Citrinin-Generated Reactive Oxygen Species Cause Cell Cycle Arrest Leading to Apoptosis via the Intrinsic Mitochondrial Pathway in Mouse Skin

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The mycotoxin, citrinin (CTN), is a contaminant of various food and feed materials. Several in vivo and in vitro studies have demonstrated that CTN has broad toxicity spectra; however, dermal toxicity is not known. In the present investigation, dermal exposure to CTN was undertaken to study oxidative stress, DNA damage, cell cycle arrest, and apoptosis in mouse skin. A single topical application of CTN caused significant change in oxidative stress markers, such as lipid peroxidation, protein carbonyl content, glutathione (GSH) content, and antioxidant enzymes in a dose-dependent (25–100 mg/mouse) and time-dependent (12–72 h) manner. Single topical application of CTN (50 mg/mouse) for 12–72 h caused significant enhancement in (1) reactive oxygen species (ROS); (2) cell cycle arrest at the G0/G1 phase (30–71%) and G2/M phase (56–65%) along with the induction of apoptosis (3.6–27%); (3) expression of p53, p21/waf1; (4) Bax/Bcl2 ratio and cytochrome c release; and (5) activities of caspase 9 (22–46%) and 3 (42–54%) as well as increased poly(ADP-ribose) polymerase cleavage. It was also observed that pretreatment with bio-antioxidants viz butylated hydroxyanisole (55 mg/100 mg), quercetin (10 mg/100 mg), or α-tocopherol (40 mg/100 mg) resulted in decreases of ROS generation, arrest in the G0/G1 phase of the cell cycle, and apoptosis. These data confirm the involvement of ROS in apoptosis and suggest that these bio-antioxidants may be useful in the prevention of CTN-induced dermal toxicity.

Key Words: citrinin; mouse skin; ROS; apoptosis; antioxidants.

The mycotoxin, citrinin (CTN), a secondary metabolite of several fungal species belonging to the genus Penicillium and Monascus, is a natural contaminant of various types of feed and foods, viz corn, wheat, rice, barley, and nuts worldwide, and is associated with environmental and human health injuries (CAST, 2003). Studies have shown that CTN triggers nephropathy and hepatotoxicity as well as renal adenoma formation in various cellular and animal models (Aleo et al., 1991; Arai and Hibino, 1983; Kitabatake et al., 1993). CTN along with ochratoxin A (OTA) has been implicated as a potential causative agent in human endemic Balkan nephropathy (Vrabcheva et al., 2000).

According to World Health Organization (WHO) guidelines, humans and animals may be exposed to mycotoxins through ingestion, inhalation, or skin contact (WHO, 1998). However, due to limited knowledge regarding epidermal carcinogenesis of mycotoxins, WHO has highlighted the need for toxicological evaluation of mycotoxins through dermal exposure (FAO, 1990). This is an important aspect from the point of view of developing countries including India where manual labor is employed during pre- and postharvest stages of agriculture, thus indicating a probable cause of exposure through dermal route. Earlier studies have shown that mycotoxins like aflatoxin-B1 (AFB1) and trichothecenes (T-2 Toxin) readily penetrates through human skin and causes systemic toxic effects in their respective target organs (Kemppainen et al., 1987, 1988). In this regard, our prior studies have revealed that dermal exposure to AFB1 and patulin resulted in toxicological injury in skin (Rastogi et al., 2006; Saxena et al., 2009). Furthermore, twice weekly dermal application of AFB1 for 25 weeks resulted in modulation of hepatic oxidative stress, xenobiotic metabolizing enzymes, and degenerative changes in hepatocytes, indicating its absorption through skin and subsequent systemic effects after dermal exposure (Rastogi et al., 2006). There are no data available on CTN following dermal exposure, but due to the widespread contamination of food and feed with this mycotoxin (Xu et al., 2006); it is likely that humans involved in agricultural practices may be exposed to CTN through their skin. Although there has been no case report regarding CTN-induced dermatological effects, nonetheless, WHO has suggested the need to study dermal toxic effects of mycotoxins including CTN. The cytotoxic effects of several mycotoxins including CTN on target tissues and cultured cells are thought to correlate with their apoptosis-inducing ability (Chan, 2007; Yu et al., 2006). It has been suggested that numerous chemical and physical treatments capable of
inducing apoptosis stimulate oxidative stress via intracellular reactive oxygen species (ROS) generation (Gutteridge and Halliwell, 2010); however, the link between ROS perturbation and CTN-induced cellular changes has not been previously examined.

