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Pre-treatment with Beta Carotene Gives Protection Against Nephrotoxicity Induced by Bromobenzene via Modulation of Antioxidant System, Pro-inflammatory Cytokines and Pro-apoptotic Factors

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Abstract

Bromobenzene is an environmental toxin which causes hepatotoxicity, and the secondary metabolites on biotransformation cause nephrotoxicity. The objective of this study was to assess the alleviation of the nephrotoxic effect of bromobenzene by beta carotene in female Wistar albino rats. Beta carotene (10 mg/kg b.w.p.o.) was delivered orally to the rats for 9 days before bromobenzene (10 mM/kg b.w.p.o.) was intragastrically intubated. Kidney markers, antioxidant status and lipid peroxidation were evaluated. In addition, the levels of TNF-α, IL-6 and IL-1β were measured in serum and in kidney tissue homogenate using ELISA. Caspase, COX-2 and NF-κB were measured with the help of Western blotting. Histopathological analysis of the kidney was done for the control and experimental rats. Bromobenzene induction caused elevation in levels of creatinine, urea, uric acid, cytokines and lipid per oxidation along with deterioration in histological observations and antioxidant status. Pre-treatment with beta carotene significantly ($*p < 0.05$) normalised the levels of kidney markers and pro-inflammatory cytokines. It also reduced oxidative stress and lipid peroxidation, as shown by improved antioxidant status. The anti-apoptotic activity was evidenced by inhibition of protein expression of caspase, COX-2 and NF-κB. This significant reversal ($*p < 0.05$) of the above variations in comparison with the control group as noticed in the bromobenzene-administered rats demonstrates that beta carotene possesses promising nephroprotective effect through its antioxidant, anti-inflammatory and anti-apoptotic activity and therefore suggests its use as a potential therapeutic agent for protection from bromobenzene and hence environmental pollutant toxicity.

Keywords Nephrotoxicity · Bromobenzene · Beta carotene · Inflammatory · Apoptosis · Antioxidant

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Introduction

Kidney plays a crucial role in eliminating drugs as well as toxic environmental pollutants. The existence of numerous xenobiotic transporters and enzymes increased blood flow including concentration of metabolites during production of urine which are factors which make the kidney a sensitive organ during xenobiotic metabolism. The conjugation of toxic environmental pollutants to glutathione leads to a cascade of reactions culminating in renal dysfunction [1]. A variety of chronic and acute nephropathies arise due to the aftereffect of a miscellany of drugs which causes nephrotoxicity associated with injury to the tubulointerstitial section. Vascular and glomerular diseases induced by drug have been reported due to cellular or immune-mediated injury to endothelial or glomerular cells [2]. Chronic kidney disease might subsequently lead to tissue fibrosis which is caused by the release of chemokines and movement of inflammatory cells to the affected site which proceeds to the activation of fibroblasts for the production of extracellular matrix [3].

Bromobenzene (BB) has been valuable in understanding the mechanisms involved in drugs, and environment pollutants induced hepatotoxicity as well as nephrotoxicity. It is a solvent used in industry as an additive in motor oils and as a flame retardant, but was identified to cause necrosis in the kidney and liver [4]. Low concentrations of BB have been found in samples of water, food and air [5]. Biotransformation of BB occurs in the liver to enhance its excretion through urine, thus producing reactive metabolites which are hepatotoxic while the secondary metabolites of BB are nephrotoxic. The contributing factors to nephrotoxicity and oxidative stress are secondary metabolites of BB which include 2-bromohydroquinone, benzoquinone bromophenoisomers and 4-bromocatechol produced in the hepatic phase II reactions [6]. The large-scale exhaustion of mitochondrial and cytosolic glutathione during the metabolism of BB leads to an elevation in reactive oxygen within mitochondria [7]. One plausible protective plan would be to control the oxidative damage to mitochondria by augmenting tissue mitochondria with antioxidants [8, 9]. The protective effect of many plant extracts against xenobiotic toxicity has been reported to be due to its capacity to increase the reduced glutathione levels [10], detoxification of ROS by non-enzymatic antioxidants [11] and enzymatic antioxidants which concerted with reduction in lipid peroxidation [12].

The role of diet rich in vegetables and fruits in the prevention of acute renal diseases was demonstrated by higher carotenoid levels in the serum and its inverse association with rapid decline in kidney function during middle adulthood [13]. Studies have suggested to take foods rich in micronutrients including beta carotene in areas highly polluted with polyaromatic hydrocarbon [14]. Beta carotene has shown remarkable protection against ammonium sulfate-induced toxicity by ameliorating the raised levels of liver enzymes and by improving oxidative stress [15].

Beta carotene (BC) is a carotenoid and an antioxidant [16] which is a normal component of human colostrum and mature milk, where it contributes to antioxidant defences in the neonate. The bioavailability of beta carotene is dependent on the concentration of fat in the meal as well as administered form, and it was shown that the best bioavailability was by the synthetic pharmaceutical forms [17]. Beta carotene synergistically augments the anti-tumour effect of drugs on carcinoma [18] and ameliorates arsenic-induced toxicity by its antioxidant and anti-genotoxic properties [19]. Beta carotene-9′,10′-oxygenase modulates the impact of dietary beta carotene on hepatic nuclear receptor-, stress- and metabolism-related gene expression [20]. Beta carotene has potent antioxidant activities in decreasing hypobaric hypoxia-induced oxidative stress [21]. The aim of the present study is to evaluate the beneficial effect of beta carotene as a protective agent against BB-induced renal toxicity by modulating biochemical, antioxidant and cytokine levels.

