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The carotenoid lycopene protects rats against DNA damage induced by Ochratoxin A

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ABSTRACT

Ochratoxin A (OTA), one of the most prevalent mycotoxins in the world, has nephrotoxic and hepatotoxic properties. Lycopene is an important carotenoid and has a high singlet-oxygen and free-radical scavenging capacity. This study was designed to investigate the possible protective effects of lycopene against the genotoxicity of OTA in rat tissues using the alkaline comet assay. Male Sprague–Dawley rats were used in the experiments. OTA (0.5 mg/kg b.w./day) was administered by gavage for 14 days, whereas lycopene was applied on the last 7 days or for 14 days of the feeding period, with OTA treatment. OTA caused marked increases in tail length, tail moment, and tail intensity vs. control both in the kidney and liver cells, but not in the lymphocytes. Lycopene administration alone for 7 and 14 days did not provide any significant change in DNA damage of the lymphocytes, renal and hepatic cells vs. controls. However, lycopene for both 7 and 14 days, with OTA exposure in renal and hepatic cells, supplied significant decreases in tail length, tail moment, and tail intensity vs. OTA-exposed rats. The effect of 14 days supplementation seemed to be more protective, particularly against hepatic cells. These results suggest that lycopene may protect hepatic and renal tissue from OTA-induced DNA damage.

1. Introduction

Ochratoxins are secondary metabolites of *Aspergillus* and *Penicillium* species (EFSA, 2006; WHO, 2001). Ochratoxin A (OTA) is a well-known nephrotoxin and a possible hepatotoxin of environmental significance (el Khoury and Atoui, 2010; Pfohl-Leszkowicz and Manderville, 2007, 2012). Several studies suggested that OTA exposure can cause morphological and functional changes in kidney and liver of several domestic and experimental animals (Atroshi et al., 2000; Gagliano et al., 2006; Pfohl-Leszkowicz and Manderville, 2007). OTA is considered to be the main causal agent of Balkan Endemic Nephropathy (BEN), a disease mainly occurring in some areas of South-Eastern Europe. BEN is characterized by progressive renal fibrosis in humans (Cavin et al., 2009; Radovanovic et al., 1991). Furthermore, in West African countries, end stage chronic interstitial nephropathy has been increasing continuously in last decades and could be related to exposure to strong nephrotoxins like OTA (Abid et al., 2003; Filali et al., 2002).

OTA has been reported to be carcinogenic, teratogenic, and immunosuppressive in several animal species (Madrigal-Santillán et al., 2010). OTA is classified as a possible carcinogen to human (Group 2B) by the International Agency for Research on Cancer (IARC, 1993). The long-term exposure to OTA may induce oxidative stress, which is a probable mechanism underlying its nephrotoxicity (Meki and Hussein, 2001; Petrik et al., 2003). Besides,

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high concentrations of OTA were suggested to provoke the inhibition of protein synthesis, alteration of mitochondrial respiration, and induction of lipid peroxidation (Meisner and Chan, 1974; Rahimtula et al., 1988; Roth et al., 1989).

Recently, the genotoxic and carcinogenic effects of OTA in humans have attracted the attention of many scientists (Ali et al., 2011). However, the molecular mechanisms involved in OTA-induced renal and hepatic toxicities are not fully elucidated. It was suggested that these effects were the result of the ability of OTA to form DNA adducts and single-strand breaks in DNA, possibly through the generation of reactive oxygen species (ROS) (Cosimi et al., 2009; Ehrlich et al., 2002; Kamp et al., 2005a; Mally and Dekant, 2005; Tozlovanu et al., 2006). Several studies show that low concentrations of OTA may induce DNA damage and apoptosis in renal and hepatic human and rodent cells (Corcuera et al., 2011; Liu et al., 2012; Meki and Hussein, 2001; Petrik et al., 2003). Liu et al. (2012) demonstrated the involvement of oxidative pathway in OTA mediated cytotoxicity, genotoxicity, consequently G1 arrest, and apoptosis in human peripheral blood mononuclear cells. Russo et al. (2005) determined that OTA produced a dose and time-dependent increase in ROS production and DNA damage in human fibroblasts. In another study, OTA (at 1 and 5 μM) evoked significant DNA damage in porcine kidney epithelial PK15 cells after 24 h (Klarić et al., 2010). Golli-Bennour et al. (2010) observed that OTA decreased cell viability markedly in a dose-dependent manner and increased fragmented DNA levels in cultured Vero cells. In addition, p53 was activated and the expression of the antiapoptotic factor bcl-2 decreased significantly in response to OTA-induced DNA damage. Besides, Arbillaga et al. (2007) reported that OTA produced single-strand DNA breaks and oxidative DNA damage in human renal proximal tubular epithelial cell line, HK-2. Moreover, the production of OTA metabolites was suggested to be involved in genotoxicity of OTA (Domijan et al., 2006; Klarić et al., 2010).

