Original Article

Studies on Apoptotic Changes in Combined Toxicity of Citrinin and Endosulfan in Pregnant Wistar Rats and Their Fetuses

N. D. Singh, A. K. Sharma[1], P. Dwivedi[1], A. G. Telang[2], M. Kumar[3], R. D. Patil[4]

COVS, GADVASU, Ludhiana, [1]Division of Pathology, [2]CADRAD, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, [3]HSADL, Bhopal, Madhya Pradesh, [4]Department of Veterinary Pathology, GC Negi COVS, CSKHPKVV, Palampur, Himachal Pradesh, India

ABSTRACT

Background: Citrinin (mycotoxin) and endosulfan (pesticide) both environmental contaminants easily enter the food chain and are caoomon causes of various toxicities. **Materials and Methods:** In the present investigation, citrinin (CIT) (10 mg/kg feed) and endosulfan (1 mg/kg body weight) were administered orally alone and in combination to pregnant Wistar rats from gestational day 6 to 20 to study their effect to cause apoptosis in the pregnant Wistar rats and their fetuses. Apoptosis was assessed in dams by agarose gel electrophoresis, flow cytometry and electron microscopy, while in the fetuses it was assessed by flow cytometry only. **Result:** Citrinin and endosulfan in the combination group caused apoptosis in an additive manner as there was increased number of apoptotic cells as compared to the individual toxin and control groups. The fetuses also showed increased number of apoptotic cells in the combination groups, which also indicated that both the toxins crossed the placental barrier. **Conclusion:** So it was concluded that apoptosis played a significant role in the pathogenesis of endosulfan and citrinin toxicity.

Key words: Apoptosis, citrinin, endosulfan, fetuses, pregnant dams

INTRODUCTION

Mycotoxins, secondary metabolites produced by moulds, are common contaminants of foods, feeds and the raw ingredients. Mycotoxin studies have gained immense importance in recent years since they are posing serious threat to human health through food chain owing to mycotoxin residues remaining in animal tissues for longer period and even being excreted in milk.

Organochlorine pesticides are a large class of multipurpose chlorinated hydrocarbon chemicals. Organochlorine pesticides break down slowly in the environment and accumulate in the fatty tissues of animals. Thus, they stay in the environment and food web long after being applied.[1]

Among the mycotoxins, aflatoxin B1 and ochratoxin A have been studied in great detail, but other mycotoxins such as citrinin (CIT) have not been given due attention in spite of its detrimental effects on reproduction and pathological effects on the kidneys and other vital organs.[2] Citrinin (CIT), a nephrotoxic mycotoxin produced by *Penicillium citrinum*, often co-occurs with ochratoxin A as a co-contaminant of various food commodities. It has also been implicated in a fatal human kidney disease, Balkan endemic nephropathy, along with ochratoxin A.[3]

Endosulfan, an organochlorine compound of the cyclodiene group, is primarily used as an insecticide and secondarily as an acaricide. Alarmingly high levels (0.055–3.875 ppm) of endosulfan were found in blood, fat, milk, vegetables, cashew leaves, soil and water from Padre village of Kasargod district of Kerala, India.[4] Further, human milk

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Address for correspondence: Dr. A. K. Sharma, Division of Pathology, Indian Veterinary Research Institute, Izatnagar - 243 122, Bareilly, Uttar Pradesh, India. E-mail: aksharmaivri@rediffmail.com

samples were also found to contain endosulfan residues.[5] The perused literature showed some information on the toxicity of CIT and endosulfan in poultry, but reports on their toxic effects in animals are limited. Moreover, no report could be traced in the literature on the combined effect of CIT and endosulfan in pregnant rats, although both may occur as co-contaminants under field conditions in certain areas. Individual cell death (apoptosis) is an important part of normal physiological functions of any multicellular organism. Apoptosis of the immune system has recently drawn interest since immune dysfunction can affect the survival of the host in various ways. There may be autoimmune diseases, cancer, immuno-pathological and immunotoxicological alterations

The present work is designed to study the role of apoptosis in the pathogenesis of CIT and endosulfan toxicity in pregnant rats and their fetuses, when fed alone and in combination during gestation days 6–20.

