β-Carotene improves intestinal barrier function by modulating proinflammatory cytokines and improving antioxidant capacity in β-lactoglobulin-sensitized mice

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Received January 9, 2020 – Accepted June 12, 2020

Increased intestinal permeability due to barrier dysfunction is supposed to cause several gastrointestinal diseases. We have previously demonstrated that a single β-carotene (BC) dose protects against increase in anaphylactic response in β-lactoglobulin (BLG)-sensitized mice with no effect on the epithelial permeability and weak recovery of villi length. Utilizing the same murine *ex vivo* intestinal model, the aim of this study was to investigate the effect of different BC doses on BLG-mediated intestinal epithelial barrier disturbances. Jejunum was harvested from BLG-sensitized mice pretreated with either one of three different doses of BC (5, 10 and 20 mg/kg body weight) and mounted on Ussing Chambers. Transepithelial electrical resistance (TER) and short-circuit current (*Isc*) were recorded as indicators of intestinal epithelial barrier function. Histopathological analysis of the intestine was carried out for the control and experimental mice. TNF-α and IL-6 levels were determined in serum using ELISA, and the analysis of antioxidant activity was performed for reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). BC was capable of enhancing the intestinal barrier function, as indicated by the increased TER and the decreased *Isc*. Intestinal damage characterized by the shortening of villi and infiltration of intestinal lymphocytes was significantly reversed by BC pretreatment. Such effects of BC were accompanied by a reduction in the levels of IL-6 and TBARS and an increase of GSH. TNF-α levels were reduced only at the lowest BC dose. These findings may encourage the use of BC-based therapies for controlling the breakdown of the intestinal barrier *in vivo*.

Intestinal barrier dysfunction initiated by various etiologies is a main contributing factor in several pathological conditions involving the gastrointestinal tract (1). A breakdown or impairment of the epithelial barrier has been implicated as a critical determinant in the predisposition to intestinal inflammation and a number of gastrointestinal diseases including inflammatory bowel disease and food allergy (2). While increased intestinal epithelial permeability can be a consequence of disease exacerbation, clinical evidence suggests that it may be a primary etiologic factor predisposing to disease development (3).

Key words: β-carotene; transepithelial resistance; TNF-α; IL-6; oxidative stress

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0393-974X (2020)

Proinflammatory cytokines such as TNF-α and IL-6 play a crucial role in the modulation of the inflammatory response in the gastrointestinal tract (4). Proinflammatory cytokines can induce the endocytosis of tight junction proteins resulting in increased intestinal permeability (5).

The role of the immune system is likely to generate an aggressive physiological response to the imbalance induced by the barrier dysfunction (6). Increased immune cell activity has been reported to result in significant free radical production (7) that may negatively impact barrier function. Excessive levels of reactive oxygen species (ROS) damage cellular proteins including cytoskeletal proteins (8) and, ultimately, disrupt gastrointestinal tract barrier to increase gut permeability which contributes to inflammation in a variety of gastrointestinal diseases (9). In this respect, antioxidants may reduce increased intestinal permeability by regulating ROS signaling.

Several studies have shown that diets rich in β-carotene (BC) are beneficial to the health of humans and may prevent the development of inflammation associated diseases. Such an effect has been attributed to the various bioactivities of BC, including antioxidant activity (10). In an earlier publication from our laboratory, we showed that supplementation with a single BC dose resulted in a significant decrease in secretory response with no effect on the epithelial permeability and weak recovery of villi length in a murine model of food allergy (11). Therefore, in the current study, we questioned whether different BC doses could improve β-lactoglobulin (BLG)-mediated intestinal barrier dysfunction. Towards this goal, we used the same *in vivo* model employed above.

MATERIALS AND METHODS

Animals and housing conditions

Female Balb/c mice (16.80±1.95 g, n = 40) were maintained at the animal facility of nutrition physiology and food safety laboratory-university Oran 1 with a 12-h light–dark cycle at 23±2°C and free access to standard laboratory feed and water. All experimental procedures involving animals were approved by the current Algerian legislation covering the protection of animals.