There is considerable interest in developing strategies preventing genotoxicity and cancer, with minimal risk or toxicity (Ferguson and Philpott, 2008). Carotenoids like lycopene are of particular interest, as they might have high potential to prevent DNA damage caused by ROS. Lycopene is an antioxidant found in tomato, and in other red fruits, and vegetables (Agarwal and Rao, 1998; Lowe et al., 1999; Story et al., 2010). Lycopene was shown to be protective against oxidation of lipids, proteins, and DNA *in vivo* (Stahl and Sies, 2005). When administered at 5, 10, and 20 mg/day to humans, lycopene provided significant decreases in serum malondialdehyde (MDA) levels and increases in serum thiol levels. However, increasing the dose of lycopene did not provide any significant decrease in lipid peroxidation or any marked increase in serum thiols, so it was suggested that 5 or 10 mg/day lycopene supplementation was sufficient to provide protection against oxidative stress (Rao and Shen, 2002). Moreover, lycopene also exerts its effects *via* other mechanisms that include gene function regulation, gap-junction communication, hormone and immune modulation, carcinogen metabolism, and metabolic pathways involving phase II drug-metabolizing enzymes (Rao and Rao, 2007). Early research suggested the protective role of lycopene against cardiovascular disease, cancer, diabetes, osteoporosis, and male infertility. Recent scientific and clinical research has been devoted to a possible correlation between lycopene consumption and general health (Story et al., 2010).

There are several studies determining the antioxidant effect of different compounds against mycotoxin toxicity (Gowda and Ledoux, 2008). However, protection studies were mostly focused on aflatoxins and available data against other mycotoxins is limited or is generally obtained from *in vitro* studies (Gross-Steinmeyer and Eaton, 2012; Singh et al., 1994). The *in vivo* chemopreventive studies against the genotoxicity of OTA are also limited and the protective role of lycopene on DNA damage induced by OTA has not been yet investigated (Arbillaga et al., 2007; Kamp et al., 2005b; Wahhab et al., 2008).

Based on this background and taking into account the high OTA exposure of general population, this study was designed to investigate the possible protective effect of lycopene against the genotoxicity of OTA in rat tissues using the alkaline comet assay.

2. Material and methods

2.1. Chemicals

OTA was obtained from Sigma (St Louis, MO, USA). Lycopene powder (10% purity) was gift from Microgen (Istanbul, Turkey). Other chemicals were purchased from the following suppliers: normal melting point agarose (NMA) and low melting point agarose (LMA) from Boehringer Manheim (Germany); sodium chloride (NaCl) and sodium hydroxide (NaOH) from Merck Chemicals (Darmstadt, Germany); dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), Triton X-100, phosphate buffered saline (PBS) tablets, ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA), N-lauroyl sarcosinate and Tris from ICN Biomedicals Inc. (Aurora, Ohio, USA).

2.2. Animals

Adult male Sprague–Dawley rats (12 weeks old, weighing 250–260 g) were obtained from Hacettepe University Experimental Animal Laboratory. The animals were handled humanely and with regard for alleviation of suffering, and the study was approved by the Hacettepe University Ethical Committee. The animals were divided randomly, and each group was housed in plastic cages with stainless-steel grid tops. The cages were maintained in a room with controlled temperature (23 °C), humidity (50%) and a 12-h light–dark cycle. Body weights (b.w.) were monitored daily.

