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Genotoxic Effects of Carotenoid Breakdown Products in Human Retinal Pigment Epithelial Cells

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Abstract

Purpose—To investigate the genotoxic effects of Lutein (LBP) & β-carotene breakdown products (β-apo-8-carotenal, BA8C) and preventive role of GSH in human retinal pigment epithelial cells (ARPE-19).

Methods—LBP- and BA8C-induced DNA damage in human retinal pigment epithelial cells (ARPE-19) was determined by Comet assay. The DNA damage was quantified by the image analysis system using Comet Score™ software. ARPE-19 cell viability was determined by CellTiter 96 AQ_{ueous} one solution cell proliferation assay kit. Intracellular GSH levels were measured by Ellman's reagent.

Results—Incubation of serum-starved ARPE-19 cells with LBP & BA8C caused significant DNA damage in a dose- and time-dependent manner. The DNA damage and cell death incurred by LBP & BA8C were significantly prevented by *N*-acetylcysteine (NAC) but not by α-Tocopherol + Ascorbic acid (T + AA). Furthermore, BSO-induced GSH depletion in ARPE-19 cells caused a significant elevation in LBP- & BA8C-induced DNA damage, whereas increased GSH levels in ARPE-19 cells prevented it.

Conclusions—Our results suggest that breakdown products of dietary carotenoids could be genotoxic in ARPE-19 cells. LBP-induced genotoxic effects could worsen oxidative stress. The intracellular GSH pool in ARPE-19 cells might play a critical role in carotenoid breakdown products-induced genotoxicity.

Keywords

Carotenoids; carotenoid breakdown products; lutein breakdown products; Antioxidants; DNA damage; Age-related Macular Degeneration

Introduction

Age-related macular degeneration (AMD) is considered to have a multifactorial pathogenesis[1–4]. The molecular events that mediate pathogenic mechanisms in AMD are not

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The authors report no conflicts of interest. The authors alone are responsible for content and writing of the paper.

clearly defined and preventive strategies have yet to be established. However, dietary antioxidants have been considered as potential agents for AMD intervention[1]. In recent years a number of investigators have suggested that carotenoids such as lutein and zeaxanthin could delay or protect against AMD by acting as an antioxidant or by elevating macular pigment levels and reducing exposure to phototoxic short wavelength light[5–8].

Like a number of other antioxidants, carotenoids are also reported to be pro-oxidants under various physiological conditions such as high oxygen tension and imbalanced intracellular redox status[9]. Under such conditions, carotenoids undergo oxidation and generate a variety of oxidized products, as demonstrated *in vitro* and *in vivo*[10–12]. Many carotenoid cleavage products characterized either *in vitro* or *in vivo*, are identified as aldehydes including retinaldehyde and apocarotenaldehyde, and mixtures of various long- and short-chained products. Besides aldehydes, such mixtures also contain epoxides, ionones, and other unidentified products[13]. The existence as well as identification of lutein, zeaxanthin and other carotenoid breakdown products (CBP) in human and primate ocular tissues has been reported[12, 14–16]. In case of lutein supplementation, the levels of lutein and its breakdown products (LBP) have been demonstrated to increase in human serum[17] as well as in plasma and ocular tissues of monkey[18]. After short-term (6 months) lutein supplementation the physiological concentration of lutein and LBP in human serum is in nM range[6, 17, 19]. However, the physiological concentration of carotenoids and CBP could reach μM range in case of long-term carotenoid supplementation. For example, ATBC[20] and CARET[21] studies have reported μM concentration of β-carotene and its breakdown products after 3 years of carotenoid supplementation. However, the effects of long-term and high dose xanthophylls (lutein & zeaxanthin) supplementation on the concentration of their breakdown products in human serum and ocular tissues are not known. The ongoing AREDS-2 study is intended to investigate the effects of long-term (5 years) and high dose xanthophyll supplementation on AMD progression. Such studies may provide data on physiological concentrations of xanthophylls and their breakdown products.

The physiological role of various CBP is not known. However, various studies have reported cyto- & genotoxic effects of CBP at physiological (nM) or abnormally higher (μM) concentrations[13, 22–29]. Lutein & zeaxanthin supplementation as an intervention therapy for AMD, has been widely advocated with little regard for the toxic effects of lutein breakdown products (LBP). Recently, we have demonstrated that LBP could cause cell death in human retinal pigment epithelial cells (ARPE-19)[22]. In the present study, we have investigated the genotoxic effects of LBP as well as β-apo-8-carotenal (BA8C, β-carotene-derived aldehyde) in ARPE-19 cells. The role of intracellular GSH in the protection against lipid aldehydes-induced cyto- & genotoxicity is well established[30–32]. Such protection is mediated by efficient conjugation between GSH and lipid aldehydes such as 4-HNE[30, 33]. However, the role of GSH in protection against CBP-induced toxicity is obscure. A preliminary study has suggested that GSH can conjugate with CBP[34]. We observed that CBP formed a thioether linkage with the sulfhydryl moiety of GSH[34]. In the present study, we have shown that increase in the cellular GSH significantly prevented CBP-induced genotoxicity in ARPE-19 cells. However, α-Tocopherol + Ascorbic acid could not prevent CBP-induced genotoxicity. These results suggest that the protection rendered by GSH could be due to its conjugation with CBP followed by its possible extracellular removal. Contrary, α-Tocopherol + Ascorbic acid may not conjugate with CBP and therefore could not prevent CBP-induced genotoxicity.

Materials and Methods

Materials

Human retinal pigment epithelial cells (ARPE-19) were purchased from American Type Culture Collection (ATCC). Phosphate-buffered saline (PBS), penicillin/streptomycin (P/S)

and fetal bovine serum (FBS) were purchased from GIBCO Inc. (Grand Island, NY). Glutathione-ethyl ester (GS-Et), Buthionine-[S,R]-sulfoximine (BSO), Trypsin/EDTA, *N*-acetylcysteine (NAC), β-apo-8-carotenal (BA8C) and Lutein were purchased from Sigma, USA. DL-all-rac-α-Tocopherol was obtained from Fluka, USA. Ascorbic acid was purchased from Mallinckrodt, USA. CellTiter96® AQ_{ueous} one solution cell proliferation assay kit was purchased from Promega. USA. The Comet Assay™ kit was purchased from Trevigen, Inc. (Gaithersburg, MD). DNA purification Kit was purchased from Qiagen Inc. USA. Ellman's Reagent was purchased from Pierce (Rockford, IL). All other chemicals were obtained from Sigma, USA.

