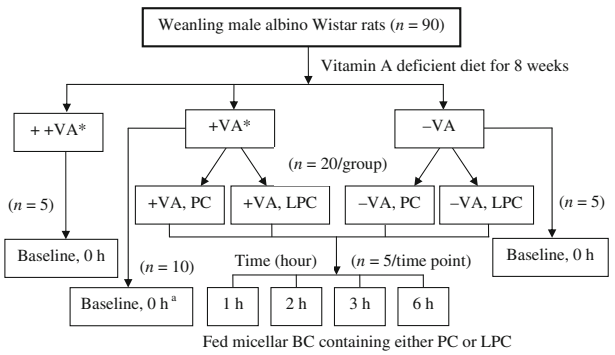

Fig. 1 Experimental plan. Weanling rats consumed vitamin A-free diet and water ad libitum for 8 weeks. The vitamin A-sufficient (+VA) and vitamin A-deficient (−VA) rats then received a single oral dose of micellar BC containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC) and killed at 1, 2, 3, and 6 h after the dose. Two separate groups of control (++VA) and −VA rats ($n = 5$), not fed with micellar BC were considered baseline (0 h) groups. *Control rats (++VA: rats fed with unrestricted diet, +VA: rats received the diet restricted to mean amount consumed by −VA rats) were fed orally with retinyl palmitate in groundnut oil to maintain their normal vitamin A reserve. The ++VA control group was used to determine the diet-induced changes on the growth and baseline (0 h) levels of retinol and plasma lipid responses. [a] Blood drawn (0.5 mL from each rat) from +VA rats ($n = 10$), before administering micellar BC served the baseline (0 h) values of plasma retinol ($n = 5$) and lipids ($n = 5$) and later, those rats were randomly assigned to treatment groups

baseline (0 h) levels of retinol and plasma lipid responses. These results were compared within the baseline groups (++VA, +VA, and −VA). The −VA rats attained the weight-plateau stage of deficiency at 8 weeks, as determined by measuring the plasma retinol level.

At the end of the 8 weeks, both −VA and +VA rats were further divided randomly into 4 groups ($n = 5$ rats/group) and fed with a single oral dose of micellar BC containing either PC or LPC. Two separate groups (not fed micellar BC), the unrestricted diet control (++VA, $n = 5$) and VA deficient (−VA, $n = 5$) rats, considered control (baseline, 0 h) groups. Since there was no separate group for baseline (0 h) +VA rats, blood (0.5 mL) drawn from +VA animals ($n = 10$) through retro-orbital plexus, treated as baseline values (retinol and plasma lipid responses, $n = 5$). This procedure was performed with the help of skilled staff from our animal house facility, before administering micellar BC dose. Later, those animals were randomly assigned to the treatment groups.

Preparation of mixed micelles and feeding to animals

Mixed micelles were prepared as described previously [15]. The stock solutions of the micelles components were prepared as follows: monooleoyl glycerol, oleic acid, PC, and LPC were dissolved in methanol: dichloromethane (2:1, v/v), BC was dissolved in hexane and the sodium taurocholate in phosphate-buffered saline (PBS, pH 7.0). Appropriate volumes of each of the components from the stock solutions were taken into the test tube to give the final concentrations of 2.5 mM monooleoyl glycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate, 200 μM BC, and 3 mM PC or LPC. The solvent was evaporated to dryness using nitrogen gas, and the residue was suspended in PBS, mixed vigorously using a vortex mixer and sonicated for 30 min to obtain an optically clear solution. Necessary measures were taken to minimize the BC oxidation and the concentration of BC in the micelles was determined by HPLC. The mixed micelles were administered to each rat (0.2 mL/rat) by direct intubation to the stomach. The volume size of intubation had no adverse effects on the rats. The amount of BC fed to each rat was 0.03 mg/100 g body wt. Time course (gavage) study up to 6 h was performed to determine the postprandial appearance of BC and its metabolites. At the termination of the experiment, the rats were sacrificed by ether anesthesia; blood, intestine, and liver were sampled.

Assay of β-carotene 15,15′-monooxygenase activity

β-Carotene 15,15′-monoxygenase (EC.1.14.99.36) activity (previously known as β-carotene 15,15′-dioxygenase) was measured [18]. In brief, the upper portions of the small intestine (jejunum) from each rat was removed and rinsed with isotonic saline. The mucosal layer was gently scraped off and homogenized with a Potter–Elvehjem homogenizer in five volumes of 50 mM HEPES–KOH buffer (pH 7.4) containing 0.154 M potassium chloride, 1 mM EDTA and 0.1 mM DTT. The homogenate was centrifuged at 10,000×g for 30 min, and the supernatant was applied to a Sephadex G-25 column (1.5 × 5.5 cm), equilibrated with 10 mM HEPES–KOH buffer (pH 7.4) containing 0.1 mM EDTA, 0.05 M potassium chloride and 0.1 mM DTT. The eluate was used to measure the enzyme activity.

The reaction mixture contained 15 μM BC, 100 mM tricine-KOH buffer (pH 8.0), 0.5 mM DTT, 0.15% Tween-40, 0.1 mM α-tocopherol and enzyme source (~1.5 mg protein) in a total volume of 0.4 mL. After pre-incubation at 37 °C for 5 min, the reaction was initiated by adding 1.2 nmol (80 μL) of BC solubilized with α-tocopherol in aqueous Tween-40 (0.15%) to 0.32 mL of the reaction mixture. The mixture was incubated at 37 °C under atmospheric oxygen for 1 h under diffused yellow light. The reaction was terminated by the addition of 0.1 mL formaldehyde (37%). The mixture was further incubated for 10 min at 37 °C, then 0.5 mL acetonitrile was added, and the mixture was cooled on ice for 5 min. The insoluble matter in the reaction mixture was removed by centrifugation at 10,000×*g* at 4 °C for 10 min and the supernatant was analyzed by HPLC to determine the retinal formed.

