Oral Supplementation of β-Carotene Significantly Ameliorates Testicular Oxidative Stress in the Streptozotocin-Diabetic Rat

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Abstract

**Background:** Our recent findings have shown that the rat testis is subjected to significant oxidative stress during the early phase of diabetes induced by Streptozotocin (STZ). In the present study, we have investigated whether oral supplementation of β-carotene (BC) to pubertal rats would provide protection against diabetes associated oxidative stress in testis and liver.

**Materials and Methods:** Male (6 wk old) rats were rendered diabetic by an acute dose of Streptozotocin (60 mg/kg bw) and were given oral BC supplements (20 mg/kg bw/d on alternate days) for 4 weeks. The modulatory potency of BC was assessed by determination of selected markers of oxidative stress in testis and liver.

**Results:** The testis of STZ-administered rats exhibited significantly elevated status of lipid peroxidation (cytosol and mitochondria) and increased ROS production compared to the non-diabetic controls. Oral supplements of BC completely normalized the oxidative damage in testis. Further, STZ-induced depletion of reduced glutathione (GSH) and elevated protein carbonyl content in testis were also restored to normalcy. The protective effects of BC in testis were also discernible in terms of restoration of activities of various antioxidant enzymes in diabetic rats. Furthermore, STZ-induced oxidative impairments in liver were also abrogated significantly by BC treatment. STZ-induced perturbations in serum and testicular lipid profiles in diabetic rats were also attenuated by BC treatment.

**Conclusion:** Collectively, our data indicate that oral supplementation of β-carotene can significantly mitigate the diabetes associated oxidative impairments in testis as well as in liver and suggest its efficacy as a complementary therapeutic agent in the management of diabetes associated oxidative stress mediated complications.

**Keywords:** β-Carotene, Diabetes, Oxidative Stress, Amelioration

Introduction

Involvement of oxidative stress (OS) and its role in the development of various diabetic complications is well known (1). Enhanced cellular OS and altered antioxidant pool have been implicated under both clinical and experimental type-I diabetes mellitus (2–5). There is evidence for multiple pathways of increased generation of reactive oxygen species (ROS) in diabetes, which may alter several redox sensitive genes and or cellular signaling pathways (5, 6). Consistent with a role for oxidative mechanisms in the pathogenesis of diabetic complications in vivo, numerous antioxidant dietary supplements such as vitamin E, C, n-acetyl cysteine, oxerutin, taurine, α-lipoic acid have been demonstrated to attenuate OS in experimental models of diabetes (7).

It is also well known that OS mechanisms are involved in xenobiotic-induced testicular dysfunctions which may lead to male infertility (8–11). Further, the free radical theory of male infertility (12) emphasizes the significant role played by OS mechanisms in the development of male infertility. Earlier, few studies have recognized the potential advantages of antioxidant therapy on human reproductive functions (13–14). Recently the role of antioxidants in the treatment of male infertility in humans was reviewed (15). Interestingly, improvement in sperm quality in smokers following oral doses of vitamin C (16) and improvements in sperm motility in asthenozoospermic subjects by oral doses of vitamin E (17) have been reported. Further, reduced glutathione (GSH) treatment protected against lipid peroxidation (LPO) and dyspermia in human spermatozoa (18).
The participation of OS mechanisms in the development of various diabetic complications is well known (1, 5). However, the involvement of OS mechanisms and their contribution to the development of testicular dysfunctions is beginning to be understood only recently (19, 20). Earlier, we have demonstrated that testis is indeed subjected to significant oxidative stress both during the acute and progressive phase of diabetes in a streptozotocin-induced diabetic model in adult rats and mice (21, 22). Recently, we have shown the efficacy of the oral supplementation of Ferulic acid, a phenolic antioxidant to ameliorate oxidative stress in testis and liver of diabetic pubertal rats (23). Hence, it was hypothesized that if oxidative stress mechanisms participate in male reproductive dysfunctions under diabetic situations, antioxidants are likely to play a significant role in the amelioration of oxidative damage in the testis milieu. Accordingly, the objective of the present study was to investigate the possibility of modulating diabetes associated testicular oxidative stress phenomenon employing oral supplementation of β-carotene (BC), a carotenoid, whose role in spermatogenesis is well known (24). For this purpose, growing rats (6 wk old) were rendered diabetic by Streptozotocin and the potential of BC to ameliorate oxidative stress in testis was assessed after one month.