Preparation of LBP

The LBP were prepared as described previously[22]. The use of sodium hypochlorite (NaOCl) to oxidize carotenoids is based on the fact that hypochlorite is a product of myeloperoxidase, hydrogen peroxide and Cl^- in activated phagocytic cells[35,36]. Since retinal pigment epithelial cells are phagocytic cells, we used NaOCl as a model system for investigating the possible harmful effects of carotenoid oxidation products. Briefly, 5 mg of lutein dissolved in 5 ml dichloromethane and 5 ml methanol were oxidized with 80 mM NaOCl in 1.25 ml water at room temperature for 15 min. The concentration of NaOCl used was similar to that reported by Lee et al[37] in activated macrophages. The LBP was extracted by a modification of the Bligh and Dyer method[38, 39]. To each one ml of oxidized carotenoid solution, 5 ml of dichloromethane were added, followed by vortexing for 1 min; 3.75 ml water was added and vortexing was continued for another minute. The samples were centrifuged for 2 min at 1000 × *g* and the lower organic phase from each sample was collected. The extraction was repeated and the organic phase from both extractions was pooled. The solvent was evaporated under Argon to approximately 0.1 ml which was diluted with sterile phosphate-buffered saline (PBS) without calcium and magnesium (pH 7.4; Cellgro) to make LBP stock solution. The excess organic phase was evaporated again. The LBP solution was ultra-filtered by centrifugation using Centricon Tube (Millipore Corp. Bedford, MA, USA) for 1 h at 10,000 × *g* at 4 °C. The concentration of LBP in the ultrafiltrate was determined by measuring the optical density at 220 nm on UV-2101 PC recording spectrophotometer (Shimadzu, Columbia, MD)[13]. The characterization of NaOCl-oxidized carotenoid products revealed the presence of apo-carotenals, epoxides, ionones and various unidentified carbonyls[10]. The LBP present in human macula were also characterized and showed existence of two prominent products, 3-hydroxy-β-ionone and 3-hydroxy-14'-apocarotenal[12]. The LBP stock solution was stored at −20° C in dark. Vehicle without Lutein was prepared by the same method.

Preparation of BA8C

The BA8C was dissolved in dichloromethane and methanol (1:1). The solution was concentrated by evaporation under Argon followed by dilution with ice-cold 50 mM sodium phosphate Buffer (pH 7.4) and the remaining solvent was evaporated again. The solution was ultra-filtered by centrifugation using Centricon Tube (Millipore Corp. Bedford, MA, USA) for 1 h at 10,000 × *g* at 4 °C. The concentration of BA8C in the ultrafiltrate was determined by measuring the optical density at 220 nm on UV-2101 PC recording spectrophotometer (Shimadzu, Columbia, MD)[13]. The stock solution was stored at −20° C in dark. For some experiments vehicle was also prepared by the same method without BA8C.

Cell culture and treatment

The ARPE-19 cells were grown to confluency in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37° C in a humidified atmosphere of 5% CO_2. Sub-confluent cells were growth-arrested in 0.1% FBS medium. The cells were subcultured using trypsin/EDTA solution with a split ratio of 1:4. The LBP or BA8C

treatment was done in serum-free medium to avoid binding with serum proteins. The pretreatment of cells with NAC (1 mM) or α-Tocopherol + Ascorbic acid (T+AA) (1 mM + 100 μM) was for 1 and 24 h respectively. Control cells were incubated with vehicle, PBS. In all the experiments, the concentration of PBS was not greater than 0.2%.

The delivery of α-Tocopherol in ARPE-19 cells was carried out as described below. The stock solution of 500mM α-Tocopherol was prepared in 100 % ethanol and stored at −80° C in dark. To prepare the working stock solution, 1 ml stock solution was diluted with 1 ml heat-inactivated fetal calf serum and 3 ml of culture media without serum. Serum increases the solubility of α-Tocopherol in hydrophilic solutions. The working stock solution was further diluted 100X with culture media (without serum but containing 100μM Ascorbic acid) to get 1 mM final concentration of α-Tocopherol. The final concentration of ethanol and fetal calf serum in the media was approximately 0.2 % each.

Treatment of the cells

ARPE-19 cells (5×10^5 cells) were treated with various concentrations of LBP or BA8C or treated for varied time periods as described in the figure legends. For negative control, the cells were treated with vehicle. After the treatment the cells were detached from the well by brief trypsinization. The released cells were subsequently washed twice with ice-cold PBS and the cell viability was determined by trypan blue exclusion method. Also an aliquot was used for comet assay.

Determination of DNA damage (Comet assay)

The comet assay was carried out as per suppliers manual. Briefly, an aliquot from cell suspension was mixed with 1% low melting point (LMP) Agarose in 1:10 ratio. Immediately, 75 μl of the cell suspension were dispersed onto Comet Slides specially treated to enhance the adherence of low melting point Agarose. The slides were kept at 4° C in dark for 30 min to allow Agarose solidification. Subsequently, the slides were immersed in cold lysis buffer (10 mM Tris–HCl, 100 mM EDTA (pH 10), 2.5 M NaCl, 1% sodium lauryl sarcosinate, 1% Triton X-100) for 1 hr. The slides were then washed twice with 1× Tris-buffered EDTA solution (TBE), placed in a horizontal electrophoresis chamber and covered with TBE buffer. Electrophoresis was carried out at the rate of 1.0 V/cm for 20 min. The slides were removed from the electrophoresis chamber, washed in deionized water for 5 min and dipped in 70% alcohol for 5 min. Subsequently, the slides were air-dried, stained with SYBR-Green (1 μl/ml; 30 μl per slide) and mounted. The comet pictures were taken using Nikon Eclipse 800 epifluorescence microscope. Photographs were taken using a Roper Scientific CoolSNAP Fx monochrome cooled CCD 12-bit digital camera. At least 40 non-overlapping comets were observed per slide and each experiment was repeated at least three times. All the steps of Comet assay were conducted under red light. In order to detect the combination of DNA single strand breaks, double strand breaks, and alkali-labile sites in the DNA of ARPE-19 cell nuclei, we performed alkaline electrophoresis using TBE buffer.

Scoring

For the analysis of DNA in comet tails, the slides were examined at 250X magnification on Nixon fluorescent microscope (Nikon Eclipse 800 epifluorescence microscope). Fourty randomly selected non-overlapping cells were scored for DNA damage using the Comet Score ™ version 1.5 analysis software (TriTek Corp.). A variety of objective measurements such as total intensity (DNA content), percent DNA in tail, tail length (measured from the leading edge of the comet head) and tail moment were made. We used % DNA in tail for statistical analysis.

Cell viability assay

The ARPE-19 cells were plated (5000 cells/well) in a 96-well plate. After 24 h, the cells were serum-starved in 0.1% FBS medium for 24 h. The cells were then washed with PBS and treated with BA8C in media without serum. Cells incubated without vehicle served as control. Cell viability was determined by CellTiter 96 AQ_{ueous} one solution cell proliferation assay kit. CellTiter reagent was added to each well, incubated for 3 h, and the absorbance was recorded at 490 nm using a 96-well plate spectra count (Packard, USA).

DNA laddering assay

The characteristic DNA ladder pattern for apoptosis or necrosis was determined by DNA laddering assay. ARPE-19 cells (5×10^6) were exposed to BA8C for 15 h without or with NAC pretreatment. The cells were detached by brief trypsinisation, gently washed with ice-cold PBS, and DNA was extracted using DNeasy Kit (Qiagen Inc.) according to manufacturer's protocol. RNA was digested during DNA purification procedure. The extracted DNA was loaded onto 1.6% Agarose gel. After electrophoresis and staining with Ethidium bromide, DNA fragments were visualized and image was captured in an automatic gel documentation system (Omega System, Ultra-Lum Inc.).