2.3. Experimental groups

The study consisted of seven groups of six animals each: **Control Group I (C7)** received 1 ml of corn oil containing 10% DMSO, by intragastric lavage (i.g.) for 7 days; **Control Group II (C14)** received 1 ml of corn oil containing 10% DMSO, i.g. for 14 days; **Lycopene Control Group I (L7)** received 5 mg/kg/day lycopene (dissolved in 10% DMSO

and then scaled to required volume with corn oil), i.g. for 7 days; **Lycopene Control Group II (L14)** received 5 mg/kg/day lycopene i.g. for 14 days; **OTA Group (OTA)** received 0.5 mg/kg/day OTA (dissolved in 10% DMSO and then scaled to required volume with corn oil), i.g. for 14 days; **OTA and Lycopene 7 Group (OTA + L7)** received 0.5 mg/kg/day OTA, i.g. for 14 days and 5 mg/kg/day lycopene, i.g. for last 7 days; **OTA and Lycopene 14 Group (OTA + L14)** received 0.5 mg/kg/day OTA, i.g. and 5 mg/kg/day lycopene, i.g. for 14 days. Therefore, each animal received 0.1 ml DMSO (in 1 ml of corn oil) during the treatment period (0.4 ml DMSO/kg bw).

Considering the results of the study of Breinholt et al. (2000), in which the researchers demonstrated that the administration of lycopene at a dose of 5 mg/kg b.w./day for 14 days peaked the antioxidant enzyme activities, we applied the same dose of lycopene with two different supplementation periods.

Throughout the experiments, all animals appeared to remain healthy. No significant difference was observed in food intake between the groups. Twenty–four hours after the last dose of OTA and/or lycopene, animals were weighed, and sacrificed by decapitation under thiopental anesthesia. Venous blood samples were taken in heparinized tubes. Liver and kidney tissues were carefully dissected from their attachments and totally excised. The heparinized blood samples and tissue samples were kept in the dark at 4 °C and processed within 6 h.

2.4. Evaluation of DNA damage by the alkaline single cell gel electrophoresis (comet assay)

The DNA damage was determined by single cell gel electrophoresis (comet assay). The basic alkaline technique of Singh et al. (1988), as further described by Anderson et al. (1994) and Collins et al. (1997), was applied. Lymphocytes from whole heparinized blood were separated by Ficoll–Hypaque density gradient and centrifugation (Boyum, 1976). Then the cells were washed with phosphate-buffered saline (PBS) buffer. A small piece of liver or kidney tissue was placed in 1 ml of cold Hanks Balanced Salt Solution (HBSS) containing 20 mM EDTA/10% DMSO and was minced into fine pieces. After it was settled for 15 min, the supernatant was used for the experiments.

The concentrations of the renal and hepatic cells and the lymphocytes were adjusted to $\sim 2 \times 10^6$ cells/ml in PBS buffer. A total of 50 μl of the cells were suspended in 75 μl of 0.5% LMA. Then the suspensions were embedded on slides pre-coated with a layer of 1% NMA. Slides were allowed to solidify on ice for 5 min. Cover slips were then removed. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO), for a minimum of 1 h at 4 °C. Then they were removed from the lysing solution, were drained and were left in the electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH 13) for 20 min at 4 °C to allow unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was conducted at 4 °C for 20 min at 24 V/300 mA. The slides were neutralized at room temperature by washing 3 times in 0.4 M Tris–HCl (pH 7.5) for 5 min. After neutralization, the slides were incubated in 50%, 75%, and 98% of alcohols for 5 min for each, respectively.

The dried microscope slides were stained with EtBr (20 μg/ml in distilled water, 60 μl/slide), covered with a cover-glass prior to analysis with a Leica (Wetzlar, Germany) fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, Version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize the DNA damage, slides were examined at 40×. For each condition 100 randomly selected comets on each slide were scored, and tail length, tail intensity, and tail moment were determined as an average of duplicate slides for each condition.

2.5. Statistical analysis

Experimental data was analyzed with the one-way analysis of variance (ANOVA) followed by the Student's *t*-test using SPSS Software version 17.0 (SPSS Inc., Chicago, IL, USA). The *p* values less than 0.05 were considered significant. All results were expressed as mean ± standard error mean (SEM).

3. Results and discussion

In the present study, using alkaline comet assay, we evaluated whether lycopene could protect rat lymphocytes, kidney and liver tissues against the genotoxicity of OTA and whether different periods of lycopene administration could have different protective effects against the detrimental effects of OTA on DNA.

There were no significant alterations in body weights in any of the groups before OTA treatment. However, we observed a time-dependent decrease in the body weight of OTA group throughout 14 days. In the first week of OTA application, we observed a ~11% decrease in the body weights and at the 10th day of exposure, the decrease was ~15%. At the termination of the experiments, body weight of OTA group was significantly lower than control group (20%).

