ORIGINAL ARTICLE

Microencapsulation of β-Carotene with Vanillic Acid Grafted Chitosan Improves Stability and Glutathione Content in Rats

K. K. Asha, K. K. Ajeeshkumar, Niladri Sekhar Chatterjee, R. Ananadan, Suseela Mathew

Department of Biochemistry and Nutrition, ICAR-Central Institute of Fisheries Technology, Cochin, India

ABSTRACT

Introduction: The most common cause of Vitamin A deficiency is insufficient intake of Vitamin A and one of the ways to deal with this challenge is using β-carotene for food fortification. In our study, we addressed the concern of instability in β-carotene molecules by microencapsulation with vanillic acid grafted chitosan.

Methods and Materials: β-Carotene was microencapsulated using vanillic acid-grafted chitosan as wall material. Microencapsulation efficiency and morphology were determined. Size and shape of encapsulated β-carotene particles were determined by a Zetasizer and scanning electron microscope, respectively. β-Carotene concentration was determined by HPLC while TBARS and glutathione in serum were assayed by spectrophotometric methods.

Results: The particles formed were confirmed to be in the microrange and also were smooth walled spherical units devoid of cracks and pores. Structures of free and encapsulated β-carotene were compared by Fourier transform-infrared spectroscopy. Encapsulated β-carotene particles showed better stability to degradation by light and exposure to oxidation. Analysis of β-carotene in liver, plasma, and urine following 1 week of feeding either free β-carotene or microencapsulated β-carotene (mBC) in two groups of rats showed significantly ($P < 0.001$) higher content in liver and plasma of mBC-fed rats. While glutathione content was raised significantly in mBC-fed rats, the level of thiobarbituric acid reacting substances was found unchanged between the groups.

Conclusions: This is the first instance of a report on microencapsulation of β-carotene with vannilic acid-grafted chitosan. Structural characterization showed that particles formed were of micro-size range with smooth walls devoid of cracks and pores.

Key words: Antioxidant property, microencapsulation, vanillic acid grafted chitosan, Vitamin A deficiency, β-carotene

INTRODUCTION

One of three people in developing countries are affected by vitamin and mineral deficiencies, according to the World Health Organization. India is today one of the most malnourished and nutrient deficient countries in the world.[1] Malnutrition in combination with micronutrient deficiency lowers the resistance of the body to infections and negatively impacts the gross domestic product as it reduces physical/cognitive growth and reduces productivity of individuals.[2] The WHO estimates that between 100 and 140 million children are Vitamin A deficient and that about 250,000–500,000 Vitamin A-deficient children become blind every year.[3]

The most common cause of Vitamin A deficiency is insufficient intake of Vitamin A. Although considerable progress has been made in controlling Vitamin A deficiency

Address for correspondence:
K. K. Asha, Biochemistry and Nutrition Division, Matsyapuri PO, ICAR-Central Institute of Fisheries Technology, Cochin, India. E-mail: asha.santhosh5@gmail.com©

worldwide,[4,5] there is still a need for additional prevention efforts in the form of dietary diversification, fortification, and supplementation.[6,7]

β-carotene (*β*, *β*-carotene, and BC) is the most prominent member of the group of carotenoids that occur in human diet.[8] In biological systems, the predominant isomer is all-*trans* β-carotene (E-isomer) and its most important function is being a precursor for Vitamin A. Considering the higher bioavailability of β-carotene from dietary supplements compared with fruits and vegetables, fortified foods have a potentially important role in supplying Vitamin A to the population.[9] With this background, it was hypothesized that β-carotene fortified fish soup powder could provide/fulfill a part of the daily requirement of Vitamin A in severely Vitamin A deficient malnourished populations. However, the utilization of supplemented β-carotene is limited due to its instability due to its highly unsaturated nature and susceptibility to rancid degradation. β-carotene is reported to undergo oxidative degradation through pathways induced by heat light, oxygen, acid, transition metal, or interactions with radical species.[10] Thermal processing reduced β-carotene content in potato cultivars by over 80% as described by Kotíková *et al*.[11] Zhang *et al*.[12] reported the protective influence of maltodextrin on β-carotene emulsions. Microencapsulation techniques are commonly used because they physically isolate susceptible bioactive material from the surrounding environment and protect the microencapsulated material from oxidation. To render protection to β-carotene from oxidative degradation, it was microencapsulated β-carotene (mBC) in our study. Authors[13] elsewhere also reported that mBC with natural polymers drastically reduced its susceptibility to isomerization and oxidation in the presence of oxygen, light, and heat, which prevented loss in color, antioxidant property, and vitamin activity.[14] Microencapsulation may also delay and increase absorption of mBC over free β-carotene (fBC).[15]