Although few studies have shown involvement of ROS in CTN-mediated toxicity in certain in vitro models (Chan, 2007; Chen and Chan, 2009; Yu et al., 2006), nonetheless, the cellular and molecular mechanisms of CTN toxicity of exposure to skin are not yet investigated. Thus, the aim of the present investigation was to gain an understanding of the cellular events involved in CTN-mediated toxicity and more specifically to study whether CTN-induced ROS are responsible for DNA damage leading to apoptosis in mouse skin. Additionally, topical treatment with several bio-antioxidants viz butylated hydroxyanisole (BHA), quercetin (Quer), and α-tocopherol (Toco) was carried out not only for assessment of potential prevention of CTN-induced dermal toxicity but also to establish the role of ROS in CTN-mediated apoptosis and cell cycle phase alterations.

**MATERIALS AND METHODS**

**Chemicals.** Citrinin (CTN), 2′,7′-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA), diithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), 2-mercaptoethanol (BME), propidium iodide (PI), RNase A, normal melting agarose, low melting point agarose (LMPA), protease inhibitor cocktail set L, ethidium bromide (EtBr), EDTA disodium salt, Triton X-100, quercetin (Quer), α-tocopherol (Toco), butylated hydroxyanisole (BHA), and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co. (St Louis, MO). Anti-β2-ad (BME), anti-cytochrome c, anti-anti-ADP-ribose polymerase (PARP), and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p21/waf1 and anti-Bax antibodies were purchased from Cell Signaling (Beverly, MA). Fluorescein isothiocyanate (FITC)–conjugated Annexin V and specific substrates for caspase 3 and 8 were procured from BD Pharmingen (SanJose, CA), whereas substrate for caspase 9 was purchased from G-Biosciences (Maryland Height, MO). All other chemicals used were of the highest purity commercially available.

**Animals.** Six- to seven-week old female Swiss albino mice (20 ± 3 g) derived from the animal breeding colony at the Indian Institute of Toxicology Research, Lucknow, were acclimatized under standard laboratory conditions and provided a commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. Animals were housed in plastic cages having rice husk as bedding and maintained in a controlled atmosphere of 12-h dark/light cycle, 22 ± 2°C temperature, and 50–60% humidity in accordance with the rules of the Animal Welfare Committee of Indian Institute of Toxicology Research. The mice were shaved with electric clippers (Oster, McMinville, TN) 1 week prior to the beginning of experiment. Mice showing no signs of hair growth were used for further experiments.

**Oxidative stress markers.** The animals were distributed randomly into five groups, with five mice per group. The animals in the first group received a single topical application of 0.2 ml acetone, and the remaining four groups received a single topical dose of 50 μg CTN/0.2 ml acetone per mouse. The animals were sacrificed after 12, 24, 48, and 72 h of CTN treatment and 2 cm<sup>2</sup> of treated skin was excised from each animal. Fat from the dermal side of skin was removed by scraping and 10% wt/vol skin homogenate was prepared in 0.2 M phosphate buffer using Ultra Turrax Polytron (IKA-Labortechnik, Staufen, Germany). A portion of the homogenate was centrifuged at 10,000 × g for 20 min at 4°C to isolate the postmitochondrial supernatant. Reduced glutathione (GSH) content was assayed in the homogenate according to the method of Ellman and Lyssy (1967). The rate of lipid peroxidation (LPO) was determined by estimating malondialdehyde (MDA) formed with thiobarbituric acid (TBA) (Utlely et al., 1967). Catalase activity was determined by the method of Sinha (Sinha, 1972). Glutathione peroxidase (GPx) was assayed according to the method of Rotruck et al. (1973).