**Materials and Methods**

**Chemicals**

Streptozotocin (STZ), thiobarbituric acid (TBA), 1, 1, 3, 3-tetramethoxypropane, 2', 7'-dichlorofluorescein diacetate (DCFH-DA), β-carotene and other fine chemicals were obtained from M/s Sigma Chemical Co., St Louis, USA. All other chemicals were of analytical grade.

**Animals and care**

Growing male rats (6 week old, CFT-Wistar strain) were randomly drawn from the stock colony of our 'Institute Animal House facility'. Rats were individually housed in polypropylene cages kept on racks built of slotted angles and kept in a controlled atmosphere with a temperature range of 25±3°C and mean relative humidity of 50±5%. The animals were maintained on commercial rat pellets (Gold Mohur, supplied by M/s Lipton India Pvt. Ltd., Mumbai, India) ad libitum and had free access to water during a week of acclimatization and throughout. All procedures with animals were conducted strictly in accordance with guidelines approved by the local "Institute Animal Ethical Committee" regulated by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, India. During the experiments, maximum care was taken to minimize animal suffering and in addition, the number of rats used was kept at a minimum.

**Experimental design and methodology**

We have employed pubertal rats for this study, since recent epidemiological findings have documented increased incidence of type II diabetes among children and adolescent males in various parts of the world (25,26). Diabetes was induced by a single intraperitoneal (i.p) injection of a buffered solution (0.1 mol/L of citrate, pH 4.5) of STZ at a dosage of 60mg/kg bw. Our preliminary studies had indicated that this dose induced significant hyperglycemia (blood glucose>350 mg/dl) (range 350-700) in young male rats measured at 72h post–treatment. Rats administered with citrate buffer served as the 'non diabetic controls'. They were randomly assigned into four groups (n=6) as follows: Group I- non diabetic control; Group II- β-Carotene control (BC, 20mg/kg bw /d); Group III- STZ (60mg/kgbw); and Group IV- STZ (60mg/kgbw) plus β-Carotene (20mg/kg bw /d). The criterion of selection of BC dose was based on our own pilot experiments using a range of doses and also taking in to consideration earlier published data (27). At the end of 4 weeks, all animals were killed by light ether anesthesia. Blood was drawn by cardiac puncture for separation of serum. Organs (liver and testis) were excised, rinsed in physiological saline, blotted, weighed and processed further for analysis. The plasma glucose level was estimated using a commercial kit based on Trinder’s method in which glucose oxidase (GOD) and peroxidase (POD) are used along with phenol and 4-aminoantipyrine (M/s Dr.Reddy’s laboratories, Hyderabad, India).

**Preparation of tissue homogenates and mitochondria**

Tissues (testes and liver) were excised and used for isolating cytosol and mitochondria by differential centrifugation as described earlier (28). Briefly, a 20% homogenate of the testis was prepared in ice-cold sucrose (0.25M) solution. The homogenate was centrifuged at 1000Xg for 10min at 4°C to obtain the nuclear pellet. Mitochondrial pellet was obtained by centrifuging the post-nuclear supernatant at 10,000Xg for 20min at 4°C. The pellet was washed thrice with 1.15% potassium chloride solution and finally suspended in sucrose solution.
Oxidative stress measurements in testis

Measurement of Lipid peroxidation
Lipid peroxidation was quantified in terms of malondialdehyde (MDA) equivalents (29). Briefly, 0.5mg of cytosolic protein was added to tubes containing 0.2 ml of 8% SDS, 1.5ml of 20% acetic acid pH 3.5 and 1.5ml of freshly prepared 0.8% thiobarbituric acid (TBA). The tubes were then kept in a boiling water bath for 45min. The MDA-TBA complex formed was extracted into n-butanol and measured spectrophotometrically at 532nm. Quantification was done using 1, 1, 3, 3-tetramethoxypropane as the standard and expressed as nmol MDA formed/mg protein.

