

## Beta-Carotene Attenuates Experimentally Induced Liver Cirrhosis in Rats

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### Abstract

**Objective:** To study whether retinolpalmitate, beta-carotene or lycopene could prevent liver cirrhosis induced by thioacetamide in rats.

**Methods:** In the control group liver cirrhosis was induced in male Wistar rats by intraperitoneal injections of TAA 200 mg/kg for 12 weeks. The three study groups received in addition to TAA either beta-carotene, lycopene or retinolpalmitate by gavage through an orogastric tube. Histopathological analysis and determination of the hydroxyproline contents of the livers were performed at the end of the protocol.

**Results:** Rats treated with beta-carotene and TAA had lower histopathologic scores and reduced levels of hepatic hydroxyproline ( $P=0.02$ ) than those treated by TAA alone. A trend of decreased fibrosis was observed in the rats treated with lycopene and TAA although this lacked statistical significance.

**Conclusions:** Beta-carotene attenuated liver cirrhosis induced by TAA in rats. The mechanism may be related to effects on hepatic stellate cells or to scavenging of free radicals by beta-carotene. Retinolpalmitate and lycopene had no significant beneficial effect.

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Hepatic stellate cell activation is a crucial step in fibrogenesis of the liver. This leads to differentiation of the HSC to a myofibroblast-like cell that may secrete a variety of extracellular matrix components. The factors responsible for HSC activation are still incompletely understood. Various cytokines such as transforming growth factor beta-1 and platelet-derived growth factor are thought to be involved [1].

Conflicting data have been reported regarding the role of retinoids in HSC activation in the liver. Normally these cells store vitamin A in the liver, but following liver injury the

vitamin A content drops. For example, hepatic vitamin A depletion has been detected in alcoholic liver injury [2]. Also, the hepatotoxin CCl<sub>4</sub> induces liver fibrosis more effectively in rats when the vitamin A content is reduced [3]. On the other hand, a significant increase in liver vitamin A may result in hepatic fibrosis in situations of vitamin A toxicity [4]. Retinol and retinoic acid have been found to inhibit HSC activation *in vitro* [5,6]. In contrast, β-carotene, one of the major biologically active carotenoids, decreases hepatic fibrosis when given orally together with CCl<sub>4</sub> [7]. It has been suggested that β-carotene exerts its beneficial effect by maintaining liver vitamin A concentration and thereby decreasing collagen synthesis by HSC. β-carotene is thought to function also as a scavenger of the free radicals produced by CCl<sub>4</sub> [3]. However, retinoids were recently found by others to exacerbate experimental liver fibrosis in rats by activating latent TGF-β [8]. β-carotene is transformed in part to retinol primarily in the intestinal mucosa [9]. In contrast to retinol, β-carotene is considered to be non-toxic even in high doses [10].

The aim of our study was to examine the effect of retinol palmitate and two carotenoids – beta-carotene and lycopene – on the development of liver fibrosis in an experimental model of cirrhosis induced by the hepatotoxin thioacetamide.

### Materials and Methods

#### Animals and Materials

Male Wistar rats (250–300 g) obtained from Tel Aviv University's Animal Breeding Center were kept in the animal breeding house of the Wolfson Medical Center and fed Purina rodent chow *ad libitum* [10]. Animals were treated according to institutional guidelines based on the principles of the Declaration of Helsinki. Beta-carotene, lycopene, retinol palmitate, thioacetamide and glycerol were purchased from Sigma Chemical Co., St. Louis, MO, USA.

#### Induction of liver cirrhosis

Liver cirrhosis was induced in rats by intraperitoneal injection of TAA, 200 mg/kg, twice a week for 12 weeks as previously described [11].