**ROS measurement and alkaline comet assay.** The treatment schedule was same as described under the section “Oxidative Stress Markers.” Single cell suspensions were prepared as described earlier (Saxena et al., 2009), and cell viability was determined by trypan blue exclusion assay. For estimation of ROS, 1 × 10<sup>6</sup> cells were incubated in the presence of 100μM H<sub>2</sub>DCFDA for 60 min at 37°C in a black 96-well plate, and relative fluorescence intensity was measured in SYNERGY-HT multiwell plate reader (Bio-Tek, Winooski, VT) using KC4 software at excitation and emission wavelengths of 485 and 528 nm, respectively.

DNA damage in mouse skin cells was evaluated by the alkaline comet assay technique as described previously (Saxena et al., 2009; Singh et al., 1998). Slides were prepared and scored with an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescence microscope (Leica, Wetlzar, Germany) equipped with appropriate filters. The microscope was connected to a computer through a charge-coupled device camera to transport images to software (Komet 5.0) for analysis. The final magnification was ×400. The parameters measured in the study were olive tail moment (arbitrary units), tail DNA (%), and tail length (migration of the DNA from the nucleus; micro meter).

**Cell cycle analysis.** The treatment schedule was same as described under the section “Oxidative Stress Markers” and single cell suspensions were prepared as described earlier (Saxena et al., 2009). Single cell suspensions were washed thrice with 0.5 ml PBS and fixed in ice-cold 70% ethanol for 2 h at −20°C for cell cycle analysis. The cells were flushed through a 21-gauge needle (3–4 times) for uniform dispersion. The fixed cells were again washed twice with PBS and incubated with propidium iodide (PI) (20 μg/ml) and RNase A (200 μg/ml) for 60 min at 37°C. Data acquisition was performed with an argon laser fluorescence–activated cell analyzer (FAC Sort, Becton-Dickinson). For each sample, 10,000 events were acquired, and the analysis was carried out using Cell Quest software version 1.22.

**Apoptosis.** Apoptosis was detected by flow cytometry of single cell suspensions by binding of annexin V-FITC, according to the manufacturer’s protocol (BD Pharmingen). As described earlier (Saxena et al., 2009), the cell pellet was suspended in 1× binding buffer followed by incubation with annexin V-FITC and PI in the dark for 20 min. The fluorescence of the cells was then analyzed by flow cytometer.

**Western blot analysis.** The treatment schedule was same as described under the section “Oxidative Stress Markers.” Dorsal skin was excised and fat was removed by scraping on ice. The scraped skin was rinsed in normal saline and homogenized in ice-cold lysin buffer (50mM Tris–HCl [pH 7.5], 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Dithiothreitol [DTT], 1mM phenylmethylene sulfonyl fluoride [PMSF], supplemented with protease inhibitor cocktail (Protease Inhibitor Cocktail Set I, Sigma Chemical Co.) kept, incubated on ice for 10 min with intermittent vortexing, and then centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was collected and protein concentration in each sample was measured by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA), using BSA as a standard. Sixty micrograms of total protein were resolved by 12% SDS-polyacrylamide gel electrophoresis and electrotransferred on polyvinylidene fluoride membranes. The blotted membrane was blocked with 5% Blotto nonfat dry milk (Santa Cruz Biotechnology) in PBS containing 0.1% Tween 20 (blocking solution) and incubated with indicated antibodies (used at dilutions indicated by the manufacturer) followed by incubation with horseradish peroxidase–conjugated secondary antibody (Sigma Chemical Co.) in blocking solution. Bound antibody was detected by enhanced chemiluminescence using Amersham ECL Western Blotting Detection Reagents, following the manufacturer’s directions (Amersham, Fairfield, CT). All the blots were stripped and reprobed with anti-β-actin to ensure equal loading of protein.
FIG. 1. Effect of topical application of CTN on oxidative stress markers in mice skin. (A) Dose-dependent effect of CTN on epidermal LPO, protein carbonyl content, GSH content, and activities of catalase and GPx of mice exposed for 24 h. Each value represents the mean ± SE of five values. (B) Time-dependent effect of CTN (50 µg/mouse) on epidermal LPO, protein carbonyl content, GSH content, and activities of catalase and GPx of mice exposed for 12–72 h. Each value represents the mean ± SE of five values. (C) Measurement of ROS generation in mouse skin following CTN (50 µg/mouse) treatment for different time intervals. Single cell suspensions from vehicle-/CTN-treated mice skin were prepared and 1 × 10^5 cells were incubated with 100 µM H_2DCFDA for 90 min at 37°C, and relative fluorescence intensity was determined by spectrofluorimetry (λ_ex = 488 nm, λ_em = 520 nm). Data are presented in terms of fold change as compared with control, as the mean ± SE of five values. *p < 0.05, significant with respect to control group.
**Results**