Peripheral blood is the first exposure sight of xenobiotics, including toxins and it sounded logical to measure DNA damage in the lymphocytes of the study groups. Although several studies conducted on peripheral blood mononuclear cells and fibroblasts determined significant increase in DNA damage by OTA exposure (Liu et al., 2012; Russo et al., 2005), we did not determine any significant change in DNA damage in the lymphocytes of OTA-exposed animals compared to control groups. Lycopene administration for both 7 and 14 days did not provide any marked change in the DNA damage of lymphocytes vs. control groups. However, in OTA + L14 group, there was a significant decrease in tail length (25% vs. C7 and C14), tail moment (34% vs. C7 and 25% vs. C 14), and tail intensity (44% vs. C7 and 39% vs. C14) compared to controls (data not shown).

In the renal cells, in contrast to the situation observed in the lymphocytes, OTA exposure caused marked increases in

tail length (78% vs C7; 80% vs. C14), tail moment (147% vs C7; 188% vs. C14), and tail intensity (92% vs C7; 87% vs. C14) when compared to control groups (Fig. 1). Lycopene application for 7 days with OTA exposure supplied 15%, 52%, and 51% decrease in tail length ($p < 0.05$), tail moment ($p < 0.05$), and tail intensity ($p < 0.05$), respectively, when compared to OTA treatment; while lycopene for 14 days with OTA application provided 29%, 42%, and 32% decrease in tail length, tail moment, and tail intensity, respectively, vs. OTA-exposed rats ($p < 0.05$, all). In both 7 and 14 days of lycopene plus OTA exposure, tail moment and tail intensity were almost at control levels (not significantly different from the C7 and C14 groups) (Fig. 1). Lycopene supplementation alone for both 7 and 14 days did not induce any significant change in the DNA damage of renal cells vs. control groups.

