IN OVO AND IN VITRO TOXICITY OF THIRAM, A COMMON BROAD SPECTRUM FUNGICIDE IN AGRICULTURE

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ABSTRACT**:** At the recent years, thiram, a dithiocarbamate compound is used as a fungicide to protect crops against a wide variety of plant pathogens. In this work, Chicken embryos were studied for the screening of skeletal malformation caused after application of thiram. On the other hand, the toxicity of thiram to chicken liver cells culture was determined. The compound was dissolved in acetone, then a volume of 50 µl of different doses of it was injected into the yolk sac after 72 hours of incubation and the embryos were harvested after 19 day of incubation The results showed that exposure of the embryos to thiram increased mortality with a LD_{50} value of 3 5 µg egg and malformations including clubfoot and unossified caudal vertebrae No significant difference was observed in wet weight of the embryos. Thiram also exhibited the cytotoxic and antiproliferative effects against liver cells with an IC_{50} value of 5 2 µg ml The present study proposes that thiram can affect the growth and ossification of the chicken embryos, which are developed outside the mother.

KEY WORDS: Embryotoxicity, thiram, malformation, cytotoxicity, dithiocarbamate, Chicken embryos.

Thiram, a heavy metal chelator, has been considered as a fungicide in agriculture and is widely used for the control of plant diseases (Guitart et al., 1996; Marikovsky, 2002; Grosicka et al., 2005). The compound has been used in the treatment of human scabies, as an accelerator vulcanizing agent during rubber processes. The application of thiram as an ingredient in sunscreen and as a bacteriostatic in medical soaps and certain antiseptic sprays has also been reported (Dalvi, 1988; Ruijtenbeek et al., 2002). On the other hand, the oral administration of thiram to mice has caused a significant inhibition in the development of glioma tumor and reduced the metastatic growth of Lewis lung carcinoma (Marikovsky, 2002).

The chick embryo (*Gallus gallus*) is a general model for the developmental physiology and toxicological studies (Altimiras & Crossley, 2000; Ruijtenbeek et al., 2002). The chickens and its embryos are cost-effective and readily available, and present an alternative approach to the treatment of pregnant mammals. The chicken embryo develops outside the mother and therefore the effects of external stresses on embryonic development can be studied without interferences of the maternal hormonal and metabolic modifications. Due to the absence of maternal metabolism, considerably smaller amount of administered substances per embryo is needed, which is particularly useful for testing rare or expensive compounds, or when the maternal toxicity is of concern (Petrovova et al., 2009). The present study deals with the embryotoxic and cytotoxic effects of thiram on chicken embryo and liver cells culture, respectively

MATERIALS AND METHODS

Materials

Thiram, 98% purity (Guaranteed by supplier), was obtained as a gift from Giah co. (Iran- Tehran). Fertile eggs (*Gallus gallus*) were obtained from a local commercial poultry source and were checked for bacterial contamination. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and alizarin red were purchased from Sigma and Merck (Germany), respectively.

Embryos preparation and treatment

The eggs were incubated at 37 7-38 C in incubators with a relative humidity of 70%. The eggs were turned three times daily to avoid the sticking of the embryo to the shell membranes. After three days, the eggs were candled and embryos of uniform size were selected randomly to be used in all experiments. Before injection, the large end of egg was wiped with a sterile cotton wool pad moistened with a 70 ethanol solution and drilled above the air cell.

A series of a fresh stock solution of thiram in minimum volume of acetone were prepared and injected aseptically into the yolk sac of the eggs in various doses (1, 2 5, 5 and 10 µg egg) after 72 hours of incubation All the tests were performed in three replications (n=10). Control eggs injected with 50 µl of acetone were run with each batch of treated eggs (Anwar, 2004). The eggs were candled at day 7 and 14 of incubation and the dead embryos were recorded before they were discarded. After 18th day of incubation, the embryos were taken out from the shells and subjected to detection of abnormalities (Natekar, 2007). Percentage of mortality was calculated using the following formula [(mortality $_{\text{treatment}}$ % - mortality $_{\text{control}}$ %) (100 – mortality $_{\text{control}}$ %)] ×100.

Skeletal study

In order to study of the skeletal elements, the specimens were prepared according to the method expressed by previous published data (Staples & Shnell, 1963). Briefly, the specimens were eviscerated and skinned. Then, they were placed into the 2 aqueous potassium hydroxide until the flesh was sufficiently clear to render the skeleton visible. The solution was changed whenever it became discolored. The specimens were then placed into 1 aqueous KOH solution to which a few drops of aqueous alizarin red solution was added. The stained specimens were stored in glycerin.

For the study of skeletal elements, The length of long bons and incidence of abnormalities of each embryo treated with various doses of thiram in all three experimental groups was measured and statistically analysed to see the degree of suppression of growth in the long bones as compared with their corresponding controls.