**Effect of Topical Application of CTN on Oxidative Stress Markers in Mouse Skin**

To observe the dose-dependent effect of dermal exposure to CTN on oxidative stress markers in mouse skin, animals were treated with a single topical application of CTN (25, 50, and 100 μg/mouse) for 24 h. It was observed that CTN at doses of 50 and 100 μg/mouse caused significant increases in LPO (160–208%) and protein carbonyl content (221–371%) along with significant decreases in GSH content (40–47%) (Fig. 1A).

The DNA-damaging potential of CTN was evaluated in mouse skin using the comet assay (Singh et al. 1998). As shown in Figure 2A, CTN treatment for 12–72 h caused significant enhancement of olive tail moment (96–232%), tail DNA (64–135%), and tail length (75–122%) with maximum values at 24 h. The DNA from epidermal cells prepared from CTN-treated animals showed the typical comet formation, whereas epidermal cells of control animals showed no damage to DNA as assessed by the defined circular nucleus shape (Fig. 2B).

**Effect of Topical Application of CTN on Cell Cycle Phase Distribution and Apoptosis**

Table 1 shows the effect of dermal exposure of CTN on cell cycle phases in mouse skin cells. Exposure of mice to CTN for 24–72 h resulted in significant increase in the proportion of cells in the G0/G1 phase (45–71%) with a concomitant decrease in the S phase (44–59%) when compared with control. However, the G2/M phase was not found to be significantly affected by CTN exposure for 12–24 h, but increased at 48–72 h (Table 1). Apoptosis was analyzed using Annexin V and PI dual staining of epidermal cells from CTN-treated mice (Fig. 3). Topical application of mice with 50 μg of CTN for 24–72 h caused significant enhancement in apoptosis (7.6- to 25-fold) when compared with control (Fig. 3). Together, these results indicate that cells are arresting at the G0/G1 and G2/M phases to repair CTN-induced DNA damage and that some of the damage was not repaired causing the cells to undergo apoptosis.

**Effect of Topical Application of CTN on Expression of p53 and p21/waf1 Proteins and Activation of the Intrinsic Pathway of Apoptosis**

The effect of a single topical application of CTN on expression of p53 and p21/waf1 proteins in mouse skin is shown in Figure 4A. Dermal wild-type p53 protein expression was significantly enhanced following CTN treatment (12–72 h) compared with control, whereas p21/waf1 protein expression was significantly enhanced from 24 to 72 h. The respective blots were stripped and probed with anti-β-actin to ensure equal protein loading. Further, topical application of mice for 24–72 h resulted in significant overexpression of Bax along with reduction of Bcl2, which was associated with an increase in cytochrome c levels in the cytoplasm (Fig. 4B).
application to mouse skin for 12–72 h also caused significant enhancement in the activities of caspase 9 (1.2- to 1.8-fold) and caspase 3 (1.7- to 2.2-fold) with maximum at 24 h but although caspase 8 activity was unaffected (Fig. 4C). Activation of caspase 3 results in PARP cleavage, which was observed in a time-dependent manner following CTN application to mouse skin (Fig. 4D).

**Effect of Scavengers of ROS on CTN-Induced ROS Generation, Cell Cycle Arrest, and Apoptosis**

Topical application of the antioxidants like Toco, BHA, or Quer to mice 30 min prior to CTN application produced a significant reduction in ROS levels by 34, 38, and 53%, respectively, after 24 h when compared with the CTN-only treated group (Fig. 5A). Pretreatment with Toco, BHA, or Quer to CTN-treated mice resulted in significant reduction (43–49%) in the proportion of cells in G0/G1 phase of cell cycle after 24 h when compared with CTN-only treated group (Fig. 5B). Similarly, pretreatment with Toco, BHA, or Quer to CTN-treated mice caused significant reduction in apoptosis (38–56%) when compared with the CTN-only treated group (Fig. 5C).