Determination of Intracellular GSH using Ellman's Reagent

ARPE-19 cells (1×10^6 cells) were treated with various concentrations of 10, 25, and 50 μM LBP for 6 h or with 100 μM BSO for 24 h or with 1 mM GS-Et for 1 h. After treatment, the cells were washed twice with ice cold PBS and scraped in 1 ml ice cold PBS. The PBS was removed after centrifugation at 1000 *g* for 5 min. at 4° C. The cells were homogenized in 0.3 ml of Reaction Buffer (0.1 M sodium phosphate, *p*H 8.0, containing 1 mM EDTA). Again the cells were centrifuged at 8000 *g* for 20 min. at 4° C. The resultant supernatant was used for the determination of intracellular GSH. The assay mixture was prepared by taking 100 μl standard or experimental supernatant into test tubes containing 20 μl of Ellman's Reagent solution (4 mg Ellman's Reagent in 1 ml of Reaction Buffer) and 1 ml of Reaction Buffer. The assay mixture was mixed gently and incubated for 15 min at room temperature. The absorbance was measured at 412 nm. A standard curve was also prepared.

Statistical Analysis

All the data represent the mean ± SD. Statistical significance of difference between untreated control and treated groups was analyzed by Student's *t*-test as well as ANOVA. Differences were considered statistically significant at $P < 0.01$.

Results

Dose- and time-dependent effect of LBP & BA8C on DNA damage in ARPE-19 cells

We examined the genotoxic effects of LBP on ARPE-19 cells. The results shown in Fig.1 suggest that LBP caused a significant DNA damage in a dose-dependent manner. The minimum concentration of LBP, 10 μM, elicited a significant (7.4 %, $P<0.05$) DNA damage which increased to approximately 25 % ($P<0.001$) with 25 μM LBP (Fig. 1 A & B). The control cells and cells treated with vehicle showed no-significant DNA damage. In order to quantify the extent of LBP-induced DNA breaks in ARPE-19 cells, the comet tail intensity was scored. As shown in Fig. 1 B, 10, 25 & 50 μM LBP caused 1.8-, 3.8- & 5.7-fold increase in % DNA in tail as compared to control cells, respectively. We also examined if a single species such as BA8C could also cause genotoxicity in retinal cells. As shown in Fig. 1, like LBP, BA8C, 10 μM, also elicited a significant 12 % ($P<0.05$) DNA damage which increased to approximately 30.6 % ($P<0.001$) with 20 μM BA8C (Fig. 1 A & B). As shown in Fig. 1 B, 10, 20 & 40 μM

BA8C caused 3.5-, 7.5- & 12-fold increase in % DNA in tail as compared to control cells, respectively (Fig. 1 A & B).

We also examined time-dependent effects of LBP and BA8C on DNA damage in ARPE-19 cells. As shown in Fig. 2, with 50 μM LBP the comet tails were visible as early as 3 h after LBP treatment but the extended incubation time caused highly significant (P<0.001) (12 fold) damage as measured by tail length which was highly significant. Moreover, with 40 μM BA8C, the comet tails were also visible as early as 3 h after BA8C treatment but the extended incubation time elicited 9.4 fold (P<0.001) DNA damage (Fig. 2 A & B).

Effect of BA8C on ARPE-19 cell viability

In our previous study[22] we demonstrated that LBP cause ARPE-19 cell death. Therefore, we examined whether single aldehyde species of carotenoid (i.e. BA8C) cause cell death in retinal cells. When ARPE-19 cells were incubated with BA8C for 24 h, a concentration-dependent (0 – 100 μM BA8C) decrease in cell viability was observed (Fig. 3 A & B). The LD_{50} for BA8C was approximately 40 μM as determined by CellTiter 96® AQ_{ueous} one solution cell proliferation assay. No apparent change in cell viability was observed when the cells were treated with vehicle. The characteristic DNA ladder pattern of apoptotic and/or necrotic cells was also determined by DNA laddering assay. DNA fragments were examined as irregular bands ("smear"), indicating BA8C-induced necrosis in a dose-dependent manner in ARPE-19 cells (Fig. 3 C). However, NAC prevented BA8C-induced cell death in ARPE-19 cells. These results suggest that CBP could be genotoxic to retinal cells.

Effect of NAC and α-Tocopherol + Ascorbic Acid on LBP- and BA8C-induced DNA damage in ARPE-19 cells

In order to examine the effect of NAC on LBP- and BA8C-induced genotoxicity, ARPE-19 cells were incubated with 1 mM NAC for 1h before LBP or BA8C treatment. As shown in Fig. 4, NAC decreased the LBP- & BA8C-induced DNA damage in ARPE-19 cells. The scoring of comet tails in terms of % DNA in tail showed significant (P<0.001) reduction in DNA damage compared to cells treated with LBP or BA8C (Fig. 4 B). The protective and neutralizing effect of NAC could involve its conjugation with carotenoid products through thioether linkage[34]. This phenomenon could be confirmed by using other physiologically relevant antioxidants which may not conjugate with carotenoid products. Therefore, we examined the effect of α-Tocopherol + Ascorbic Acid on DNA damage induced by LBP in ARPE-19 cells. The incubation with α-Tocopherol + Ascorbic Acid prior to LBP or BA8C treatment could not prevent the DNA damage induced by LBP (Fig. 4). These results indicate that the difference in protective action of NAC as well as α-Tocopherol + Ascorbic Acid could be determined by their ability to conjugate with the carotenoid aldehydes.

Role of cellular GSH in LBP- & BA8C-induced DNA damage in ARPE-19 cells

It is demonstrated that GSH regulates 4-hydroxynonenal-induced genotoxicity in cancer cells[30]. Therefore, we examined if cellular GSH could also regulate LBP- & BA8C-induced genotoxicity in retinal cells. The effects of LBP on intracellular GSH levels in ARPE-19 cells were examined first. As shown in Fig. 5 A, LBP caused significant decline in GSH pool in retinal cells after 6 h of treatment. The decline in GSH was ~22% (73 nmol/mg protein, p<0.05), ~30% (65 nmol/mg protein, p<0.05), and ~61% (36 nmol/mg protein, p<0.001) by 10, 25, and 50 μM LBP, respectively. The effects of CDA on DNA of retinal cells with increased or decreased GSH levels were determined by treating the cells with GS-Et or BSO, respectively. The GSH levels (94 nmol/mg protein) in ARPE-19 cells were depleted (~77%) by pretreatment with 100 μM BSO for 24 h (Fig. 5 B). The GSH levels in ARPE-19 cells were elevated significantly (~23%, p<0.01, without BSO treatment and ~213%, p<0.001, with BSO treatment) after 1 h incubation with 1mM GS-Et (Fig. 5 C). When the intracellular GSH was

depleted by BSO, there was a significant increase in DNA damage by LBP as well as BA8C. However, the DNA damage decreased significantly when the intracellular GSH was elevated. As shown in Fig. 5D, LBP- as well as BA8C-induced DNA damage was significantly ($P<0.001$ and $P<0.01$, respectively) enhanced due to pretreatment of BSO as compared to BSO untreated cells. These results demonstrate that GSH could play a pivotal role in the protection of ARPE-19 cells against CBP-induced genotoxicity. The comet scores in terms of % DNA in tail increased in cells pretreated with BSO prior to LBP or BA8C treatment compared to cells treated with LBP or BA8C only. Moreover, GS-Et-pretreatment significantly ($P<0.01$ and $P<0.001$ respectively) prevented LBP- and BA8C-induced DNA damage in cells treated without or with BSO (Fig. 5 D). The results demonstrate that adequate cellular GSH levels could protect against LBP- and BA8C-induced DNA damage in ARPE-19 cells.