Materials and Methods

Chemicals and Reagents

Bromobenzene was procured from Sigma Chemical Company. Commercially available beta carotene was bought from Natural Remedies, Bangalore, India. The standard drug for hepatoprotection, silymarin, was procured from Micro Labs Ltd., Goa, India. The remaining chemicals and reagents used were procured locally and of analytical standard. Previous studies formed the basis for deciding the effectual dosage of BB and beta carotene [22, 23]. Ten millimetres per kilogram b.w.p.o. of BB was administered after dissolution in coconut oil, and 10 mg/kg. b.w.p.o. of beta carotene was administered after dissolution in sterile distilled water. Twenty-five milligrams per kilogram b.w.p.o. of silymarin (SLY) was made into an aqueous suspension in double distilled water and administered to rats. Analysis of serum kidney function markers was performed with commercial diagnostic kits procured from Span Diagnostics Ltd., Surat, Gujarat, India. Commercial ELISA kits were procured from Sigma-Aldrich, Bangalore, India, for the analysis of IL-6, TNF-α and IL-1β.

Animals

Thirty female Wistar albino rats which were 6–8 weeks old and had a weight of 120–150 g were acquired from the animal house of VIT University, Vellore, Tamil Nadu, India, and were maintained in a thermo- and photo-regulated room. The rats were housed six in a cage and habituated for a week prior to the inception of the experiment. The animals under study were given free access to water and commercial pelleted rat feed obtained from Hindustan Lever Ltd., Mumbai, India. Approval was given by the ethical committee of the institution, VIT University, Vellore, India (VIT/IAEC/13/Feb13/20), for the experimental procedure. The animals were treated as below after randomly allocating six animals each into five groups.

Group 1: normal control—0.1 ml coconut oil was administered only once by intragastric intubation.
Group 2: BB treated—10 mM/kg b.w.p.o. of BB in 0.1 ml coconut oil was administered by intragastric intubation once on the ninth day.
Group 3: beta carotene pre-treated—10 mg/kg. b.w.p.o. of beta carotene was administered orally for 9 days, and a single dose of 10 mM/kg b.w.p.o. of BB in 0.1 ml coconut oil was administered by intragastric intubation on the ninth day.
Group 4: SLY pre-treated—25 mg/kg. b.w.p.o. of SLY was administered orally for 9 days, and a single dose of 10 mM/kg b.w.p.o. of BB in 0.1 ml coconut oil was administered by intragastric intubation, on the ninth day.
Group 5: beta carotene alone treated—10 mg/kg. b.w.p.o. of beta carotene was administered orally for 9 days.

Sacrifice of experimental rats was done using ether anesthesia after last dosage. Samples of blood were collected and centrifuged for 10 min at 2000 rpm to separate serum. Samples of the kidney were procured for histopathological examination. Twenty percent *w*/*v* homogenate of kidney tissue was made by homogenising approximately 0.05–0.1 g of the tissue in phosphate buffer of pH 7.4. Centrifugation of this homogenate was performed at 4 °C for 10 min at 3000 rpm, and the supernatant was stored at − 20 °C for analysis.

Analysis of Nephroprotective Activity

Analysis of serum creatinine (Span-4000015408), urea (Span-4000015316) and uric acid (Span-4000015141) were carried out utilising the serum procured from experimental rats with commercial diagnostic kits from AutoSpan Diagnostics Ltd., India. Performance of assays was done as stated in the manufacturer's protocol.

Estimation of Antioxidant Activity

Analysis of antioxidant activity was performed for superoxide dismutase (SOD) [24], catalase [25], glutathione-S-transferase (GST) [26], glutathione peroxidase (GPx) [27], reduced glutathione (GSH) [28] and thiobarbituric acid reactive substances (TBARS) [29] utilising kidney homogenate in 0.1 M phosphate-buffered saline (PBS).

Assessment of Histopathological Changes

The kidney of animals under study was collected and washed with 0.1 M ice-cold PBS, and a portion of it was processed after fixing in 10% formalin. Sections of tissues thus processed were stained with eosin and haematoxylin and assessed for histopathological variations.

Analysis of Serum and Kidney TNF-α, IL-6 and IL-1β

Commercial kits procured from Sigma-Aldrich, Bangalore, India, were used to analyse the concentration of tumour necrosis factor-alpha (TNF-α) (Sigma-RAB0479), interleukin-6 (IL-6) (Sigma-RAB0311) and interleukin-1 beta (IL-1β) (Sigma-RAB0246) in the serum and kidney by ELISA following manufacturer's protocol, and the results were expressed in picograms per millilitre.

