ASSESSMENT OF SKIN TUMORIGENIC POTENTIAL OF OCHRATOXIN A IN MICE: ELUCIDATION OF MOLECULAR MECHANISM(S)

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Mycotoxins are unavoidable contaminants in food and feed and their toxicological impact on public health and food security has been the cause of global concern. Ochratoxin A (OTA) is a mycotoxin produced by several food-borne species of *Aspergillus* and *Penicillium* fungi. Due to its wide occurrence and consequent exposure, OTA has attracted significant public health attention over the past few years. Owing to the adverse effects of OTA in animals and its relevance to human health, authorities in USA and Europe considered OTA as a contaminant in food and prescribed 20 ppb and 5 ppb maximum permissible levels, respectively (USFDA, 2004; EU, 2003). Moreover, Indian temperate climate is conducive for fungal growth, but OTA contamination in Indian food produce has not been surveyed so far and due to lack of data under the Prevention of Food Adulteration Act (PFA) of India, OTA level has not been prescribed. Therefore it is important to generate the surveillance data of OTA that may provide an insight to the Indian food industry.

In spite of the growing evidences regarding the toxic and carcinogenic potential of OTA, understanding of the molecular mechanism(s) involved in OTA-mediated carcinogenicity is still inadequate. In this regard, various modes of action have been suggested for OTA-mediated carcinogenicity. Some studies suggest that genotoxicity is likely to play a role in OTA carcinogenicity; however, it is not clear whether OTA acts as a direct genotoxic carcinogen or carcinogenicity is related to indirect mechanisms, such as induction of cytotoxicity, oxidative cell damage and increased cellular proliferation as a consequence of tissue injury. Genotoxicity of OTA has been postulated due to observed modifications in DNA. However, covalently bound DNA adducts of OTA or its active metabolites have not been identified. The studies suggest that observed DNA lesions are not due to binding of OTA or its metabolites to DNA but occur through products derived from OTA-mediated toxicity such as reactive oxygen species (ROS). It was also reported that additional treatment with antioxidants reduced OTA-dependent toxic effects and formation of DNA lesions. Recently, it has been
shown that OTA exposure leads to oxidative stress and DNA damage in target tissue by inhibiting the Nrf2 transcription factor, the master regulator of antioxidant defense system. Furthermore, DNA damaging potential of OTA was found to be pronounced in male animals as compared to females and susceptibility for OTA-mediated carcinogenicity differ substantially in different animal species. From these studies it can be derived that the marked sex- and species-differences associated with OTA toxicity may not be directly related to genotoxic action. Therefore, it is likely that several potential epigenetic pathways may be involved in toxic and carcinogenic potential of OTA which requires elucidation. It has been suspected that induction of cell proliferation following OTA exposure could be one of the mechanisms. A close co relation between cell proliferation and carcinogenesis has been well established and earlier reports suggest that OTA can induce the phosphorylation and subsequent activation of ERK, p38 and JNK MAPKs and Akt signaling molecules in kidney, liver and several cell systems. These findings indicate the involvement of epigenetic pathway in OTA carcinogenicity.

It was argued that if OTA has DNA damaging and cell proliferative potential, it may act as both tumor initiator as well as tumor promoter. Therefore, it is of importance to gain better understanding of the events involved in OTA-mediated carcinogenicity. For these reasons, the two stage mouse skin carcinogenesis model, an ideal system to study the sequential and stepwise development of tumors as the initiation and promotion stages can be distinctly separated both operationally and mechanistically, was chosen to investigate the molecular mechanism of OTA-induced skin carcinogenicity. Further, a number of short-term markers of skin carcinogenesis following a single topical application of OTA were performed to determine the stage at which it affect tumorigenesis and give clues about the molecular mechanism involved therein. Although ingestion is the most vulnerable routes of exposure in humans yet exposure through dermal route cannot be ignored, especially in tropical countries including India where manual labor is
employed without any protective measure during pre- and post-harvest stages of agriculture, thereby causing a probable risk through dermal route. WHO has also highlighted the need for toxicological studies through dermal exposure, as limited data is available regarding epidermal exposure risk to mycotoxins. Therefore, the main objective of this dissertation is to explore the surveillance of OTA in staple food crops, and to assess the dermal exposure risk along with mechanism of action in the exposed tissue.

Hence, following studies are proposed as a part of this dissertation to fill up the above mentioned lacuna in OTA intoxication.

1. Detection of Ochratoxin A in cereal samples in different regions of India.
2. Evaluation of DNA damage and tumor initiation in mouse skin by topical exposure of Ochratoxin A.
3. Evaluation of cell proliferation and tumor promotion in mouse skin by topical exposure of Ochratoxin A.

