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9-*cis* β-carotene Inhibits Atherosclerosis Development in Female LDLR-/- Mice

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ABSTRACT:

Background: Several epidemiological studies have shown that diets rich in carotenoids are associated with reduced risk for cardiovascular disease. However, administration of synthetic all-*trans* β-carotene was reported to have no effect on cardiovascular disease. We previously demonstrated that the 9-*cis* β-carotene-rich powder of the alga *Dunaliella bardawil* inhibits atherogenesis and reduces plasma non-HDL cholesterol levels in mice.

Context and purpose of this study: We sought to investigate whether isolated 9-*cis* β-carotene inhibits atherogenesis in a murine model of atherosclerosis.

Results: Twelve-week-old female LDL receptor knockout mice (LDLR-/-) were pretreated for 2 weeks with regular chow diet fortified with the alga *Dunaliella* powder, 9-*cis* β-carotene isomer, all-*trans* β-carotene isomer, or 9-*cis* retinoic acid, followed by 10 weeks of a high-fat diet with the same fortifications. In contrast to *Dunaliella*, 9-*cis* β-carotene did not inhibit the high fat diet-induced elevation of plasma cholesterol. Additionally, diet fortification with *Dunaliella* powder, β-carotene isomers, or 9-*cis* retinoic acid did not change the plasma retinol or retinoic acid levels. Nevertheless, 9-*cis* β-carotene significantly inhibited atherogenesis compared to the control mice (39% reduction).

Conclusions: The results suggest that 9-*cis* β-carotene should be considered as an anti-atherogenic agent in the human diet.

Key words: Atherosclerosis, *Dunaliella*, 9CBC, LDLR-/- mice

INTRODUCTION:

β-carotene is a precursor of retinoids, including retinal, retinol, and retinoic acid. The 9-*cis* β-carotene (9CBC) isomer is a precursor of all-*trans* (ATRA) and 9-*cis* isomers of retinoic acid, both *in vitro* and *in vivo* [1-3]. While the two retinoic acid isomers serve as ligands for the nuclear retinoic acid receptor (RAR), only the 9-*cis* retinoic acid is a ligand of the retinoid X receptor (RXR) [4]; as a result, it can coordinate regulation of lipid metabolism via several pathways [5].

Several epidemiological studies have shown that diets rich in carotenoids are associated with reduced risk for cancer and cardiovascular disease [6, 7]. However, administration of synthetic all-*trans* β-carotene (ATBC) to smokers was associated with increased incidence of lung cancer in one study [8], and it was reported to have no effect on cancer and cardiovascular disease in another study [9]. This suggests that a mixture of natural isomers of β-carotene and other carotenoids, instead of just pure ATBC, may have a beneficial impact on the occurrence of malignancies and atherosclerosis.

While the 9CBC content of fruits and vegetables in a regular diet is low, (relative to ATBC), a remarkably high level of 9CBC was observed in the unicellular halo-tolerant green alga, *Dunaliella bardawil* [10]. When cultivated under appropriate conditions, β-carotene comprises up to 10% of the algal dry weight, which contains roughly 50% of 9CBC and 50% ATBC [11]. Based on this significantly high β-carotene content, we used *Dunaliella* powder as the source for natural β-carotene isomers in previously published studies. We determined that 9CBC-rich *Dunaliella* powder augmented the effect of fibrates on plasma HDL cholesterol and triglyceride (TG) levels in humans. It also improved the HDL-cholesterol raising effect of fibrates in human apo-lipoproteinAI (apoAI) transgenic mice [12]. Moreover, we demonstrated that high-dose synthetic ATBC accelerated atherosclerosis, while 9CBC-rich *Dunaliella* inhibited atherogenesis, reduced non-HDL plasma cholesterol, and inhibited fatty liver development and liver inflammatory response in LDL receptor knockout mice (LDLR-/-) [13]. In a recent study, we found that 9CBC-rich *Dunaliella* powder lowered plasma cholesterol levels and slowed atherosclerosis progression in apoE deficient mice (apoE-/-) [14].

Retinoic acid, a metabolite of β-carotene, is known to modify various metabolic pathways that are involved in atherogenesis [15]. Therefore, we reasoned that 9CBC has the potential to inhibit atherogenesis via its potential conversion to 9-*cis* retinoic acid and/or other potential retinoids. To further substantiate the favorable effect of *Dunaliella* and to clarify its potential mechanism, we sought to study the regulatory role of algal-derived, isolated 9CBC on plasma lipid and lipoprotein levels, in addition to atherosclerosis, when given in a purified form to an LDLR-/- murine model for atherosclerosis.