MATERIALS AND METHODS

Production and analysis of citrinin

Citrinin was produced by culturing *Penicillium citrinum* (NRRL-5907), procured from National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois, USA, on sterile maize and groundnut[6] (1:1). Thin layer chromatography (TLC) and spectrophotometry were used for the quantitative determination of the toxin[7] against the standard CIT procured from Sigma Chemicals Ltd., USA.

Experimental animals

The present study is carried out using sexually mature (180±10 g) Wistar female rats, procured from the Laboratory Animal Resource (LAR) Section of the Institute. All the animals were kept as per the standard conditions.[7] All the experimental procedures were conducted as per the guidelines of the Institute Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). After an acclimatization period of one week, females were mated with mature males of the same strain. The day on which a vaginal plug was found or spermatozoa were observed in the vaginal smears was designated as day 0 of pregnancy. After mating, the female rats were individually housed in polypropylene cages.

Treatment of pregnant rats

Pregnant females were weighed and randomly distributed into four groups and treated as follows: Group I, citrinin (10 mg kg^{-1} feed); group II, endosulfan (1 mg kg^{-1} body weight) dissolved in olive oil by oral intubation; group III, citrinin (10 mg kg^{-1} feed) plus endosulfan (1 mg kg^{-1} body weight); group IV, control receiving the basal feed tested negative for CIT and olive oil (0.1 ml $100g^{-1}$ b.w.) orally. The dose selection criteria for citrinin was based on the $1/20^{th}$ oral lethal (LD_{50} i.e. 50 mg kg^{-1} body weight) dose of citrinin in rats keeping in view the body weight and daily feed consumption. The dose of endosulfan (1 mg kg^{-1} body weight) employed in the present study was lower than the dose established by the World Health Organization (WHO) for reproductive toxicity assessment in rats.[8] The suggested NOEL (no-observed-adverse-effect level) dose for endosulfan in rat is 1.5 mg kg^{-1} body weight. Since the animals used in the present experiment were pregnant, the dose was reduced to 1.0 mg kg^{-1} body weight in view of pregnancy stress. The toxins were given from day 6 to 20 of gestation to prevent the pre-implantation and early post-implantation losses during days 0 to 5 of gestation.

Treatment of toxicated feed

Cultured substrate containing a known amount of CIT was added to the basal ration (tested negative for mycotoxin contamination) in such a proportion that the concentration of CIT in the diet was 10 mg kg^{-1} feed. Aliquots were taken from the mixed diet and the toxin was quantified by thin layer chromatography and spectrophotometry to ensure proper mixing of the toxin. The toxicated feed was freshly prepared daily and given to the pregnant rats from gestation day 6–20.

Technical grade (>99.98% pure crystalline form) endosulfan procured from Shriram Chemicals Ltd, India, was dissolved in olive oil (vehicle) and orally intubated to pregnant rats at the rate of 1 mg kg^{-1} body weight daily from days 6 to 20 of pregnancy. The treatment volume was 0.1 ml 100 g^{-1} body weight. A fresh solution of endosulfan was prepared on each day of treatment. The control animals received an equal volume of olive oil similar to those treated with endosulfan.

Electron microscopy

All the animals (dams) from each group were euthanized using overdose of ketamine anesthesia at 60 days post treatment. Kidneys were weighed and small tissue pieces were immediately collected into a petri dish containing chilled 2.5% glutaraldehyde in cacodylate buffer (pH 7.4), trimmed to cubes measuring approximately 1 mm^3 and fixed for 6 h at 4°C. The tissues were washed twice (30 min each) with cold 0.2 M phosphate buffer (pH 7.4) and fixed in 1% osmium tetraoxide for 4 h at 4°C. The tissue pieces were then dehydrated in ethyl alcohol, cleared and embedded in epon-araldite resin. Ultra thin sections (600 A°) were cut employing an ultra microtome (Ultracut, Reichert-Jung, Austria), mounted onto copper grids, and stained with uranyl acetate and subsequently with lead citrate.[9] The grids were examined under an

electron microscope (Philips M-10, Holland) at the All India Institute of Medical Sciences (AIIMS), New Delhi.

Assessment of apoptosis

Apoptosis was assessed in dams by agarose gel electrophoresis, flow cytometry and electron microscopy whereas in fetuses it was done by flow cytometry alone owing to very small size of the fetal organs.