TAA = thioacetamide  
HSC = hepatic stellate cells

TGF-β = transforming growth factor-beta

## Experimental design

TAA was dissolved in 0.9% NaCl and a volume of 2 ml was injected intraperitoneally at a dosage of 200 mg/kg/rat twice weekly. Six groups of five rats each were treated as follows: One group received only TAA for 12 weeks. Four groups received TAA and either  $\beta$ -carotene 75 mg/rat, retinol palmitate 5 mg/rat, or lycopene 75 mg/rat, diluted in glycerol. All the compounds were given in a volume of 1.5 ml by gavage through an orogastric tube. The sixth group of five rats served as a control and received only 1.5 ml glycerol orally using the same tube. All the rats received the above mentioned substances for 12 weeks.

## Analysis of liver histopathology

The rats were sacrificed at the completion of the treatment protocols, their livers were removed and midsections of the left lobes of the livers were processed for light microscopy. This processing consisted of fixing the specimens in a 5% neutral formol solution, embedding the specimens in paraffin, cutting sections of 5  $\mu$ m thickness and staining the sections with hematoxylin and eosin and Masson trichrome. This staining facilitates accurate assessment of the degree of hepatic fibrosis. The tissue slices were scanned and scored semiquantitatively by two expert pathologists who were not aware which sample belonged to which group. The degree of inflammation and fibrosis was expressed as the mean of 10 different fields in each slide, which had been classified on a scale of 0–3 according to Muller et al. [12]. Pathological alterations consistent with fibrosis, enlargement, inflammatory infiltration and breaking up of the hepatocellular limiting plates were observed in the portal tracts. Changes observed in the intra-acinar mesenchyma included diffuse or spotty inflammation, nodule formation, spotty fibrosis and the presence of fibrotic septa.

## Hydroxyproline content in the liver

Small samples of liver tissue were hydrolyzed by adding HCl to a final concentration of 6 N. The samples were sealed in small Pyrex test tubes and hydrolyzed for 3 hours at 130°C. The tubes were then opened and washed thoroughly with water and the washings were combined with the hydrolyzate. Several drops of the methyl red indicator were added followed by the theoretical amount of 2.5 N NaOH required for neutralization. Final adjustments were made with diluted HCl and NaOH until the indicator turned slightly yellow, corresponding to pH 6–7. The concentrations of NaCl in the final dilutions did not exceed 0.4 M as required. Samples of 2 ml were then oxidized by adding 1 ml chloramine T (Sodium p-Toluenesulfonylchloramide). After 20 min of mixing and shaking the test tubes 1 ml perchloric acid was added to each tube. After 5 min 1 ml p-dimethylamino-benzaldehyde solution was added and the mixture was shaken for 5 min. The tubes were placed in a 60°C water bath for 20 min and then cooled in tap water for 5 min. The absorbance of the solutions was determined spectrophotometrically at 557 nm. The hydroxyproline values were then determined directly from the standard curve [13].

## Statistical analysis

The data are presented as means  $\pm$  SD. The significance of differences among different groups was determined by ANOVA followed by a post-hoc test.

## Results

Intraperitoneal injections of TAA for 12 weeks resulted in a uniform nodularity of the surface of the rat livers [Table 1]. Microscopic analysis revealed cirrhosis-like structural patterns characterized by mixed-sized fibrotic nodules [Figure 1A]. The oral administration of  $\beta$ -carotene dissolved in glycerol for 12 weeks reduced the degree of liver fibrosis [Figure 1B]. Microscopic analysis showed a mean degree of fibrosis of  $1.7 \pm 0.9$  in the TAA +  $\beta$ -carotene group compared to  $3.0 \pm 0.7$  in the group of TAA alone. The hydroxyproline content (mg/g protein) was  $6.2 \pm 1.0$  in the  $\beta$ -carotene group versus  $7.7 \pm 0.9$  in the TAA-alone group. These differences in the hydroxyproline contents were statistically significant ( $P=0.02$ ). In the TAA + lycopene group both the mean fibrosis score and the mean hydroxyproline content ( $2.1 \pm 0.6$  versus  $3.0 \pm 0.7$  and  $6.8 \pm 1.6$  vs.  $7.7 \pm 0.9$  mg/g protein, respectively) were lower than in the TAA-alone group, but this difference was not statistically significant ( $P=0.25$ ).