MATERIALS AND METHODS:

Mice

Female, 12-week-old LDLR-/- (C57BL6 background, Jackson Laboratories) mice were used. The mice were housed in plastic cages on a 12 h light/12 h dark cycle with free access to feed and water. The mice were killed with isoflurane. The Animal Care and Use committee of Sheba Medical Center, Tel-Hashomer, approved all animal protocols (506/09).

Diets

Two commercial diets were used: a non-purified, low-fat diet (18% protein, 5% fat; TD2018, Harlan Teklad) and a semi-purified high fat diet (17.3% protein, 21.2% fat, 0.15% cholesterol; TD88137, Harlan Teklad). To enrich the diet with β-carotene, we used three preparations: a powder of the alga *Dunaliella bardawil,* containing 6% β-carotene (weight/weight), comprised of 50% ATBC and 50% 9CBC [10] (a gift from Nikken Sohonsha, Japan), 9CBC, isolated from the alga *Dunaliella bardawil,* and finally synthetic ATBC (Sigma Aldrich). To prepare the feed, 0.25 L of distilled hot water was mixed with 14 g of gelatin until the solution was clear. Then, 1 kg of powdered feed and *Dunaliella* powder (80 g/kg feed, containing 3 g/kg ATBC and 3 g/kg 9CBC) or β-carotene isomers (3 g/kg feed) were thoroughly mixed with the warm gelatin solution. After solidification, the feed was divided into tablets and stored at -20°C in the freezer. The feed was replaced every other day to minimize the oxidation and degradation of its ingredients.

Study design

Seventy, 12-week-old female LDLR-/- mice were allocated into five groups, 14 animals per group, all with similar body weight, plasma cholesterol and plasma triglyceride (TG) levels. The mice were fed with the specified supplementations for two weeks with a chow diet (pretreatment) and an additional 10 weeks with a high fat diet to induce atherosclerosis. The control group was fed a regular diet with no supplementations. The *Dunaliella* group was fed diets fortified with the algal powder. The 9CBC supplemented group (G-9CBC) was fed diets fortified with the purified 9CBC. The ATBC group (G-ATBC) was fed diets fortified with the synthetic ATBC, and the 9-*cis* retinoic acid group (G-9CRA) was fed a regular diet given every morning with 9-*cis* retinoic-acid dissolved in 100 μL PBS pH 8.5 (7.2 μg/mouse/day). Blood was drawn from each mouse after a 12 hour fast in the morning. After 12 weeks, the mice were killed by CO_2 inhalation and blood was drawn. After perfusion with PBS, the liver and the heart were removed for further analysis.

Lipid analysis

We used a colorimetric enzymatic procedure to measure total plasma cholesterol (CHOL, Roche/Hitachi, Roche Diagnostics) and TG (Triglyceride liquid, Senitinel).

Carotenoid analysis

β-carotene isomer levels in the plasma and in the liver were determined by HPLC, according to the method described by Shaish et al. [17]. Mass spectrometry (MS) analysis was taken in an Autoflex III Smartbeam (MALDI, Bruker) reflectron positive mode. The nuclear magnetic resonance (NMR) spectrum was taken in a Bruker Avance-III-700 instrument, in $CDCl_3$ containing TMS as the internal reference, at 300K.

Retinoic acid analysis

Concentrations of retinoic acids (RAs) were determined in the mouse plasma by the LC-MS method [18]. In summary, 100 μL of plasma was diluted with a threefold volume of isopropanol and was then vortexed for 10 seconds, put in an ultrasonic bath for 5 minutes, shaken for 6

minutes, and centrifuged at 13,000 rpm in a Heraeus BIOFUGE Fresco at 4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were re-suspended with 60 µL of methanol, vortexed, shaken, diluted with 40 µL of 60 mM aqueous ammonium acetate solution, transferred into the auto-sampler, and subsequently analyzed.

Assessment of atherosclerosis in the aortic sinus

Atherosclerotic fatty streak lesions were quantified by calculating the lesion areas in the aortic sinus [19].

Fast protein liquid chromatography analysis of lipoproteins

The plasma from five mice was pooled and the serum lipoproteins were separated by size exclusion chromatography using a superose-6 column (30 cm) on fast protein liquid chromatography [20].