### DISCUSSION

Mycotoxins are among the most significant food contaminants with regard to public health and food security. Although the oral route is the most common means of human exposure to these toxins, it was recently suggested by WHO that in the developing countries, humans may also be exposed through their skin because manual labor is involved during pre- and postharvest stages of agriculture (WHO, 1998). The rationale for conducting the present study was that CTN contamination in various food crops has been detected at the levels of 93 µg/kg in a barley sample from the Czech Republic, 460 µg/kg in a wheat sample from Argentina, and 280–920 µg/kg in fruit produce of European countries (Xu et al., 2006). Thus, human exposure through skin may occur at these levels during pre- and postharvest stages, which is relevant to the present study where 25–100 µg/animal doses were used. Several studies have demonstrated that intracellular oxidative stress is responsible for CTN-induced toxicity (Liu et al., 2003; Yu et al., 2006). It is well known that cells have developed adaptive dynamic programs to counteract environmental stresses imposed by intrinsic and extrinsic oxidants and electrophiles. Glutathione,

<table>
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<tr>
<th>Groups</th>
<th>G0/G1 phase</th>
<th>S phase</th>
<th>G2/M phase</th>
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<tbody>
<tr>
<td>Control</td>
<td>35.48 ± 0.4</td>
<td>2.29 ± 0.08</td>
<td>1.32 ± 0.02</td>
</tr>
<tr>
<td>Citrinin (12 h)</td>
<td>46.11 ± 0.2</td>
<td>1.31 ± 0.04* (42% ↓)</td>
<td>1.44 ± 0.03</td>
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<tr>
<td>Citrinin (24 h)</td>
<td>59.05 ± 0.6* (66% ↑)</td>
<td>1.27 ± 0.03* (44% ↓)</td>
<td>1.49 ± 0.02</td>
</tr>
<tr>
<td>Citrinin (48 h)</td>
<td>51.71 ± 0.4* (45% ↑)</td>
<td>1.06 ± 0.05* (53% ↓)</td>
<td>2.18 ± 0.06* (65% ↑)</td>
</tr>
<tr>
<td>Citrinin (72 h)</td>
<td>60.79 ± 0.9* (71% ↑)</td>
<td>0.93 ± 0.07* (59% ↓)</td>
<td>2.06 ± 0.09* (56% ↑)</td>
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*Note. Data represent mean ± SE of five animals per group. Values in parentheses indicate percent increase (↑) or decrease (↓). Details of treatment schedule and processing of cells are described in the Materials and Methods. *p < 0.05, significant when compared with control.
a bio-antioxidant tripeptide, is present in cells in reduced form. During several disease conditions or chemical exposures, this tripeptide acts as a nucleophile to scavenge the enhanced generation of free radicals, followed by depletion and the manifestation of toxicity (Scholz et al., 1989). Also, there are several enzymes which help to counteract oxidative stress in cells, e.g., catalase, GPx, etc. (Fridovich and Freeman, 1986; Khan et al., 2010). Enhanced free radical generation may cause oxidative damage to cellular macromolecules such as lipids, proteins, and DNA, thus altering their structure and function (Das et al., 2005a; Martin and Barrett, 2002). Our present in vivo study showed that topical application of CTN caused enhanced generation of ROS, which indicates that CTN causes oxidative stress. Further, significant depletion of GSH as well as inhibition of GPx and catalase activities, along with an increase in LPO and protein carbonyl content, following CTN treatment reemphasizes the occurrence of oxidative stress.