Discussion

Lutein and zeaxanthin are widely used as prophylactic agents in AMD[5–8]. They can undergo oxidation and form various short and long chain cleavage products. The effects of CBP have largely been ignored. This study demonstrates that the CBP of dietary carotenoids can be genotoxic. This may have implications for dietary supplementation with carotenoids in AMD.

The physiological concentration of lutein and LBP in human serum has been reported to be in nM range after short term lutein supplementation for 6 months[6, 17,19], but their actual concentration in human serum and ocular tissues after long-term lutein supplementation is not known. The ongoing AREDS-2 study (5 years) may provide data on physiological concentration of xanthophylls (lutein and zeaxanthin) and their breakdown products after long-term xanthophylls supplementation. Nevertheless, the ATBC[20] and CARET[21] studies have reported μM concentration of β-carotene and its breakdown products (For example, average Vit. A concentration - 2.2 μM) after 3 years of carotenoid supplementation. Moreover, van Helden et al[23] demonstrated that β-carotene breakdown products could enhance inflammation-induced oxidative DNA damage in lung epithelial cells at physiologically relevant concentrations. Based on this fact, in the present study, we used μM concentration of LBP and BA8C to investigate their effect on DNA damage in ARPE-19 cells. The concentrations of breakdown products though high based on short term lutein supplementation studies but may be in physiological range based upon long term use of xanthophylls.

The DNA damage and genetic alterations are major risk factors in various oxidative stress-related diseases including AMD[40, 41]. The results of present study demonstrate that LBP as well as BA8C contribute to DNA damage in a concentration- and time-dependent manner (Fig. 1 & Fig. 2). To assess the quantity and extent of DNA damage in ARPE-19 cells by CBP, a highly sensitive comet assay method was used to demonstrate the severity of genotoxicity. We used LD_{50} dose for LBP as well as BA8C to study genotoxic effects as reported by us earlier in ARPE-19 cells[22]. The genotoxic effects of β-carotene (20 μM) and BA8C (2, 5, and 20 μM) have been demonstrated in A549 cells[29]. Moreover, BA8C is also demonstrated to form 1,N^2 –etheno-2'-deoxyguanosine adduct which could lead to DNA strand breaks and mutations[42]. These results are concordant with our findings which show significant DNA damage in ARPE-19 cells treated with similar concentrations of LBP and BA8C. The mechanism of DNA damage could be either direct through apoptosis[22], or indirect through damaging plasma membrane and inducing necrosis as observed in this study. Thus, genotoxic effects of LBP at concentrations used in this study could potentially cause or exacerbate retinal pathology.

The extent of geno- & cytotoxicity induced by various agents is dependent on cellular GSH levels.[30, 43]. It is well known that under oxidative stress, GSH is oxidized to GSSG and transported out[44, 45] which eventually results in significantly lower cellular GSH levels. The

increased carotenoid oxidation and CBP formation coupled with decreased availability of GSH could exacerbate the genotoxicity of CBP. Since GSH is known to be the first line of defense in various tissues against oxidative stress, we investigated the effects of increased as well as decreased intracellular GSH levels in CBP-induced genotoxicity in retinal cells. When the cells with basal cytosolic GSH levels were treated with CBP, there was a dose-dependent increase in the DNA damage (Fig. 1) which corresponded to decline in basal GSH levels in cytosol (Fig. 5 A). These results indicate that the extent of CBP-induced DNA damage in ARPE-19 cells is associated with cytosolic GSH levels. The CBP, being hydrophobic, could interact with hydrophilic molecules such as GSH which can be translocated to nucleus where it could cause increased DNA damage and thereby decreased intracellular GSH levels. The conjugate of GSH and CBP formed by thioether linkage could also be readily translocated out of the cells thereby causing decreased intracellular levels of GSH as well as CBP. This would be the protective role of GSH against the genotoxic effects of CBP. This hypothesis is supported by our observation that BSO treatment, which decreased the GSH levels, significantly increased DNA damage (Fig. 5 D). However, it also suggests that CBP translocation to nucleus, under depleted GSH conditions, could also be facilitated by other mechanisms. Although, the precise mechanism for the transportation of CBP from cytosol to nucleus is not known, β-carotene and its breakdown products have been demonstrated to accumulate in crude nuclei of NCL-H69 cells[46]. Furthermore, the GSH supplementation significantly prevented the CBP-induced DNA damage in the ARPE-19 cells (Fig. 5 D). Our results thus indicate that increasing GSH levels in the cells could attenuate CBP-induced DNA damage.

During oxidative stress GSH readily and rapidly conjugates with lipid aldehydes, such as HNE, catalysed by Glutathione-*S*-transferase (GST)[47,48]. The GST-mediated conjugation of HNE with GSH is a major detoxification pathway against lipid aldehyde-induced DNA damage[30]. Thus GST could be the body's major defense against lipid aldehyde-induced protein alteration as well as DNA damage through conjugation of aldehydes. Reduction of lipid aldehydes to corresponding alcohols by Aldose reductase (AR) and transporting lipid aldehydes out of cells could be the major defense of cells[30]. Glutathione conjugates with CBP could also be the major defense of cells against products of carotenoid oxidation. In case of carotenoid products, we have observed efficient conjugation of CBP with GSH or NAC[34]. However, the role of GST in expediting conjugation and protection of cells against CBP is not known. Moreover, the role of AR in lipid aldehydes-induced DNA damage as well as cytotoxicity is well established[30, 33, 49]. Further studies are required to investigate the possible role of AR and other reductases in the protection against CBP-induced genotoxicity or cytotoxicity.

In summary, the results of this study suggest that (1) LBP could contribute to dose- and time-dependent increase of DNA damage in ARPE-19 cells, (2) short term dietary supplementation with lutein, which results in only nM serum levels, may be quite safe since μM levels are required to cause damage and, (3) the modulation of cellular GSH levels could ameliorate LBP-induced genotoxic effects in retinal cells. Randomized long-term studies such as AREDS-2 would greatly help in understanding the beneficial versus harmful effects of lutein and zeaxanthin commonly used in the prevention and treatment of AMD.