Statistical analyses

One-way ANOVA was used was to compare the treatment effect on atherogenesis, and the post hoc Tukey method was used for multiple pairwise comparisons. Repeated measures ANOVA analysis was applied to compare changes in the weight gain between the treatment groups over the course of the study period. Significance was considered as $p<0.05$. The values in the text are means ± SE.

RESULTS:

Characterization of the 9CBC isolated from the alga *Dunaliella bardawil*

We first characterized the 9CBC-enriched fraction isolated from the *Dunaliella* extract. This preparation had the expected absorption spectrum of 9CBC with a maximum absorbance at 448 nm (Figure 1A). HPLC analysis showed that 90% of the total carotenoids in the preparation were 9CBC with a retention time of 17.5 minutes using the C18 HPLC column (Figure 1B).

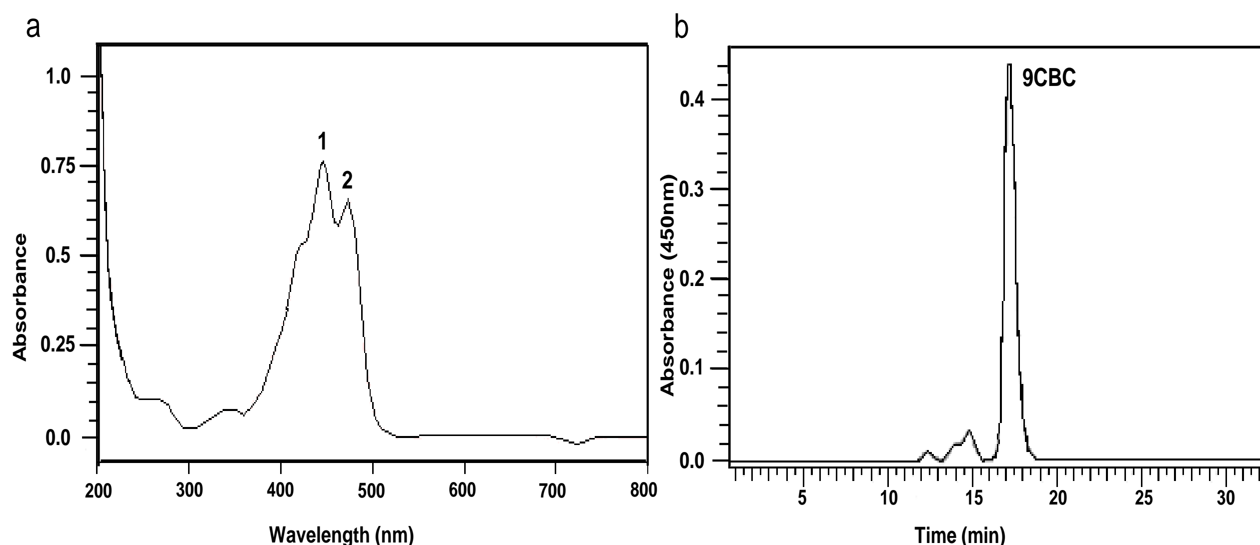


Figure 1. 9CBC isomer characterization.

Absorption spectrum of the 9CBC preparation, 1 – 448 nm, 2 – 474 nm (A), HPLC chromatogram with detection at 450 nm (B)

Mass spectrometry (MS) analysis revealed that the fraction corresponding to 9-*cis* β-carotene had the expected molecular mass of 536 m/z, and "Nuclear Magnetic Resonance" (NMR) analysis confirmed that the preparation did not contain a detectable amount of all-*trans* β-carotene (data not shown). To ensure suitability for oral administration, we mixed algal 9-*cis* with the mouse feed, as described in the Methods section, and measured its stability after two days at room temperature, while also exposing the feed to ambient light and ambient air. HPLC analysis showed that under these conditions 9CBC was stable, did not degrade, and was not converted to other isomers (Figure 2 A, B).