The bioavailability of provitamin A carotenoids like β-carotene from specific foods as well as from microencapsulated particles is not well understood. Before we continued with fortification of fish soup powder with mBC, we presumed that it is essential to assess whether microencapsulated provitamin A carotenoids can positively contribute to β-carotene content of the supplemented rats. Many factors influence β-carotene absorption and bioconversion.[16] Measuring the change in serum, liver, and urinary β-carotene concentrations following intervention has been used previously. Numerous factors affect results from this approach including carotenoid and Vitamin A regulation in the blood and carotenoid bioconversion to Vitamin A preceding entry into the bloodstream.[17] The conversion of b-carotene to Vitamin A is tightly regulated and dependent on Vitamin A status and the amount administered in the dose or meal.[18]

In this study, the levels of fBC and mBC in plasma, liver, and urine post-dosing which would reflect the bioavailability of β-carotene were compared. In view of the antioxidant nature and health benefits demonstrated by β-carotene, the effect of beta-carotene supplementation (free and encapsulated) on the content of glutathione (GSH), an intracellular antioxidant molecule and thiobarbituric acid reactive substances (TBARS), an index of oxidative damage in plasma were measured. The outcome of the study is expected to support the eventual purpose of β-carotene fortification as a strategy to address Vitamin A deficiency.

MATERIALS AND METHODS

Chemicals: β-carotene for fortification was obtained from, Fluka Chemie GmbH (Steinheim, Germany). Solvents used for separation on high-performance liquid chromatography (HPLC) were of HPLC grade. Standards reduced GSH and 1, 1, 3, 3-tetraethoxypropane (TEP) were procured from Sigma (St. Louis, MO). All other reagents used for analyses were of analytical grade and the purest quality available. All experiments were carried out in minimal, indirect light.

mBC

β-carotene was microencapsulated using vanillic acid grafted chitosan (VACS) as wall material. The VACS was synthesized following procedure as reported by our research group earlier.[19] 100 mg of β-carotene was dissolved in 12 g of sunflower oil under nitrogen environment, by applying low heat (55°C for 5 min). This constituted the oil phase while the aqueous phase consisted (3000 mL) of VACS (30 g) and tween 20 (0.1%). An oil in water emulsion was prepared by blending the oil and aqueous phase for 5 min with a high-speed homogenizer at a speed of 12,000 rpm. The emulsion was observed under optical microscope (Leica ICC50 HD) to confirm micelle formation and their uniform distribution. The emulsion was spray dried using a pilot plant spray dryer (S M Scientech, Kolkata) to yield the mBC powder. The inlet and outlet temperatures were set at 140°C and 77°C, respectively. The feed pump rpm was set at 18. The powder obtained was transferred immediately into a cold glass jar. Stability of the β-carotene-loaded particles (BVACS) against oxidation and light was studied for 21 days under controlled conditions. A set of samples was exposed to light by storing in stoppered and sealed transparent glass vials inside an illuminated BOD chamber at a temperature of 28 ± 2°C. Another set of samples was exposed to air, however, was protected from light by keeping in dark BOD chamber, maintained at a temperature of 28 ± 2°C. After 21 days of storage, β-carotene content was determined and expressed as percentage degraded with respect to the initial amount. The spray-dried β-carotene powder was used for conducting feeding studies for 2 weeks in male Wistar rats.