Experimental protocol

At 4 weeks of age, mice were randomly divided into four groups of ten animals each: one control group (Vehicle) and three experimental groups (5BC, 10BC and 20BC, respectively). The experimental groups received daily doses of 5, 10, and 20 mg/kg of body weight/day of BC (Sigma, France), respectively, for 2 weeks through the gavage method; while the control group was given only corn oil (Sigma, France) over the same period of time. BC was prepared as suspension in 0.2 ml corn oil used as vehicle. On the 16th day of the experiment, mice in all groups were sensitized intraperitoneally with BLG, as described previously (11). During the 50-day experimental period, the mice body weights were monitored weekly, however, there were no significant differences in body weight among the groups in the study (data not shown).

It has to be noted that the applied doses exceed the levels from any natural sources of BC. Although mice readily convert BC to vitamin A, the bioavailability of the molecule is low in case of absorption through the gut, requiring administration of BC at higher doses than physiologically required (12). The experiments were made to partly offset this limitation

Sample collection and processing

On day 50, the mice were anesthetized by pentobarbital sodium (5 mg/kg) and sacrificed. The small intestine and the liver were carefully collected. After discarding the duodenum and the ileum, the intestine was rinsed with phosphate buffer saline (PBS) solution in order to remove fecal content. A 5 cm portion of the middle of the intestine was placed in cassettes and fixed in 10% buffered formalin solution pH 7.2, and the consecutive following segment was opened lengthwise along the mesenteric line and immediately mounted as sheets in Ussing chambers. Serum samples, collected after removing blood cells, and liver were stored at -80°C until analysis.

From each mouse, 4 to 8 pieces of intestine were used for *ex vivo* Ussing chamber experiments as previously described by our group (11, 13, 14). Briefly, the spontaneous potential difference (PD) was monitored via agar-salt bridges connected to calomel electrodes, and the appropriate short-circuit current (*Isc*) was added via Ag-AgCl electrodes to maintain a zero PD through an automatic voltage clamp device (model VCC MC8; Physiologic Instruments, San Diego, CA, USA). The

transepithelial electrical resistance (TER) was calculated from the spontaneous PD and *Isc* by means of Ohm's law. The transmembrane resistance is a sensitive marker of the epithelial barrier function, and a decline of the value reflects a break in the barrier (15). *Isc* is considered an index of electrogenic ion movement. Changes in TER and *Isc* during experimental conditions were calculated as a percentage of corresponding basal values.

Intestinal tissues were dehydrated by gradually soaking in alcohol and xylene, paraffin-embedded, and stained with hematoxylin and eosin for histological analysis. Tissues were visualized for changes in intestinal pathology. Villus length was measured using an optical microscope equipped with a micrometer (Optica Axiom 5000, Beijing, China).

Serum IL-6 (Cat. No. RAB0308) and TNF-α (Cat. No. RAB0477) were analyzed by using commercial ELISA kits according to the manufacturer's protocol and instruction. The levels of TBARS in serum and liver were determined according to previously described methods (16). The results are expressed as μmol malondialdehyde (MDA)/mg protein. GSH content was determined by 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as an indicator (17). GSH content was expressed as μmol/mg protein. The protein content was determined using Lowry's method measuring absorbance at 750 nm (18). Bovine serum albumin was used as a standard.

Statistical analysis

Results are given as mean±standard error of the mean (SEM). Differences between means were determined using one-way ANOVA (analysis of variance) with Tukey's post-hoc test using GraphPad Prism 5 software. A p value of less than 0.05 was considered statistically significant.

RESULTS

BC improved BLG-induced disruption of barrier function ex vivo

Three concentrations of BC were tested, and their effects on transepithelial electrical resistance and short current circuit were measured. *Isc* and TER were followed over time.

Sensitization with BLG induced significant decline of transepithelial resistance (Fig. 1a, b) and increase of short current circuit (Fig. 1d, e), whereas BC pretreatment at different doses significantly inhibited these effects. By increasing concentrations of BC, the peak amplitude of ΔTER increased by 45.27, 61.62 and 59.45%, respectively, and Δ*Isc* decreased by 16.37, 29.16 and 24.92%, respectively.