Evaluation of Protein Expression

Caspase-3 (CSB-MA000320), cyclooxygenase-2 (COX-2) (CSB-MA080171) and necrosis factor kappa B (NF-κB) (ab176648) were purchased from Sigma-Aldrich, Bangalore, and measured with the help of Western blotting. The same quantity of total protein from the serum and kidneys of five different groups was denatured, electrophoresed using 8% SDS PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked before being incubated with specific primary antibodies at 4 °C overnight. Afterwards, the secondary antibodies were kept for incubation at 25 °C for 1 h. The immune reactive proteins of the samples were detected, and intensity of bands was analysed using a densitometer. Western blotting analysis result was expressed by the ratio of target gene with the housekeeping gene. Each measurement was conducted in triplicate.

Statistical Analysis

Values were presented as mean ± SD. One-way analysis of variance (ANOVA) was performed using GraphPad InStat3 software for further statistical analysis. Significant ($p < 0.05$) differences between groups were determined using Student-Newman-Keuls test.

Results

Nephroprotective Capacity of Beta Carotene on Serum Kidney Function Markers in BB-Induced Rats

The assay of kidney function markers in BB-treated rats showed significant ($*p < 0.05$) rise in creatinine, urea and uric acid levels (Table 1). Oral administration of beta carotene brought about significant ($*p < 0.05$) decrease in the increased levels of these parameters. The normalisation of kidney function markers was more pronounced in the beta carotene pre-treated rats in comparison with SLY pre-treated animals in BB-induced rats. Similar to control group 1, beta carotene solely treated group showed the normal range of renal markers.

Outcome of Beta Carotene on Nephrotic Antioxidant Status of BB-Treated Rats

BB-treated rats showed significant reduction in the activities of the antioxidant enzymes, SOD, catalase, GST, GPx and also hepatic glutathione content (Table 2). The diminished antioxidant levels in the kidney tissue of BB-treated rats were restored to near normalcy by the oral pre-treatment with beta carotene. Similar normalisation in levels of antioxidant was shown in groups treated with silymarin. The catalase activity in the kidney was effectively increased by the pre-treatment of beta carotene and was significantly more ($*p < 0.05$) when compared with silymarin pre-treatment for BB-induced rats. There was significant increase in TBARS in the liver tissue homogenates of BB-induced rats which was brought down effectively to normal values by beta carotene and silymarin in BB-induced rats. Groups treated solely with beta carotene showed antioxidant levels in the normal range similar to the normal control group.

Protective Outcome of Beta Carotene on Kidney Tissue Morphology of BB-Induced Rats

The kidneys of the control group showed normal tubules but slight interstitial oedema. The kidneys from group 2 rats treated with BB revealed changes in the renal medulla with diffuse morphological changes in tubules giving impression of toxicity. There was interstitial infiltration of inflammatory cells, congestion and tubular necrosis. In group 3 rats pre-treated with beta carotene, the cross section of the renal medulla revealed tubular epithelium showing mild to moderate degenerative changes and increase in Bowman's space in glomeruli. In group 4 rats pre-treated with silymarin, the cross section of the renal cortex revealed the tubular epithelium showing mild to moderate degenerative cytoplasmic changes. In group 5 rats which were pre-treated with beta carotene alone, the cross section of renal tubules showed a normal epithelium of tubules with prominent nuclei and no degeneration with normal architecture of tubules (Fig. 1).

Outcome of Beta Carotene on the Pro-inflammatory Cytokines TNF-α, IL-6 and IL-1β in the Kidney of BB-Induced Rats

Exposure to BB resulted in increased levels of TNF-α, IL-6 and IL-1β in the kidney of BB-induced rats. On beta carotene pre-treatment, the TNF-α, IL-6 and IL-1β levels in the kidney were found to be decreased due to the anti-inflammatory effect of beta carotene (Fig. 2). Pre-treatment with beta carotene by oral administration significantly reduced the levels of cytokines TNF-α, IL-6 and IL-1β in the kidney in a more effective manner when compared with

Table 1 Nephroprotective capacity of beta carotene on serum kidney function markers in BB-induced rats

Parameters	Group 1 Normal control	Group 2 BB (10 mM/kg. b.w.p.o.)	Group 3 BB + beta carotene (10 mg/kg b.wt.p.o.)	Group 4 BB + silymarin (25 mg/kg. b.w.p.o.)	Group 5 Beta carotene (10 mg/kg. b.w.p.o.)
Urea (mg/dl)	13.34 ± 0.53	71.31 ± 1.51^{a*}	18.43 ± 0.47^{a*b*}	20.30 ± 0.57^{a*b*}	14.44 ± 0.42^{b*c*d*}
Creatinine (mg/dl)	0.81 ± 0.03	3.42 ± 0.06^{a*}	0.92 ± 0.04^{b*}	1.15 ± 0.09^{a*b*c*}	0.78 ± 0.03^{b*d*}
Uric acid (mg/dl)	7.12 ± 0.15	24.60 ± 0.38^{a*}	8.23 ± 0.03^{a*b*}	8.33 ± 0.13^{a*b*}	6.79 ± 0.09^{b*c*d*}

Each value constitutes the mean SD of six rats. Comparisons were made as follows: [a] group 1 vs groups 2, 3, 4 and 5; [b] group 2 vs groups 3, 4 and 5; [c] group 3 vs groups 4 and 5; [d] group 4 vs group 5. Statistical significance at $*p < 0.05$ is represented by the symbols. One-way ANOVA was used to calculate statistical analysis followed by the Student-Newman-Keuls test