Determination of ROS levels
ROS was determined spectrofluorometrically using the membrane-permeable fluorescent dye, 2', 7’–dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, 0.2mg cytosolic protein was suspended in locke’s buffer (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO3, 2.0mM CaCl2, 10mM D-glucose and 5mM HEPES. pH 7.4) and allowed to equilibrate to room temperature for 5min. DCFH-DA was then added to a final concentration of 10μM, mixed and allowed to stand for 15min at room temperature to allow the DCFH to be converted into fluorescent product DCF. Fluorescence was measured with excitation at 485nm and emission at 530nm. Background fluorescence was corrected by inclusion of parallel blanks. ROS production was quantified from DCF standard curve and results were expressed as pmol DCF formed/min/mg protein (30).

Antioxidant enzyme assays
The activity of Glutathione peroxidase was determined using the t-butyl hydroperoxide as the substrate (31) and the activity was expressed as μmoles of NADPH oxidized /min/mg protein (ε340=6.22mM/cm). Glutathione transferase was assayed (32) at 340nm by measuring the rate of enzyme catalyzed conjugation of reduced glutathione with 1-chloro-2,4-dinitro benzene (CDNB) and the activity was expressed as μmoles of 2, 4-dinitrophenyl glutathione formed/min/mg protein. Glutathione reductase was determined by monitoring the oxidation of NADPH at 340nm (33) and the activity is expressed as μmoles of GSSG reduced/min/mg protein. The protein concentration was determined using bovine serum albumin as the standard (34).

Glutathione and oxidative damage to proteins
To measure reduced GSH, testis samples were homogenized in phosphate -EDTA buffer (pH 8.0) and 25% HPO3, centrifuged at 4°C at 1,00,000Xg for 30min to obtain the supernatant. Measurement of reduced GSH was done according to the fluorimetric procedure using o-phthalaldehyde (35). Protein carbonyl content was quantified in supernatants obtained after centrifugation of tissue homogenates at 10,000Xg for 15min by measuring the hydrazone derivatives between 360 and 390nm (36).

Determination of lipid profile in serum and testis
Lipids from serum and testis were extracted following the procedure of Folch (37). Total cholesterol was estimated by the method of Searcy and Bergquist (38). The color intensity was measured by spectrophotometer at 540nm and samples were analyzed using the cholesterol reference standard. Phospholipids were analyzed by ferrous ammonium thiocyanate method using dipalmitoylphosphatidyl choline as reference standard (39). Following the phase separation, absorbance of chloroform phase was measured spectrophotometrically at 488nm. Triglycerides were estimated by Fletcher’s method (40) spectrophotometrically at 405nm using triolein as a reference standard.

Statistical analysis
Data are expressed as mean ± SD. Data were analyzed by ANOVA (SPSS 10.0, SPSS Inc., Chicago, IL) and a P value less than 0.05 was set as the minimum level of significance.

Results
Effects on growth, plasma glucose and testis weights
Data on the plasma glucose levels, absolute body weights and testicular weights of control and various treatment groups are presented in Table 1. Terminally, the average body weight gain among STZ administered rats was significantly lower (30g) compared to that of control rats (100g). The plasma glucose levels in STZ rats were significantly elevated (3.1 fold higher) compared with non-diabetic controls. Oral treatment with BC per se had no obvious effect on the blood glucose levels in non-diabetic rats. However, marginal (12%) lowering of blood glucose levels was evident in diabetic rats given oral supplements BC. Further, BC did not significantly affect the body weight gain of diabetic rats and also had no appreciable effect on the testicular weights.
Oxidative stress markers: lipid peroxidation and ROS levels

In general, oral supplements of BC to non-diabetic rats had no effect on both TBARS and ROS levels as they were comparable to those of non-diabetic controls. Induction of diabetes caused a significant increase in MDA as well as ROS levels in both testis and liver (Fig 1). In testis, STZ caused a marked (98%) elevation in MDA levels. BC supplements in diabetic rats markedly reduced (81%) the testicular MDA levels, while the degree of protection was only marginal (13%) in the liver (Fig 1).