Microencapsulation efficiency

Microencapsulation efficiency or loading capacity was assessed according to Nunes and Mercadante.[20] Briefly, for exhaustive extraction of total carotenoid with dichloromethane, the microcapsules were previously dissolved in a 2:3 mixture of water:methanol. The carotenoids located on the surface of the microcapsules were directly extracted with dichloromethane from powdered microcapsules. In both analyses, the organic phases were recovered in a separation funnel and dried with anhydrous Na_2SO_4. Afterward, the organic solvent was removed under vacuum in a rotary evaporator, and the carotenoids were redissolved in petroleum ether for quantification by HPLC.[21]

Microcapsule morphology

Particle size analysis of the β-carotene-loaded microparticles was performed in a Zetasizer (Malvern). Surface morphology was observed under scanning electron microscope (SEM, JeolJSM 5800 LV apparatus, Tokyo, Japan). Fourier transform-infrared (FTIR) analysis of the mBC was outsourced from Sophisticated Test and Instrumentation Center, Cochin University P.O., Cochin.

Animals

Male Wistar rats that were 120-day-old weighing about 350 ± 20 g, housed individually in polypropylene cages were used. The present study was implemented according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India, and authorized by the Institute Animal Ethics Committee, the approval number being CIFT/B&N/IAEC/2016-1(3).

Experimental design

A total of 36 rats divided into three groups of 12 animals each, were taken for the study. All animals were held in standard metabolic cages at an animal facility in our institute at constant room temperature (22°C), under a 12-h light/dark cycle. The animals were recruited for the study following acclimatization for 48 h. Water and feed were given *ad libitum* and animals were weighed weekly. The groups were as follows: Group I, control was given placebo; Group II, fBC fed at the level of 20 mg/kg body weight; and Group III, mBC at the level of 20 mg/kg body weight (before feeding of Group III rats, weight of β-carotene was corrected for encapsulation efficiency which was 30%). All groups were fed standard commercial diet. mBC and fBC dispersed in oil phase by ultrasonication for 30 s were administered by gavage and the study lasted for a period of 14 days. On day 7 of the study period, after 4 h of the day's dosing, half the number of rats in each group were sacrificed following chloroform anesthesia. Blood was collected with 1% EDTA and plasma was separated for analysis of β-carotene in plasma. Liver was excised, weighed, cut into small pieces of approximately 100–200 mg, frozen immediately, and stored at −20°C until analyzed for β-carotene. Concentration of β-carotene was also determined in urine that was collected from rats housed in metabolic cages. After the last dosing on day 14, rats were fasted overnight and sacrificed following chloroform anesthesia. Blood and liver samples were processed as described above and used for biochemical analyses, namely reduced GSH and TBARS.

HPLC analysis

About 5 mg of the mBC was dissolved in 5 ml 1% acetic acid and vortexed for 2 min. The released β-carotene was extracted with hexane (1.0 mL). HPLC was used to determine its concentration with 452 nm as a detection wavelength.[21] Concentration of β-carotene in plasma, liver, and urine samples was determined using a standard curve created from a series of β-carotene standard solutions of different concentrations. Individual working standard solutions were freshly prepared from individual stock standard solutions by diluting in hexane.

Biochemical analysis

The quantitative determination of the total amount of (GSH + GSSG) in plasma samples was carried out by the method of Ellman, 1959.[22] Briefly, the reaction of GSH with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid gives rise to a yellow product that can be quantified spectrophotometrically at 412 nm. This reaction is used to measure the reduction of GSSG to GSH, and the rate of the reaction is proportional to the GSH and GSSG concentration. Lipid peroxidation in liver and plasma was assessed by TBARS formation as described by Okhawa *et al*., 1979.[23] Briefly, samples were incubated with 0.5 ml of 20% acetic acid, pH 3.5 and 0.5 ml of 0.78% aqueous solution of thiobarbituric acid. After heating at 95°C for 45 min, the samples were centrifuged at 4000 rpm for 5 min. The pink pigment in the supernatant fractions was estimated by absorbance at 532 nm. A calibration curve was prepared with TEP standard. Results were expressed as nmol MDA/mg protein. All samples gave results which were within the linear portion of the TEP standard curve.