Liver tissue culture

The embryo livers were aseptically harvested from 12-day-old embryonated chicken eggs. The liver tissue was washed, minced and placed in a 50 ml flask containing 2-3 ml of phosphate buffered saline. The minced liver tissue was trypsinized at room temperature for 15 minutes and the undigested tissue was allowed to settle. In order to inactivation of the enzyme, the supernatant fraction was transferred to a tube containing RPMI1640 medium supplemented with 20% fetal bovine serum The remaining tissues were repeatedly trypsinized until all the tissue pieces were digested The embryo liver cells were cultured in RPMI1640 medium, supplemented with 10 FCS and pencillin streptomycine (100 u ml, 100

μg ml), at 37 C in a humidified, CO_2-controlled (5) incubator (Bissell & Tilles, 1971).

Cytotoxicity assay

The MTT colorimetric assay was used to evaluation of thiram cytotoxicity (Carmichael et al., 1987; Zahri et al., 2009). The freshly prepared cells were suspended in a fresh medium and dispensed into 24-well microplates at 3×10^5 cells/well. After 24h of incubation, the fungicide was dissolved in DMSO and added to the cells at different concentrations (0, 0.25, 0.5, 1, 2.5 and 5 μg/ml) in the volume of 500 μl/well. Cells in the control wells were treated with the same volume of medium containing DMSO. After 16h, the medium was removed and the cells were incubated with 50μl of sterile aqueous solution of MTT 5 mg ml) for 3 h. The blue formazan precipitation was dissolved in DMSO and the absorbances of resulting solutions were measured at 540 nm The 50% inhibition concentration (IC_{50}) values were defined as the concentration of an agent required to induce a 50% reduction in absorbance. Viability percentage was evaluated as "$(OD_{treatment} / OD_{control})\times 100$".

Statistically analysis

One-way ANOVA was used to determine the difference between groups and controls. A P-value of < 0.05 indicated significance. The analysis was performed using SPSS V. 14.

RESULTS

The in-ovo embrotoxicity studies showed that treatment of the embryos with thiram at the concentrations of 2.5, 5 and 10 μg egg increased embryos mortality by 22%, 44% and 80%, respectively. However, no significant effect was observed at concentration of 1 μg egg. The results showed that thiram exhibited an embryo toxicity effect in a dose-dependent manner with a LD_{50} (lethal dose, 50%) value of 3 5 μg egg (Fig. 2). However, the wet weight of embryos was not significantly affected by thiram On the other hand, typical morphologic malformations were observed at the concentrations of 2 5 and 5 μg egg In the concentration of 2.5 and 5 μg egg, the reduced body size and clubfoots were detected in 12 5 and 20% of the embryos (Fig. 3A).

Detailed study of skeleton by alizarin red staining showed that the treatment of the eggs with a 2 5μg egg of thiram induces wavy pubis and retarded skeletal growth in 12 5 of the embryos, which were characterized by unossified vertebrae (Fig. 3B). Incidence of unossified vertebrates was increased to 60 by dose-dependent increament of the fungicide up to 5μg egg (Fig. 2C). Treatment with the 10 μg egg of thiram was not led to any abnormalities, due likely to the excess mortality of the embryos.

Liver tissue culture as a major detoxification organ was used to evaluate of cytotoxic effects of the fungicide. Treating of the embryos liver cell culture with the various concentrations of thiram showed a significant decrease in the cell viability in a time- and dose-dependent manner ($P < 0.05$) In the concentration of 5.2 μg/ml thiram, the viability of 50% (IC_{50}) of liver cells was inhibited (Fig. 4). The growth of treated cells was clearly inhibited and analyses of cell shapes showed significant alterations in the elevated concentrations of thiram. The treated liver cells reduced to the condensed form and intercellular connections were lost.

The difference of mortality rates between control and 1 and 2.5 µg/egg was not significant and the liver cell cytotoxicity assays showed that > 60% of the cells were alive after treatment with the 2.5 µg/ml of thiram.

DISCUSSION

Thiram is a broad spectrum agriculture fungicide that is widely used to control seed decay and seedling blights. Because the maternal metabolism degrades the compound, some previous data have not been shown any teratogenic effects of the fungicide against mammals and it did not present any genotoxic hazard to humans (Larsen, 1992). These experiments showed that the administration of thiram (2.5 and 5 µg/egg) extensively reduces the body size and retard skeletal growth. Previous reports have shown that thiram induces apoptosis and inhibits angiogenesis and DNA synthesis, leading to the cell death in rapidly proliferating embryonic tissue (Ritter et al., 1971; Izaguirre et al., 2000; Hammond et al., 2001; Marikovsky, 2002; Grosicka et al., 2005). The observed retardation of the growth and reduction in the size of body may occur by induced apoptosis in embryo by the fungicide. Anthrogryposis or clubfoot is the prevalent imperfection in the treated groups and the cause of this phenomenon was postulated as muscular hypoplasia (Landauer, 1975). It has been suggested that this substances probably compete with acetylcholine for the possession of the acetylcholine receptors resulting in a pronounced cholinergic effect (Forsyth et al., 1994; Paul et al., 1999).