Extraction and HPLC analysis of BC and retinoids

β-Carotene and retinoids were extracted as described previously [15]. In brief, 3 mL of dichloromethane: methanol (1:2, v/v) containing 2 mM α-tocopherol was added to 0.8 mL of plasma. β-Apo-8′-carotenal (0.1 nmol) was added as an internal standard. After mixing, 1.5 mL of hexane was added, vortexed and the mixture was centrifuged at 1000×*g* for 15 min. The upper hexane/dichloromethane layer was collected and the extractions were repeated twice with 1 mL of dichloromethane and 1.5 mL of hexane. The extracts were pooled and evaporated to dryness using nitrogen gas. The residue was dissolved in 0.1 mL dichloromethane: methanol (2:1, v/v) and analyzed the BC and retinoids by HPLC. Liver samples (1 g) were homogenized with 10 mL ice-cold isotonic saline with a Potter–Elvehjem homogenizer. BC was extracted from the liver homogenate (0.8 mL) and analyzed as described for plasma. Sample handling, homogenization, and extraction were carried out under ice-cold condition and dim yellow light to minimize isomerization and oxidation of BC.

β-Carotene and retinoids were analyzed by an HPLC system (LC-10Avp, Shimadzu, Kyoto, Japan) equipped with Shimadzu photodiode array detector (SPD-M20A). Analytes were separated on a TSK gel ODS-80TS column (Tosoh, Stuttgart, Germany), 4.6 × 150 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco Inc., Bellefonte, PA) using acetonitrile: methanol: dichloromethane (70:20:10, v/v/v) containing 0.1% ammonium acetate as the mobile phase. Injection volume was 20 μL. An isocratic analysis was performed at a flow rate of 1 mL/min by monitoring at 450 nm (BC), 460 nm (β-apo-8′-carotenal), 325 nm (retinol), 380 nm (retinal) and 350 nm (RP). Peak identities were confirmed by comparing their retention times and characteristic UV–visible spectra with respective standards and quantified using reference curve. Working solutions of standard BC, β-apo-8′-carotenal, retinol, retinal, and RP were prepared using appropriate solvents in a range between 20 and 100 ng/mL and injected to HPLC. The calibration curve was plotted using their peak area vs. concentration of standards. The response was linear ($R^2 = 0.987$) for all the calibrated compounds. The limits of detection for this method were as follows: BC, 5 ng/mL; and retinoids (RP, β-apo-8′-carotenal, retinal, and retinol), 10 ng/mL. The accuracy and precision of the extraction was determined by the recovery of β-apo-8′-carotenal (internal standard) used at the beginning of extraction. The recovery of the internal standard was found to be >98%.

Plasma lipid extraction, evaluation of triglyceride, total cholesterol and phospholipids

Plasma lipids were extracted (0.5 mL plasma) using the method of Folch et al. [19]. Aliquots of the lipid extract (0.2 mL) were used to determine the phospholipids, total cholesterol, triglycerides, and fatty acids. Triglycerides were estimated by the method of Fletcher [20] using triolein as standard. Total cholesterol was measured by the method of Rudel and Morris [21]. Phospholipids were estimated by the method of Stewart [22] using dipalmitoyl phosphatidylcholine as standard and quantified by their respective standard curves obtained using Shimadzu model 1601 spectrophotometer.

Fatty acid analysis

Fatty acid methyl esters were prepared from the plasma lipid extracts using boron trifluoride in methanol [23] and analyzed (sample size, 1 μL) by gas chromatography (Shimadzu 14B-GC), equipped with flame ionization detector (FID) using a fused silica capillary column 25 m × 0.25 mm (Parma bond FFAP-DF-0.25: Machery-Nagel Gm BH Co. Duren, Germany). The initial column temperature was 160 °C, injector temperature was 210 °C and detector temperature was 250 °C. The column temperature was programmed to increase 6 °C per min to the final temperature of 240 °C. The carrier gas (nitrogen) was maintained at a flow rate of 1 mL/min (column inlet pressure, 1500 Psi). Hydrogen and compressed air used for FID were maintained at 1.0 and 0.1 KgF/cm^2 (before ignition) and 0.5 KgF/cm^2 for both (after ignition); with split ratio, 1:4. Individual fatty acids were identified by their retention times with that of authentic standards (Nu-Chek Prep. Inc., Elysian, MN) and were quantified by an online chromatopack CR-6A integrator using internal standard (C15:0, methyl pentadecanoate).

Statistical analyses

Analyses were performed using GraphPad Prism version 3.00 for Windows (San Diego, CA). Area under the curve values for plasma BC, RP, and liver BC were calculated by trapezoidal approximations. Data were tested for the homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, every data set were tested by one-way ANOVA and significant differences in mean values among groups and at different time points were evaluated by Tukey's test. The differences in the data set were analyzed nonparametrically by the Kruskal–Wallis test, and significant differences in means were evaluated by the Mann–Whitney U test. The percent differences between the control and experimental groups were calculated and considered significant at $P < 0.05$.