Statistical analysis

Statistical analysis was performed using SPSS software, version 10.0. Values for each parameter analyzed are means of triplicate determinations.

RESULTS AND DISCUSSION

Following mBC, the encapsulation efficiency or loading capacity was determined as 600 mg/100 g of powder. mBC was successfully accomplished as evidenced by microcapsule morphology studies. Microscopic structure of the emulsion did not show any coalescence or flocculation. Coalescence is an irreversible process involving rupture of the interfacial film resulting in the fusion of two or more droplets forming a larger droplet. Whereas flocculation is a process that leads droplets to come together to form aggregates, while retaining their individual integrity as the interfacial membrane is conserved.[24] These two phenomena lead to increase in

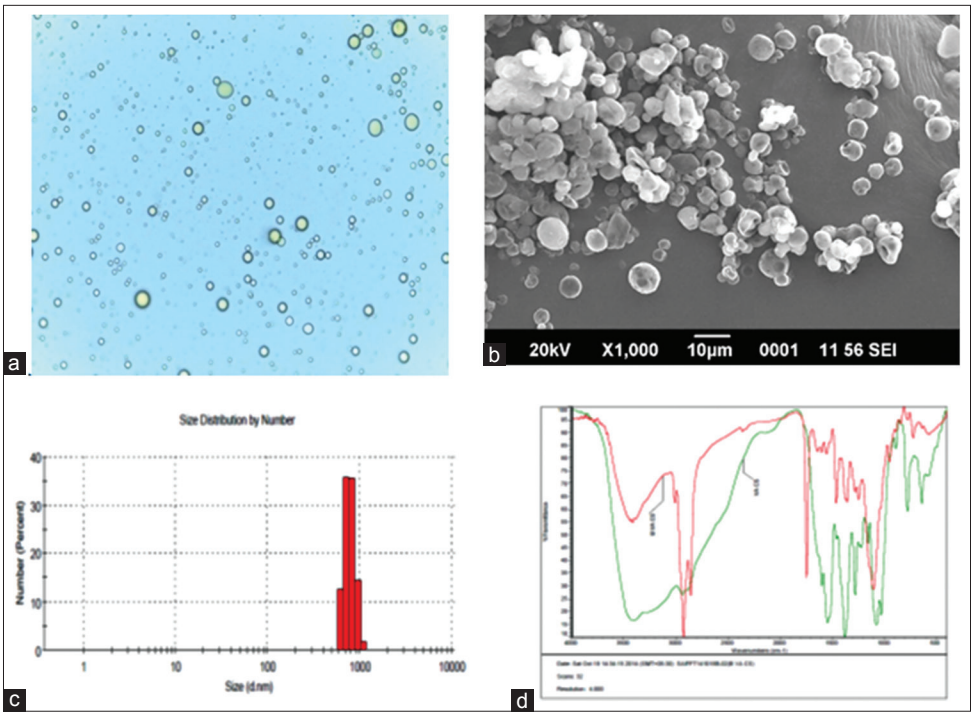

Figure 1: (a) Microstructure of the oil in water emulsion. (b) Surface morphology of the particles as observed under scanning electron microscope. (c) Panel showing size distribution by number as observed in a Zetasizer. (d) Image of overlaid Fourier transform infrared spectra of vanillic acid grafted chitosan and β -carotene-loaded particles.

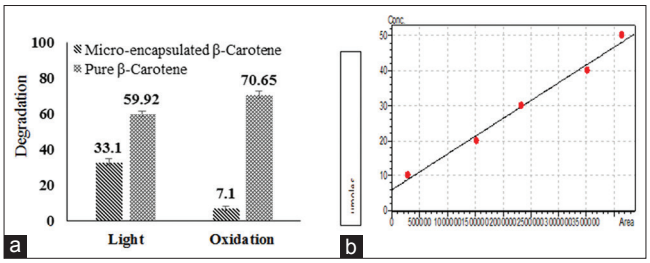

Figure 2: (a) Stability of β-carotene-loaded microparticles during storage. (b) Standard graph generated from a series of standard concentrations

droplet size. Figure 1a shows uniformly distributed micelles without coalescence/flocculation phenomena indicating efficacy of the emulsification process.