**Fig 1:** Effect of oral supplementation of β-carotene on cytosolic MDA levels in testis and liver of growing male rats rendered diabetic by an acute dose of STZ. Values are mean±SD (n=6); Data analyzed by ANOVA; mean with different superscripts are significantly different from each other (α<0.05).

ROS levels in testis of diabetic rats were significantly enhanced (28%) compared with non-diabetic rats (Fig 2A). BC supplements resulted in complete normalization of testicular ROS levels (100% protection), while only a marginal decrease was evident in liver ROS levels. In mitochondria of testis, the ROS levels were markedly enhanced (63%) in STZ administered rats compared to non-diabetic controls (Fig 2B). However, BC treatment offered only a marginal protection in testis (17%) and liver (28%) against the diabetes-induced increase in ROS levels.

**Fig 2:** Effect of oral supplementation of β-carotene on testicular cytosolic (A) and mitochondrial (B) ROS levels in growing male rats rendered diabetic by an acute dose of STZ. Values are mean ± SD (n=6); Data analyzed by ANOVA; mean with different superscripts are significantly different from each other (α<0.05).

Effect on antioxidant enzyme activities

In non-diabetic rats, BC supplements did not appreciably alter the testicular Glutathione S transferase (GST) activity since the activities were comparable with those of controls (Fig 3). Further, the supplements had no effect on the STZ-induced...
increase (27%) in GST activity in testis. However, in liver BC treatment significantly normalized the STZ-induced changes in GST activity. Supplementation of non-diabetic rats with BC marginally (11%) enhanced the testicular Glutathione peroxidase (GPx) activity (Fig 4). While STZ-induced a significant decrease in testicular GPx activity, BC treatment significantly restored the GPx activity. Likewise, in non-diabetic rats, BC supplements did not substantially alter the testicular Glutathione reductase (GR) activity. The supplements significantly normalized the STZ-induced decrease (20%) in GR activity.

Modulation of reduced GSH
In general, BC supplements per se had no measurable effect on the testicular or liver GSH levels in non-diabetic rats. In diabetic rats, testicular GSH levels were significantly decreased compared to those of non-diabetic controls (Table 2). BC treatment in diabetic rats significantly offset the decrease in GSH levels in both testis and liver.

Modulation on protein carbonyls
BC supplements marginally decreased the protein carbonyl content in testis of non-diabetic rats. The carbonyl content was significantly elevated in testis and liver of diabetic rats (Table 2). Interestingly, BC treatments substantially reduced the carbonyl levels in testis (68%) and liver (42%) in diabetic rats.

Effects on lipid profile in serum and testis
The serum lipid profile of rats given oral supplements of BC to both non-diabetic and diabetic groups is presented in Table 3.

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**Table 2:** Effect of oral supplementation of β-carotene on reduced glutathione (GSH) and protein carbonyl in testis and liver of pubertal rats rendered diabetic by STZ

<table>
<thead>
<tr>
<th>Protein carbonyls</th>
<th>Testis</th>
<th>Liver</th>
<th>Testis</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>4.03±0.51</td>
<td>3.93±0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol carbonyls/mg protein)</td>
<td>4.33±0.54</td>
<td>4.20±0.58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD (n=6); Data analyzed by ANOVA; Mean with different superscripts are significantly different from each other (α≤0.05).