Results

Effect of feeding vitamin A-deficient diet on growth of rats

The experimental rats were put on VA-free diet, the food intake was monitored everyday and weight gain was recorded once in a week. It is common experience that one of the most pronounced effects of VA deprivation is gradual retardation and eventual cessation of growth of an animal. After 4 weeks, the rats started showing VAD symptoms, indicated by the weight-loss and reduced diet consumption (measured by left over diet) and took 8 weeks to deplete their liver VA stores. This was confirmed by measuring the plasma VA. The weight gain of the rats increased slowly up to 5 weeks and then decreased gradually. After 5 weeks of VA-free diet, VA-deficient (−VA) rats showed significantly reduced body weight compared to control (+VA and ++VA) rats. At the end of 8 weeks, the average gain in weight of the −VA, +VA, and ++VA rats were 103 ± 12, 184 ± 18, and 244 ± 24 g, respectively (Fig. 2). During the study, no animals suffered from diarrhea; however, −VA rats' noses were bloody and often blood droplets noticed in their cages. Baseline (0 h) values for the plasma retinol (pmol/mL) of −VA, +VA, and ++VA rats were 62 ± 8, 1138 ± 87, and 1234 ± 156, respectively.

Plasma BC, RP, and liver BC level and their area under the curve (AUC) values

The plasma level of absorbed BC peaked within 3 h and decreased thereafter up to 6 h in all the groups to the post-dose of micellar BC with phospholipids. In +VA rats, the plasma BC level (pmol/mL) was maximum at 2 h in PC (1330 ± 124) and at 1 h in LPC (1576 ± 144) groups. In −VA rats, plasma BC level was maximum at 3 h in PC (1621 ± 158) and LPC (2248 ± 675) groups. BC was not detected in the plasma of baseline (0 h) −VA, +VA, and ++VA groups. Interestingly, plasma BC level of −VA rats was significantly ($P < 0.05$) higher compared to +VA rats. The BC level was higher ($P < 0.05$) in both −VA and +VA rats fed with micellar BC with LPC than PC (Fig. 3A).

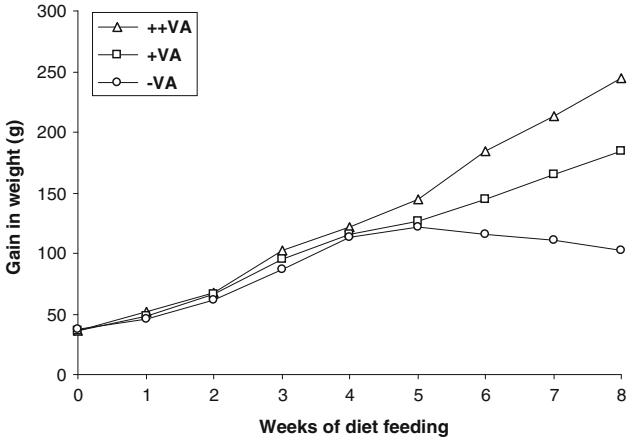

Fig. 2 Effect of feeding vitamin A-free diet on growth curve of experimental control (++VA, +VA) and vitamin A-deficient (−VA) rats. Data represent the average group weight in grams plotted from first day of the week to 8 weeks. Control rats (++VA: rats fed with unrestricted diet, +VA: rats received the diet restricted to mean amount consumed by the −VA rats) were fed orally with retinyl palmitate in groundnut oil to maintain their normal vitamin A reserve

The plasma RP was not detected in baseline (0 h) −VA rats, but, after the micellar BC dose with phospholipids its level increased significantly in both −VA and +VA rats. The plasma RP level (pmol/mL) was maximum at 3 h in PC (97 ± 18) and at 2 h in LPC (126 ± 14) groups of +VA rats. In −VA rats, plasma RP level was maximum at 1 h in the PC (92 ± 13) and LPC (134 ± 27) groups. The RP level was higher ($P < 0.05$) in both +VA and −VA rats fed with micellar BC with LPC than PC (Fig. 3B).

After the BC dose, the liver BC level (pmol/g) of +VA rats reached maximum at 1 h in PC (218 ± 32) and LPC (249 ± 24) groups. In −VA rats, the BC level was maximum at 2 h in PC (228 ± 23) and at 3 h in LPC (277 ± 18) groups. BC was not detected in the liver of baseline (0 h) −VA, +VA, and ++VA groups. The BC level of −VA rats was higher ($P < 0.05$) compared to +VA rats after the dose of micellar BC with phospholipids (Fig. 4).

The AUC values of plasma BC (pmol/mL/h) in PC and LPC groups of +VA rats were 713 ± 93 and 760 ± 74, respectively and this was 6% higher in LPC group compared to PC group. In −VA rats, the AUC values of plasma BC in PC and LPC groups were 1251 ± 149 and