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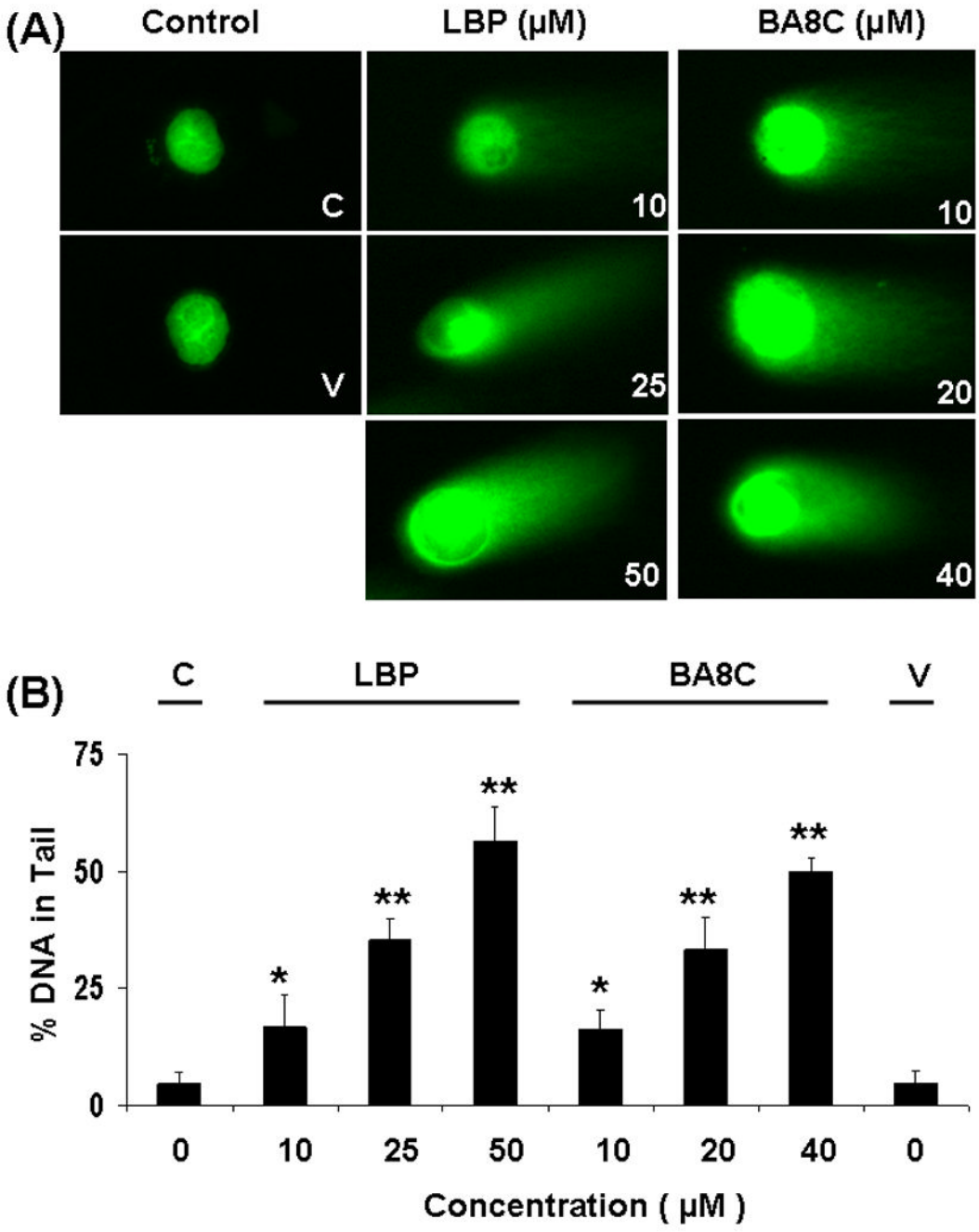


Fig. 1.

Dose-dependent genotoxic effects induced by LBP & BA8C in ARPE-19 cells. (A) ARPE-19 cells were incubated with 10, 25 & 50 μM LBP and 10, 20 & 40 μM BA8C and vehicle for 9 h and single cell gel electrophoresis was performed to determine the DNA damage. The figure shows the representative comet tails indicative of DNA damage. (B) The bars indicate %DNA in tail (mean ± SD) from three independent experiments. Mean was calculated from three to four parallel slides, 40 comets were evaluated per slide (*$P < 0.05$, **$P < 0.001$ vs. control).