The jejunal sheets isolated from vehicle-sensitized control mice had a basal TER of 45.38±9.96 $\Omega.cm^2$ and a basal *Isc* of 75.84±11.10 $\mu A.cm^{-2}$. Oral administration of BC at doses of 5, 10 and 20 mg/kg induced a significant increase in the mean value of basal TER (63.82±4.29, 68.98±5.40 and 68.69±3.30 $\Omega.cm^2$, respectively) and decrease of *Isc* (66.33±6.23, 48.33±3.35 and 46.22±4.28 $\mu A.cm^{-2}$, respectively) (Fig. 1 c, f).

BC ameliorated BLG-induced mucosal damage

Histological examination of the control group showed in most cases preservation of structure (Fig. 2 a, b). In the intestinal tissues of BLG-sensitized mice receiving or not corn oil, there was obvious intestinal inflammation characterized by an increased lymphocytes infiltration and remarkable villus atrophy (Fig. 2 c-f). After two weeks of treatment, BC improved the mucosal inflammation induced by BLG (Fig. 2 g-i). The decrease of the villus length induced by BLG was significantly inhibited by BC pretreatment with a maximal inhibition at BC 20 mg/kg (Fig. 2j). Villus height was increased by 23.54, 30.92 and 62.76% in the jejunum of mice receiving 5, 10 and 20 mg/kg of BC respectively.

As shown in Fig. 3a, the serum level of IL-6 was markedly inhibited by all the investigated doses of BC. For TNF-α, only pretreatment of BLG-sensitized mice with 5 mg/ml of BC per day significantly attenuated the level of this cytokine, whereas 20 mg/kg BC significantly increased TNF-α content in serum (Fig. 3b). BC treatment significantly decreased TBARS levels and elevated GSH content in the liver when compared with vehicle-sensitized control mice (Fig. 4a, c), whereas there was no effect on the serum TBARS level (Fig. 4b).

DISCUSSION

We have previously shown that supplementation

with a single BC dose resulted in a significant decrease in secretory response with no effect on the epithelial permeability and weak recovery of villi length (11). Therefore, in the present study, we investigated whether different BC doses could regulate intestinal barrier function in our mouse model of food allergy by examining changes in the transepithelial electrical resistance and short current circuit. An important factor is the poor bioavailability of carotenoids, including BC, in case of the absorption from the gut requiring the administration of BC at higher doses than physiologically relevant (12). In our study, three concentrations were tested (5, 10 and 20 mg/kg) in order to achieve maximal barrier enhancement. The basal values for *Isc* and TER recorded in our experiments are well within