Table 2 Effect of beta carotene on nephrotic antioxidant status of BB-treated rats

Parameters	Group 1 Normal control	Group 2 BB (10 mM/kg b.w.p.o)	Group 3 BB + beta carotene (10 mg/kg b.w.p.o.)	Group 4 BB + silymarin (25 mg/kg b.w.p.o.)	Group 5 Beta carotene (10 mg/kg b.w.p.o.)
SOD (U/min/mg protein)	64.69 ± 1.10	23.71 ± 0.51^{a*}	66.74 ± 1.30^{b*}	68.02 ± 1.15^{a*b*}	66.19 ± 1.05^{b*}
Catalase (U/min/mg protein)	66.72 ± 1.27	23.75 ± 0.44^{a*}	61.77 ± 1.08^{a*b*}	58.17 ± 0.62^{a*b*c*}	66.25 ± 1.73^{b*c*d*}
GPx (mg of GSH utilised/min/mg protein)	26.68 ± 1.28	16.75 ± 0.56^{a*}	24.89 ± 1.13^{b*}	25.27 ± 0.81^{b*}	27.43 ± 1.12^{b*}
GSH (nM/mg protein)	8.28 ± 0.20	3.82 ± 0.15^{a*}	7.23 ± 0.21^{a*b*}	7.64 ± 0.28^{a*b*}	8.65 ± 0.29^{b*c*d*}
GST (nM of CDNB-GSH conjugate formed/min/mg protein)	23.64 ± 1.21	5.76 ± 0.29^{a*}	18.66 ± 0.45^{a*b*}	19.51 ± 0.43^{a*b*}	26.13 ± 1.14^{a*b*c*d*}
TBARS (mM/TBARS/100 g of wet tissue)	1.57 ± 0.03	3.51 ± 0.11^{a*}	1.62 ± 0.03^{b*}	1.65 ± 0.06^{b*}	1.57 ± 0.03^{b*}

Each value constitutes the mean SD of six rats. Comparisons were made as follows: [a] group 1 vs groups 2, 3, 4 and 5; [b] group 2 vs groups 3, 4 and 5; [c] group 3 vs groups 4 and 5; [d] group 4 vs group 5. Statistical significance at $*p < 0.05$ is represented by the symbols. One-way ANOVA was used to calculate statistical analysis followed by the Student-Newman-Keuls test

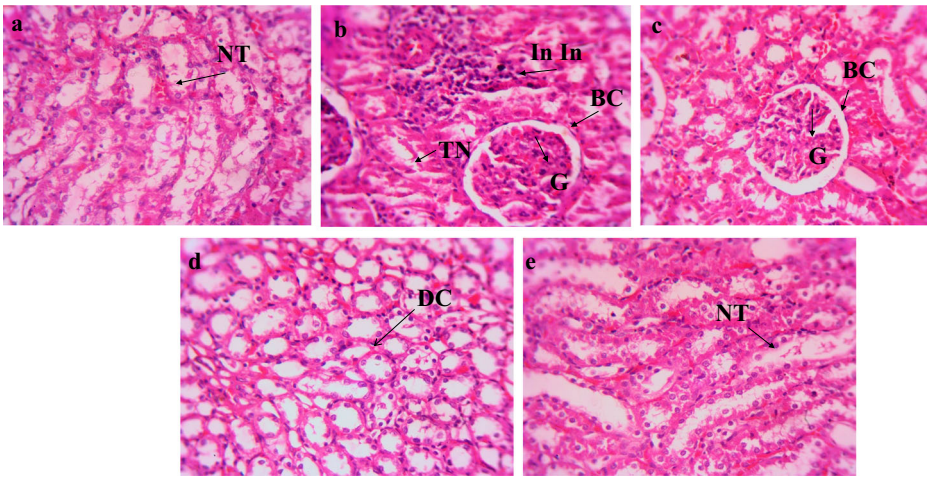

Fig. 1 Protective outcome of beta carotene on kidney tissue morphology of BB-induced rats. Histopathological monograph of the kidney. **a** Group 1 (normal). **b** Group 2 (bromobenzene). **c** Group 3 (beta carotene + bromobenzene). **d** Group 4 (silymarin + bromobenzene). **e** Group 5 (beta carotene); all the pictures are taken under × 400 magnification. NT, normal tubules; InIn, inflammatory infiltration; TN, tubular necrosis; BC, Bowman's capsule; G, glomerulus; DC, degenerative cytoplasm