Retinol palmitate had no beneficial effect in reducing fibrosis in our model, but in contrast to a recent report [8] it did not exacerbate liver fibrosis, as judged by the mean hydroxyproline content of the rats livers. All rats in the control group, which received the same amount of glycerol alone, had normal livers microscopically and the mean hydroxyproline content in those livers was  $2.0 \pm 0.4$  mg/g protein.

## Discussion

In the present work, we studied the effect of retinolpalmitate and two carotenoids on the development of TAA-induced liver fibrosis in rats.  $\beta$ -carotene, in addition to having the strongest provitamin A activity of all carotenoids, is an efficient scavenger of peroxy radicals, especially in low oxygen tension [14]. Recently, Okuno et al. [8] found that retinoids exacerbate liver fibrosis by activating latent TGF- $\beta$  in hepatic stellate cells. We also found that retinolpalmitate did not exert any beneficial

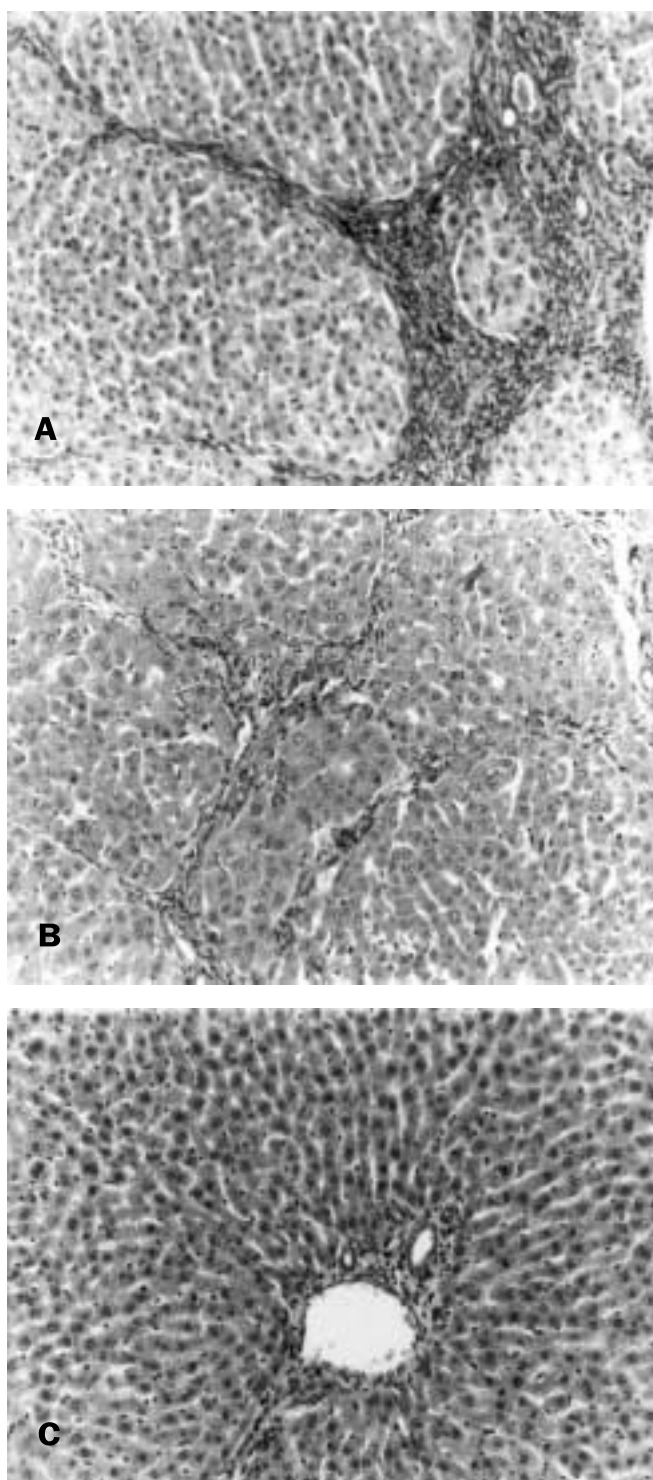
**Table 1.** Effects of retinolpalmitate, beta-carotene and lycopene on TAA-induced liver cirrhosis