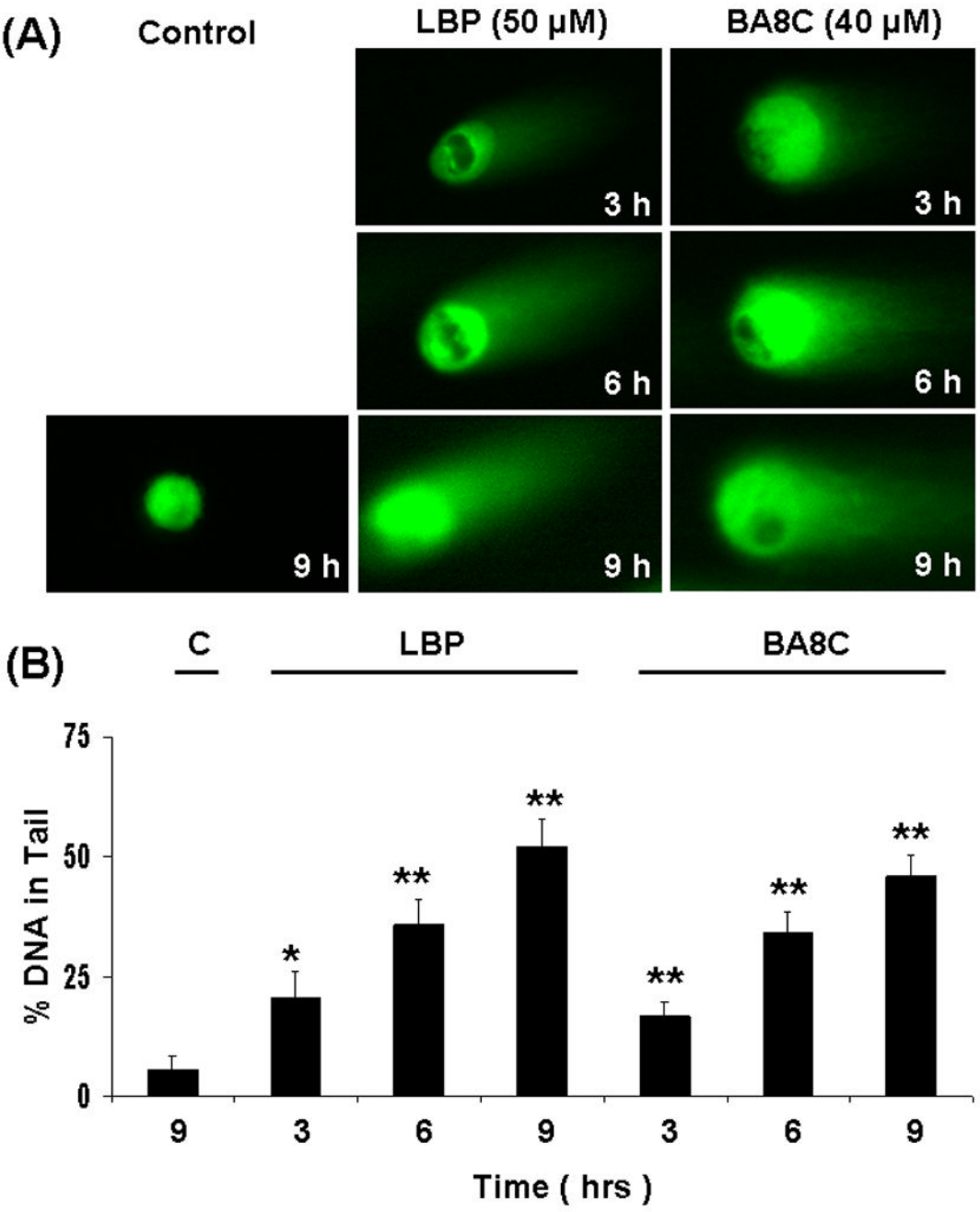
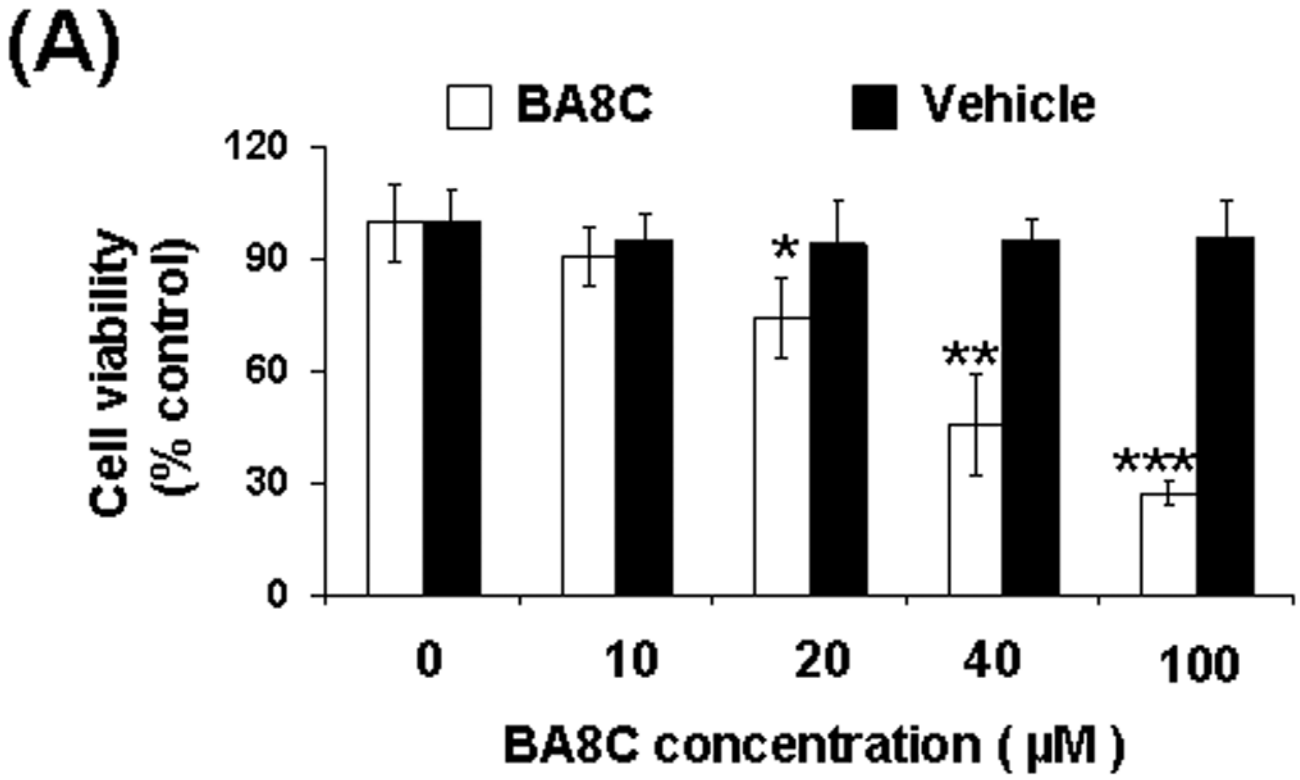
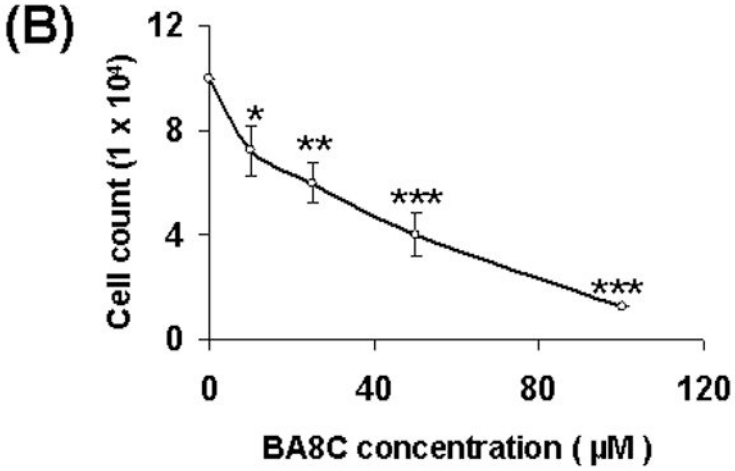


Fig. 2.

Time-dependent genotoxic effects induced by LBP & BA8C in ARPE-19 cells. (A) ARPE-19 cells were incubated with 50 µM LBP and 40 µM BA8C for 3, 6 and 9 h and single cell gel electrophoresis was performed to assess the DNA damage. The figure shows the representative comet tails indicative of DNA damage. (B) The bar diagram shows tail intensity (%DNA in tail) (mean ± SD) from three independent experiments. The results are given as mean ± SD. The mean in each case was calculated from three to four parallel slides, 40 comets were evaluated per slide (*$P < 0.05$, **$P < 0.001$ vs. control).





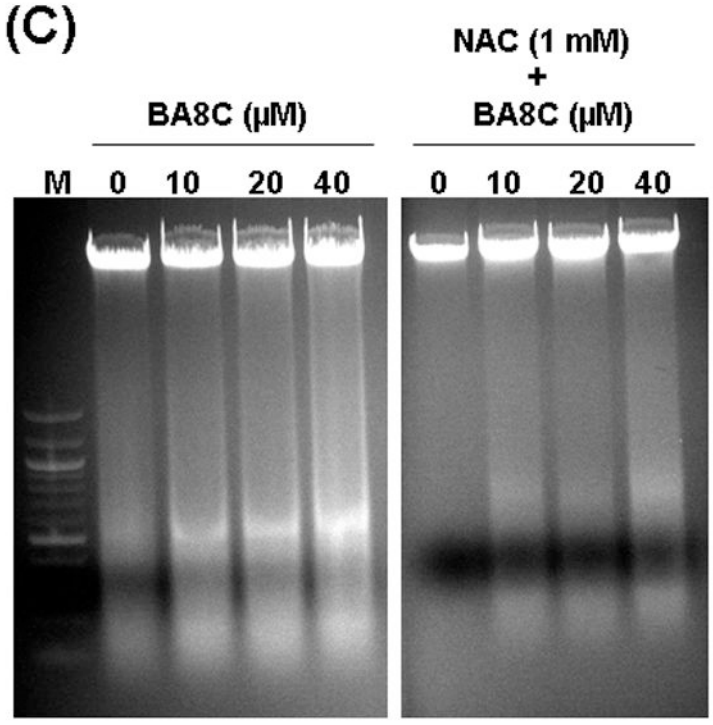


Fig. 3.