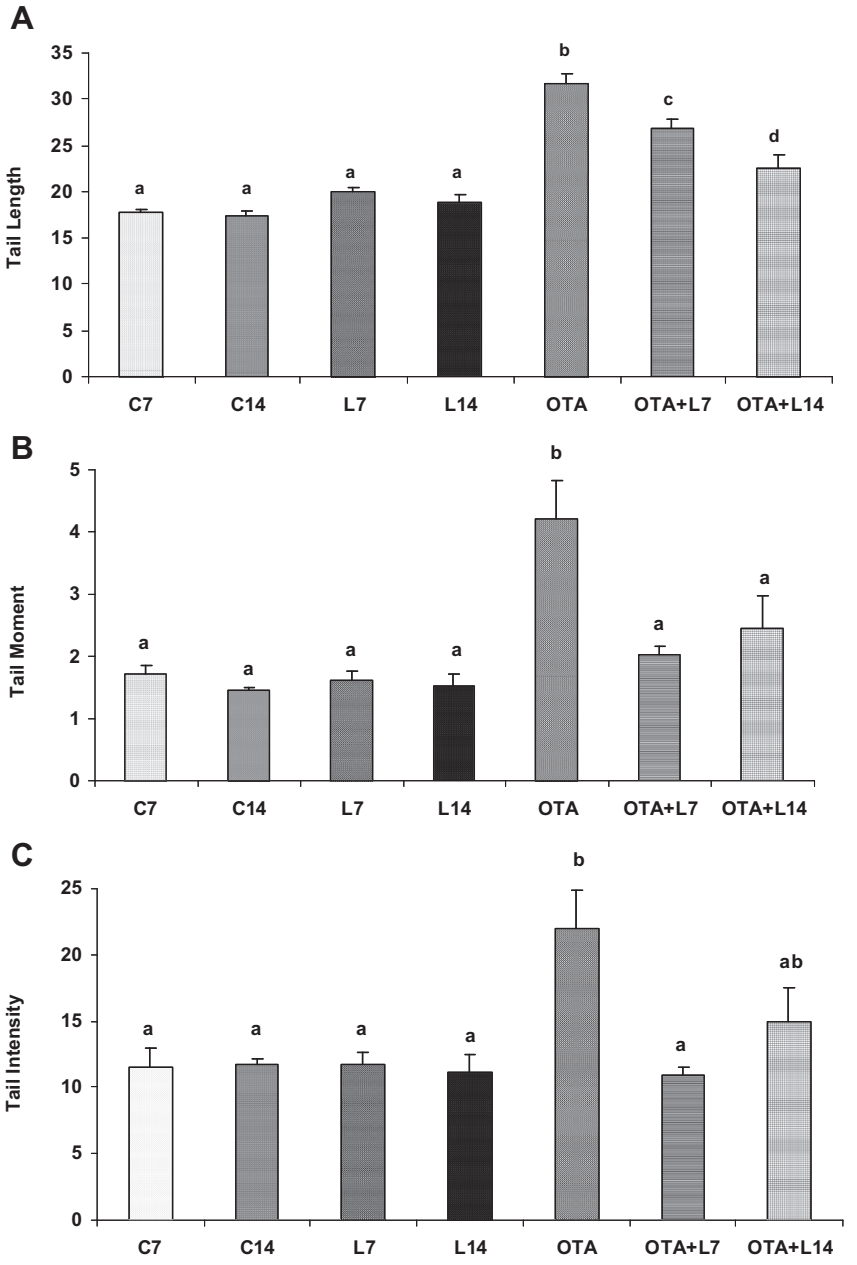

Fig. 1. DNA damage in kidney. The study consisted of seven groups of six animals each: Control Group I (C7) received 1 ml of corn oil, by intragastric lavage (i.g.) for 7 days; Control Group II (C14) received 1 ml of corn oil, i.g. for 14 days; Lycopene Control Group I (L7) received 5 mg/kg/day lycopene (dissolved in 10% DMSO and then scaled to required volume with corn oil), i.g. for 7 days; Lycopene Control Group II (L14) received 5 mg/kg/day lycopene i.g. for 14 days; OTA Group (OTA) received 0.5 mg/kg/day OTA (dissolved in 10% DMSO and then scaled to required volume with corn oil), i.g. for 14 days; OTA and Lycopene 7 Group (OTA + L7) received 0.5 mg/kg/day OTA, i.g. for 14 days and 5 mg/kg/day lycopene, i.g. for last 7 days; OTA and Lycopene 14 Group (OTA + L14) received 0.5 mg/kg/day OTA, i.g. and 5 mg/kg/day lycopene, i.g. for 14 days. Bars that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

In vivo studies investigating DNA damage caused by OTA using comet assay are limited (Mally and Dekant, 2005; Zeljezić et al., 2006; Klarić et al., 2010; Robbiano et al., 2004). Robbiano et al. (2004) reported that a single high oral dose (10 mg/kg b.w) of OTA-induced DNA damage in rat kidney. Zeljezić et al. (2006) evaluated the genotoxicity potential of OTA (0.5 mg/kg b.w./day, i.p., for 7, 14, 21 days) in the kidney of female Wistar rats using alkaline comet assay and observed significantly higher tail length and tail moment after 14 and 21 days of exposure compared to 7 days, with the highest tail intensity after 21 days.

In the liver cells, OTA exposure also caused marked increases in tail length (87% vs C7; 72% vs. C14), tail moment (245% vs C7; 237% vs. C14), and tail intensity (107% vs 17?18 C7; 125% vs. C24) when compared to control groups (Fig. 2). Lycopene supplementation (both for 7 days and 14 days)