DETECTION OF OCHRATOXIN A IN CEREAL SAMPLES IN DIFFERENT REGIONS OF INDIA

Ochratoxin A (OTA), a mycotoxin produced by different species of Aspergillus and Penicillium spp of fungi, and appreciable levels of OTA contamination in wheat, corn, grapes and their byproducts like beer, wine have been reported from various parts of world. However, OTA in wheat or other cereals from the Indian food produce have not been studied. It is a matter of concern as cereals are major source of dietary carbohydrate and protein for majority of Indian population and the consumers are unaware of the exposure risk to OTA because of inadequate data. Moreover, due to the widespread nature of fungi in the environment, mycotoxins are considered unavoidable contaminants in foods and feeds; therefore one of the most effective measures to protect the public health is to establish regulatory levels of these toxins on the basis of valid toxicological data. Therefore, an attempt has been made to analyze OTA contamination in wheat, maize and barley, and assesses the likely intake of OTA in Indian population.
A total of 50 wheat samples, 15 maize and 15 barley samples were collected from northern states (Uttar Pradesh, Haryana, Punjab, Delhi, Bihar) of India for investigation of OTA contamination. Cereal samples were extracted according to the earlier published method. In brief, cereal samples were grinded and 10 g of each ground samples were extracted with 30 ml chloroform and 2.5 ml 0.1 M phosphoric acid for 30 min. The extract was filtered using whatman number 1 filter paper and 10 ml was collected and evaporated under nitrogen stream. The residue was reconstituted in 600 µl of acetonitrile–water–acetic acid (41:58:1, v/v) by vortexing for 1 min. The reconstituted extract was defatted with 1 ml n-hexane by vortexing for another 1 min followed by centrifugation at 6000 rpm for 20 min. The lower phase was collected and filtered for further analysis.

Quantitative analysis of OTA in cereal samples was performed using HPLC attached with a fluorescence detector utilizing acetonitrile–water–acetic acid (99:99:2, v/v/v) mixture as mobile phase. The Limit of Quantitation (LOQ) was 0.71 µg kg⁻¹, and the average recovery values of OTA ranged from 71 to 92%. It was observed that 29 (58%) wheat samples were contaminated with detectable quantity of OTA ranging from 1.36-21.17 µg kg⁻¹ including 13 (26%) samples that exceeded the level of 5 µg kg⁻¹ suggested by European Union. Out of 15 maize samples, only 2 (13%) samples from Uttar Pradesh were found to be contaminated with OTA. Out of 15 barley samples, 7 (46%) samples were also found to be contaminated with OTA ranging from 1.08-5.12 µg kg⁻¹.

Based on the consumption data of wheat in northern states of India, the intake of OTA was found to saturate the provisional tolerable weekly intake (PTWI) to an extent of 0.97–2.49 fold for mean values in adult population. Overall, the study provides evidence for contamination of Indian cereals with higher levels of OTA in some samples, thereby leading to exposure risk in Indian population.

**EVALUATION OF DNA DAMAGE AND TUMOR INITIATION IN MOUSE SKIN BY TOPICAL EXPOSURE OF OCHRATOXIN A.**
The carcinogenic potential of OTA is well documented in literature; however, the mode of action is not well understood. OTA has been shown to be genotoxic which may play an important role in OTA carcinogenicity. The actual molecular mechanism of action either through DNA adducts formation or through epigenetic pathway(s) is still debatable. As DNA damage is one of the most important event of initiation of carcinogenesis, OTA may act as tumor initiator. To evaluate this, a two stage mouse skin carcinogenesis model, which is an ideal system to study the sequential and stepwise development of tumors along with the associated molecular events, has been employed.

Our study showed that a single topical application of OTA (200 nmol/mouse) caused significant DNA damage as detected by alkaline comet assay with enhancement of Olive tail moment (OTM) (176%), tail DNA (144%), and tail length (164%) when compared to control. The DNA from skin cells prepared from OTA treated animals showed typical formation of comet, while skin cells of control animals showed no DNA damage with well defined circular nucleus. Further, the level of γ-H2AX, a marker of DNA damage, was also found to be significantly elevated (5.5 fold) in mouse skin following OTA (200 nmol/mouse) exposure. OTA at the doses of 50 and 100 nmol/mouse caused no significant effect on comet assay parameters and γ-H2AX level in mouse skin.