Isolation of apoptotic DNA

Apoptotic DNA was isolated from organs (liver, kidneys and spleen) of treated and control group dams.[10]

Agarose gel electrophoresis

The resolution and purity of the DNA was further analyzed by agarose gel electrophoresis in a submarine horizontal electrophoresis unit (Genei, Banaglore). Two percent (2%) agarose gel was prepared by boiling analytical grade agarose (low EEO, Promega) in ×1 Tris acetic acid EDTA (TAE) buffer to dissolve it completely. After cooling to about 50°C, ethidium bromide (Amresco) was added to the agarose solution to obtain final concentration of 0.5 μg/ml. The gel-casting platform was placed on a leveled surface and the open sides were sealed with adhesive tape. The gel comb was then placed across the gel-casting platform, so that the teeth of the comb remained about 1 mm from the base of the platform. The molten agarose was then poured on to the gel-casting platform and it was kept undisturbed for about 35 min to solidify the gel. After the gel got solidified, the comb was taken out and the adhesive tape was removed. The gel-casting platform with the set gel was submerged in the electrophoresis tank with sufficient quantity (about 1 mm level) of electrophoresis buffer (TAE ×1 with pH 8.5) above the surface of the gel. The test DNA sample (1 μl) was mixed with 4 μl of sterile distilled water and 1 μl of 6x-bromophenol blue, loading dye (Promega) and loaded into the well with micropipette. Electrophoresis was performed at 45 volts/cm for 1 h and progress of mobility was monitored by the migration of dye. The DNA migration and resolution pattern was examined by UV transillumination technique and the picture was documented by photography.

Fluorescence activated cell sorter analysis

Liver, kidneys and spleen of pregnant dams and their fetuses were used for the flow cytometric analysis using fluorescence activated cell sorter (FACS) calibur (Becton Dickinson, San Jose, CA). The single cell suspension of liver, kidneys and spleen cells was prepared.[11] Cells were kept at room temperature for 4 min, in the dark and analyzed by FACS calibur. Forward (FSC for size) and side (SSC for granularity) scatter count was recorded using an excitation wavelength of 448 nm and emission wavelength of 530 nm.

RESULTS AND DISCUSSION

Electron microscopy

In dams, ultrastructurally, in group I (Citrinin), there were severe degenerative changes and nuclear condensation along with loss of cytoplasm in renal epithelial cells, which showed initiation of apoptosis [Figures 1-3]. In group II (endosulfan), in liver there was vacuolation of cells with mitochondrial damage along with initiation of nuclear condensation which suggested apoptosis [Figure 4]. In the combination group i.e. group III (combination), there was clear nuclear fragmentation and formation of apoptotic bodies [Figure 5] in liver.

Agarose gel electrophoresis

Gel electrophoresis of DNA from liver and kidney failed to reveal a clear cut ladder pattern in all the three groups. In group I (CIT), DNA gel electrophoresis of spleen revealed a clear ladder pattern suggesting apoptotic properties of CIT [Figure 4], whereas, in group II (Endosulfan) by DNA electrophoresis technique, a clear cut ladder pattern could not be observed [Figure 4]. However, group III i.e. the combination group showed a clear cut ladder pattern [Figure 4].

Fluorescence activated cell sorter analysis

In group I (CIT), when fluorescence activated cell sorter analysis was performed on liver, kidneys and spleen tissue samples of dams [Figure 5], there was significant increase in the percentage of apoptotic cells in liver, kidneys and spleen (1.46; 2.0 and 2.0%) as compared to those in the control (0.84; 0.70 and 1.05%) [Table 1 and Figure 5]. In group II (endosulfan), there was significant increase in the percentage of apoptotic cells in liver (2.06%) as

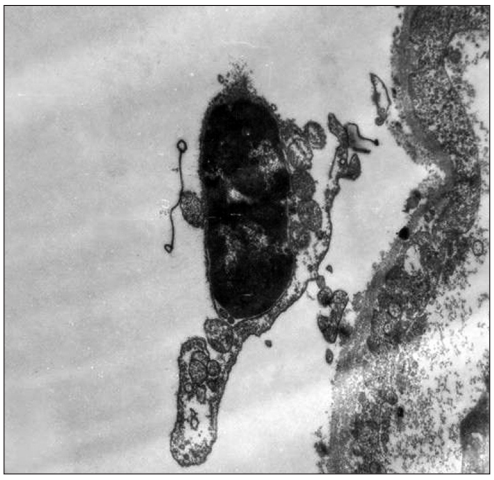

Figure 1: Group I (CIT), Kidney; In Bowman's space, nuclear condensation (apoptosis) with loss of cell organelles (uranyl acetate and lead citrate ×31000)