SEM images [Figure 1b] reveal spherical particles without apparent cracks and pores. The smooth surfaces ensure barrier against oxidation. The size distribution by number observed in Zetasizer [Figure 1c] showed uniform distribution of the particles. The Z-average diameter of the particles was determined to be 6 µ. A polydispersity index of 0.58 and zeta potential of + 17 were observed. This indicates that the loaded particles are dispersible in water and will produce moderately stable dispersions. The overlaid FTIR spectra of VACS and BVACS are presented in Figure 1d. The FTIR spectra of the microparticles showed emergence of new bands signifying cross-linking and folding of polymer lattice.

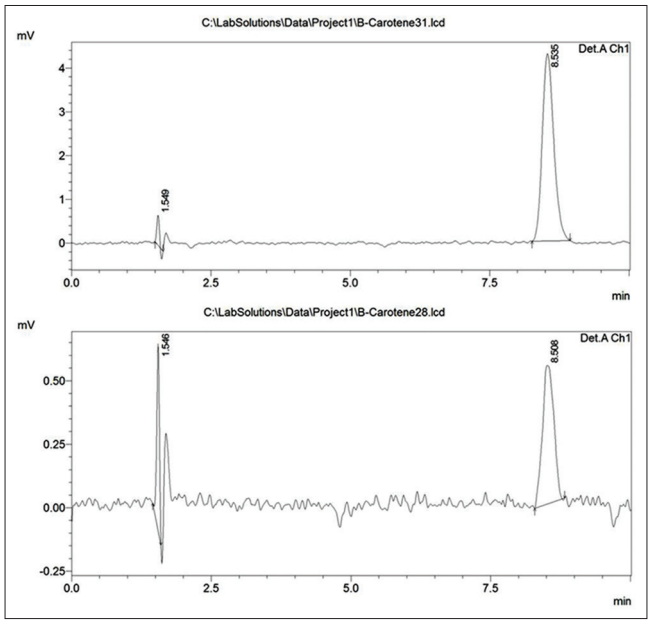

Figure 3: High-performance liquid chromatography chromatogram of β-carotene, with (bottom) and without exposure to light

Figure 2a shows that microencapsulation imparted stability to β-carotene as evidenced by the extent of degradation on exposure to light seen in fBC (nearly 60%) and mBC (33%). Similarly, degradation due to oxidation was more than 70% in fBC but was only 7% in mBC.

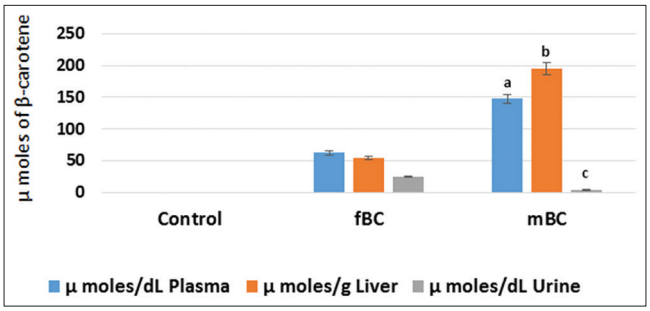

Figure 4: Levels of β-carotene in plasma, liver, and urine of control and experimental groups of rats.Control group did not get any supplementation of β-carotene, whereas free β-carotene (fBC) and microencapsulated β-carotene (mBC) groups were supplemented with 20 mg/kg body weight of either fBC or mBC, respectively. (a) a value significantly different from group fBC at $P < 0.001$ for plasma levels of β-carotene, (b) a value significantly different from group fBC at $P < 0.001$ for liver levels of β-carotene, and (c) value significantly different from group fBC at $P < 0.001$ for urinary levels of β-carotene

HPLC chromatograms before and after exposure to light are depicted in Figure 3.