**Table 3:** Effect of oral supplementation of β-carotene on serum and testis lipid profile of pubertal rats rendered diabetic by an acute dose of STZ

<table>
<thead>
<tr>
<th>Group</th>
<th>CHL</th>
<th>TG</th>
<th>PL</th>
<th>CHL</th>
<th>TG</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>68.85±3.46</td>
<td>79.44±3.78</td>
<td>86.87±5.90</td>
<td>3.58±0.38</td>
<td>4.16±0.36</td>
<td>4.90±0.35</td>
</tr>
<tr>
<td>BC</td>
<td>45.67±6.45</td>
<td>57.08±4.56</td>
<td>84.75±6.71</td>
<td>3.35±0.31</td>
<td>4.46±0.45</td>
<td>4.44±0.46</td>
</tr>
<tr>
<td>STZ</td>
<td>81.97±3.80</td>
<td>94.29±3.33</td>
<td>115.9±0.37</td>
<td>4.32±0.44</td>
<td>8.25±0.53</td>
<td>5.39±0.38</td>
</tr>
<tr>
<td>STZ+BC</td>
<td>47.11±2.87</td>
<td>58.37±2.43</td>
<td>90.37±8.54</td>
<td>3.97±0.20</td>
<td>7.77±0.43</td>
<td>5.54±0.31</td>
</tr>
</tbody>
</table>

CHL: cholesterol; TG: triglyceride; PL: phospholipids (mg/g tissue); Values are mean ± SD (n=6); Data analyzed by ANOVA; Mean with different superscripts are significantly different from each other (α≤0.05).
In general, BC had a significant hypocholesterolemic effect (30%) in non-diabetic rats as well as diabetic rats. However, the serum of diabetic rats showed significantly higher (19%) cholesterol levels compared to non-diabetic group. In general, supplementation of BC dramatically lowered the elevated cholesterol levels in diabetic rats. Supplementation of BC per se reduced the triglyceride (TG) levels (25 and 28% respectively) compared to the non-diabetic rats. However, a significant increase (33%) was observed in the phospholipid (PL) levels among the diabetic rats (Table 3). The elevated PL levels were markedly (88%) reduced by oral supplementation of BC among the diabetic rats.

In testis, a marginal increase (21%) in the total cholesterol levels was evident in diabetic rats compared to those of non-diabetic controls. However, with BC treatment, the cholesterol levels of diabetic rats were significantly reduced (47%). Further, in diabetic rats, a significant increase (66%) in the triglyceride levels was observed compared to those of non-diabetic controls. With BC treatment, the triglyceride levels of diabetic rats were significantly lowered (41%). However, no significant alterations were evident in the testicular phospholipid levels among diabetic and non-diabetic animals with or without BC treatment.

**Discussion**

Currently, much attention is focused on the protective properties of naturally occurring antioxidants in biological systems (41). Our recent findings have adequately demonstrated the occurrence of oxidative impairments in the testis of adult rats/mice rendered diabetic by streptozotocin (21, 22). Since the oxidative impairments in the testis were consistent and progressive with progression of diabetes, we hypothesized that it may be amenable for modulation by antioxidant therapy. Hence in the present study, we chose to examine the status of oxidative damage markers in the testis (and liver) of pubertal rats which were provided with oral supplementation of BC on alternate days. Epidemiological evidence suggests that serum carotenoids are potent antioxidants and may play a protective role in the development of chronic diseases including cancers, cardiovascular disease and inflammatory diseases. Numerous workers have demonstrated the protective effects of BC against oxidative stress induced by gamma radiation (42) and various xenobiotics (43, 44) and STZ-induced diabetes (45). BC is shown to be an effective antioxidant not only against singlet oxygen (46) but also against lipid peroxidation and the highly destructive hydroxyl radical, which is implicated in many diseases such as cancer and heart disease (47). Since BC is a lipophilic substance, it exerts its action in hydrophobic environment such as the lipid core of membranes. In the present study, BC per se neither showed any hypoglycemic effect nor had any significant effect on the oxidative stress biomarkers among non-diabetic rats excepting for significant hypolipidemic activity. Interestingly, BC marginally reduced the protein carbonyl levels in both testis and liver of diabetic as well as non-diabetic rats. In diabetic rats, BC supplements offered varying degree of protection against STZ-induced testicular oxidative stress. While BC completely normalized the ROS levels in diabetic testis, it provided only 80% protection against lipid peroxidation. These findings are consistent with the basic concept that BC can significantly mitigate varying situations of oxidative stress conditions in vivo in general and diabetes induced oxidative stress (44, 45, 48, 49).