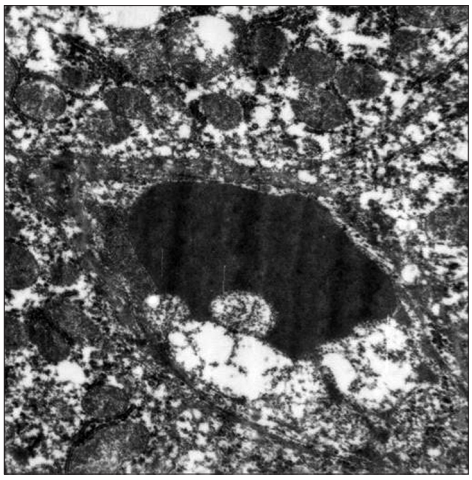

Figure 2: Group II (endosulfan), Liver: The hepatocyte showing chromatin condensation in nucleus along with cellular vacuolation and mitochondrial damage (uranyl acetate and lead citrate ×15000)

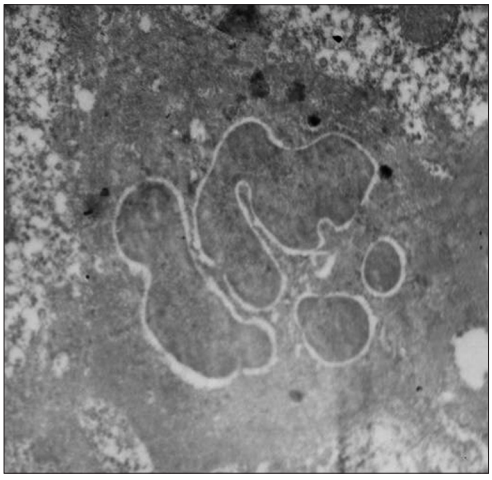

Figure 3: Group III (combination), Liver: Hepatocyte showing typical nuclear fragmentation along with apoptotic bodies (uranyl acetate and lead citrate ×19000)

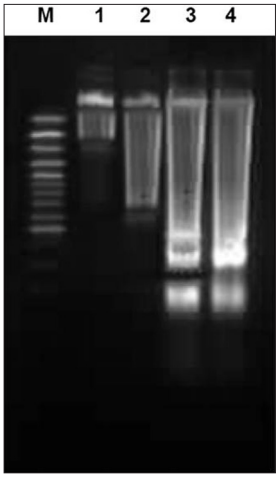

Figure 4: Agarose gel electrophoretic pattern of DNA isolated from spleen of toxin treated and control pregnant rats. Lane M - 100 bp marker. Lane 1 - Gr. IV Control. Lane 2 - Gr. II Endosulfan. Lane 3 - Gr. I Citrinin (CIT) treated. Lane 4 - Gr. III Citrinin+endosulfan (C+E)

Table 1: Fluorescence activated cell sorter analysis (FACS) for percent apoptotic cells in various organs of dams and fetuses

Organs	Groups			
	I	II	III	IV
Dam				
Liver	1.46 ± 0.11^{b}	2.06 ± 0.09^{a}	1.64 ± 0.10^{b}	0.84 ± 0.16^{c}
Kidney	2.00 ± 0.11^{b}	0.50 ± 0.03^{c}	3.10 ± 0.19^{a}	0.70 ± 0.19^{c}
Spleen	2.00 ± 0.07^{b}	1.20 ± 0.16^{c}	6.79 ± 0.16^{a}	1.05 ± 0.12^{c}
Fetus				
Liver	1.30 ± 0.01^{bc}	1.57 ± 0.03^{a}	1.39 ± 0.01^{b}	1.40 ± 0.05^{b}
Kidney	3.82 ± 0.03^{b}	3.40 ± 0.01^{c}	5.79 ± 0.03^{a}	1.44 ± 0.14^{d}

Gr. I: Citrinin (10 ppm); Gr. II: Endosulfan (1 mg/kg b.w.);
Gr. III: Citrinin+endosulfan (10 ppm+1 mg/kg b.w.); Gr. IV: Control. Means bearing at least one common superscript do not vary significantly between group (P<0.05)

compared to that in the control (0.84%). The values of apoptotic cells percentage in spleen and kidneys were, however, comparable to those of control [Table 1 and Figure 2]. In combination group (group III), there was significant increase in the percentage of apoptotic cells in liver, kidneys and spleen (1.64%; 3.1% and 6.79%) as compared to those of the control (0.84; 0.70 and 1.05%) [Table 1 and Figure 5].