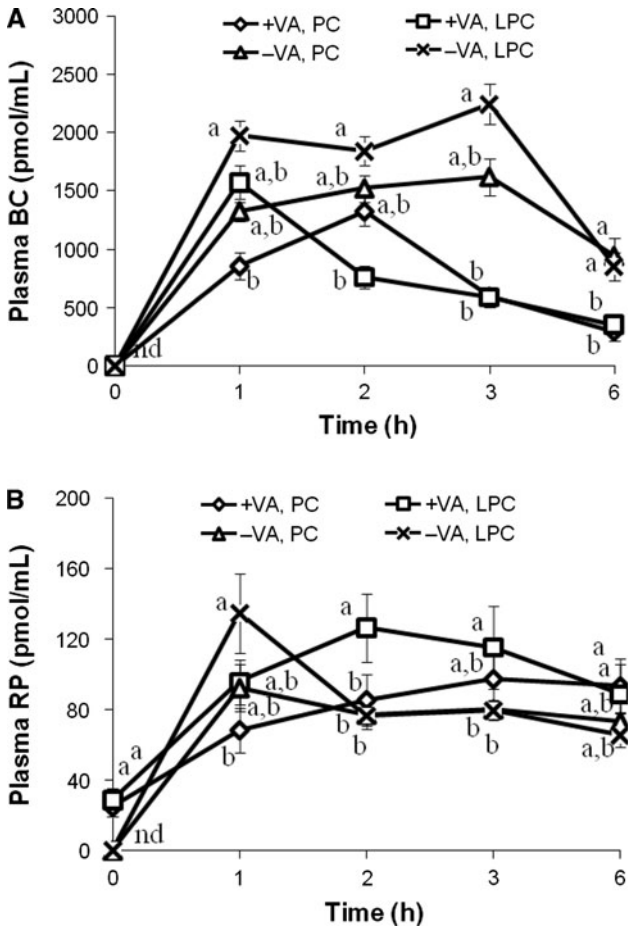

Fig. 3 Plasma β-carotene (**A**) and retinyl palmitate (**B**) level in rats. Values are mean ± SD ($n = 5$ rats/time point). β-Carotene (BC) was not detected (nd) in the control (+VA, 0 h) rats. Retinyl palmitate (RP) was not detected (nd) in the vitamin A-deficient (−VA, 0 h) rats. Values not sharing a common letter at each time point are different ($P < 0.05$) between groups as determined by one-way ANOVA followed by Tukey's test. Vitamin A-sufficient (+VA) and Vitamin A-deficient (−VA) rats were fed with micellar BC containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC)

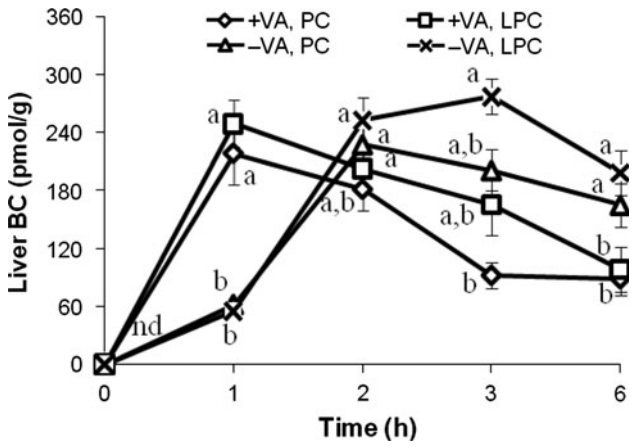

Fig. 4 Liver β-carotene (BC) level in rats. Values are mean ± SD ($n = 5$ rats/time point). BC was not detected (nd) in control (+VA, 0 h) rats. Values not sharing a common letter at each time point are different ($P < 0.05$) between groups as determined by one-way ANOVA followed by Tukey's test. Vitamin A-sufficient (+VA) and Vitamin A-deficient (−VA) rats were fed with micellar BC containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC)

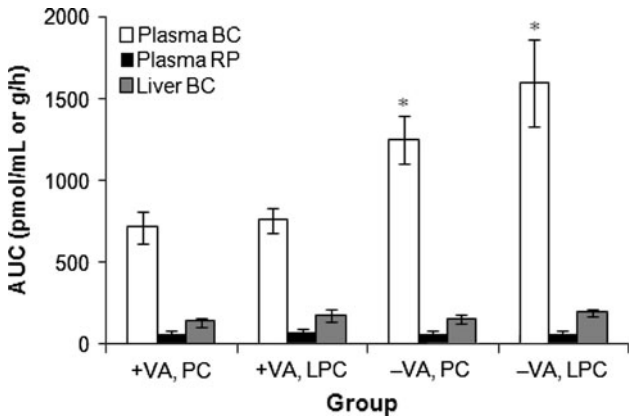

Fig. 5 Area under the curve (AUC) values of plasma BC, RP, and liver BC in rats. Vitamin A-sufficient (+VA) and Vitamin A-deficient (−VA) rats were fed with micellar BC containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC). *Values are different ($P < 0.05$) compared to their respective groups of +VA rats as determined by one-way ANOVA followed by Tukey's test. *BC* β-carotene, *RP* retinyl palmitate

1600 ± 265, respectively and this was 22% higher in LPC group compared to PC group. The AUC values of plasma BC in −VA rats were higher ($P < 0.05$) compared to +VA rats (Fig. 5).

The AUC values of plasma RP (pmol/mL/h) in PC and LPC of +VA groups were 66 ± 15 and 79 ± 12, respectively. In −VA rats, the values were 67 ± 13 and 66 ± 16 in PC and LPC groups, respectively. The AUC value of plasma RP in LPC group of +VA rats was 16% higher than that of LPC group of −VA rats. Although there was no significant difference in RP values (AUC) among the +VA and −VA rats, it is noteworthy, that the RP level of −VA rats was normalized to +VA rats, indicating the intestinal conversion of absorbed BC to RP (Fig. 5).

The AUC values of liver BC (pmol/g/h) in PC and LPC of +VA groups were 138 ± 26 and 176 ± 34, respectively. In −VA rats, the values were 156 ± 22 and 193 ± 25 in PC and LPC groups, respectively. The level was 11% and 8% higher in PC and LPC groups of −VA rats compared with their respective +VA rats (PC and LPC), indicating the accumulation of absorbed BC in the liver to the post-dose of micellar BC (Fig. 5).