BC supplements per se had no significant effect on any oxidative stress biomarkers such as endogenous MDA, ROS and GSH levels in non-diabetic rats except for a significant hypocholesterolemic effect in testis among both non-diabetic and diabetic rats. While it completely decreased the MDA and ROS levels in testis cytosol to a normal range, the mitochondrial ROS levels were still significantly diminished. However, BC appeared to protect hepatic lipid peroxidation to a lesser degree, since only a marginal degree of protection was evident in terms of both MDA and ROS levels. Although the reason is not clear, it may be related to the fact that the degree of oxidative stress in the liver was more robust compared to the liver in the present study. Nevertheless, our major finding that BC alleviates testicular oxidative damage under diabetic situations is a new finding. Although the precise mechanism of action of BC in diabetic testis cannot be defined from this study, it is attributable to its antioxidant properties. Earlier, under experimentally–induced diabetic situations, numerous workers have reported protective effects of BC in somatic organs such as liver, kidney, heart and retina (45). Multiple mechanisms have been suggested to explain the protective action of BC in somatic organs under experimentally induced diabetic conditions which include: (I) the reversal of lipid peroxidation indicating lower levels of damage caused by free radicals, which is attributable to physical quenching of free radicals, (II) interaction with BC with GSH pathway and (III) possible enhancement of antioxidant defenses. In the present study, the ameliorative effects observed with BC...
on testicular oxidative damage could be speculated to involve any of the above three mechanisms. GSH and its oxidized counterpart GSSG constitute a major redox buffer system (50). GSH can act as a non-enzymic antioxidant by direct interaction of –SH group with ROS or it can be involved in the detoxification reaction for ROS, as a cofactor or coenzyme. In the present study, testis GSH levels in diabetic rats were significantly depleted indicating the importance of this non-enzymic antioxidant. Interestingly, BC supplements however, restored the GSH levels to normal endogenous levels. A similar trend was also evident in the hepatic tissue of diabetic rats suggesting that the protective ability of BC could be partly ascribed to this effect. Furthermore, in this study, the levels of testicular enzymes such as GR and GPX were also significantly reduced in diabetic rats. BC supplements however, markedly restored the GPX and the GR levels in testis of diabetic rats to normal levels suggesting the important role played by these redox enzymes under diabetes induced OS. It is quite apparent that the decreased activity of testicular GR may be an important factor for the depletion of GSH content in diabetic testis. Protein carbonyls are employed as useful biomarkers of ROS mediated protein oxidation (51) and elevated levels of oxidized proteins in animal tissues under various oxidative stress situations are documented (52). ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkage and oxidation of protein backbone resulting in protein fragmentation (53). Further, protein carbonyls are introduced into the protein by reaction with aldehydes such as MDA and 4-HNE which are end products of lipid peroxidation. In the present study, among diabetic rats both testis and liver showed higher levels of protein carbonyls suggesting high rate of protein oxidation. BC treatment in diabetic rats could substantially ameliorate the degree of protein carbonyl formation.

**Conclusion**

In conclusion, we have presented significant evidence which suggest that in STZ-diabetic rats, oral supplementation (on alternate days) of β-carotene has the potential to markedly mitigate oxidative stress in the testis and liver. The restoration of oxidative impairments in testis of diabetic rats supports our hypothesis that antioxidants such as β-carotene significantly attenuate diabetes induced oxidative damage in testis. However, further studies are required to identify the extent of protection provided by this antioxidant to different cell types of testis. Further, it may be worthwhile to investigate the protective effects of β-carotene in combination with phenolic acids (eg., ferulic acid) and other antioxidant vitamins (eg. Vitamin C and E) under diabetic induced oxidative stress in testis. Such studies are in progress in our laboratory.

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