Apoptosis appeared to play an important role in the pathology and pathogenesis of endosulfan toxicity in the present study. Increased number of apoptotic cells in liver, kidneys and spleen were detected by FACS and was also evident histopathologically in lymphocytes of spleen and Peyer's patches.[2] Earlier workers have reported that endosulfan caused apoptosis in human T-cell leukemic cells[12] and poultry lymphocytes;[13] however, DNA fragments were not clear in agarose gel electrophoresis.

In CIT group also, there was an increase in the number of apoptotic cell percentages in kidneys, liver and spleen. It was in agreement with earlier reports regarding CIT causing apoptosis in kidneys.[14] However, no report is available regarding CIT causing apoptosis in liver and spleen. Occurrence of increase in apoptosis can be attributed to generation of superoxide radicals which might have led to cell death.[15] Oxidative stress has been considered to play a key role in the process of apoptosis as it has been reported that CIT directly evokes intracellular oxidative stress which leads to ROS mediated apoptosis.[16] Citrinin-induced apoptosis might be due to interference with electron transport system of mitochondria and initiation of cellular messenger system.[17–20]

There was significant increase in the percentage of apoptotic cells in kidneys and spleen in the combination group, but apoptotic cells in liver were comparable to those of the endosulfan group indicating an additive effect of endosulfan and CIT on kidneys (nephrotoxicity) and

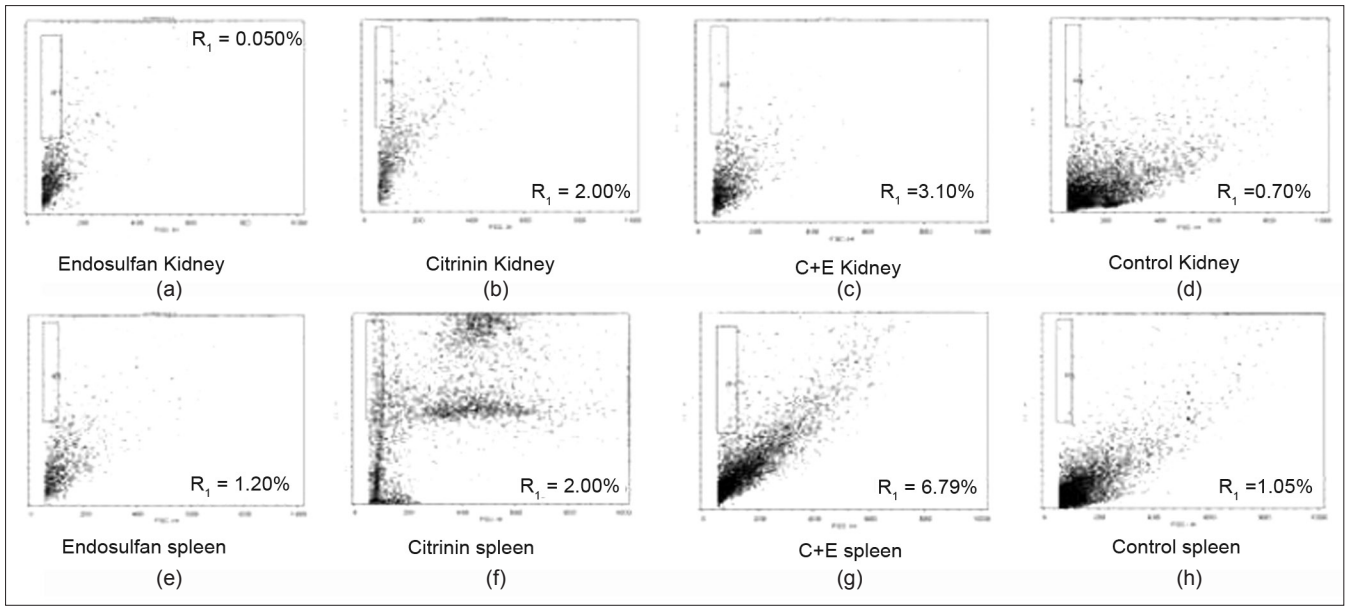

Figure 5: Representative histogram of flow cytometry output showing percent apoptotic cells (R1) in kidneys and spleen of pregnant dams after staining with propidium iodide

