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# Study on combined effects of ochratoxin A and endosulfan on antioxidant enzymes in rats

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**Abstract**

The aim of the present study was to determine the effects of ochratoxin A (OTA) and endosulfan, alone and in combination, on oxidative stress in rats. In total of 40 male Wistar rats were randomly divided into four groups of 10 rats each: Control (Standard diet); OTA (at 4ppm through diet); endosulfan (at 5mg $kg^{-1}$ body weight in corn oil by oral intubation) and OTA plus endosulfan (at 4ppm OTA plus 5mg $kg^{-1}$ body weight endosulfan). The level of lipid peroxidation (LPO), catalase, superoxide dismutase (SOD) and reduced glutathione (GSH) were measured in erythrocytes as well as in tissue homogenates of liver and kidneys, after 30 days post-intoxication. OTA and endosulfan treatment caused significant elevation in LPO levels in erythrocytes (62%), liver (28%) and kidneys (125%). However, the level of antioxidant enzymes such as catalase, SOD and GSH decreased (20% to 50%) in all the treated groups. It was concluded that oxidative stress was more severe in case of combination treatment, which might be due additive toxic effect of endosulfan and OTA in rats.

**Key words**

Antioxidant enzymes, Endosulfan, Lipid peroxidation, Ochratoxin A, Wistar rat

## Introduction

Due to rapid industrialization and corresponding increase in the use of chemicals, there has been substantial increase in the number of dangerous or toxic substances in the environment. Heavy metals, pesticides, mycotoxins, phthalates, volatile organic compounds and dioxin have been considered as the most common toxins in the environment affecting health of animals and human beings (Maresca and Fantini, 2010; Kumar *et al*., 2011). Ochratoxin A (OTA) is a nephrotoxic mycotoxin, mainly produced by *Aspergillus ochraceus* in tropical regions and by *Penicillium verrucosum* in temperate areas (Pitt, 2000). In addition, its chemical stability against heat and during industrial food processing, makes OTA one of the most abundant food-contaminating mycotoxins. In human and animals, the major source of exposure to OTA is via ingestion of contaminated food/feed commodities. Human exposure to OTA has been clearly demonstrated by its detection in blood and breast milk due to its long serum half-life (Haighton *et al*., 2012). It has also been well documented to be genotoxic (Pfohl-Leszkowicz and Manderville, 2012), immunotoxic (Liu *et al*., 2012), carcinogenic (Schilter *et al*., 2005) and teratogenic (Patil *et al*., 2006) in rodents. OTA has also been reported to cause endemic nephropathies in porcine and human beings (Stove *et al*., 2001; Castegnaro *et al*., 2006). The ubiquitous presence of OTA has been reported earlier in various agricultural commodities and their products such as cereal grains (wheat, sorghum, oats, rice, corn etc.), food, feed, beans, peanuts, wine, beer, juices, green coffee, cheese and milk (Meulenberg, 2012).

Endosulfan is one of the most commonly used pesticides and is a member of organochlorine insecticide. It has been shown

that endosulfan induces T-cell apoptosis (Kannan *et al.,* 2000), immunotoxicity (Pistl *et al.,* 2001), hepatotoxicity (Uboh *et al*., 2011) and mutagenicity (Bajpayee *et al*., 2006). It is neurotoxic to rats and considered to be an endocrine disruptor and also induces hepatic tumours in rodents. In India, endosulfan residues have been reported to occur at high levels in various samples of cashew, fruits, milk, butter, coconut oil, soil, ground water and even in human blood in Kasargod district of Kerala (Jayashree and Vasudevan, 2007).

Both endosulfan and OTA induce increased lipid peroxidation by stimulating the production of reactive oxygen species (ROS) that lead to oxidative damage (Yenilmez *et al*., 2010; Shao *et al*., 2012). Despite rigorous attempts for preventing environmental contamination, both OTA and endosulfan have remained a problem of human health concern in several parts of the world including developed countries like India. On perusal of literature, not a single report is available for simultaneous exposure of OTA and endosulfan in male rats. Moreover, the study of toxic effects of OTA and endosulfan under low dose exposure regimen is extremely important in order to have more data for human risk assessment. Therefore, the present work was undertaken to study the effect on antioxidant enzymes by OTA and endosulfan, alone and in combination in male rats when given orally for 30 days.