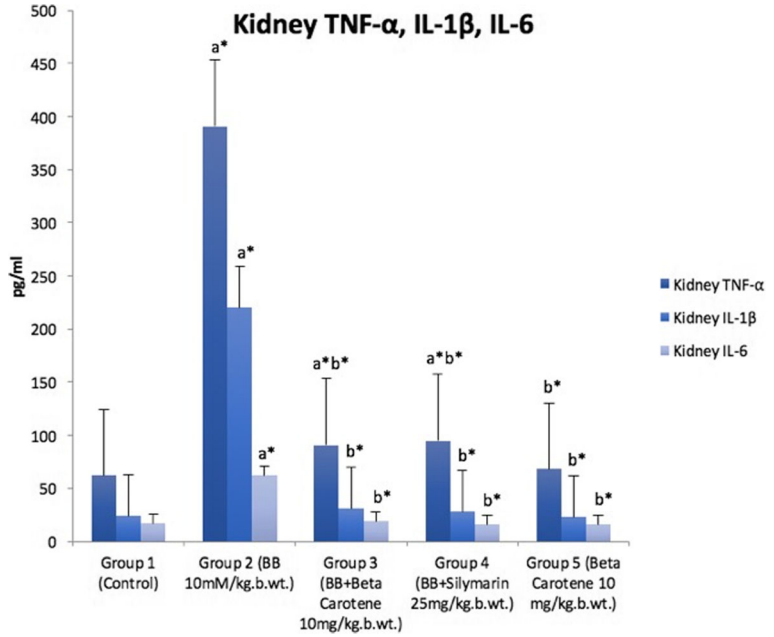

Fig. 2 Outcome of beta carotene on TNF-α, IL-1β and IL-6 in the kidney of BB-induced rats. Each value represents the mean SD of six rats. Comparisons were made as follows: a, group 1 vs groups 2, 3, 4 and 5; b, group 2 vs groups 3, 4 and 5; c, group 3 vs groups 4 and 5; d, group 4 vs group 5. Statistical significance at $*p < 0.05$ is represented by the symbols. One-way ANOVA was used to calculate statistical analysis followed by the Student-Newman-Keuls test.

SLY in BB-treated rats. The group solely treated with beta carotene exhibited normal range of cytokine levels similar to the control group.

Outcome of Beta Carotene on the Pro-inflammatory Cytokines TNF-α, IL-6 and IL-1β in Serum of BB-Induced Rats

Exposure to BB resulted in increased levels of TNF-α, IL-6 and IL-1β in the serum of BB-induced rats. The levels of TNF-α, IL-6 and IL-1β levels in serum were found to be decreased on pre-treatment with beta carotene due to the anti-inflammatory effect of beta carotene (Fig. 3). Pre-treatment with beta carotene by oral administration significantly reduced the levels of cytokines TNF-α, IL-6 and IL-1β in serum in a more effective manner when compared with SLY in BB-treated rats. The group solely treated with beta carotene exhibited normal range of cytokine levels in serum similar to the control group.

Effect of Beta Carotene on Protein Expression Levels of COX-2, NF-κB and Caspase-3 in the Liver of BB-Induced Rats

The ratio of the target gene to housekeeping gene was identified to be normal for group 1, normal control. In rats induced with BB, the expression of protein levels of COX-2, caspase-3 and NF-κB was significantly elevated ($*p < 0.05$). Beta carotene pre-treatment was capable of normalising the elevated levels in protein expression of COX-2, caspase-3 and NF-κB which was induced by BB (Fig. 4). Silymarin pre-treated groups showed normal levels of protein expression. Rats in group 5 which were pre-treated with beta carotene alone also showed normal levels of COX-2, caspase-3 and NF-κB protein expression.

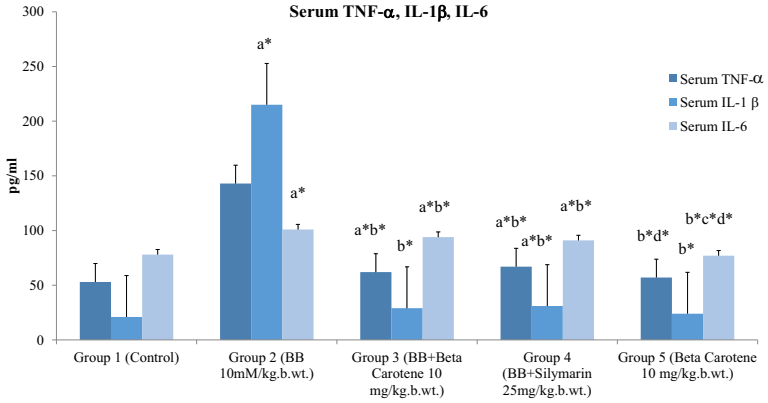

Fig. 3 Outcome of beta carotene on TNF-α, IL-1β and IL-6 in serum of BB-induced rats. Each value represents the mean SD of six rats. Comparisons were made as follows: a, group 1 vs groups 2, 3, 4 and 5; b, group 2 vs groups 3, 4 and 5; c, group 3 vs groups 4 and 5; d, group 4 vs group 5. Statistical significance at $*p < 0.05$ is represented by the symbols. One-way ANOVA was used to calculate statistical analysis followed by the Student-Newman-Keuls test.