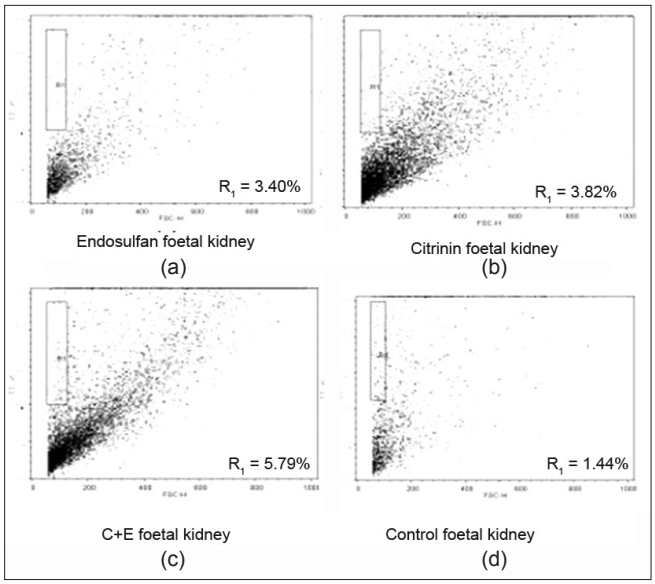

Figure 6: Representative histogram of flow cytometry output showing percent apoptotic cells (R1) in kidneys of fetuses after staining with propidium iodide

spleen (immunosuppression). DNA fragments were clear in agarose gel electrophoresis, substantiating the claim.

Fetuses

Fluorescence activated cell sorter analysis was performed on liver and kidney of fetuses of all the treatment groups as well as control [Table 1]. In group I (CIT), there was significant increase in the percentage of apoptotic cells in kidneys (3.82%±0.03) as compared to that in the control (1.44%±0.14); however, in liver the percentage of apoptotic cells was comparable to those of the control [Table 1 and Figure 6]. In group II (endosulfan), there was significant increase in the percentage of apoptotic cells in the liver (1.57%) as compared to the control (1.40%). Similarly, significant difference was seen in the percentage of apoptotic cells in kidneys (3.40%) as compared to that in the control (1.44%) [Table 1 and Figure 6]. In the combination group, group III (CIT+endosulfan), revealed significant increase in the percentage of apoptotic cells in kidneys (5.79%) as compared to that in the control (1.44%). The percentage of apoptotic cells in liver was, however, comparable to that of the control [Table 1 and Figure 6]. Negligible apoptosis was observed in different organs of the control group. The percent apoptotic cells in liver, kidneys and spleen were 0.84±0.16, 0.70±0.19 and 1.05±0.12, respectively.

Although apoptosis is a normal phenomenon in the fetal organs, yet in endosulfan, there was an increase in apoptotic cells in liver and kidneys as compared to that in the control. It is likely that endosulfan and CIT might be reaching fetal circulation through dam generating free radicals (SOD) which led to increased apoptosis.[12] However, no such finding has been reported earlier regarding apoptosis in fetal organs.

In CIT group, there was an increase in the percentage of apoptotic cells in fetal kidneys; however, liver was comparable to that of the control. CIT has been reported to reach the fetal circulation through dam, generating superoxide anions (ROS) which might have led to apoptosis.[15] However, no report is available regarding studies on apoptosis in organs of fetuses of CIT treated pregnant rats.

There was significant increase in the apoptotic cells percentage in kidneys, whereas fetal liver was comparable

to endosulfan group indicating additive action of CIT and endosulfan on fetal kidneys.

On the basis of the present study, it can be concluded that CIT is more nephrotoxic than hepatotoxic and endosulfan is both hepatotoxic and nephrotoxic in pregnant rats. The present findings regarding apoptosis in tissues of dams and fetuses due to CIT and endosulfan exposure during pregnancy opens a new area for further research regarding the potential toxicity of these two environmental co-contaminants through dietary exposure which might cause deleterious effects in animal and human populations.

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