Previous reports have been described that thiram is rapidly degraded to more polar products in the adult rats and some other mammals and the LD_{50} was very high (2000-4000 mg/kg bw) (Thouin, 1985; Larsen, 1992), however, our results showed that the chicken embryos, which develop outside of the mother, were sensitive to toxicity of thiram and their grows and ossification was affected by the fungicide. Liver tissue is a major site for detoxification and xenobiotic metabolism in animals and our results show that thiram also possess cytotoxic effect against the liver tissue culture.

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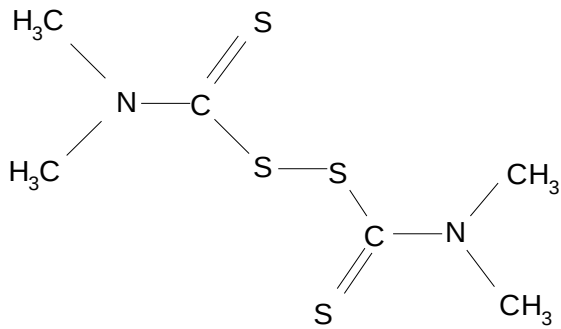

Figure 1. The structure of Thiram.

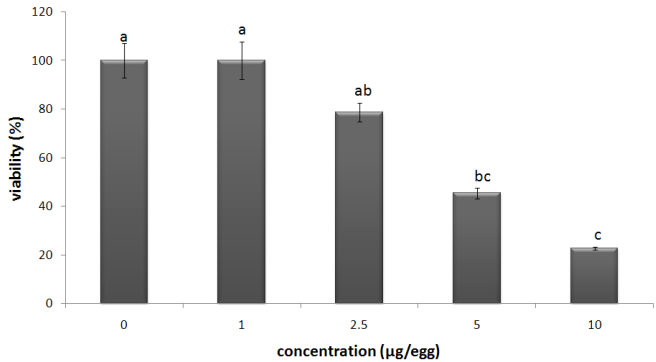

Figure 2 Effects of in ovo injection of various doses (1, 2 5, 5 and 10 µg egg) of thiram in vehicle (50µl of acetone) or vehicle alone in yolk sac of the fertile eggs prior to incubation Same letters indicates nonsignificant difference between groups.

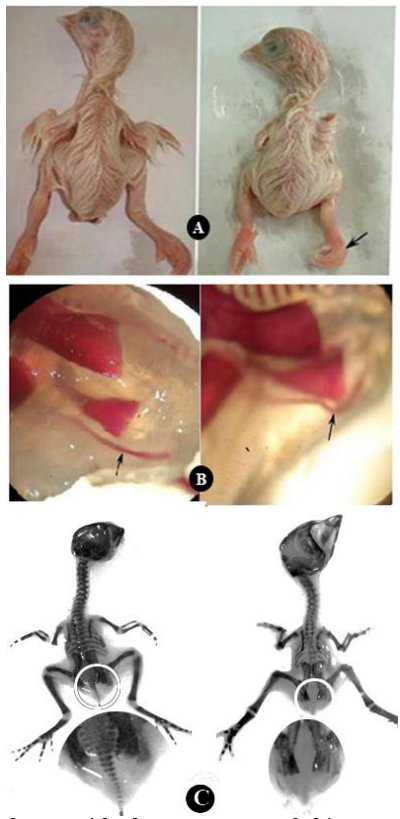

Figure 3. A: Treated embryos with the 2.5 µg egg of thiram prior to incubation which exhibits the reduced body size and clubfoot (right) and Control (left). (B) Control embryo (left) ant the embryo which was treated with the 2.5 µg egg displaying wavy pubis (right). (C) Control embryo (left) and the embryo which was treated with the 5µg egg showing unossified caudal vertebrae (right).

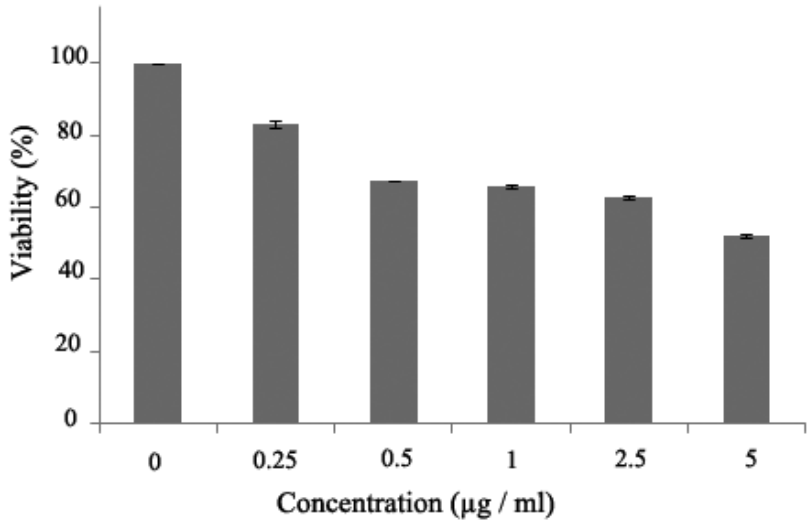

Figure 4. In vitro cytotoxic effects of the various doses (0 25, 0 5, 1, 2 5, 5 µg ml) of thiram against the liver cell culture after 16 h incubation.