## Materials and Methods

**Production and analysis of ochratoxin A :** A pure culture of *Aspergillus ochraceus* NRRL-3174, originally procured from the National Centre for Agriculture Research (NCAUR-3174) Peoria, Illinois, USA was grown on sterilized maize following the method of Trenk *et al*. (1971). The extraction and clean up of the toxin sample was done as per method of AOAC (1995). Cultured maize powder containing known amount of OTA was added to basal ration in such a proportion that the final concentration of OTA was adjusted to 4 ppm level in the feed.

**Animals and experimental design :** Male Wistar rats (n=40) with average weight of 160±10g were procured from Laboratory Animal Resource Section of Indian Veterinary Research Institute, Izatnagar, Bareilly, India. Animals were housed in polypropylene cages, in an artificially illuminated room (12 hr light: 12-hr dark cycle) free from any source of chemical contamination. The temperature and relative humidity of the room were maintained at 22±3°C and about 50-60%, respectively. Rice bran was used as bedding material which was changed on every alternate day. The experiment on rats was approved by the Animal Ethics Committee of IVRI, Bareilly. The rats were provided with standard laboratory animal feed and water *ad libitum*. After fifteen days of acclimatization, they were randomly assigned into four groups viz. Group-I served as a negative control and rats received standard toxin-free feed; Group-II rats received diet containing OTA alone at a dietary level of 4 ppm daily (as explained above); Group-III rats received endosulfan alone dissolved in corn oil at a concentration of 5 mg $kg^{-1}$ b. wt. by oral in tubation daily; and Group-IV rats received both OTA (4 ppm) plus endosulfan (5 mg $kg^{-1}$ b. wt.) throughout the experiment.

The dose selection criteria for the present study was decided on the basis of oral median lethal dose ($LD_{50}$) of OTA (20-30 mg $kg^{-1}$ b. wt.) and endosulfan (80 mg $kg^{-1}$ b. wt.) in rats. Rats weighing 160g, daily consuming about 25g diet containing 4µg OTA $g^{-1}$ diet, approximately, 1/30$^{th}$ oral lethal ($LD_{50}$) dose levels for OTA (4 ppm) and 1/15$^{th}$ oral lethal ($LD_{50}$) dose levels for endosulfan (5 mg $kg^{-1}$ b. wt.) were selected and used in the present investigation. At the end of the study *i.e.* after 30 days post-intoxication, the blood samples were collected by cardiac puncture into heparinized vials during the sacrifice of animals. At necropsy, the liver and kidney tissue samples were also collected. All the estimations were carried out on the same day of tissue/ blood collection.

**Analysis of antioxidants :** The hemolysate containing 33% packed erythrocytes was used for LPO and GSH estimation. Hemolysate (10%) was further prepared by diluting packed erythrocytes in phosphate buffer saline (PBS) and used for determination of activities of other antioxidant enzymes. For preparation of tissue homogenate, 200 mg each tissue samples from liver and kidney were taken in 2ml ice-cold normal saline. Another aliquot of 200 mg each from tissue samples were also taken in 2ml of 0.02M ethylene diamine tetraacetic acid for GSH estimation. Tissue homogenate (10%) was prepared using electronic tissue homogenizer (IKA, Germany) under ice-cold condition. The homogenate was centrifuged for 10 min at 3000 rpm. The resultant supernatant was used for estimation of biochemical attributes.

Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) level, based on the reaction of MDA with thiobarbituric acid (Shafiq-Ur- Rehman, 1984). In brief, 1ml of homogenate or packed erythrocytes (33%) was incubated at 37±0.5°C for 2 hr. To each sample, 1ml of 10% w/v trichloroacetic acid was added. After through mixing, the mixture was centrifuged at 2000 rpm for 10 min. To 1ml of supernatant, an equal volume of 0.67% thiobarbituric acid was added and kept in boiling water bath for 10 min. After cooling, it was diluted with 1ml of distilled water. The absorbance was read 535 nm wavelength by spectrophotometer. Results were expressed as nM MDA $g^{-1}$ of wet tissue or nM MDA $ml^{-1}$ packed erythrocytes.