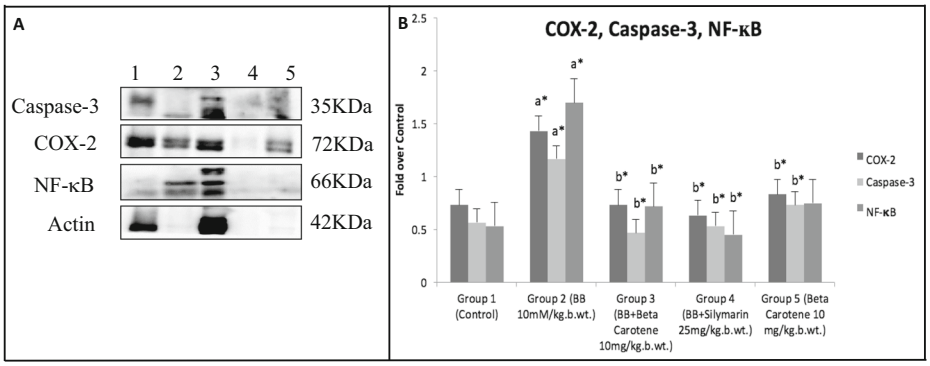

Fig. 4 Outcome of beta carotene on protein expression levels of caspase-3, COX-2 and NF-κB in the liver of BB-induced rats. Western blot analysis. **a** The protein expression in Western blots taking actin as control. **b** Quantitative assay of protein expression after normalising to the control. It is represented in fold difference with the mean ± SD of six rats. Comparisons were made as follows: a, group 1 vs groups 2, 3, 4 and 5; b, group 2 vs groups 3, 4 and 5; c, group 3 vs groups 4 and 5; d, group 4 vs group 5. Statistical significance at $*p < 0.05$ is represented by the symbols. One-way ANOVA was used to calculate statistical analysis followed by the Student-Newman-Keuls test.

Discussion

The result of exposure to xenobiotics in the cells of proximal tubules is production of oxidative stress manifested by diminution in free radical scavenger levels and intracellular GSH. This is due to the retardation in activity of various antioxidant enzymes which are involved in free radical detoxification and hence leads to an increased generation of reactive oxygen species (ROS) [30]. Oxidative stress could thus be indirectly caused by the depletion of cellular levels of glutathione, inactivation of antioxidant enzymes, lipid peroxidation and DNA damage in cells [31].

Mono- and di-substituted metabolites produced by the conjugation of glutathione with secondary phase metabolites of bromobenzene get concentrated in the kidney resulting in necrosis [23]. The after effect of necrosis is a reduction in the rate of glomerular filtration culminating in the reduction in the clearance of uric acid, urea and creatinine which ultimately elevates the concentration of these in blood [5]. In our study, there was significant elevation in the serum uric acid, urea and creatinine in the group of rats intubated intragastrically with bromobenzene alone denoting the renal toxicity induced by BB. In the group which was pre-treated with beta carotene, the serum creatinine, urea and uric acid were brought down significantly to near normal levels. Creatinine levels were brought down more effectively by beta carotene when compared with silymarin. Creatinine has been shown to be a more reliable indicator of nephrotoxicity as its levels are increased in the first phase of kidney disease [32], and our results point out the ameliorative effect of beta carotene for mitigating kidney toxicity induced by BB.

The occurrence of oxidative stress is due to multiple factors like the imbalance between the formation of ROS with activation of c-Jun N-terminal kinase (JNK) by factors like environmental stress and the ability to voluntarily eliminate the reactive metabolites produced by detoxification promoted by the antioxidant defence systems [33]. Reports show a significant variance in the levels of pro-oxidants vs antioxidants in patients with kidney dysfunction [34]. A deficit in the crucial antioxidant systems was found to cause an elevation in the generation of ROS in patients diagnosed with acute kidney failure [35]. The activity of antioxidant enzymes,

SOD, GPx, CAT and glutathione reductase, along with non-enzymatic shielding systems is involved in safeguarding from oxygen free radicals [36, 37]. Hence, a balance in the combined actions of these is vital to attain more effective protection against damage caused by oxidative stress [38]. The superoxide ion is changed to hydrogen peroxide by SOD while hydrogen peroxide is broken down to products which are non-toxic, thus protecting the tissue from highly reactive hydroxyl radicals. In the present study, the elevation in the antioxidant enzymes, SOD, CAT and GPx, brought about by the administration of BB was reverted back to normalcy on pre-treatment with beta carotene. This demonstrates the effect of beta carotene on oxygen free radicals and reactive hydroxyl radicals, thereby imparting protection against oxidative stress damage which is a root cause for renal pathology.

Conjugation of quinones with glutathione results in the generation of products which cause renal toxicity during excretion via the kidney [39]. GST is a detoxifying enzyme of phase II which catalyses the conjugation of electrophiles with GSH, thus providing protection against toxic products of xenobiotic metabolism and therefore regarded as marker for toxicity in imperative organs. Nephrotoxicity is linked to depletion of glutathione and generation of free radicals. In the current study, the levels of GST were significantly reduced in rats treated with BB which was elevated to near normal levels on pre-treatment with beta carotene comparable to that of silymarin. Pre-treatment with beta carotene alone showed elevated levels of GST when compared with that of the normal group pointing to the protective antioxidant effect of beta carotene.