β-Carotene 15,15′-monooxygenase activity

The β-carotene 15,15′-monooxygenase activity (pmol retinal/h/mg protein) was higher by ∼50% in the baseline

−VA (16 ± 3) rats compared to control (8 ± 2) rats (0 h, not fed with micellar BC) group. However, after the micellar BC dose with phospholipids, the activity was increased significantly, peaked within 2 h and decreased up to 6 h in the PC and LPC groups of both −VA and +VA rats. The maximal enzyme activities were observed at 1 h in the PC (17 ± 5), LPC (20 ± 5) groups of +VA rats and at 2 h in the PC (26 ± 4), LPC (28 ± 5) groups of −VA rats. The BC cleavage enzyme activity was higher ($P < 0.05$) in −VA rats compared to +VA rats (Fig. 6).

Plasma lipid profile

Results show that VAD affected the circulating plasma lipids. The baseline (0 h, not fed with micellar BC) plasma triglycerides (mg/dL) values of −VA, +VA, and ++VA groups were 88 ± 3, 121 ± 5, and 113 ± 2, respectively. The baseline triglycerides level of −VA rats was reduced by 36.9% compared to +VA rats. After the micellar BC dose with phospholipids, the plasma triglycerides level was increased ($P < 0.05$) in −VA rats close to the baseline control (0 h, +VA) rats. The maximal level (mg/dL) of triglycerides was observed at 1 h in PC (196 ± 15) and at 3 h in LPC (191 ± 12) groups of +VA rats (Table 2). In −VA rats, the triglycerides level was maximum at 3 h in the PC (121 ± 12) and LPC (130 ± 14) groups (Table 3).

The baseline plasma phospholipids (mg/dL) values of −VA, +VA, and ++VA groups were 27 ± 1, 38 ± 3, and 35 ± 2, respectively. The baseline phospholipids level of −VA rats was reduced by 41.9% compared to +VA rats. After the micellar BC dose with phospholipids, the plasma phospholipids level was improved ($P < 0.05$) in −VA rats close to the baseline control (0 h, +VA) rats. The maximal level (mg/dL) of phospholipids was observed at 2 h in the PC (41 ± 4) and LPC (45 ± 6) groups of +VA rats (Table 2). In −VA rats, the phospholipids level was maximum at 2 h in the PC (38 ± 0.4) and LPC (35 ± 0.2) groups (Table 3).

The baseline plasma total cholesterol (mg/dL) values of −VA, +VA, and ++VA groups were 33 ± 3, 45 ± 4, and 47 ± 4, respectively. The baseline plasma total cholesterol level of −VA rats was decreased by 33.3% compared to +VA group. However, after the micellar BC dose with phospholipids, the total plasma cholesterol level was increased ($P < 0.05$) in the PC and LPC groups of both −VA and +VA rats compared to the baseline control (0 h) rats. This may be due to the transport of absorbed BC by cholesterol molecules to the target organs. Interestingly, after 6 h the cholesterol level was dropped to the baseline values (Tables 2 and 3).

The fatty acids (oleic and palmitic) of the PC and LPC were reflected in the plasma of rats in a time-dependant manner after the dose. No significant differences were observed in the baseline (0 h) values of +VA and ++VA rats (data not shown), whereas the plasma fatty acid profile of the −VA (0 h) rats was altered compared to control +VA (0 h) rats. Reduced oleic (18:1, n-9) and linoleic (18:2, n-6) acids were observed in the −VA rats compared to +VA rats. The proportion of arachidonic acid (20:4, n-6) was increased in the plasma of −VA rats compared to +VA rats. But, after the micellar BC dose with phospholipids, the plasma fatty acid level was reinstated in the −VA rats close to the control (0 h, +VA) rats. The effect was higher ($P < 0.05$) in +VA rats compared to −VA rats after the micellar BC dose with phospholipids (Tables 4 and 5). However, the proportion of oleic and linoleic acids were restored in −VA rats. Similarly, the arachidonic acid level become normalized ($P < 0.05$) in the −VA rats close to the baseline control (0 h, +VA) rats to the post-dose of micellar BC with phospholipids (Tables 4 and 5).

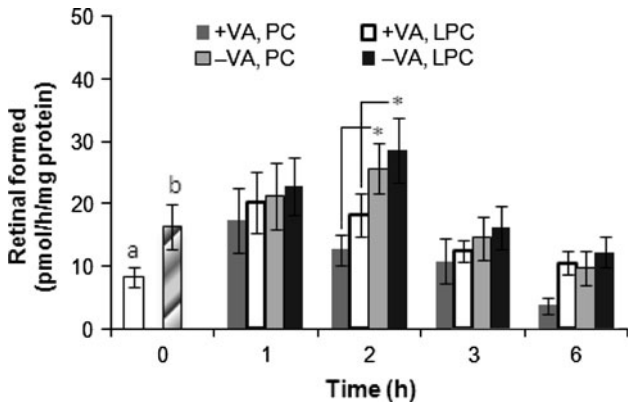

Fig. 6 β-Carotene 15,15′-monooxygenase activity. Values are mean ± SD ($n = 5$ rats). Values at 0 h are the baseline enzyme activities of the control vitamin A-sufficient (++VA, a) and vitamin A-deficient (−VA, b) rats, which were not fed with micellar BC. *Values are different ($P < 0.05$) between groups after the micellar BC dose as determined by one-way ANOVA followed by Tukey's test. Vitamin A-sufficient (+VA) and Vitamin A-deficient (−VA) rats were fed with micellar BC containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC)