(A & B) Effect of BA8C on ARPE-19 cell viability. BA8C caused concentration-dependent decrease in ARPE-19 cell viability. Cells were exposed to various concentrations of BA8C (0–100 μM) as well as corresponding amount of vehicle and after 24 h cell viability was determined by MTT assay (A) or trypan blue exclusion method (B). Data represents the mean ± S.D. of three experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (C) Effects of BA8C on DNA in ARPE-19 cells. The figure shows Ethidium bromide stained gels containing DNA from ARPE-19 cells treated with 10, 20 & 40 μM BA8C for 15 h without or with pretreatment of 1 mM NAC for 1 h. DNA fragmentation is observed as a smear from BA8C-treated cells which was prevented by NAC. Lane 1. DNA marker; Lanes 2 - 4. DNA from BA8C (0, 10, 20 & 40

µM, respectively) treated cells. Lanes 5 - 8. DNA from BA8C (0, 10, 20 & 40 µM respectively)-treated cells with pretreatment of NAC (1mM).

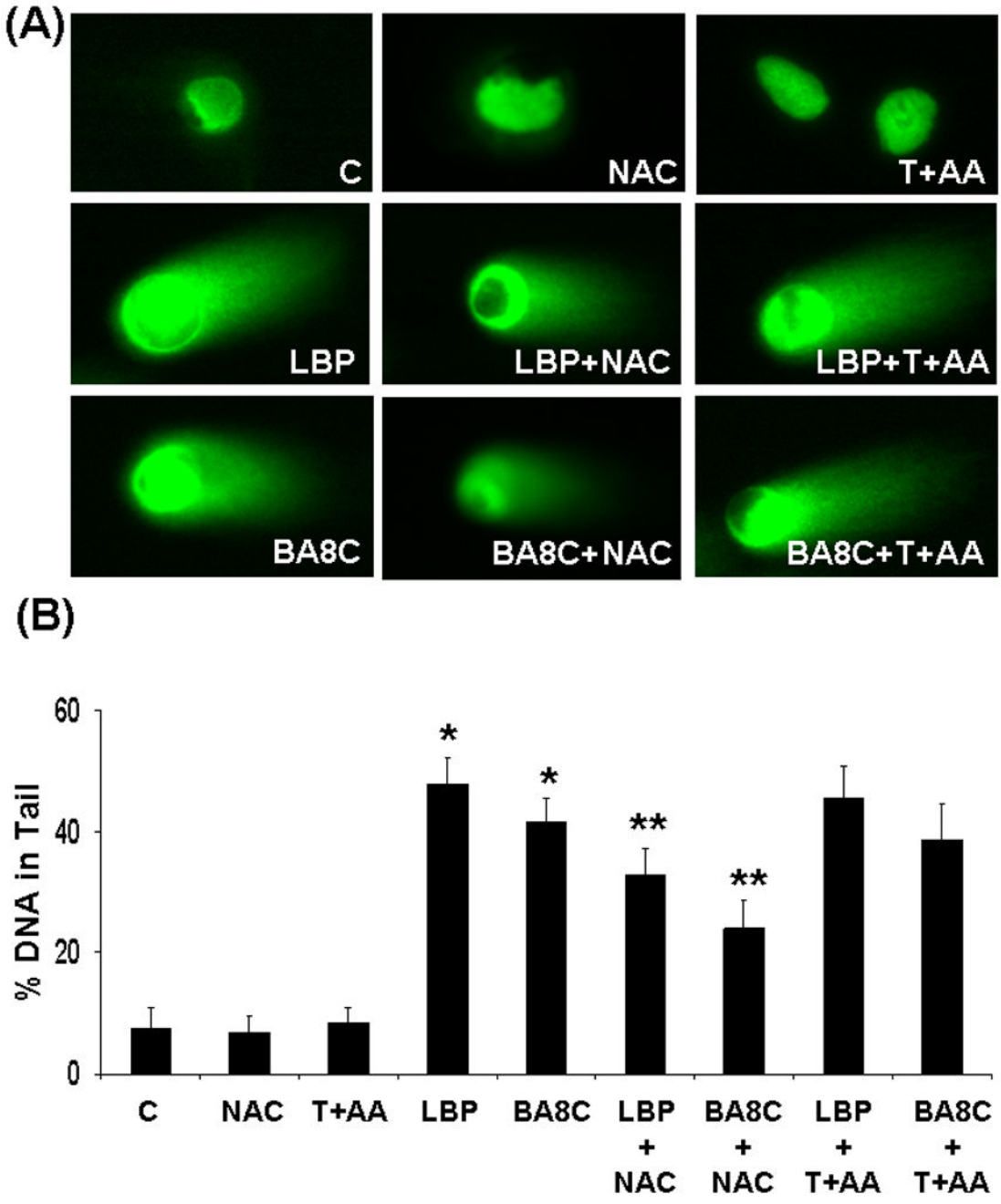
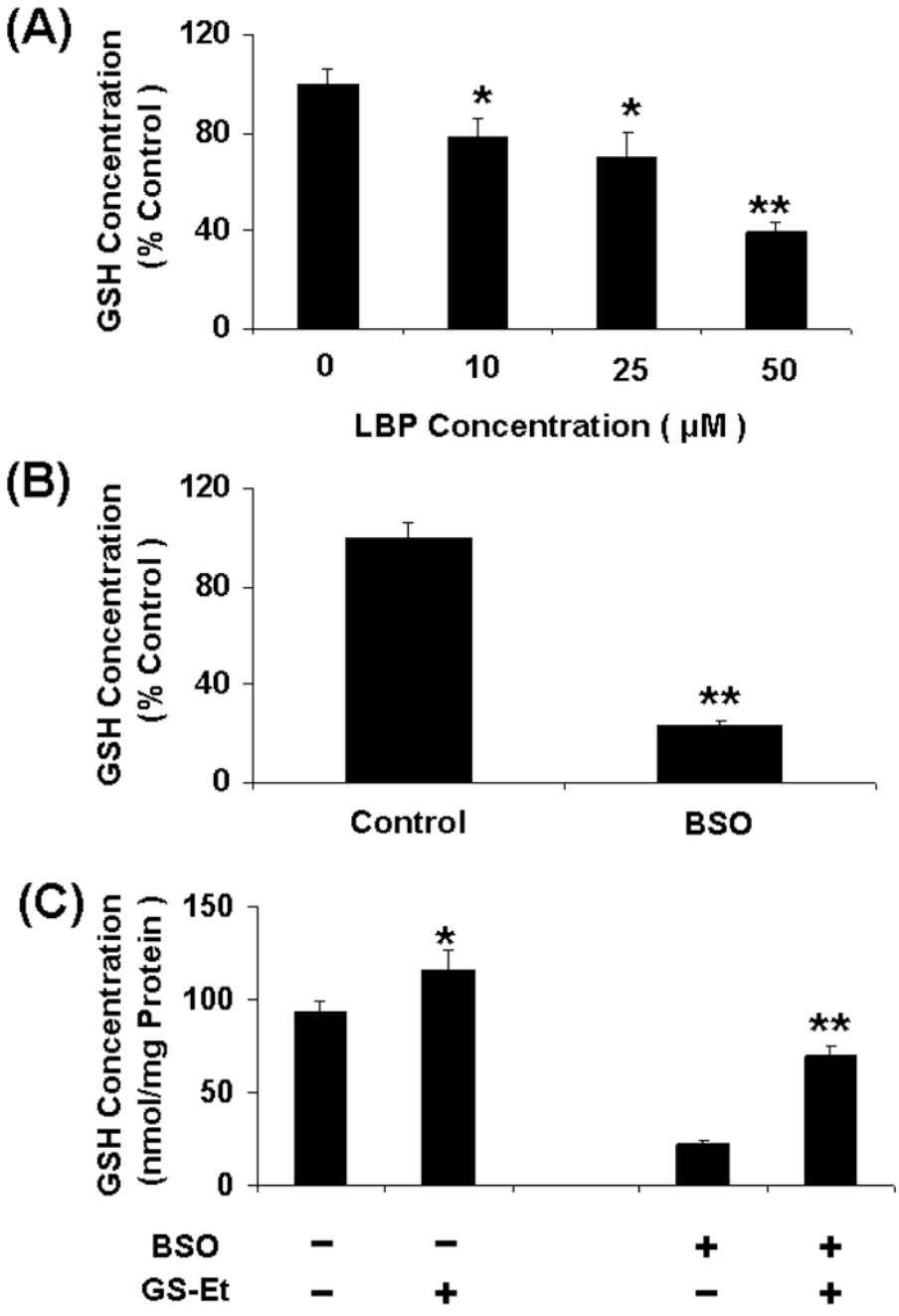