Superoxide dismutase (SOD) activity was measured by the method of Madesh and Balasubramanian (1998). In brief, the reaction mixture contained 0.65ml PBS (pH 7.4), 30µl 3-(4-5-dimethylthiazol 2-xl) 2,5-diphenyltetrazolium bromide (MTT; 1.25 mM), 75µl pyrogallol (100 µM) and 10µl homogenate or hemolysate (10%). The mixture was incubated at room temperature for 5 min and the reaction was stopped by adding

0.75ml of dimethyl sulfoxide. The absorbance was read at 570 nm wavelength by spectrophotometer against distilled water and the activity has been expressed as Unit. One unit was microgram of protein or hemoglobin required to inhibit MTT reduction by 50%.

Catalase activity was assayed by the method of Aebi (1983) and expressed as k $g^{-1}$ of wet tissue or k $mg^{-1}$ hemoglobin. One k stands for nM $H_2O_2$ utilized per min. Briefly, 2ml PBS (50mM, pH 7.4) and 10µl homogenate or hemolysate (10%) were taken in a cuvette. Reaction was started by adding 1ml $H_2O_2$ (20mM) and the absorbance was recorded at every 10 sec for 1 min at 240 nm wavelength against water blank.

Reduced glutathione (GSH) content in liver and kidney was determined by estimating free –SH groups, using 5, 5' dithiobis-2-nirobenzoic (DTNB) acid method of Sedlak and Lindsay (1968) and expressed as mM GSH $g^{-1}$ of wet tissue. Briefly, to 1ml supernatant, 0.8ml of distilled water and 0.2ml of 50% trichloroacetic acid were added and incubated at room temperature for 15 min; centrifuged at 3000 rpm for 15 min. From this, 0.4ml supernatant was added to 0.8ml of 1M tris buffer (pH 8.9) followed by 0.2ml of DTNB (0.01M) and absorbance was read at 412 nm wavelength within 5 min. GSH content in erythrocytes was estimated by the method of Prins and Loos (1969). Briefly, an erythrocyte pack of 0.2ml (33%) was mixed with 4ml of 0.08N $H_2SO_4$. After 10 min, 0.5ml of tungstate solution was added and mixed vigorously for 5 min to clear the brown hemolysate. After allowing it to stand for 5 min to avoid crust formation on top of the supernatant, it was centrifuged for 15 min at 2000 rpm at room temperature. 2ml of supernatant was mixed with 2.5ml of Tris buffer (pH 8.0) and 0.2ml of DTNB reagent. Within a minute, absorbance was read at 412 nm. The GSH level was expressed as mM GSH $ml^{-1}$ packed erythrocytes.

Hemoglobin concentration in erythrocytes was determined spectrophotometrically by cyanomethaemoglobin method (VanKampen and Zijlstra, 1961). Protein level in tissue homogenate was measured by the method of Lowry *et al*. (1951), using bovine serum albumin as standard.

**Statistical analysis :** Data generated in the present study were subjected to statistical test for comparison of mean values among different groups using one-way analysis of variance (ANOVA) and Duncan's multiple range tests (Snedecor and Cochran, 1989). Experimental results were expressed as mean ± SE and accompanied by a number of observations (n=10). All the level of significance were based on 95% level of probability.

## Results and Discussion

LPO is one of the main processes induced during oxidative stress *in vivo*. It results in generation of reactive oxygen species (ROS) and electrophilic aldehydes, which target lipoproteins and DNA (Jia and Misra, 2007). In the present study level of lipid peroxidation in erythrocytes of OTA (31.96%) and endosulfan (28.09%) treated animals were raised as compared to those in control group. However, significant increase (61.98%) in lipid peroxidation level in erythrocytes were recorded in animals exposed simultaneously to both OTA and endosulfan. Similar patterns were also observed in liver and kidney tissues, which revealed 94.90% and 125.12% elevation in LPO level on administration of combinated of OTA and endosulfan to rats as compared to those in the individual treatment groups (Group II and III). LPO results in disruption of the structural integrity of cell membrane and formation of potentially harmful molecular species (Ganesan *et al*., 2011). The effects of OTA and endosulfan, alone and in combination, on antioxidant enzyme activities of male rats, have been summarized in Table 1.