Discussion

In the present study, we determined the β-carotene (BC) metabolism and restoration of the altered plasma lipids in vitamin A (VA)-deficient rats and its improvement in VA-sufficient rats to a single micellar BC dose containing phospholipids. Specific phospholipids in the mixed micelles modulate the absorption of carotenoids in vitro and in vivo [15, 24]. Apart from the dietary fat, nutritional status of the individuals affects the BC bioefficacy. Based on our previous studies [14, 25], rats were able to absorb and metabolize significant portion of ingested carotenoids

Table 2 Plasma lipid profile (mg/dL) of vitamin A-sufficient rats

Plasma lipids	Group	Baseline, 0 h	1 h	2 h	3 h	6 h
Triglycerides	++VA	112.7 ± 2.5[a]				
	+VA	121.3 ± 5.3**				
	+VA, PC		196.8 ± 15.8*	181.6 ± 14.3	144.9 ± 22.1	177.6 ± 24.5
	+VA, LPC		147.3 ± 16.2	188.4 ± 17.6	191.4 ± 12.8*	172.3 ± 15.3
Phospholipids	++VA	35.2 ± 2.4[a]				
	+VA	38.6 ± 3.2**				
	+VA, PC		32.6 ± 2.3	41.6 ± 3.7*	36.5 ± 2.5	37.5 ± 3.2
	+VA, LPC		42.2 ± 4.3	45.1 ± 5.8*	38.3 ± 4.6	42.6 ± 5.6
Total cholesterol	++VA	47.5 ± 4.5[a]				
	+VA	44.8 ± 3.7**				
	+VA, PC		73.4 ± 8.1	73.5 ± 6.5	66.5 ± 7.6	73.6 ± 9.7*
	+VA, LPC		62.8 ± 5.3	67.6 ± 8.7	64.3 ± 5.2	71.3 ± 7.5*

Values are mean ± SD ($n = 5$ rats). Vitamin A-sufficient (+VA) rats were fed with micellar β-carotene (BC) containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC). Blood drawn (0.5 mL from each rat) from +VA rats ($n = 5$), before administering micellar BC served baseline (0 h, +VA) value

* Values for each parameter are different, $P < 0.05$ compared to baseline (0 h **) values

[a] A separate group of vitamin A-sufficient rats (++VA, $n = 5$), not fed with micellar BC was treated as control to determine baseline (0 h) value

Table 3 Plasma lipid profile (mg/dL) of vitamin A-deficient rats

Plasma lipids	Group	Baseline, 0 h	1 h	2 h	3 h	6 h
Triglycerides	−VA	88.6 ± 3.5**				
	−VA, PC		109.6 ± 4.3	116.4 ± 8.6	121.5 ± 12.3*	112.9 ± 9.3
	−VA, LPC		107.3 ± 4.2	117.2 ± 7.4	129.8 ± 14.3*	117.4 ± 6.5
Phospholipids	−VA	27.2 ± 1.5**				
	−VA, PC		31.3 ± 2.5	38.2 ± 0.4*	32.2 ± 0.3	29.3 ± 0.2
	−VA, LPC		33.8 ± 4.2	35.3 ± 0.2*	33.2 ± 0.9	31.4 ± 0.8
Total cholesterol	−VA	33.6 ± 3.3**				
	−VA, PC		41.2 ± 1.2	62 ± 2.9	64.2 ± 2.3	59.8 ± 3.2*
	−VA, LPC		42.7 ± 2.3	53.2 ± 2.4	62.2 ± 1.3	49.3 ± 2.2*

Values are mean ± SD ($n = 5$ rats). Vitamin A-deficient (−VA) rats were fed with micellar β-carotene (BC) containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC). A separate group of vitamin A-deficient rats (−VA, $n = 5$), not fed micellar BC was treated as control to determine baseline (0 h) value

* Values for each parameter are different, $P < 0.05$ compared to baseline (0 h **) values

and thus, rats could be used to study the BC bioavailability under nutritional deficiency conditions. Thus, in this study, we compared the effects of PC and LPC on BC metabolism in VA-deficient (−VA) and VA-sufficient (+VA) rats to a post-dose of micellar BC.

As expected, to the post-dose of micellar BC with phospholipids, BC absorption and its conversion into retinol was improved in both −VA and +VA rats in a time-dependant manner. This is consistent with our previous study in normal rats [14] and the effect was more prominent in rats fed with micellar BC containing LPC than PC. Notably, plasma BC level was significantly higher in −VA rats compared to +VA rats. Others reported only a small amount (<5%) of BC absorption and conversion in rats of different VA status after a dose of BC and suggested that single dose of BC might not be an effective way to raise VA status in rats [26, 27]. These differences may be due to the vehicle used to deliver BC, as they used vegetable oil and aqueous dispersions and also small group of animals. In this study, we used micellar phospholipids as the carrier of BC, which resulted in enhanced BC uptake and metabolism in rats. However, Erdman and associates reported impaired BC uptake during VA deficiency. They report decreased uptake of micellar BC by brush border