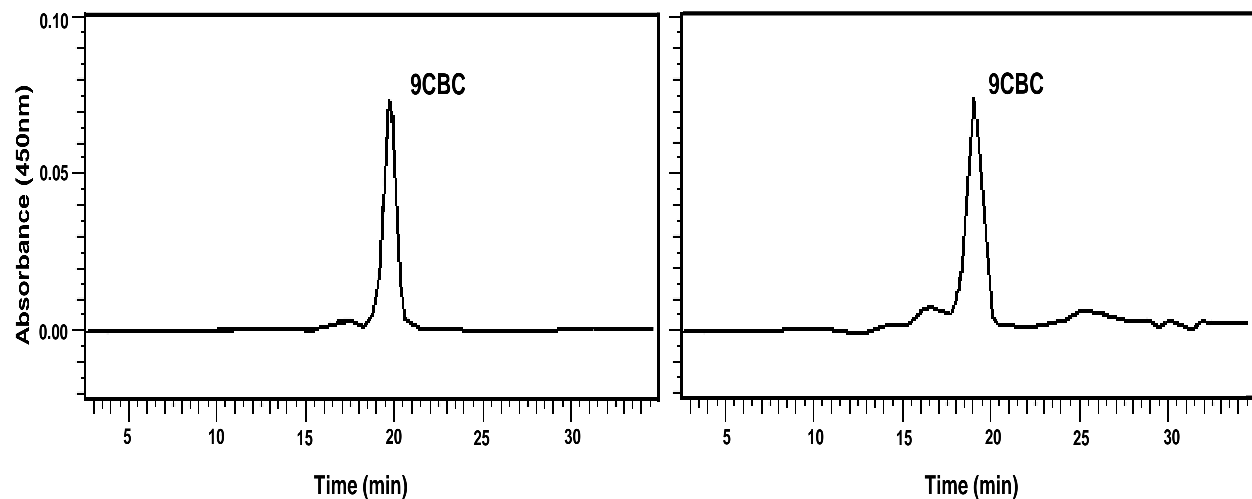


Figure 2. 9CBC was stable in the fortified feed.

HPLC chromatograms show 9CBC isomer analyzed from the G-9CBC group. Fresh feed (A) and after two days of exposure to light and air (B). Separation was conducted on C18 column with and detection at 450 nm.

Accumulation of AT- and 9C-BC in mouse liver

In the G-ATBC group, only ATBC was accumulated in the liver, while 9CBC was not detected (retention time ~11.5 minutes) (Figure 3C). Furthermore, small quantities of non-identified carotenoids were detected at a retention time of 13 minutes.

Liver samples of the G-9CBC group mainly accumulated 9CBC with a retention time of 14.5 minutes. A considerable amount of the other carotenoids was detected occurring at a retention time of 12 minutes. Despite a retention time close to that of ATBC, UV-visible spectra of the two carotenoids differed distinctively with the main absorbance peak of the non-identified carotenoid being at 440 nm (Figure 3B). It remains to be confirmed whether this non-identified carotenoid represents a metabolite of 9CBC or if it originated directly from the algal powder. In the liver of the *Dunaliella*-treated mice, we identified all three *Dunaliella* major carotenoids, i.e., ATBC, 9CBC, and to a lesser extent, α-carotene (Figure 3A). The ratio of 9CBC/ATBC in livers of mice from the *Dunaliell*a group was lower compared to this ratio in the feed. As expected, the carotenoids were under the detection limit in the liver samples of the control mice and the 9-*cis* retinoic acid-treated mice (Table 1). The data shows that orally administered 9CBC is efficiently absorbed and accumulates in the liver of the mice. The data also demonstrates that ATBC does not convert to 9CBC in liver samples in this animal model.