Hydrogen peroxides as well as hydroperoxides are reduced inside and outside the cells by non-enzymatic antioxidant GSH by detoxification through redox reaction [40]. GSH is oxidized by GPx and is converted back to the reduced form GSH by glutathione reductase. Administration of increased doses of BB leads to the formation of epoxides produced by the biotransformation of BB, which undergoes conjugation with GSH as part of xenobiotic metabolism leading to the diminution of GSH pool [41]. These events expose the protection against ROS as well as hazardous products of xenobiotic metabolism which then spurts a series of secondary episodes that drastically lead to lipid peroxidation, dysfunction of mitochondria, ATP depletion, energy imbalance [42, 43]and differed levels of intracellular calcium. GSH levels were reduced significantly in rats induced with BB in our studies, which were increased to near normalcy as with SLY. This shows the capacity of beta carotene to increase the concentration of the non-enzymatic antioxidant, GSH, which facilitates the detoxification of hydrogen peroxide and hydroperoxides as well as aiding in conjugation with products of biotransformation thereby sequestering ROS and thus shielding against dangerous metabolites of xenobiotics. The normal levels of GSH, SOD, CAT and GPx in beta carotene-alone-treated group as in the control group in our studies showed that beta carotene alone does not elicit any change in enzymatic and non-enzymatic antioxidants. Oxidative damage caused by xenobiotic-induced toxicity is manifested by diminution in the antioxidant levels and lipid peroxidation which modifies biochemical and physiological characteristics of biological networks [44]. ROS generated via oxidative stress might oxidize the polyunsaturated fatty acids which comprise the lipid bilayer causing disruption and liberation of enzymes into the extracellular fluid [45]. The elevation in TBARS values in our results correlates with the increased lipid peroxidation on induction with BB which was reported earlier for studies with BB [23]. There was a significant reduction in lipid peroxidation in our studies when rats were pre-treated with beta carotene before being induced by BB pointing out the capacity of beta carotene in modulating the biochemical events which lead to lipid peroxidation. The reduction was similar to that treated with silymarin, the standard drug. A variety of toxic aldehydes like

4-hydroxynonenal produced by lipid peroxidation peculiarly binds to thiol groups of proteins and enzymes thereby masking the thiols essential for the transport of Ca^{2+} across membranes. This leads to disruption in the homeostasis of calcium in cells which eventually culminates in cell death [46, 47]. Lethal cell injury is provoked by lipid peroxidation, preceded by mitochondrial dysfunction caused by BB [48]. The elevation in the antioxidant enzymes together with reduced glutathione and the decline in the lipid peroxidation on treatment with beta carotene in the present study lead us to decipher that beta carotene plays a remarkable role in mitigating cell injury caused by the disrupted cellular calcium levels inflicted indirectly by lipid peroxidation by oxidative stress, amplified by a reduction of antioxidant enzymes induced by BB.

Reactive nitrogen species (RNS) as well as ROS is produced in both renal vascular and tubular cells during innumerable stresses in the cell and tissue. Damage to cells occurs resulting in the impairment of tissue function [49, 50] when the levels of ROS and RNS increase. This was evidenced in the kidney tissue morphology of the rats administered with BB in our study as it revealed changes in the renal medulla with diffuse morphological changes in tubules giving impression of toxicity. Oxidative stress in the vascular cells plays a prime role in advancement of renal damage during the development of a disease. The endothelium becomes particularly vulnerable to oxidative stress, and as the levels of ROS in the blood vessels increase from moderate to severe, it encounters an alteration in phenotype. This unfavourable effect on the endothelium stimulates inflammation, along with elevated production of cytokine and enhanced expression of surface adhesion molecules [51] effectually boosting vascular remodelling. These could explain the reason for interstitial infiltration of inflammatory cells, congestion and tubular necrosis as noticed in our study on administration with BB. In our study, the cross section of the renal medulla of rats pre-treated with beta carotene revealed a tubular epithelium showing mild to moderate degenerative changes and increase in Bowman's space in the glomeruli. This implies that the tubular epithelium is recovering from the degenerative changes induced by BB due to the protective effect of beta carotene. The pathogenesis of acute renal injury has been connected with epithelial and endothelial cell damage. Alteration in cell polarity of tubular cells concomitant with mislocalisation of membrane transporters and T junction proteins ultimately leads to the development of apoptosis and necrosis [30]. Thus, pre-treatment with beta carotene could serve as a protection against renal damage caused by BB.

It has been reported that the role of beta carotene 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 [52]. Inflammation is characterized by the inherent production of pro-inflammatory cytokines, IL-1α, IL-1ß and TNFα, along with activation of immune cells. The binding of the ligand to a receptor generates a signal which communicates for variance in expression of genes which proceeds to elevated expression of cell adhesion molecules and cytokines which are the effector molecules. Immunomodulatory effect can be tracked by the elevation or repression of pro-inflammatory (IL-6, IL-8, TNF-α and NF-κB) cytokines and their gene expression [53]. IL-1β shows excellent pro-inflammatory activities by enhancing the expression of cell adhesion molecules as well as stimulating stromal cells to generate chemokines which promote the assemblage of inflammatory cells [54]. This is marked by the rise in IL-1β and permeation of inflammatory cells in our study involving rats administered with BB. Beta carotene has been reported to have reduced the production of pro-inflammatory cytokines, involving IL-1β, IL-18, TNF-α and COX-2, and obstructed the activation of NF-κB pathway thereby causing impediment to the activation of astrocyte in the spinal cord