Table 4 Plasma fatty acid profile (%) of vitamin A-sufficient rats

Fatty acid	+VA	+VA, PC				+VA, LPC			
	Baseline, 0 h[a]	1 h	2 h	3 h	6 h	1 h	2 h	3 h	6 h
14:0	3.7 ± 0.7	5.3 ± 0.6	6.2 ± 1.2	5.5 ± 0.2	4.7 ± 0.2	6.2 ± 1.2	4.6 ± 0.5	5.2 ± 1.2	4.6 ± 1.1
16:0	18.5 ± 2.5**	21.7 ± 3.4*	19.3 ± 1.3	16.2 ± 3.3	15.4 ± 1.6	19.3 ± 3.1	21.6 ± 1.3	22.4 ± 2*	16.3 ± 2.3
16:1 (n-7)	5.7 ± 2.4	5.8 ± 0.2	5.1 ± 2.1	6.2 ± 1.3	6.5 ± 1.5	5.4 ± 1.3	5.8 ± 0.9	6.4 ± 1.1	5.8 ± 1.7
18:0	17.6 ± 1.3	13.8 ± 1.4	18.1 ± 2.8	20.2 ± 4.4	16.8 ± 2.7	18.4 ± 4.1	16.4 ± 2.4	14.6 ± 0.9	15.2 ± 1.8
18:1 (n-9)	24.8 ± 2.2**	22.9 ± 1.5	26.3 ± 3.5*	23.2 ± 1.7	25.7 ± 2.5	23.2 ± 2.3	25.7 ± 2.8	26.4 ± 3.6	27.2 ± 4.2*
18:2 (n-6)	16.3 ± 2.3**	15.7 ± 4.4	14.1 ± 2.2	15.3 ± 2.2	16.6 ± 2.6*	15.3 ± 2.7	14.4 ± 3.2	15.1 ± 1.4	16.2 ± 2.9*
18:3 (n-3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:4 (n-6)	13.2 ± 3.1**	14.3 ± 2.5*	10.5 ± 3.2	13.5 ± 3.1	12.5 ± 3.2	11.4 ± 2.5	10.4 ± 2.1	11.3 ± 1.7	14.2 ± 1.5*

Values are mean ± SD ($n = 5$ rats). Vitamin A-sufficient (+VA) rats were fed with micellar β-carotene (BC) containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC)

ND not detected

* Values are different, $P < 0.05$ compared to baseline (0 h **) values

[a] Blood drawn (0.5 mL from each rat) from +VA rats ($n = 5$), before administering micellar BC served baseline (0 h, +VA) value

Table 5 Plasma fatty acid profile (%) of vitamin A-deficient rats

Fatty acid	−VA	−VA, PC				−VA, LPC			
	Baseline, 0 h	1 h	2 h	3 h	6 h	1 h	2 h	3 h	6 h
14:0	7.5 ± 0.4	2.5 ± 0.4	3.4 ± 0.3	4.6 ± 0.4	6.3 ± 1.4	3.8 ± 0.2	4.2 ± 0.7	3.6 ± 0.4	4.6 ± 0.8
16:0	22.1 ± 1.5**	23.2 ± 1.2	24.2 ± 2.1	22.2 ± 3.5	25.9 ± 6.6*	23.5 ± 1.1*	18.2 ± 0.5	20.1 ± 4.6	19.8 ± 3.3
16:1 (n-7)	6.3 ± 0.6	4.9 ± 0.8	4.6 ± 0.5	5.3 ± 1.4	6.1 ± 1.5	4.1 ± 0.9	5.5 ± 0.8	5.2 ± 1.7	6.8 ± 0.7
18:0	14.5 ± 0.3	20.5 ± 6.5	22.1 ± 7.4	20.4 ± 2.9	12.8 ± 0.7	20.8 ± 5.5	22.4 ± 2.4	21.9 ± 9.2	14.9 ± 1.8
18:1 (n-9)	11.2 ± 0.8**	24.6 ± 9.8*	21.2 ± 8.4	23.2 ± 2.1	20.5 ± 2.4	22.6 ± 8.1	23.8 ± 2.1*	21.4 ± 9.1	21.5 ± 1.4
18:2 (n-6)	12.3 ± 0.5**	12.3 ± 1.5	13.1 ± 0.6	12.9 ± 0.5	15.6 ± 3.2*	14.1 ± 1.8	14.6 ± 2.3	15.6 ± 5.1	19.3 ± 0.9*
18:3 (n-3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:4 (n-6)	25.5 ± 0.4**	11.9 ± 1.1	11.2 ± 0.8	11.3 ± 0.2	12.7 ± 2.9*	11.2 ± 2.3	11.4 ± 0.7	11.9 ± 6.3	12.9 ± 1.5*

Values are mean ± SD ($n = 5$ rats). Vitamin A-deficient (−VA) rats were fed with micellar β-carotene (BC) containing either phosphatidylcholine (PC) or lysophosphatidylcholine (LPC). A separate group of vitamin A-deficient rats (−VA, $n = 5$) not fed with micellar BC was treated as control to determine baseline (0 h) value

ND not detected

* Values are different, $P < 0.05$ compared to baseline (0 h **) values

membrane vesicles isolated from the VA-deficient gerbils and rats compared to membrane preparations of VA adequate animals [12, 28]. These differences may be attributed to the in vitro method, as the membrane vesicles become saturated in short time and reached equilibration with BC uptake or due to VA deficiency induced structural changes in the intestinal cells and other biochemical alterations.