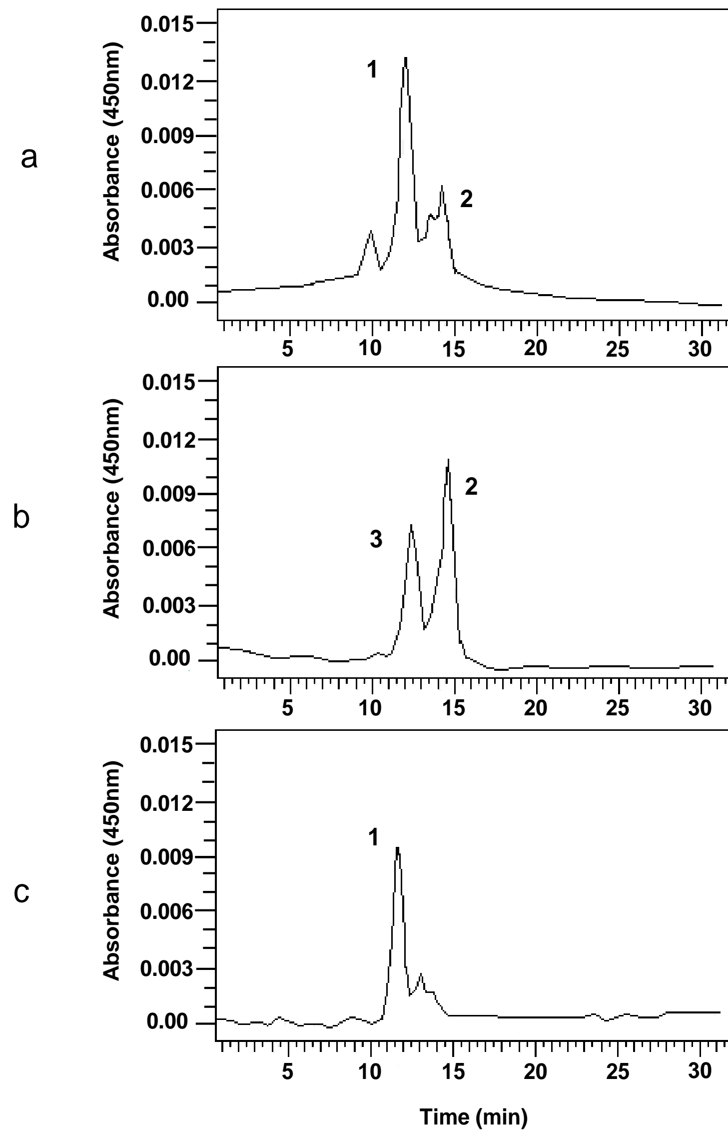


Figure 3. 9C- and AT-BC in mouse liver

HPLC chromatograms show β-carotene isomers in mouse liver. Liver carotenoids were extracted following 12 weeks of treatments. *Dunaliella* (A), G-9CBC (B), and G-ATBC (C). 1 – ATBC, 2 – 9CBC, 3 – non-identified carotenoid. Separation was conducted on C18 column with detection at 450 nm. 9CBC-treated group, G-9CBC; ATBC-treated group, G-ATBC.

Table 1. 9CBC and ATBC concentrations in mouse liver.

Liver carotenoids were extracted following 12 weeks of treatments. Values are means ± SE, n=7.

Group	9CBC	ATBC	9C- / AT-BC
		------- µg/g liver -------	
Control	0	0	-
Dunaliella	0.38±0.09	0.7±0.16	0.53
G-9CBC*	0.48±0.2	0	-
G-ATBC**	0	0.63±0.2	-

*G-9CBC: 9CBC-treated group

**G-ATBC: ATBC-treated group

Dietary carotenoids do not increase plasma levels of retinol or retinoic acid

The plasma levels of retinol and retinoic acid were measured by LC-MS/MS. The plasma levels of retinol were similar in all the examined groups and the all-*trans* retinoic acid levels were comparable in all the groups (Figure 4A, B). Interestingly, 9-*cis* retinoic acid was not detected (detection limit of 0.1 ng/ml) [18] in the plasma of any of the groups, including mice treated with 9-*cis* retinoic acid.

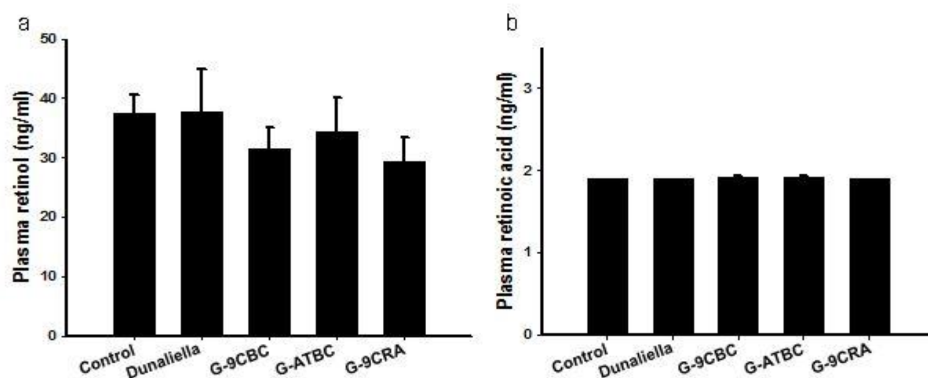
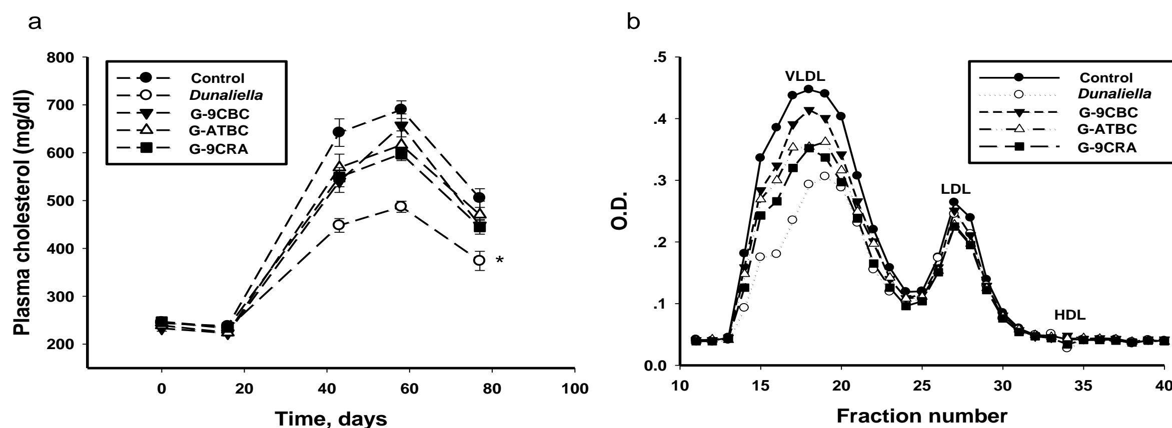