An increase in the production of MDA in tissues and RBCs was observed at 30 days post-exposure to OTA or endosulfan, alone and in combination in male Wistar rats, which might be attributed to direct generation of free radicals and reduction in antioxidant defense system, leading to disruption of peroxidant-antioxidant balance.

Earlier studies have reported that OTA enhances LPO *in vitro* and *in vivo* in a dose-dependent manner (Omar and Rahimtula, 1993; Abdel-Wahhab *et al*., 2005). In the present study an increase in LPO was observed in liver and kidneys of rats exposed to 4 ppm OTA in diet for 30 days. Moreover, earlier report of Petrik *et al*. (2003) also found elevated LPO levels in rat kidneys on administration of OTA at a dose level of 120 µg $kg^{-1}$ for 60 days. In the present study, elevated LPO levels were observed not only in kidneys but also in liver of rats exposed to endosulfan.

Exposure to OTA resulted in reduction in catalase activity by 15.36, 16.99 and 20.66% in erythrocytes, kidneys and liver. On the other hand, treatment with endosulfan decreased catalase activity by 10.65, 28.15 and 12.28% in erythrocytes, liver and kidneys, respectively. Exposure to combination of both OTA and endosulfan in rats caused more pronounced reduction catalase activity as compared to individual treatments. Recently, Palabiyki and co-workers (2013) reported that OTA induced oxidative stress in kidney was associated with marked decrease in CAT (35%) activity and GSH levels (44%) as well as an increase in SOD activity (22%) in comparison to control. On the contrary, present study revealed a decrease in SOD activity in erythrocytes, liver and kidneys, up to 33.5% in OTA treated group and up to 25.4% in endosulfan treated group. The effect was substantial, resulting up to 52.7% decline in SOD levels in the combined treated animals in kidneys followed by 45.8% in liver and 39.7% in erythrocytes. Similar results were also reported by Saxena *et al.* (2011) and Alva *et al.* (2012) who observed significant decrease in SOD activity and catalase in a dose-dependent pattern in rats exposed to endosulfan. The reduced levels of GSH were observed up to 36.36% in group II and up to 22.73% in group III. However, the magnitude of reduction was 1.5 times more in combination group (Group IV) as compared to

**Table 1:** Oxidative stress-related indices in male Wistar rats treated with Ochratoxin A and endosulfan, alone and in combination for 30 days