β-carotene determinations were facilitated by the generation of a standard graph using a series of standard β-carotene concentrations [Figure 2b]. β-carotene content in plasma, liver, and urine of rats 4 h following the administration of a fBC and mBC at 20 mg/kg body weight level for 7 days is shown in Figure 4. β-carotene was not detected in plasma, liver, and urine of control rats. Plasma content of β-carotene was significantly higher ($P < 0.001$) and urine content was significantly ($P < 0.001$) lower in mBC administered group when compared with fBC-treated group. This may be explained by the possibility that encapsulation of β-carotene allowed better intestinal bioavailability.[25] In a study on microencapsulated curcumin, bioavailability from enriched bread the authors inferred that encapsulation delayed and increased curcumin absorption as compared to the free ingredient.[26] They also observed that increase in curcumin bioavailability could be because microencapsulation prevented or slowed their biotransformation; in the current study, the similar possibilities as mentioned above could have contributed to the increase in the levels of β-carotene in plasma of mBC-administered group. Several reports exist that substantiate the enhanced dispersibility rendered by microencapsulation.[27] It was demonstrated in a study[28] that the bioavailability of n-3 PUFA from microencapsulated fish oil added to the food matrix was as effective from fish oil capsules signifying that microencapsulation does not adversely affect bioavailability. For wall materials to be ideal, they must be able to protect the encapsulated compound and release only small amounts of the core material under gastric conditions. However, under intestinal conditions, they should completely and gradually release the core material to facilitate better absorption of the nutrients in the intestine.[29] Rutz *et al*.[13] reported that the chitosan/TPP encapsulated β-carotene microparticles showed inadequate release in aqueous media and gastric conditions but showed greatest (81–98%) release under intestinal conditions. In the current study, though release of β-carotene microparticles under simulated intestinal conditions was not monitored, we consider that mBC reached the intestine where either absorption or cleavage to Vitamin A occurred. β-carotene being a long chain hydrocarbon with multiple conjugated double bonds is very susceptible to oxidative degradation. Encapsulation might have provided protection to mBC, whereas the fBC could have easily undergone oxidative degradation in the rather unfavorable milieu of the digestive tract. The fact that β-carotene was detected in plasma and liver signifies that not all administered fBC/mBC underwent intestinal cleavage to form dimers. Numerous studies have shown that a substantial amount of the absorbed carotenoids including β-carotene is not cleaved by the intestinal beta-carotene monooxygenase 1 (BCMO1) enzyme.[30] A proportion of the carotenoids (up to 60% of dietary intake) that escape BCMO1 enzyme activity is incorporated in chylomicrons together with lipids and circulate in association with plasma lipoproteins and hence can be taken up by the respective receptors for lipoproteins[31] which explains their presence in plasma and liver. Rutz *et al*., 2013,[32] have evaluated the release profile of carotenoids derived from purple Brazilian cherry juice that was encapsulated using a xanthan-tara hydrogel. They observed a <20% release of total carotenoids under simulated gastric conditions (pH 2.0, pepsin enzyme) and more than 85% release under simulated intestinal conditions (pH 8.00, pancreatic enzyme).

Urinary levels of β-carotene [Figure 4] were not detected in control group rats; conversely, their levels were significantly high in group fBC rats when compared to mBC, suggesting that the loss of fBC through urinary excretion was much more when compared to the rats administered encapsulated β-carotene. Low levels of β-carotene in the urine of mBC-fed group of rats may also be due to the apparently slow release and slow and sustained absorption of beta-carotene from microencapsulated particles in the intestine. β-carotene is poorly absorbed in humans; about 30–90% is excreted in the feces and a small amount in the urine[33] which in part can explain the undetectable levels in urine of control rats.