Figure 4. Plasma levels of retinol and ATRA

Plasma retinol levels (A) and all-*trans* retinoic acid levels (B) in ng/mL. Retinol and all-*trans* retinoic acid were analyzed following 12 weeks of treatments. Values are means ±SE, n=5. 9CBC-treated group, G-9CBC; ATBC-treated group, G-ATBC, 9-*cis* retinoic acid-treated group, G-9CRA.

9CBC did not prevent plasma cholesterol elevation in Western diet-fed mice

The plasma cholesterol and TG levels were measured every two-to-three weeks throughout the experiments (Figure 5 A). As anticipated, *Dunaliella* retarded Western diet-induced plasma cholesterol elevation; however, 9CBC did not reverse this elevation. The ATBC and 9-*cis* retinoic acid administration had no effect on the plasma cholesterol levels, and all of the treatments had no effect on the plasma TG levels (data not shown). Lipoprotein separation



showed a decrease in VLDL cholesterol content in the *Dunaliella* treated group versus control while no effect was seen in the 9CBC treated group (Figure 5 b).

Figure 5. Plasma cholesterol concentrations

Mice were fed a chow diet for two weeks following 10 weeks of a Western diet fortified with β-carotene isomers. Plasma cholesterol levels (A) and lipoprotein separation (B). Values are means ± SE, n=13-14. * $P<0.01$, *Dunaliella versus* Control. 9CBC-treated group, G-9CBC; ATBC-treated group, G-ATBC; 9-*cis* retinoic acid-treated group, G-9CRA.

9CBC and *Dunaliella* powder inhibited atherogenesis in LDLR-/- mice

We determined the effect of 9CBC on the atherosclerotic lesion area in the aortic sinus. The 9CBC treatment was associated with a 39% reduction (Figure 6A, B) in the lesion area compared to the control group (384,906 μm^2 vs. 623,357 μm^2). A comparable decrease was observed after treatment with 9-*cis* retinoic acid or *Dunaliella* (36% and 55% compared to the control, respectively, p<0.05), while the ATBC supplementation did not reduce the lesion area.