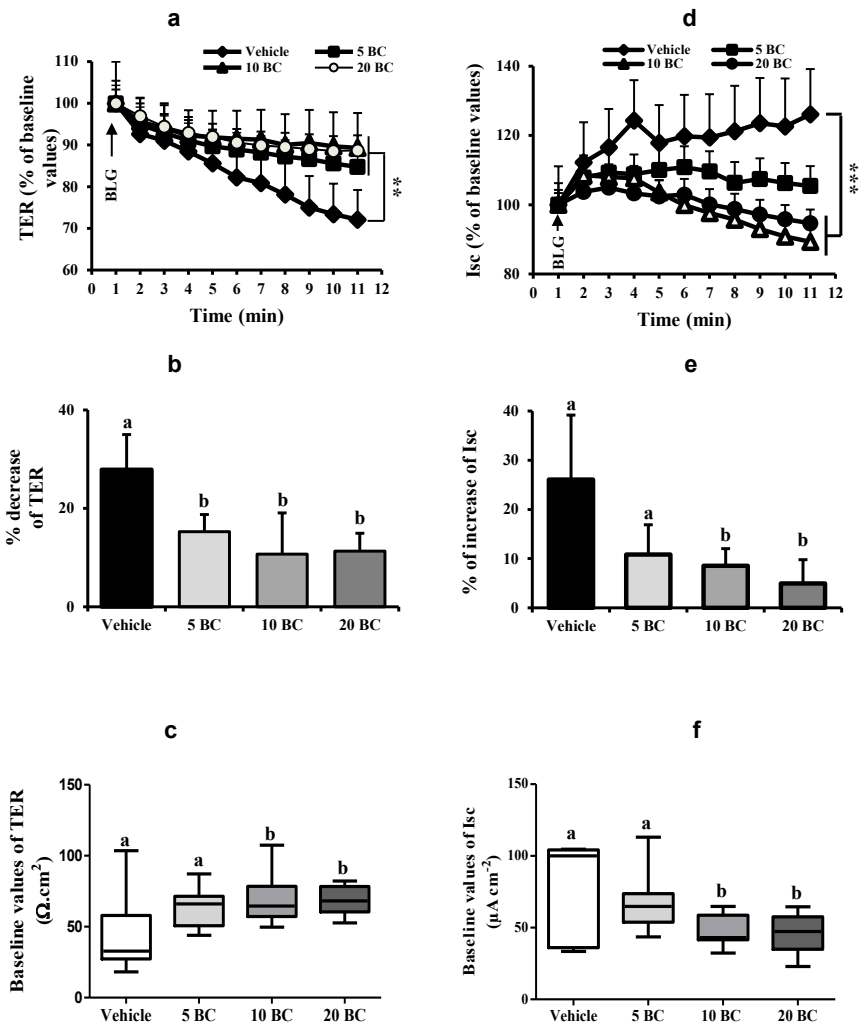

Fig. 1. *Effects of BC administration on intestinal barrier function during serosal side exposure to BLG. Murine intestine pretreated with either one of three different doses of BC (5, 10 and 20 mg/kg body weight) was placed on Ussing Chambers and the TER and Isc were measured over a 10-minute period after BLG (60 µg/ml) challenge.* ***A)*** *Percentage (%) of baseline TER values measured at various time points after BLG challenge (**p < 0.01).* ***B)*** *Percentage (%) TER decrease after BLG challenge; the decrease in TER is the difference between the peak value after BLG challenge and the baseline value.* ***C)*** *The mean value of basal TER of tissues from different groups.* ***D)*** *Percentage (%) of baseline Isc values measured at various time points after BLG challenge (***p < 0.001).* ***E)*** *Percent (%) Isc increase after BLG challenge; the increase in Isc is the difference between the peak value after BLG challenge and the baseline value.* ***F)*** *The mean value of basal Isc of tissues from different groups. Values (means±SEM, n = 10) not sharing a common letter are significantly different (*p < 0.05). Comparisons were made between different doses of the same treatment and all doses vs Vehicle*

the range found by our group and other groups working with mouse intestine (19, 20). Interestingly, all BC concentrations were optimal in terms of both elevating TER and decreasing *Isc*. More specifically, TER significantly increased at BC 10 mg/kg. TER is one of the commonly useful indicators for permeability of intestinal epithelial cells. It could reflect the opening of the tight junctions between epithelial cells and the paracellular permeability of the intestinal mucosa (21). The present study supports previous observations in that low serum carotenoids were suggested as good markers for the intestinal barrier function (22, 23). Vieira et al. (23) suggested that carotenoids ameliorate disrupted intestinal barrier function, via scavenging free oxygen radicals or via maintenance of tight