The polyene chain of β-carotene that essentially imparts the carotenoids the well-recognized properties of these compounds including their antioxidant property, also makes them susceptible to degradation from a number of agents.[34] The major cause of carotenoid destruction during storage or processing of food is oxidation. Therefore, the oxidative damage of β-carotene in foods fortified with it would leave the functional food devoid of the bioactivity it was intended to possess. The process of microencapsulation permits the

development of encapsulated β-carotene that increases the functional and nutritional quality of the fortified food and improves the delivery of β-carotene *in vivo*. There are increased chances of mBC contributing to the Vitamin A status of an individual than fBC. Among other limitations of utilizing β-carotene as a fortificant are its low chemical stability and poor water solubility. Furthermore, β-carotene is highly prone to chemical degradation during food processing and storage. Stabilizing it with the microencapsulation can improve its stability and solubility.[15,35] Gomes *et al*.[35] demonstrated enhanced stability and bioavailability of β-carotene using solid lipid microparticles of stearic acid as encapsulant while Aissa *et al*.[15] used gum Arabic for microencapsulation. In our study, usage of the encapsulant VACS is a novel approach, wherein the polymer is modified by reaction with an antioxidant molecule to enhance its bioactivity. Our results demonstrate that the strategic approach not only improved stability of β-carotene but it also contributed significantly to improvement in the antioxidant status of the experimental animals.

β-carotene is known to act as an antioxidant that can reduce the singlet oxygen produced during several metabolic processes.[36] In the current study, GSH levels [Figure 5] were significantly enhanced in plasma of both fBC ($P < 0.005$) and mBC ($P < 0.001$) groups when compared with control group. The increase recorded in the mBC group rats was also significantly more when compared to fBC group showing that encapsulation of β-carotene has not only retained its bioactive potential but also presented improved activity in terms of enhancing GSH levels. It has been reported that the addition of beta-carotene to a culture medium for a murine macrophage cell line resulted in enhanced intracellular GSH concentration.[37]

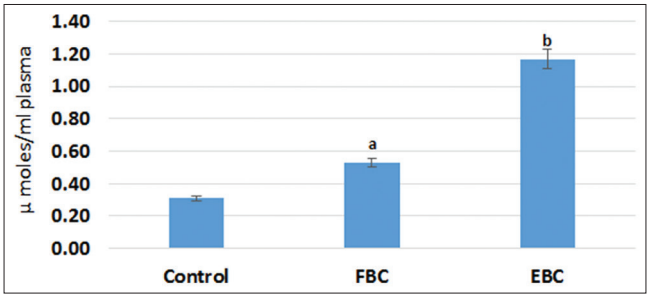

Figure 5: Levels of reduced glutathione in plasma of control and experimental rats. In the graph, (a) a value significantly different from control group at $P < 0.05$, (b) a value significantly different from control and fBC groups at $P < 0.001$. Group designations are same as shown in Figure 4

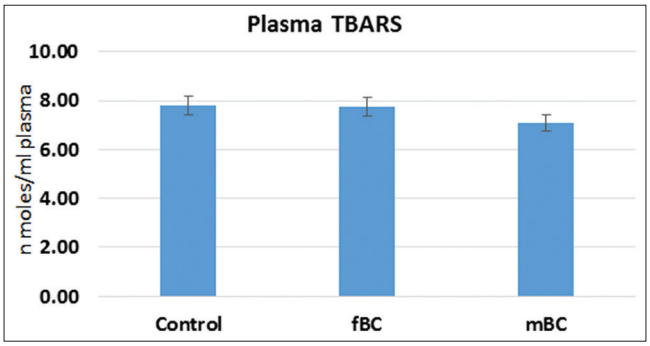

Figure 6: Levels of thiobarbituric acid reactive substances in plasma of control and experimental rats. Values in groups named fBC and mBC are not significantly different from the control group; group designations are the same as those described in Figure 4

TBARS assayed in liver and plasma of the experimental rats was essentially unchanged across all groups [Figure 6]. This is a significant outcome of the study as it establishes that at the dose of β-carotene employed in the study, it is not prooxidant in contrary to some reference reports that purport β-carotene's prooxidant nature at high doses.[38]

Studies show that the intake of Vitamin A is critically low and does not reach the recommended levels in several developing parts of the world. mBC by virtue of enhanced stability, absorption, and gradual release when supplemented through diet might help to balance inadequate retinol supply in significant parts of the world. Fortification with mBC will address the issue of Vitamin A deficiency in children more effectively than fBC.

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