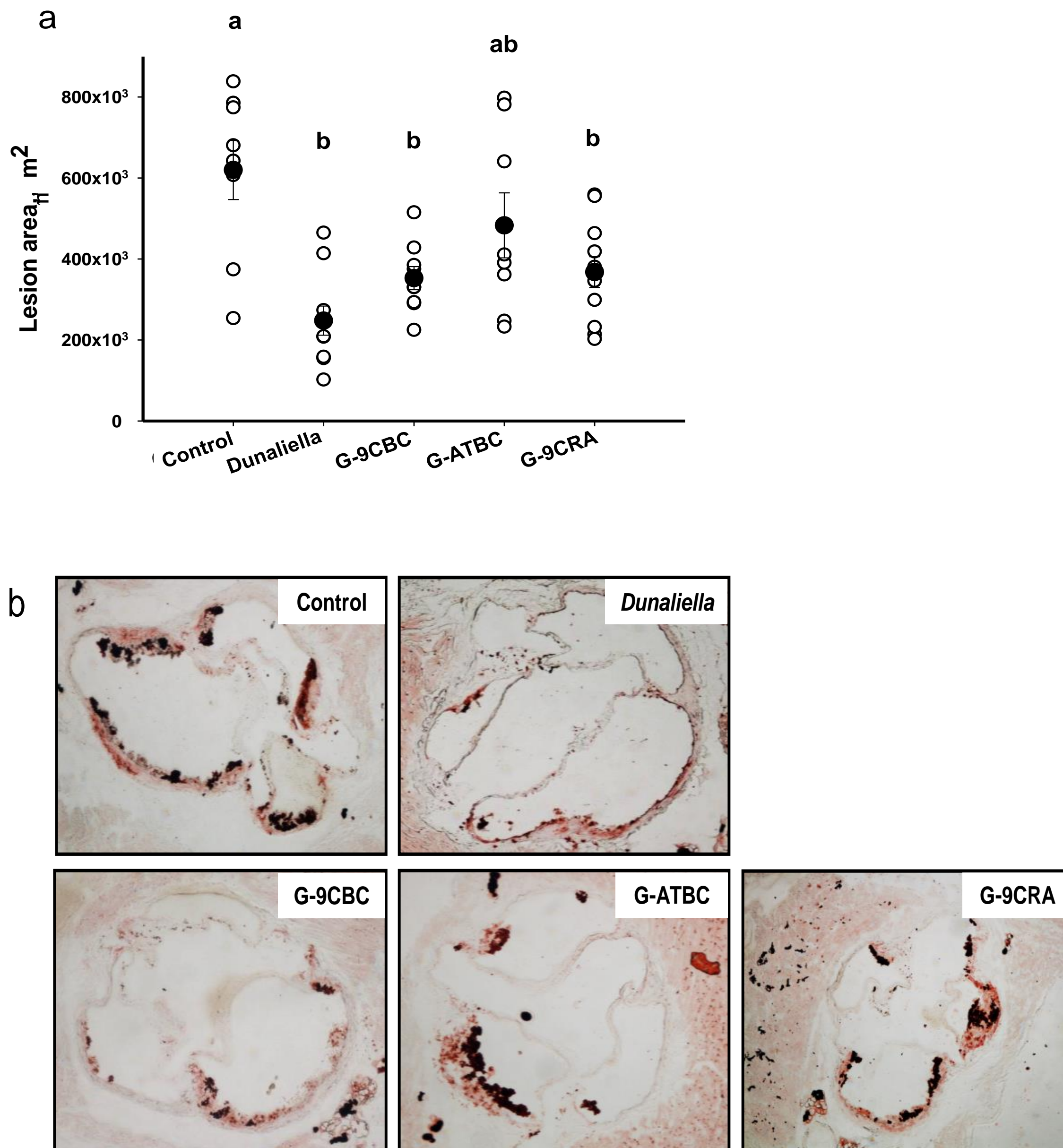


Figure 6. 9CBC, *Dunaliella*, and 9-*cis* retinoic acid supplementation reduced atherosclerotic lesion area.

Lesions were quantified after 12 weeks of treatment (A), one representative aortic sinus lesion section is shown for each treatment group (magnification X40) (B). Values are means ± SE, n=8-12. The notations, [a,b], within the graph means without a common letter difference, $P < 0.05$. 9CBC-treated group, G-9CBC; ATBC-treated group, G-ATBC; 9-*cis* retinoic acid-treated group, G-9CRA.

DISCUSSION:

This study investigated the effect of purified 9CBC on atherosclerosis in female LDLR-/- mice. To the best of our knowledge, this is the first study demonstrating that highly purified 9CBC from natural origin inhibits atherosclerosis in a mouse model.

In previous studies, we revealed that 9CBC-rich powder of *Dunaliella* inhibited atherogenesis and fatty liver formation in LDLR-/- mice [13]. It also inhibited atherosclerosis development in apoE-/- mice [14]. We further demonstrated that a powder containing a high ratio of 9C- to AT-BC is required to achieve this favorable effect [13]. These results led to our hypothesis that 9CBC is the main component in the algal powder responsible for these favorable effects, and that the potential conversion to all-*trans* and 9-*cis* retinoic acids may be the key for the mediation of these effects. In our current study, we used 90% pure 9CBC to test this hypothesis and we found that 9CBC potently inhibited atherogenesis in female LDLR-/- mice. However, it was considered a slightly less potent compared to whole *Dunaliella* powder that contained both isomers, although this difference did not have significant statistical results when using same molar amounts administered. It is worth noting that *Dunaliella* powder also contains other carotenoids, such as zeaxanthin and lutein. [21] It also contains low levels of phytoene and phytofluene, in addition to the two β-carotene isomers. We assume that the presence of other carotenoids besides 9CBC or other derivatives present in algal powder (in combination with 9CBC in the algal powder) led to the apparently enhanced effect of the whole algal powder.