[55]. Reports show that 23.1% of the children under study who were deficient in beta carotene possessed higher levels of inflammatory biomarker IL-6 levels, compared with those with normal beta carotene concentrations [56]. This further clarifies the capacity of beta carotene to bring down the pro-inflammatory cytokines and thus reduce inflammation as evidenced in our study. Suppression of pro-inflammatory cytokines including TNF-α and IL-6 which activate extracellular matrix inhibits inflammation [57]. The levels of TNF-α, IL-6 and IL-1β in serum and kidney tissue homogenates were found to be significantly elevated on induction with BB in our study. These were brought back to normal levels on pre-treatment with beta carotene. This further demonstrates from our studies the ability of beta carotene to possess anti-inflammatory activity by inhibiting pro-inflammatory cytokines TNF-α, IL-6 and IL-1β thereby inhibiting the signals which give communication for changes in gene expression which might elevate the expression of cell adhesion molecules and inducing release of chemokines that stimulate the aggregation of inflammatory cells.

The caspase family belongs to the protease family that is responsible for the degeneration of apoptotic cells, thus disturbing renal function [58]. Caspase-9 encounters self-agitation in the spectre of other proteins and stimulates downstream caspase factors, including caspase-3, which leads to a cascade-amplifying effect, causing cell apoptosis [59]. One of the most prominent executors of apoptosis in the caspase family is caspase-3, which is also an important effector in apoptotic process, and therefore, activation of caspase-3 denotes that apoptosis goes into a phase which is irreversible [60]. There was an elevation in caspase-3 in rats induced with BB in this study which might be one of the reasons for renal function imbalance caused by cell apoptosis. The groups pre-treated with beta carotene showed a reduction in caspase-3 on induction with BB pointing to the fact that beta carotene alleviates nephrotoxicity by modulating apoptotic factors including caspase-3. One of the mediators of pain and inflammation in the kidney is COX-2. The upregulation of COX-2 stimulates an elevation in the prostaglandin levels which is accountable for tissue inflammation including elevation in ROS and generation of hydrogen peroxide [61] leading to damaging effect in kidney histopathology [62]. Administration of BB caused the upregulation of COX-2 in our studies which would be indirectly responsible for the diffuse morphological changes including interstitial penetration of inflammatory cells due to increased prostaglandin levels stimulated by COX-2.

It has been reported that lead enhances pro-inflammatory processes through activation of NF-κB, which stimulates intrarenal renin-angiotensin system which in turn induces macrophages, generating an interstitial inflammatory process in the kidney [63]. The protein complexes which control cytokine production, DNA transcription and cell survival are NF-κB. The dissociation of inhibitor protein I kappa B (IkB) from NF-κB is increased by the activation of TNF stimulated by ROS which in turn translocates this transcription factor into nucleus inducing the generation of chemokines, cytokines, acute-phase proteins and cell adhesion molecules. It initiates transcriptions of specific target genes, TNF-α, IL-1β and COX-2 [64]. As an alternative to this, NF-κB may be directly increased by intracellular ROS [65]. Hence, ROS plays an important role in eliciting primary immune response; contrarily, antioxidants can produce the inverse effect. Studies have shown that antioxidative stress capacity could be enhanced through activated nuclear factor (erythroid derived 2)-like 2 (Nrf2) by blocking the activation of NF-κB [57]. It has been reported that the existence of carotenoids in subcellular organelles could shield the immune cells from oxidative injury and assure ideal cellular functions, including gene regulation, cell signalling and apoptosis [65]. In the current study, there was an elevation in serum and kidney TNF which would have stimulated the dissociation of IκB from NF-κB. Oxidative stress and inflammation were shown to be attenuated by

reducing the serum pro-inflammatory cytokines and expression of NF-κB and COX2 in mice [66]. The protective capacity of beta carotene towards BB-induced renal toxicity might be through inhibition of NF-κB-mediated COX-2 expression and may be through the oxidative stress-dependent BAX/BCl-2/caspase-3 apoptotic pathway [67] as reported in earlier studies. As our results point out, beta carotene exercises anti-inflammatory and anti-apoptotic effects by repressing NF-κB activation and attenuating the BB-induced elevation of TNF-α, IL-1β and COX-2 in renal tissues.

Conclusion

The results of our present studies lead us to conclude that pre-treatment of rats with beta carotene offers significant protection against bromobenzene-induced nephrotoxicity. Beta carotene was shown to normalise the elevated kidney function markers and decreased antioxidant levels by concomitant administration. Beta carotene reduced the production of pro-inflammatory cytokines, emphasizing its anti-inflammatory properties, and its pre-treatment markedly inhibited the NF-κB pathway activation, COX-2 and caspase-3 leading us to conclude that beta carotene might invoke its protective role by hindering these important steps in the cascade of signalling pathways involved in regulation of apoptosis stimulated by oxidative stress and xenobiotic metabolism. However, further pharmacological evidence supporting the role of beta carotene towards bromobenzene-induced renal injury is needed to understand the mechanism underlying the alleviation of nephrotoxicity by beta carotene against BB and its role as a protective agent.

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Compliance with Ethical Standards

Approval was given by the ethical committee of the institution, VIT University, Vellore, India (VIT/IAEC/13/Feb13/20), for the experimental procedure.

Conflict of Interest The authors declare that they have no conflicts of interest.

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