It is well documented that generation of intracellular ROS may also lead to DNA damage (Wang et al., 2001); hence, the DNA-damaging potential of CTN was evaluated in mouse skin using the comet assay. CTN treatment of mice for 12–72 h caused significant DNA damage, suggesting its genotoxic and mutagenic potential in skin. These results are consistent with the earlier findings where CTN was shown to be genotoxic in other models (Knasmüller et al., 2004; Liu et al., 2003). Although the severity of DNA damage following toxic insult

**FIG. 3.** Effect of topical application of CTN on apoptosis of mouse epidermal cells. A single cell suspension was prepared and apoptosis was detected by an annexin V-FITC kit through flow cytometry. Each dot blot represents 10,000 events (cells) and data are presented as the mean ± SE of five values. *p < 0.05, significant with respect to control group.
may vary, and if not fully repaired it can result in adverse effects (Essers et al., 2006). The early response to DNA damage is to arrest cell cycle progression to allow time for repair of the damage (Jiang et al., 2010). However, when cells are unable to repair their damaged DNA, they may undergo apoptosis. Exposure of mice to CTN resulted in a significant increase in the proportion of cells in the G0/G1 and G2/M phases with a concomitant decrease in S phase followed by an increase in apoptosis. Similarly, CTN has been reported to cause apoptosis in HL-60, HepG2, Vero, and mouse embryonic cells (Chan, 2007; Chen and Chan, 2009; Yu et al., 2006). In HEK293 cells, however, CTN behaves differently in that lower concentration, such as 50 μM, triggered the G2/M phase arrest, whereas concentrations exceeding 150 μM causes apoptosis (Chang et al., 2011).

The p53 protein plays a key role in the DNA damage response pathway by transmitting a variety of stress signals associated with antiproliferative cellular responses that lead to apoptosis (Jiang et al., 2010). In response to CTN-induced DNA damage, overexpression of p53 protein leading to upregulation of p21/waf1 was also observed, which results in arrest of the cell cycle progression at the G0/G1 or G2/M phases (Abbas and Dutta, 2009). CTN exposure also leads to upregulation of the proapoptotic protein Bax and suppression of antiapoptotic protein Bcl2, thus shifting the balance toward Bax, which would form homo-multimers and create protein permeable pores in the outer membrane of mitochondria, thereby releasing cytochrome c protein to the cytoplasm (Jurgensmeier et al., 1998). Cytochrome c release in the cytoplasm activates apoptosis protease activating factor 1 (Apaf1), which leads to auto-cleavage of procaspase 9 to functional caspase 9, which further cleaves procaspase 3 to its activated form caspase 3, the execution caspase (Cecconi, 1999). Subsequently, caspase 3 cleaves PARP protein, a DNA repair enzyme in the cell and among the first target of executioner caspases. Thus, PARP cleavage is considered as a marker of apoptosis. In our study, CTN application to mouse skin resulted in significant enhancement in the activities of caspase 9 and caspase 3, but not caspase 8. The lack of enhancement in caspase 8 activity indicates that the extrinsic or death receptor pathway of apoptosis was not activated by CTN in mouse skin. In continuation of caspase 3 activation, PARP cleavage was also observed after CTN application to mice skin. Similarly, several investigators found that CTN induces apoptosis in various cell lines that was mediated through the mitochondria-dependent pathway, which corroborates our in vivo findings (Chan, 2007; Chen and Chan, 2009; Yu et al., 2006). The observed induction of apoptosis by CTN is of significance because this phenomenon is a physiological process that functions as an essential mechanism of tissue formation and development.

FIG. 4. Effect of topical application of CTN on apoptotic biochemical parameters in mouse epidermal cells. (A) p53 and p21/waf1 levels in skin from vehicle-/CTN-treated (50 μg/mouse) mice. (B) Bax, Bcl2, and cytochrome c levels in cytosolic fraction of mice skin. (C) Caspase 3, 8, and 9 activities in mouse skin treated with CTN was measured using specific substrates in terms of relative fluorescence unit. Data, in terms of fold change as compared with control values, are presented as the mean ± SE of five values. *p < 0.05, significant with respect to control. (D) Cleavage of PARP was analyzed by immunoblotting using anti-PARP antibody in whole cell lysate of skin from vehicle-/CTN-treated (50 μg/mouse) mice.
homeostasis and is regarded as the preferred way to eliminate damaged cells. On the other hand, it is quite likely that CTN exposure could lead to toxicity by enhancing apoptosis of normal skin cells which involves a cascade of events including cell cycle arrest at G0/G1 as well as G2/M phases. The cell cycle arrest by CTN may permit DNA repair, but if it is faulty may allow proliferation of mutated cells, which is generally observed in case of tumorigenesis (Das et al., 2005b). Studies have shown that coexistence of OTA and CTN can occur in same agricultural crops and may exerts synergistic toxic effects even though the mechanism of action of these two mycotoxins is different (Cavin et al., 2007; Knasmüller et al., 2004; Vrabcheva et al., 2000). Thus, it is quite possible that during pre- and postharvesting of crops, human can also get coexposure to OTA along with CTN through dermal route which could pose a serious concern.