Alteration in oxidative stress markers such as lipid peroxidation (LPO), protein carbonyl, glutathione (GSH) content and antioxidant enzymes such as catalase, glutathione reductase (GR) and glutathione-s-transferase (GST) was observed in a dose (50-200 nmol/mouse) and time-dependent (12-72 h) manner. OTA at the doses of 100 and 200 nmol/mouse caused significant increase in LPO (34-78%) and protein carbonyl content (53-168%) along with significant decrease in GSH content (19-47%), while the activities of catalase (31%), GR (58%) and GST (34%) were found to be significantly inhibited only at the dose of 200 nmol/mouse. A single topical application of OTA (200 nmol/mouse) resulted in a time-dependent enhancement of LPO (9-121%)
and protein carbonyl content (32-328%) along with significant decrease in GSH content (13-68%) and inhibition of catalase (16-70%), GR (31-84%) and GST (39-58%) activities. The oxidative stress was further emphasized by the suppression of Nrf2, the master regulator of antioxidant defense system, translocation to nucleus following a single topical application of OTA (200 nmol/mouse) after 24 h. These results suggest the oxidative stress and DNA damaging capability of OTA.

The present study suggests that OTA (200 nmol/mouse) application for 12-72 h to skin caused significant enhancement in- (a) reactive oxygen species (ROS) generation, (b) cell cycle arrest at G0/G1 phase (37-67%), (c) induction of apoptosis (2.0-11.0 fold), (e) expression of p53, p21/waf1, (f) Bax/Bcl-2 ratio, (g) cytochrome c release, (h) activities of caspase 9 (1.2-1.8 fold) and 3 (1.7-2.2 fold) as well as poly ADP ribose polymerase cleavage. The observed cell cycle arrest by OTA may pass some of the cells for damage repair and if it is faulty in nature, it may leads to mutation in DNA and the mutated cells may gain a selective advantage and proliferate, which is generally observed in case of tumorigenesis process. In a two stage mouse skin carcinogenesis protocol, OTA (200 nmol/mouse) when tested as tumor initiator resulted in mutation in p53 protein and tumor formation, and showed all the characteristic features of tumor initiator similar to that of DMBA, a well known skin tumor initiator having DNA damaging and oxidative stress capability. These results suggest that OTA has skin tumor initiating property which may be related to oxidative stress signaling and DNA damage.

**Evaluation of cell proliferation and tumor promotion in mouse skin by topical exposure of Ochratoxin A.**

It has been shown in the earlier chapter of the present dissertation that OTA possesses tumor initiating property which is attributed to ROS generation and DNA damage. However, literature suggests that beside this OTA may also cause tumorigenesis by epigenetic pathways such as alteration in signal transduction pathways and cell proliferation. Further, a co-relation between
stimulation of cell division and tumorigenicity is well established as sustained induction of cell proliferation may lead to cell transformation and tumor development and majority of identified tumor promoters are reported to be cell proliferative agents. In the past, several studies have been reported that OTA has ability to induced cell proliferation in kidney, liver and several cell systems involving ERK1/2 pathway of MAPKs. Therefore, in the present investigation skin tumor promoting activity of OTA and the mechanism/(s) involved therein was undertaken.

The short term studies reveal that a single topical application of OTA caused significant enhancement in ornithine decarboxylase (ODC) activity, a marker enzyme of cell proliferation, in a dose- and time-dependent manner. Since, OTA at the dose of 100 nmol/ mouse resulted in significant enhancement in ODC activity, this dose was used in the subsequent experiments. A single topical application of OTA (100 nmol/ mouse) resulted in significant increase in DNA synthesis, hyperplasia as well as expression of Cyclin D1 and COX-2 protein, markers for cell proliferation, in mouse skin in a time-dependent manner. Further, in two stage mouse skin tumorigenesis protocol, twice weekly exposure of OTA (50 nmol/mouse) to DMBA (120 nmol/mouse) initiated mice for 24 weeks leads to tumor formation showing the tumor promoting potential of OTA.

Furthermore, to study the molecular mechanism involved in OTA-mediated tumor promotion, primary mouse keratinocytes (PMKs) were cultured and non-cytotoxic doses of OTA was identified by MTT assay. It was found that no cytotoxicity was observed up to 10 µM concentrations. Primary mouse keratinocytes (PMKs) exposure with non-cytotoxic dose of OTA (5 µM) caused- (1) significant enhancement of DNA synthesis (2) enhanced phosphorylation and subsequent activation of epidermal growth factor receptor (EGFR) and its downstream signaling pathways viz AKT, ERK1/2, p38 and JNK Mitogen Activated Protein Kinases (MAPKs), (3) enhanced phosphorylation and activation of NF-κB transcription factor, (4) enhanced over expression of subunits of AP-1 transcription factor c-jun and c-fos, (5)
significant enhancement in the activity of Cyclin D1 and COX-2 promoter which leads to over expression of Cyclin D1 and COX-2 genes at mRNA as well as protein level, and (6) increased binding of NF-κB and AP-1 transcription factors to the promoter region of Cyclin D1 and COX-2 genes.