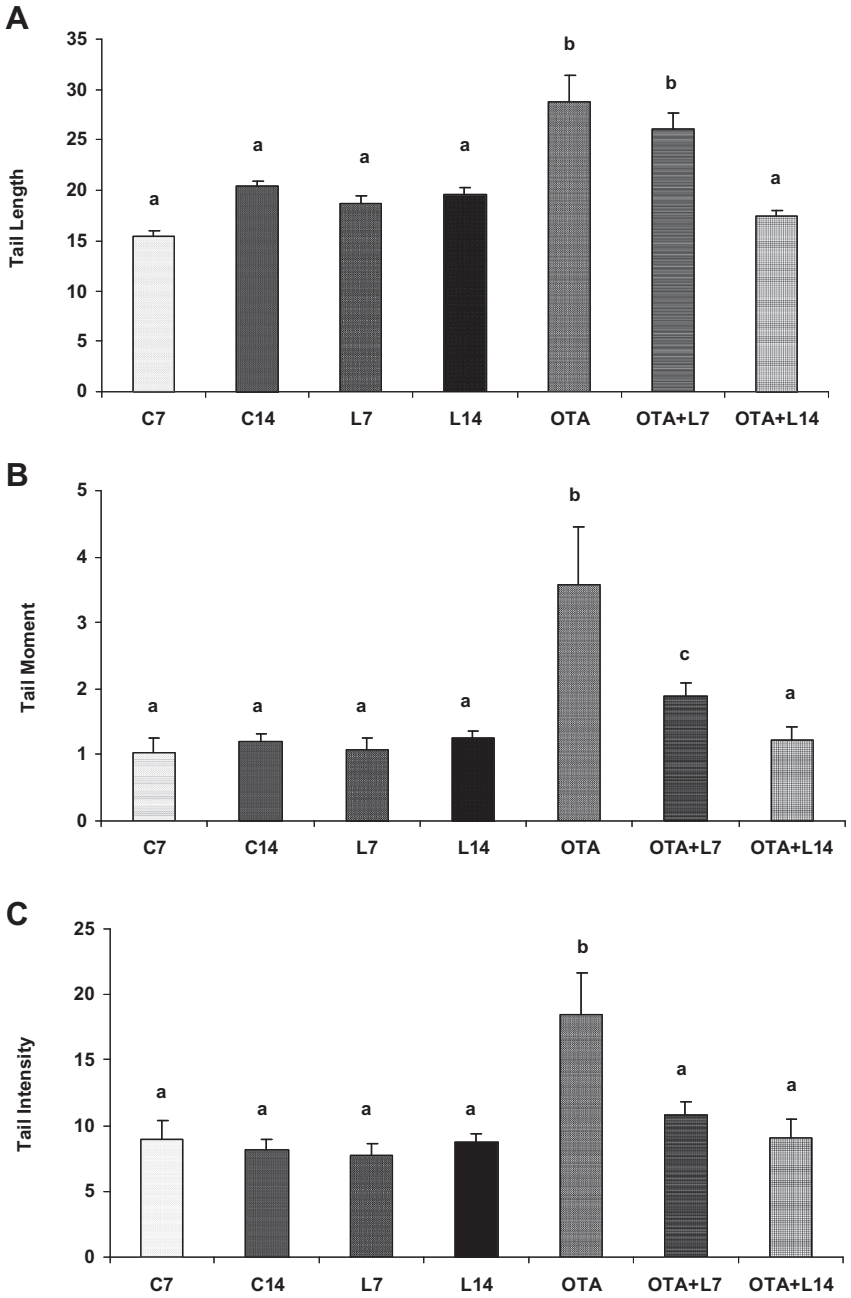

Fig. 2. DNA damage in liver. The study consisted of seven groups of six animals each: Control Group I (C7) received 1 ml of corn oil, by intragastric lavage (i.g.) for 7 days; Control Group II (C14) received 1 ml of corn oil, i.g. for 14 days; Lycopene Control Group I (L7) received 5 mg/kg/day lycopene (dissolved in 10% DMSO and then scaled to required volume with corn oil), i.g. for 7 days; Lycopene Control Group II (L14) received 5 mg/kg/day lycopene i.g. for 14 days; OTA Group (OTA) received 0.5 mg/kg/day OTA (dissolved in 10% DMSO and then scaled to required volume with corn oil), i.g. for 14 days; OTA and Lycopene 7 Group (OTA + L7) received 0.5 mg/kg/day OTA, i.g. for 14 days and 5 mg/kg/day lycopene, i.g. for last 7 days; OTA and Lycopene 14 Group (OTA + L14) received 0.5 mg/kg/day OTA, i.g. and 5 mg/kg/day lycopene, i.g. for 14 days. Bars that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

plus OTA treatment provided marked decreases in all DNA damage parameters vs. OTA group. Lycopene administration for 7 days with OTA provided 14%, 41%, and 50% decrease in tail length ($p < 0.05$), tail moment ($p < 0.05$), and tail intensity ($p < 0.05$), respectively, when compared to OTA group, while lycopene application for 14 days supplied more protection against DNA damage caused by OTA [40%, 66%, and 51% decrease in tail length, tail moment, and tail intensity, respectively, vs. OTA-exposed rats ($p < 0.05$, all)]. By 7 days of lycopene plus OTA exposure, only tail intensity was almost at control levels, while after 14 days of lycopene plus OTA exposure, all DNA damage parameters were almost at control levels (not significantly different than the C7 and C14 groups) (Fig. 2). Lycopene treatment for 14 days along with OTA provided better protection, particularly in the liver compared to the lymphocytes and the renal cells. However, lycopene application alone for both 7 and 14 days did not provide any significant decrease in the DNA damage of liver cells vs. control groups. Mally and Dekant (2005) demonstrated that OTA treatment (250, 500, 1000, 2000 μg/kg b.w. for 2 weeks) caused the formation of DNA strand breaks in both target and non-target organs; however DNA damage was not permanent and stable; DNA adducts did not form, suggesting DNA damage detected was the result of oxidative stress. In our recent studies, we have determined that OTA exposure at 0.5 mg/kg b.w. for 14 days decreased catalase (CAT) and superoxide dismutase (SOD) activities, and total glutathione (GSH) levels in rat kidney (Aydın et al., 2010) and rat liver (Palabiyik et al., 2012), supporting this hypothesis. However, as reported by Mally and Dekant (2005), additional mechanisms could also be involved in the carcinogenesis of OTA.