| Parameter | Tissue samples | Experimental group | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | I | II | %Change | III | %Change | IV | %Change |
| **Lipid peroxidation** (nM MDA/g of wet tissue or nM MDA/ml packed RBCs) | **RBCs** | 4.13 ± 0.20a | 5.45± 0.39ab | (+) 31.96 | 5.29± 0.38ab | (+) 28.09 | 6.69 ± 0.42b | (+) 61.98 |
| | **Liver** | 27.27 ± 0.24a | 45.98± 1.27c | (+) 68.61 | 34.84 ± 1.84b | (+) 27.76 | 53.15 ± 2.02d | (+) 94.90 |
| | **Kidney** | 28.39 ± 0.61a | 51.85 ± 1.99c | (+) 82.64 | 43.56 ± 1.30b | (+) 53.42 | 63.91 ± 3.11d | (+) 125.12 |
| **Catalase** (k $g^{-1}$ wet tissue or k $mg^{-1}$ hemoglobin) | **RBCs** | 139.68± 2.1a | 118.23± 2.36bc | (-) 15.36 | 124.8 ± 3.66b | (-) 10.65 | 110.51 ± 3.35c | (-) 20.88 |
| | **Liver** | 474.2± 2.48a | 376.21 ± 8.53b | (-) 20.66 | 340.71 ± 12.04c | (-) 28.15 | 299.02 ± 6.15d | (-) 36.94 |
| | **Kidney** | 345.04± 2.4a | 286.41 ± 5.23b | (-) 16.99 | 302.68 ± 5.29b | (-) 12.28 | 211.72 ± 4.25c | (-) 38.64 |
| **Superoxide dismutase** (Unit) | **RBCs** | 6.30± 0.31a | 4.19± 0.16bc | (-) 33.49 | 4.93 ± 0.26b | (-) 21.75 | 3.80 ± 0.28c | (-) 39.68 |
| | **Liver** | 32.74± 0.57a | 25.93± 0.76b | (-) 20.80 | 24.42±0.97b | (-) 25.41 | 17.75 ± 0.76c | (-) 45.79 |
| | **Kidney** | 30.45± 0.43a | 23.28± 0.55b | (-) 23.55 | 27.44 ± 0.62c | (-) 09.89 | 14.41 ± 0.65d | (-) 52.68 |
| **Reduced glutathione** (mM $g^{-1}$ wet tissue or mM $ml^{-1}$ packed RBCs) | **RBCs** | 0.44 ± 0.03a | 0.28± 0.02bc | (-) 36.36 | 0.34± 0.05ba | (-) 22.73 | 0.19 ± 0.03c | (-) 56.82 |
| | **Liver** | 0.090 ± 0.002a | 0.064± 0.001b | (-) 28.89 | 0.070± 0.002c | (-) 22.22 | 0.045 ± 0.001d | (-) 50.00 |
| | **Kidney** | 0.089± 0.004a | 0.064± 0.001b | (-) 28.09 | 0.073± 0.002c | (-) 17.98 | 0.045 ± 0.002d | (-) 49.44 |

Group-I: Healthy control group; Group-II: Ochratoxin A alone treated group (OTA at 4 ppm in diet); Group-III: Endosulfan alone treated group (Endosulfan at 5 mg $kg^{-1}$ b. wt.); Group-IV: Ochratoxin A and endosulfan treated group (OTA at 4 ppm plus endosulfan at 5 mg $kg^{-1}$ b. wt.). MDA: Malondialdehyde; RBCs: Red Blood Cells. Mean bearing common superscript do not differ significantly between groups (P d" 0.05). Values are mean ± SE from 10 observations in each group.

those in individual treated groups on comparing combined effect of OTA and endosulfan on erythrocytes and tissue samples revealed more perceptible effects on LPO, SOD and catalase in liver and kidneys, however, the changes in GSH levels were more pronounced in erythrocytes.

Reduced activities of antioxidant enzymes like catalase, SOD and GSH in RBCs, liver and kidneys of rats reflected the adverse effect of OTA and endosulfan on antioxidant system in different tissues. SOD metabolizes super-oxide anion ($O^{-2}$) into molecular oxygen; while catalase detoxifies hydrogen peroxide ($H_2O_2$) into $H_2O$. Hydroperoxides and $H_2O_2$ are detoxified in the cytosolic and mitochondrial compartments by peroxidases (Abdel-Wahhab *et al*., 2005). One of the important aspects of antioxidant enzyme systems is synergistic functioning. Similarly, endosulfan has been shown to deplete erythrocyte, hepatic and lung SOD and GSH in liver and lung tissues in rats when administered for 30 days (Bebe and Panemangalore, 2003). Increase in LPO levels accompanied by marked decrease in activities of SOD, catalase and GSH levels further indicates that sub-acute exposure to endosulfan and/or OTA results in generation of ROS, which induces oxidative stress in tissues and erythrocytes.

Sub-acute to chronic exposure to agrochemicals may affect enzyme activity that are involved in oxidative stress. Toxicants such as pesticides or heavy metals may interact with cytochrome $P_{450}$ activity and disrupt catalytic cycle to induce formation of ROS (Jia and Misra, 2007). Interaction of endosulfan with cytochrome $P_{450}$ mediating cortisol synthesis may then be responsible, by induction of oxidative stress, for the loss of activity of steroidogenic enzymes (Leblond *et al*., 2001; Kumar *et al*., 2011).

The findings of the present study suggested that simultaneous exposure to both OTA and endosulfan produced additive effects on oxidative stress in male Wistar rats and there is a strong possibility of occurrence of such kind of interaction in animals or human beings in field conditions.

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