Fig. 4.

Effect of NAC and α-Tocopherol + Ascorbic Acid (T+AA) on genotoxic effects induced by LBP & BA8C in ARPE-19 cells. (A) ARPE-19 cells pretreated with NAC (1 mM) or T+AA (1 mM + 100 μM) for 1 or 24 h respectively and incubated with 50 μM LBP or 40 μM BA8C for 9 h and single cell gel electrophoresis was done to assess the DNA damage. The figure shows representative comet tails from each group indicative of DNA damage. (B) The bar diagram shows tail intensity (%DNA in tail) (mean ± SD) from three independent experiments. The results are given as mean ± SD. The mean in each case was calculated from three to four parallel slides, 40 comets were evaluated per slide (*$P < 0.001$ vs. control; **$P < 0.001$ vs. LBP or BA8C).



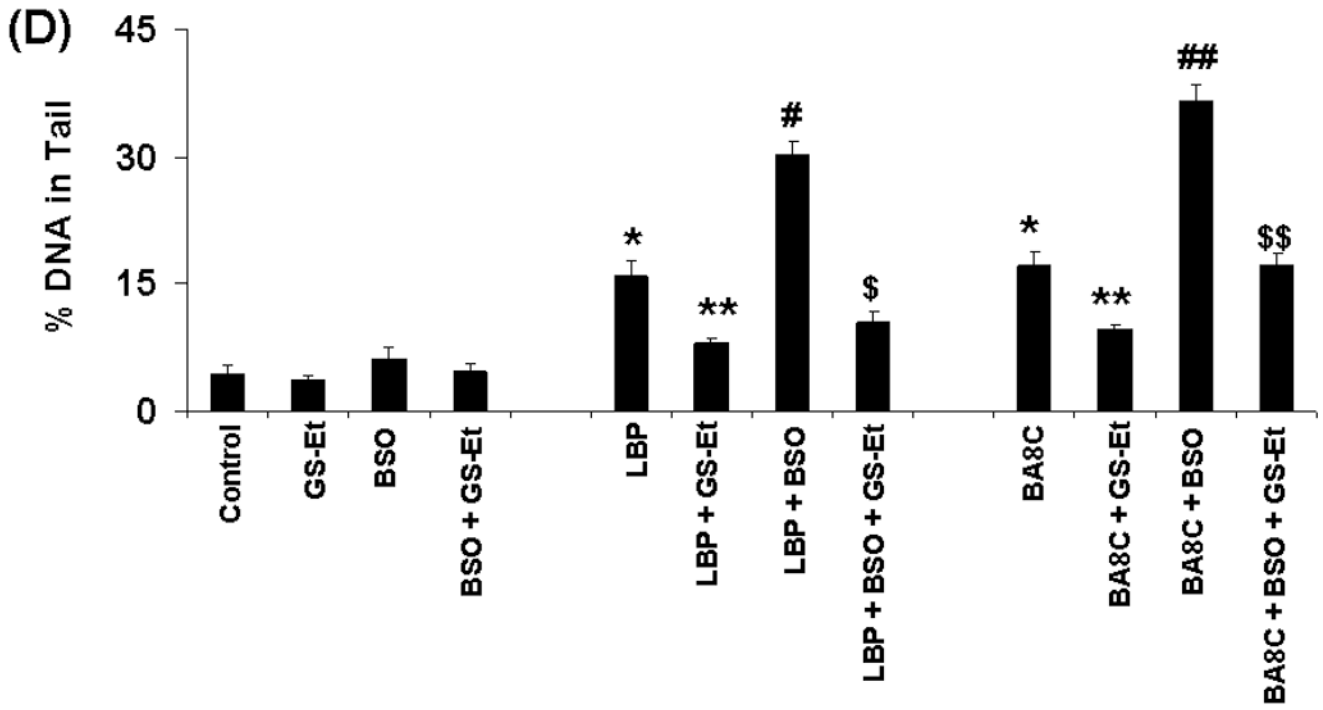


Fig. 5.

Role of GSH in CBP-induced genotoxicity. (A & B) Depletion of GSH levels induced by LBP or BSO in ARPE-19 cells. The GSH levels were determined in the cytosolic fraction of the cells treated with 10, 25 & 50 μM LBP or 100 μM BSO for 6 h or 24 h, respectively. Data represents the mean ± S.D. of three experiments (*$P < 0.05$, **$P < 0.001$). (C) Elevation of GSH levels after glutathione-ethyl-ester (GS-Et) treatment in ARPE-19 cells. The GSH levels were determined in the cytosolic fraction of the cells treated without or with 100 μM BSO for 24 h, followed by incubation with 1mM GS-Et for 1 h. Data represents the mean ± S.D. of three experiments (*$P < 0.01$ vs control, **$P < 0.001$ vs BSO control). (D) Effect of GSH depletion and/or supplementation on LBP- & BA8C-induced genotoxicity in ARPE-19 cells. For GSH depletion the cells were pretreated with 100 μM BSO for 20 h. For GSH supplementation the cells were pretreated with 1mM GS-Et for 1 h. After pretreatment the cells were treated with 10 μM LBP or 10 μM BA8C for 9 h and single cell gel electrophoresis was done to assess the DNA damage. The bar diagram shows tail intensity (%DNA in tail) (mean ± SD) from three independent experiments. The results are given as mean ± SD. The mean in each case was calculated from three to four parallel slides, 40 comets were evaluated per slide (*$P < 0.01$ vs. control; **$P < 0.01$ vs. LBP or BA8C only; #$P < 0.001$ vs. LBP only, ¤$P < 0.01$ vs. LBP + BSO, ##$P < 0.01$ vs. BA8C only, ¤¤$P < 0.001$ vs. BA8C + BSO).