It is important to note that the purified 9CBC inhibited atherogenesis, but did not inhibit the high-fat diet-induced plasma cholesterol elevation. On the other hand, *Dunaliella* powder affected both parameters in this work and in our previous studies [13]. Because *Dunaliella* powder contains, as mentioned above, additional carotenoids, we can assume that 9-cis should work with these carotenoids to achieve the cholesterol lowering effect. Alternatively, other ingredients in the algal powder, such as alpha-linoleic acid, which was previously reported to lower plasma cholesterol levels [22], might be responsible for this effect.

In order to determine the effects of consuming carotenoid-rich vegetables on the active vitamin A metabolite ATRA, there was a human trial which measured ATRA concentrations after different vegetables consumption. The study showed that consumption of carrot juice, which contained high concentrations of the pro-vitamin A carotenoid β-carotene, resulted in strong, significantly increased plasma concentrations of all-*trans* retinoic acid [23]. However, study with the isolated 9CBC has not been performed. Whether 9CBC is simply absorbed or is further isomerized to ATBC and/or other carotenoid-isomers or further metabolites is a topic of ongoing debate [24-27]. In contrast to humans, mouse plasma does not contain detectable amounts of carotenoids. Therefore, we measured carotenoid levels in the liver of the various supplementation groups. In the control mice, the liver carotenoids were below the detectable levels. 9CBC, but not ATBC, was detected in the livers of the G-9CBC animals. Also, ATBC, but not 9CBC, was detected in the livers of G-ATBC mice. In disagreement with previous reports, these results suggest that 9-*cis*-isomers and all-*trans*-isomers are not inter-convertible in the murine organism. In the G-9CBC group, in addition to 9CBC, we detected a previously non-reported carotenoid with an absorbance maximum at 440 nm. We assumed that this carotenoid is a metabolite of 9CBC. In the G-ATBC group, we also detected an additional carotenoid with an absorbance spectrum similar to that of 15-*cis* β-carotene. This peak can also be detected in the

synthetic ATBC preparations, but its relative levels were higher in the liver as compared to the levels in the feed. Whether this isomer has a beneficial or harmful effect on lipid metabolism and atherosclerosis remains to be confirmed.

We expected enrichment of the feed with isolated 9CBC to increase the plasma levels of retinol and retinoic acid. However, LC-MS/MS analysis showed that the plasma retinol levels were similar in all groups. These results contrast previous studies in hens and rats, which are known to absorb carotenoids well; those studies showed that supplementation of β-carotene caused significant elevations of plasma retinol [28, 29]. Furthermore, the plasma levels of ATRA were also similar in all the examined groups. Additionally, regardless of the high 9CBC content in the feed of the G-9CBC and *Dunaliella* groups, 9-*cis* retinoic acid could not be detected. These results do not support our initial hypothesis that 9CBC affects atherosclerosis via conversion to retinoic acids, but we cannot rule out the possibility that in the target organ the levels of retinoic acids may be increased and that other known or unknown bioactive retinoids are involved.

This work examined, for the first time, the effect of isolated, highly purified 9CBC on atherogenesis in a mouse model. These results suggest that 9CBC is the main active compound from alga for atherogenesis prevention. We suggest that additional factors in the algal powder are required to achieve optimized efficiency in inhibiting atherogenesis. The mode of action of 9CBC as anti-atherogenic agent is beyond the scope of the present study. Since 9CBC is not found in the plasma, it is reasonable to assume that it does not work as an antioxidant in LDL or other plasma lipoproteins. As retinoids confer anti-inflammatory effects in many biological systems [30], 9CBC has the potential to affect, via its conversion to retinoids, inflammation as well. Future studies in our laboratory will focus on 9CBC-induced effects on biological processes related to atherogenesis, which have been shown to be affected by retinoids, such as the formation of foam cells and reverse cholesterol transport from macrophages [31]. Additional studies will focus on 9CBC-metabolism to further bioactive retinoids and activation of RXR-mediated signaling.

Abbreviations: 9CBC, 9-*cis* β-carotene; ATBC, all-*trans* β-carotene; LDLR-/-, LDL receptor knockout mice.

Author's Contributions: All authors contributed to this study.

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