Compound	Hydroxyproline (mg/g protein)	Fibrosis (0-3) ***
Thioacetamide	$7.7 \pm 0.9$	$3.0 \pm 0.7$
Glycerol	$2.0 \pm 0.4$	0
Retinolpalmitate	$7.3 \pm 1.4$	$3.0 \pm 0.6$
Beta-carotene	$6.2 \pm 1.0^*$	$1.7 \pm 0.9$
Lycopene	$6.8 \pm 1.6^{**}$	$2.1 \pm 0.6$

\*  $P=0.02$  compared to TAA.

\*\*  $P=0.25$  compared to TAA. Mean  $\pm$  SD, n=5.

\*\*\* Fibrosis score: 0=no change, 1=mild changes, 2=moderate changes, 3=severe changes.



**Figure 1.** Effects of TAA and beta-carotene on the histopathology of the rat liver. (hematoxylin & eosin, x 80.) **[A]** Liver section from a rat that received TAA for 12 weeks. Note the cirrhosis with mixed sized nodules and fibrotic septa. **[B]** Liver section from a rat that received TAA + beta-carotene for 12 weeks. Note less nodular formation and fibrotic septa. **[C]** Liver section from a rat that received oral doses of glycerol only for 12 weeks (control group).

effect in the experimental model of cirrhosis induced by TAA. However, our results suggest that  $\beta$ -carotene attenuated liver fibrosis in this model. The second carotenoid, lycopene, showed a trend of decreasing fibrosis and hydroxyproline content in the liver, although it did not reach statistical significance.

About 90% of the total vitamin A is stored in the liver, with the kidney and the adrenal gland serving as minor storage sites [15]. The HSC serves as the primary storage of retinoids in the liver, and about 88% of total hepatic vitamin A content is found within these cells [16]. Activation of HSC changes them into myofibroblast-like cells expressing cytokine receptors for TGF- $\beta$  and platelet-derived growth factor. In response to these cytokines, the activated HSC proliferate and deposit matrix components into the extracellular space [17,18]. Along with the development of liver fibrosis, HSC lose their retinol droplets [19]. Therefore, the vitamin A level in the liver may be decreased in most forms of liver injury [1]. Paradoxically, prolonged intake of even therapeutic doses of vitamin A in humans can cause hepatic fibrosis [4]. The exact role of vitamin A in HSC activation and the mechanism of vitamin A-induced hepatic toxicity are still obscure.

Lycopene suppresses cell proliferation as evidenced by thymidine incorporation assays [20]. Both carotenoids are capable of inducing intercellular communication via gap junctions, which has been associated with inhibition of transformed cell proliferation [20]. Yet, the ability of lycopene to increase gap junctional communication is less pronounced [21]. Another possible explanation of the favorable effect of  $\beta$ -carotene and lycopene in attenuating hepatic fibrosis is their strong interaction with reactive oxygen species [21]. Free oxygen radicals can cause tissue damage by reacting with polyunsaturated fatty acids in cell membrane, nucleotides and critical sulfhydryl bonds in proteins. The extent of tissue damage depends on available antioxidants such as tocopherol, ascorbic acid, beta-carotene, glutathione, selenium and superoxide dismutase [22]. Oxidative stress plays an important role also in HSC activation as implied by the induction of c-myc expression and NF kappa B activity. This activation could be prevented by certain antioxidants [23]. Moreover, lipid peroxidation increases collagen alpha 1(I) gene expression by HSC in liver cirrhosis induced by  $\text{CCl}_4$  in rats [24]. Inflammation and resulting lipid peroxidation have been suggested to activate the HSC [25,26]. If so, substances with potent antioxidant properties such as lycopene might prevent HSC activation. This possibility should be explored using carotenoids in isolated HSC, and may be a subject of future studies

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