Our study suggests that the toxicological manifestations observed by CTN are attributed to its ROS generating potential and induction of oxidative stress, which possibly leads to apoptosis. Several studies have implicated the importance of antioxidants such as vitamins and plant polyphenols in protecting living organisms from the toxicity of environmental factors (Berton et al., 1998). The protective effect of antioxidants is attributed to its potential to quench a particular reactive electron rich moiety. Therefore, in the current study, we selected three different antioxidants viz BHA, Quer, and Toco, which have quenching capacity against different free radicals. Toco has the ability to directly quench free radicals particularly singlet oxygen \[ ^1O_2 \] and also functions as a cellular membrane stabilizer and prevents LPO (Berton et al., 1998). Quer is a bioflavonoid reported to protect cells from cytotoxic oxidative stress induced by depletion of cellular GSH by inhibiting the generation of superoxide ion \[ O_2^- \] and hydroxyl radicals \[ HO^* \], subsequently decreasing the production of lipid peroxides in cells (Das and Ray, 1988). BHA is an antioxidant that functions by stabilizing and sequestering free radicals as well as \( \text{H}_2\text{O}_2 \), which prevents a further cascade of free radical reactions (Cherouny et al., 1989). Several studies suggest that antioxidants protect cells against apoptosis induced by various stimuli including ROS. Therefore, bio-antioxidants viz Toco, BHA, and Quer were investigated for their ability to mitigate the ROS-mediated CTN-induced toxicity in mouse skin. Interestingly, topical application of Toco, BHA, and Quer to mouse skin, followed by CTN application, resulted in a significant reduction in ROS levels, the proportion of cells in the G0/G1 phase of cell cycle and apoptosis, when compared with the CTN-only

![FIG. 5. Effects of bio-antioxidants (BHA, Quer, and Toco) on CTN-induced toxicological manifestation in mouse epidermal cells. Antioxidants were topically applied 30 min prior to CTN application and animals were sacrificed after 24 h as described in the Materials and Methods. (A) Effect of antioxidants on CTN-induced ROS generation in mouse skin. Data, in terms of fold change as compared with control values, are presented as the mean ± SE of five values. (B) Effect of antioxidants on CTN-induced cell cycle distribution. Data represent the mean ± SE of five values. (C) Effect of antioxidants on CTN-induced apoptosis. Data represent the mean ± SE of five values. *p < 0.05, significant with respect to the control group. #p < 0.05, significant with respect to CTN-only treated group.](image-url)
treatment group. This suggests that CTN-induced cell cycle arrest and apoptosis are mediated through ROS and bio-antioxidants can be used to mitigate the cytotoxic effects of CTN. In the agreement with our findings, one study recently showed that resveratrol and the ROS scavengers, N-acetyl cysteine and Toco, abolished CTN-stimulated intracellular oxidative stress and apoptosis in HepG2 cells (Chen and Chan, 2009).

In conclusion, the present study reveals for the first time that CTN under in vivo condition has the ability to cause oxidative stress and ROS-mediated DNA damage in mouse skin upon topical exposure leading to enhanced expression of p53, p21/waf1, and Bax proteins that causes cell cycle arrest at the G0/G1 as well as G2/M phases and caused apoptosis through the mitochondria-mediated pathway. In addition, topical treatment of bio-antioxidants like Quer, Toco, and BHA abolished CTN-induced oxidative stress, cell cycle arrest, and apoptosis, confirming the direct involvement of ROS in CTN-induced toxicological manifestations in mouse skin.

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