The present data show that increased plasma RP level in both −VA and +VA rats to the post-dose of micellar BC with phospholipids. Though the RP level was significantly higher in +VA rats compared to −VA rats, it is remarkable that the RP level in −VA rats reached to normal as in the +VA rats to a single dose of BC indicating the conversion and transformation of the absorbed BC. This is consistent with the studies, which reported the body's VA reserve as the primary determinant to the intestinal BC bioconversion [10, 29]. VA deficiency has been shown to increase the BC cleavage enzyme activity [30, 31]. This is in agreement with the present study, in which the homogenates of intestinal mucosa of rats were assayed for BC cleavage enzyme activity. The baseline (0 h) enzyme activity of the −VA rats was significantly higher compared to control rats (0 h, not fed with micellar BC). However, after the micellar BC dose, the intestinal homogenates of −VA rats showed significantly higher BC cleavage activity compared to +VA rats, supporting the improved plasma RP level in −VA rats. Similarly, Parvin and Sivakumar [11] reported an increased BC cleavage enzyme activity in −VA rats, but lower in protein-deficient rats. These studies indicated the role of nutritional status on the BC metabolism in rats.

Another interesting observation is that, the significantly higher liver BC level in −VA rats compared to +VA rats indicated the accumulation of absorbed BC in the liver after the micellar BC dose with phospholipids. PC, with two long-chain acyl moieties, is more hydrophobic than LPC and the PC has been shown to suppress carotenoids accumulation in the liver of normal rats [15], but in this study, PC has shown improved BC bioefficacy in both +VA and −VA rats. This could be probably due to hydrolysis of PC to LPC by phospholipase A2, which facilitated the BC incorporation to micelles and improved BC uptake by the cells of jejunum. Therefore, PC needs an additional hydrolysis step to become LPC and thereby improves BC metabolism. Whereas LPC, with one acyl moiety and a free hydroxyl group, can be taken up by the cells of jejunum along with the carotenoid and facilitate its diffusion across the water layer from micelles to the brush border membrane of the intestinal cells. It is also possible that VA deficiency affected the various fat-metabolizing enzymes in the small intestine, which resulted in modulatory effect of PC and LPC on BC metabolism. Clearly, these hypotheses warrant further investigation. Studies with Caco-2 cells and perfused rat small intestine determined the role of fatty acyl chain length of triglycerides and phospholipids on the micellarization of carotenoids and the carotenoids uptake [32, 33]. The dietary lecithin has been shown to increase the biliary output of bile acid, cholesterol, and phospholipids [34]. We have also reported an improved plasma lipid profile in rats fed with an equimolar dose of BC and lutein with phospholipids [13]. Overall, the role of PC and LPC on intestinal uptake and metabolism of BC under different nutritional conditions is noteworthy. Nevertheless, this draws particular attention that, apart from nutritional status, the nature of dietary lipid is a key factor in improving the BC bioavailability from the diet, maybe by modifying the enzymes involved in fat digestion.

We compared the baseline (0 h) plasma lipid responses of the −VA and control (+VA and ++VA) animals, which were not fed with micellar BC. Significantly altered plasma lipid composition was observed in the baseline (0 h) −VA rats compared to +VA rats. In particular, decreased plasma triglycerides, total cholesterol and phospholipids level was observed in −VA rats compared to control rats of the baseline (0 h) groups. Similar hypolipidemic effects were observed by others in −VA rats, suggesting that the change in the membrane fluidity and possible role of VA depletion on decomposition of structural lipids [35]. Warden et al. [36] reported the mild structural changes and functions in the intestine of −VA rats compared to equally malnourished pair fed rats. However, in this study, after a single dose of micellar BC with phospholipids, the plasma lipids of −VA rats were normalized to control rats. Interestingly, the total plasma cholesterol level was increased in both −VA and +VA rats in a time-dependant manner, after the micellar BC dose with phospholipids. Similar trend was observed with the plasma triglycerides and phospholipids responses to the post-dose of micellar BC. This suggests that the possible role of triglyceride-rich lipoproteins on BC transport to the target organs. Though the intestinal lipid composition gives more appropriate explanation for this observation, we did not determine the intestinal lipid in this study, but our subsequent studies supported this hypothesis (data not shown). The oleic (18:1, n-9) and linoleic (18:2, n-6) acids composition was reduced in the −VA rats, whereas such differences were not observed among the baseline control rats (0 h, +VA and ++VA). This may be due to an additional weekly dose of oleic and linoleic acids rich groundnut oil (as carrier of retinyl palmitate) to those rats. A significantly higher arachidonic acid (20:4, n-6) was observed in the plasma of baseline control (0 h, −VA) rats. To a post-dose of micellar BC with phospholipids, the fatty acid composition of the mixed micelles was reflected in the plasma of rats. The plasma fatty acid composition was restored in micellar BC treated −VA rats, particularly, oleic and linoleic acids were improved and the arachidonic acid was normalized compared to baseline control (+VA) rats. PC and LPC are rich sources of oleic (18:1, n-9) and palmitic (16:0) acids and their level was reflected in plasma of both −VA and +VA rats, indicating the metabolism of micellar phospholipids by the intestinal lipases. The plasma fatty acid composition clearly supported the possible role of fat-metabolizing enzymes on BC uptake from the mixed micelles containing phospholipids.

In conclusion, results demonstrate that micellar phospholipids facilitated the absorption and metabolism of BC and mitigate the VA deficiency–related ill effects, possibly by modifying altered intestinal membrane structure. Furthermore, alteration in the lipid profile during VA deficiency can be managed by the phospholipids, which are hydrolyzed by the intestinal lipases to release fatty acids into the circulation. Although further efforts are needed on other possible fat-metabolizing enzymes to define the precise role of phospholipids on BC metabolism, micellar phospholipids could be promising molecules in improving BC metabolism and overcoming ill effects caused by the VA deficiency.

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Conflict of interest The authors have no conflict of interest.

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