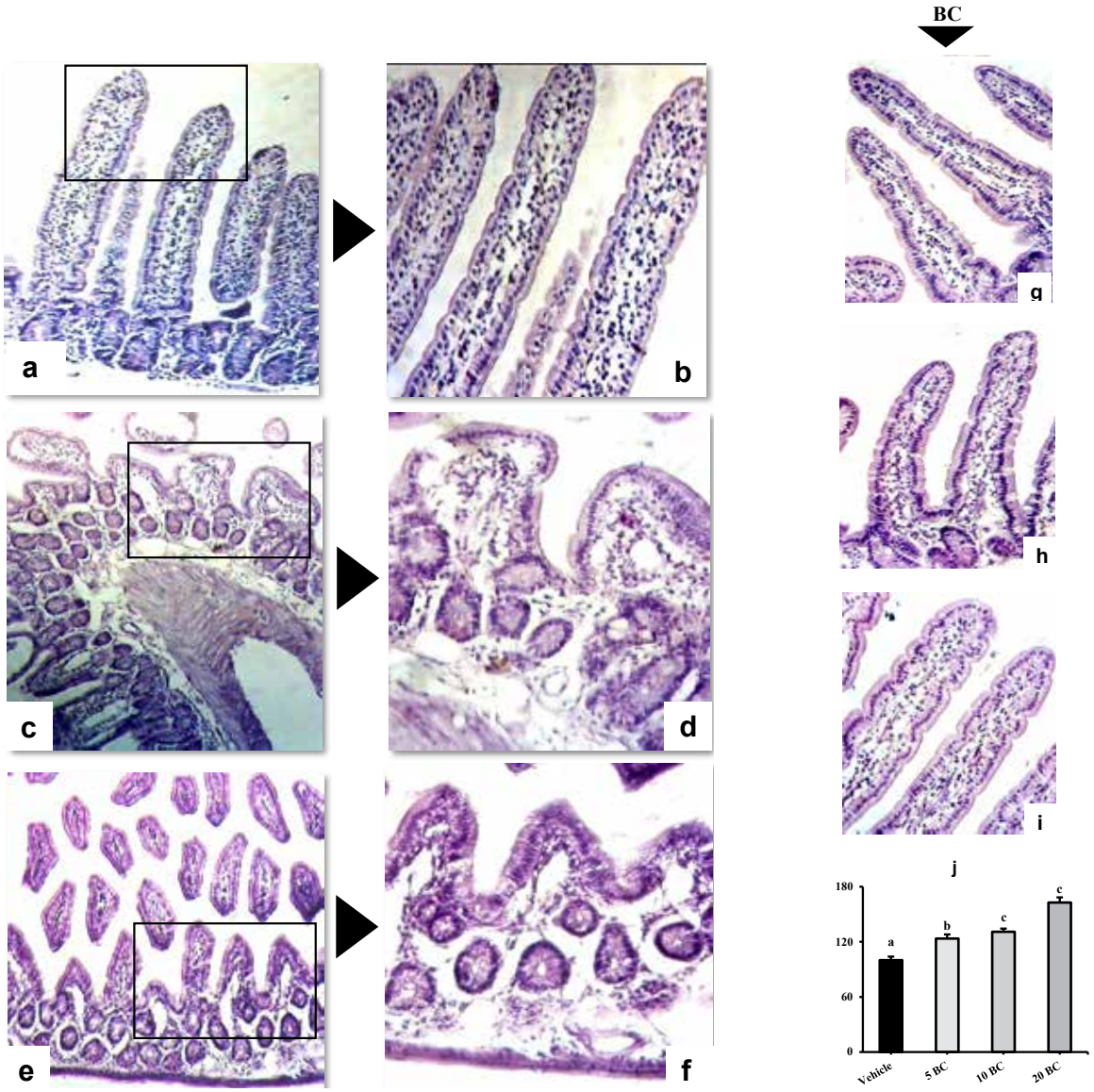

Fig. 2. *H&E staining of small intestine section from a representative mouse from each group.* ***A, B****) Naïve mice (X 100, X 400, respectively).* ***C, D****) BLG-sensitized mice (X 100, X 400, respectively).* ***E, F****) Vehicle-sensitized control mice (X 100, X 400, respectively).* ***G-I****) Mice receiving 5, 10 and 20 mg/kg of BC, respectively (X 250).* ***J****) Effect of BC pretreatment on villus length, measured from villus-crypt junction to villus tip (n = 21). n is the number of tissues studied from four mice per group. Values (means±SEM) not sharing a common letter are significantly different (p < 0.05). Comparisons were made between different doses of the same treatment and all doses vs Vehicle*