To further confirm the involvement of NF-κB, AP-1, Cyclin D1 and COX-2 in OTA-mediated cell proliferation, we used specific siRNA against mRNA of Cyclin D1 and COX-2 along with the transcription factors NF-κB and AP-1 subunits c-jun and c-fos. The results showed that knocking down the expression of NF-κB, c-jun, c-fos, Cyclin D1 and COX-2 results in significant inhibition in OTA-induced PMKs proliferation. The involvement of ERK1/2, p38 and JNK MAPKs in OTA-induced cell proliferation in mouse skin was also confirmed by using specific pharmacological inhibitors such as PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor). The results showed that inhibition of any of the three MAPK significantly inhibit the OTA-induced [³H]-thymidine uptake in mouse skin DNA. Overall, this study suggest that OTA has skin tumor promoting potential as well which involves EGFR-mediated MAPKs and Akt pathways along with NF-κB and AP-1 transcription factors and that COX-2 and Cyclin D1 are the target genes responsible for tumor-promoting activity of OTA.

Taken together, the findings of the present dissertation can be summarized as:

1. 50 wheat, 15 maize and 15 barley samples were analyzed for OTA contamination by HPLC and 29 wheat, 2 maize and 7 barley samples were found to be contaminated with appreciable level of OTA. Intake of OTA through wheat consumption alone in various northern states of India was found to saturate permissible tolerable weekly intake (PTWI) to an extent of 97-249%. The study provides evidence for contamination of Indian cereal produce with OTA and possible exposure risk to OTA in Indian population.
2. It was observed that a single topical application of OTA (200 nmol/mouse) caused oxidative stress, DNA damage and p53-mediated cell cycle arrest and apoptosis in mouse skin.

3. OTA when tested as tumor initiator in a two stage mouse skin tumorigenesis protocol resulted in tumor formation and the tumor suppressor protein p53 was found to be mutated. The observed mutation of p53 protein results in oncogenic stress.

4. At lower doses, a single topical application of OTA (100 nmol/mouse) was found to significantly enhance the short-term markers of cell proliferation and inflammation in mouse skin. OTA when tested as tumor promoter in a two stage mouse skin tumorigenesis protocol resulted in tumor formation suggesting the tumor promoting potential of OTA as well.

5. For elucidation of molecular mechanism(s) involved in OTA-induced cell proliferation and tumor promotion in mouse skin, primary mouse keratinocytes (PMKs) were cultured and exposed to OTA and it was observed that up to 10.0 µM concentrations OTA is non-cytotoxic to these cells. It was also observed that OTA (5.0 µM) exposure leads to enhanced proliferation of PMKs which may be responsible for its skin tumor promoting potential. The OTA-induced cell proliferation in PMKs was found to be mediated by the activation of EGFR, Akt and MAPKs such as ERK1/2, p38 and JNK signaling pathways.

6. The downstream target signaling molecules of EGFR, AKT and MAPKs signaling pathways such as NF-κB and AP-1 transcription factors were also found to be activated in PMKs following OTA exposure.

7. The activity of Cyclin D1 and COX-2 promoter in PMKs was significantly enhanced following OTA exposure along with the over expression of both the genes at transcriptional as well as translational levels, which was due to the enhanced binding of NF-κB and AP-1 transcription factors to the regulatory regions of their promoters.
8. Knock down of NF-κB, AP-1, Cyclin D1 and COX-2 mRNA expression by using specific siRNA significantly inhibit the OTA-induced PMKs proliferation.

9. The inhibition of ERK1/2, p38 and JNK MAPKs by pre-topical application of specific pharmacological inhibitors such as PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) significantly inhibit the OTA-induced cell proliferation in mouse skin.

Overall, the present study provides evidence for contamination of Indian cereal produce with OTA and possible exposure risk to Indian population. It was also found that OTA interfere at multiple stage of carcinogenesis process as it showed both tumor initiating as well as tumor promoting activity. At higher doses OTA caused oxidative stress and DNA damage in mouse skin which may contribute to its tumor initiating activity. While, at lower doses it enhance cell proliferation by modulating EGFR, AKT and MAPKs signaling pathways along with the NF-κB and AP-1 transcription factors and thus causing over expression of Cyclin D1 and COX-2 which may contribute to its tumor promoting activity.