In the present study, we observed that both 7 and 14 days of lycopene treatment along with OTA exposure provided significant protection in both rat kidney and liver, while 14 days of lycopene treatment was more protective against OTA-induced hepatic DNA damage. In animal studies, lycopene was shown to have a large margin of safety. In Wistar albino rats, no observed effect level (NOEL) of lycopene was found to be 586 mg/kg b.w./day for males and 616 mg/kg b.w./day for females in a ninety day oral toxicity study and no toxic effects were observed in rats treated with lycopene 2 g/kg/day for 28 days (Jonker et al., 2003).

The lycopene content of tomatoes changes according to the soil type, the season, the climate, and harvesting timing. Tomatoes usually contain 8.8–42 mg lycopene/kg wet weight (mean = ~25 mg/kg tomato) while red carrot contains 5 mg lycopene/vegetable (Rao and Rao, 2007). Concerning the dose of 5 mg/kg b.w./day used in the study, a 70-kg person should take 350 mg lycopene with his diet in order to reach to this dose. However, taking this amount is possible with a healthy diet, as other than tomatoes and red carrots, lycopene is also present in several products made from tomato and several other red fruits and vegetables (red bell peppers, watermelons, red grapefruit) (Agarwal and Rao, 1998; Lowe et al., 1999; Story et al., 2010). In a physiological Phase I model study, lycopene was delivered as a tomato beverage formulation in five graded doses (10, 30, 60, 90, or 120 mg) to healthy male subjects (five per dose). It was reported that the percent absorption at the 10 mg dose (33.9 ± 8.1%) was significantly greater than the higher doses; however, the amount of lycopene absorbed (mg) was not statistically different (mean: 4.69 ± 0.55 mg) between these doses, suggesting a possible saturation of absorptive mechanisms (Diwadkar-Navsariwala et al., 2003).

In the aforementioned study, Arbillaga et al. (2007) also evaluated the ability of N-acetyl-L-cysteine (NAC) to ameliorate OTA-induced toxicity in HK-2 human kidney cells and found that NAC was protective against ROS elevation, cytotoxicity and DNA damage. These results suggested that OTA was not a direct genotoxic carcinogen and oxidative stress had an important role in the genotoxicity and carcinogenicity of OTA. Russo et al. (2005) also showed the protective effect of cyanidin 3-O-h-d-glucoside (an anthocyanin present in pigmented oranges, red wines, fruits, and vegetables), against OTA-induced DNA damage in human fibroblasts by comet assay and the results of this study also confirmed the involvement of oxidative stress in the OTA genotoxicity. In agreement with these studies, our findings suggesting the protective role of lycopene against DNA damage induced by OTA further support this hypothesis indicating that its genotoxic effects might occur through a mechanism that may involve oxidative stress.

4. Conclusions

In summary, we observed that OTA caused DNA damage in both liver and kidney of rats and lycopene provided a protective effect against OTA-induced DNA damage as evidenced by decreased tail moment and intensity in both rat kidney and liver cells. We may postulate that oxidative stress precedes genotoxicity, and plays an important role in OTA nephrotoxicity, hepatotoxicity, and carcinogenicity when lycopene, an effective ROS quencher, was administered to rats (Turesky, 2005). As lycopene supplementation for longer period produced slightly higher protection against OTA-induced hepatic and renal DNA damage, we may suggest that lycopene should be taken routinely by diet especially from natural sources to exert enhanced protection. Our future aim is to investigate the possible protective effects of lycopene pretreatment against OTA exposure in rats. Moreover, we plan to perform more mechanistical studies with lycopene and with other antioxidants against OTA toxicity in rodent models.

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Conflict of interest

The authors declare no conflicts of interest.

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