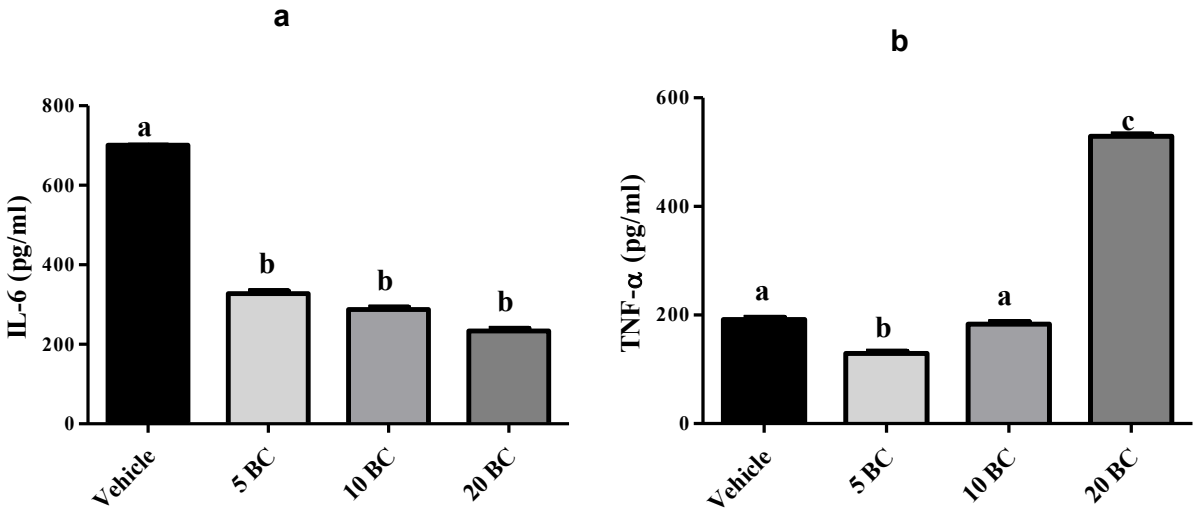

Fig. 3. *Effects of BC administration on proinflammatory cytokines. **A)** IL-6 levels. **B)** TNF-α levels. Values (means±SEM, n = 10) not sharing a common letter are significantly different (*p < 0.05). Comparisons were made between different doses of the same treatment and all doses vs Vehicle*

junctions and adherence junction proteins. The underlying mechanisms for this remain unknown at present; however, it is speculated that the beneficial effects of consumed BC are thought to be due to its ability to be converted to vitamin A (24). A study by He et al. (25) showed that vitamin A improves intestinal epithelial barrier function by enhancing the expression of tight junction proteins. Vitamin A metabolites could also affect some aspects of the epithelial barrier function. Retinoic acid, the most biologically active form of vitamin A (26), was found to be partially but significantly able to attenuate the disruption of barrier properties of MDCK monolayer (1). Interestingly, Rybakovsky et al. (27) have emphasized the ability of certain micronutrients to enhance one or more aspects of tight junction barrier function in Gie-3B11 human epithelial cell culture model. At concentrations that produced optimal improvement of barrier function, retinoic acid produced significant decreases in claudins-1 and -2, and significant increases in claudins-4 and -5.

As stated above, villi length increased as treatment concentration increased. To our knowledge, our study is believed to be the first to show that BC is effective in ameliorating epithelial damage and restoring epithelial function in *in vivo* model. Whereas, in a series of experiments aimed at examining the impact of BC on oxidative stress-induced cerebral, cardiac and hepatic damage, Esrefoglu et al. (28) demonstrated that BC could reduce stress-induced organ damage by both inhibiting lipid oxidation and supporting the cellular antioxidant defense system.

To determine whether these BC-induced improvements in barrier function were due in part to changes in proinflammatory cytokines, we examined the BC effects on TNF-α and IL-6 levels. As indicated above, BC pretreatment significantly inhibited BLG□induced IL-6 increase in a dose-dependent manner, while TNF-α levels decreased only at BC 5 mg/kg. TNF-α and IL-6 are believed to be among the most important in inducing the intestinal epithelial barrier dysfunction and leading to increased intestinal permeability (29). BC exerted an inhibitory effect on proinflammatory cytokines. It blocked nuclear translocation of the NF-κB p65 subunit which is correlated with its inhibitory effect on phosphorylation and degradation of the NF-κB inhibitor (30). Recently, a major advance in

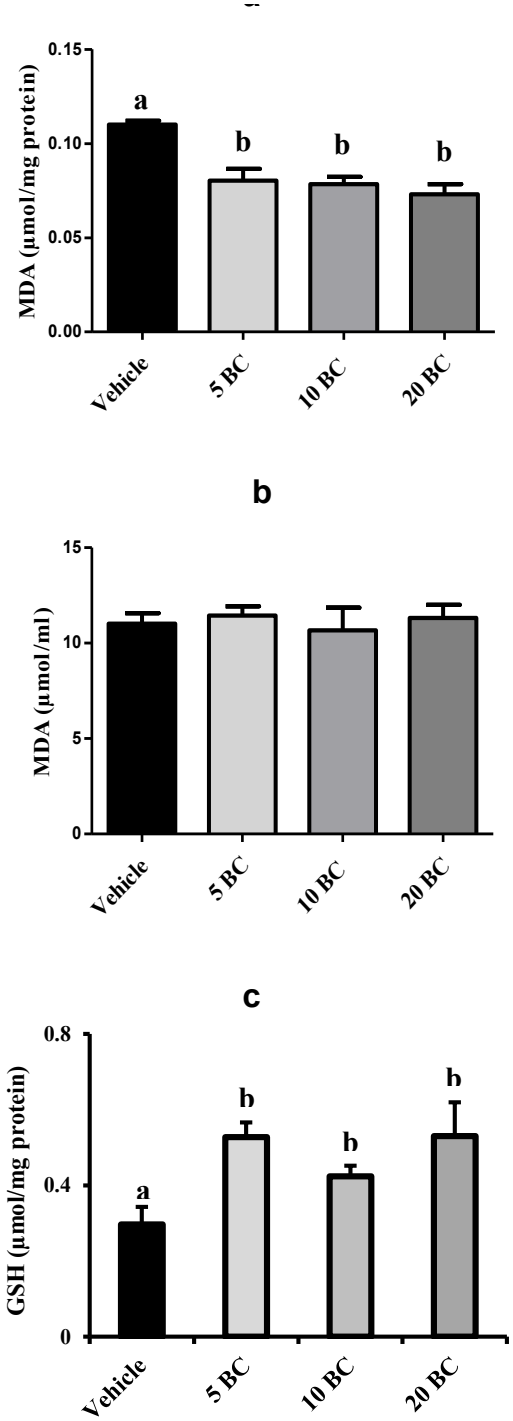

Fig. 4. *Effects of BC administration on the antioxidant status of BLG-sensitized mice.* ***A, B****) TBARS levels in the liver and the serum, respectively.* ***C****) Hepatic GSH content of BLG-sensitized mice fed either one of three different doses of BC. Values (means±SEM, n = 10) not sharing a common letter are significantly different (*p < 0.05). Comparisons were made between different doses of the same treatment and all doses vs Vehicle*

understanding the anti-inflammatory activities of BC was made by the work of Li et al. (31). They reported that the anti-inflammatory effect of BC is achieved by inhibiting multiple signaling pathways, such as JAK2/STAT3, NF-κB and JNK/p38 MAPK but not ERK1/2**.** Our results revealed that BC at dose of 20 mg/kg per day in the given model, and using the described protocol, increased TNF-α content in serum. Due to the complexity of the immune system and response, the anti-inflammatory effect of BC requires further investigation in our future study.

It has been postulated that the role of BC as an anti-inflammatory agent may be due to its capacity to scavenge ROS and may be attributed to the electrophilicity of ROS-induced carotenoid intermediates (32). Given this possibility, we felt it necessary to examine the antioxidant redox activity of BC. Thus, we assessed TBARS levels as a marker of oxidative stress. In our study, BC significantly decreased the TBARS content in the liver. The inhibition of oxidative stress was reportedly closely related to the improvement in the non-enzymatic and enzymatic antioxidant status. To our knowledge, GSH is one of the most important non-enzymatic antioxidants (33). As alluded to above, the GSH levels were significantly restored in BC-treated mice. The activity of GSH is quite important for immunological functions. In an *in vitro* cell culture model using the human alveolar macrophages, it was shown that the production of TNF-α, IL-6, and IL-8 was inhibited by addition of GSH to the medium (34). In addition, chronic depletion of mucosal GSH by buthionine sulfoximine, a specific inhibitor of glutamate cysteine ligase, has recently been shown to cause severe degeneration of epithelial cells from jejunum and colon, which was prevented by oral GSH or GSH monoester (35).

On the basis of the results discussed in this study, we believe that BC improved BLG-induced intestinal barrier disruption, through reducing epithelial permeability, decreasing intestinal mucosa atrophy, modulating the cytokine levels, and enhancing intracellular antioxidative protection. Further studies in this domain, examining different carotenoids, and perhaps mixtures representing more natural conditions, are warranted.

ACKNOWLEDGEMENT

This research was supported by the Directorate General for Scientific Research and Technological Development (DGRSDT, MESRS, Algeria).

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