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Research Article

The Teratogenic Effects of Fungicide Copper Oxychloride in Female Albino Rats

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Received: October 05, 2019; **Published:** November 15, 2019

Abstract

Copper oxychloride (COC) is used extensively in greenhouses in Egypt. To better understand the possibility of COC developmental toxicity, we done this experiment in pregnant female albino rats. COC administered orally at different doses (1/10 and 1/20 LD50 of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) daily from day 6th to day 15th of pregnancy. The pregnant dams were sacrificed at day 20th of gestation and fetuses were weighed and its lengths were measured moreover assed for the external, visceral and skeletal malformations. The results showed a significant dose dependent decrease in the fetal weights and lengths in respect to the control group besides a representative increase in the skeletal malformations in a dose dependent manner as delayed ossification of the skull bone plates, misshaped skull, delayed ossification of sternbrae, delayed ossification of vertebrae, defect in the rib cage, delayed ossification of fore limb bones and hind limb and delayed ossification and absence of the phalanges. Notably, visceral malformations was found in a dose dependent manner in fetuses of treated female rats when compared with the control group such as cerebral hemorrhage, dilatation of the ventricles, cleft palate, thickening in the myocardium, abnormal shaped and positioned kidneys besides defect in shape and lobulation of livers and lungs respectively. Microscopically the fetal livers showed a clear increase in the hematopoietic stem cells (HSC) proliferation occluding the liver blood spaces as lymphoblasts, monoblasts and megakaryocytes. Results revealed the potential teratogenicity of COC fungicide in a dose dependent manner.

Keywords: *Copper Oxychloride; Greenhouse Fungicide; Developmental Toxicity; Teratogenesis; Female Albino Rats*

Introduction

Pesticides for centuries used in agricultural practice to improve food production through controlling of unwanted pests [1] and used extensively in greenhouses. Pesticides' effect on environment and the biological system must be considered especially the chronic impact from such pesticides that may cause cancer, adverse reproductive and developmental effects besides skin and neurologic diseases [2].

Many extensively used pesticides are copper-based such as copper oxychloride and most of them have fungicidal properties and applied annually worldwide with millions of tons so it can harm human health causing different cancer types, reproductive, hematological, neurological and immune disorders [3].

Copper is a vital trace element to all living as it participates in various metabolic processes. Copper metabolism is in the body controlled via complicated homeostatic processes to provide living organisms with this micronutrient, but excess of copper has deleterious effect in organisms as it stimulates free radical production in the living cell, lipid peroxidation and disturbs the whole body antioxidant capacity [4].

Greenhouses are microcosms that provide a suitable physical environment with controlled temperature, humidity, light intensity and carbon dioxide levels may be handled to encourage growth and survival of plants in greenhouses [5]. Additionally, greenhouses on the other hand encourage the fungal infection that needs intensive fungicides' application system with different fungicides as copper oxychloride that in turn are highly toxic to human and population health but keeping workers under general provisions upon exposure and occupational hygiene may minimize the potential ill health risks [5].

The known mechanism of copper oxychloride toxicity is through promoting the oxidative stress with cellular damage due to release of ROS through Fenton reaction [6].

Liver considered as the primary target organ in copper toxicity as liver involved in the process of copper homeostasis, synthesis and storing of copper-containing protein ceruloplasmin) so many animals can cope with excess copper exposure by liver but overwhelming of such mechanism may occur with repeated exposure resulting in hepatotoxicity [7].

Excess copper or deficiency has a significant effect upon the normal development rat, mouse, chick and hamster embryos [8].

Excess dietary Cu at dose level 500 µg Cu/g diet fed to pregnant rats revealed no abnormal outcomes in embryos but fetal viability reduced with growth retardation that may only be due to decrease in the food intake [9].

In a developmental toxicity study of R6 fungicide (mixture of copper oxychloride and cymoxanil) at concentration of 2.5 µg/ml on sea urchin showed that a significant increase of larval malformations with derangement of differentiation and embryogenesis [10].

The teratogenic effect of Copper investigated *in vivo* in both mouse and chick embryos and results showed that excess copper has a significant deleterious effect with developmental retardation, decrease in crown rump length and limbs anomalies in mouse embryo besides a high rate of mortality, limb dysgenesis and tailness syndrome in a 5 days post incubation chick embryo [11,12].

The teratogenic effect of copper salts (sulphate and citrate) investigated in hamster results showed that there was an increased levels embryonic resorption and a high incidence of malformations furthermore its confirmed radioactive copper passed through the placenta during the organogenesis critical stages [13].

In *in vitro* study in 9th days' mouse embryos cultured for 48 hours in rat serum then exposed to different concentration of $CuCl_2$ showed failure of neural tube closure in the anterior region and exencephalia with significant embryonic developmental retardation and decrease in somites average numbers [14].

The fetal skeleton of mice embryo was also investigated from day 9 to day 19 of gestation with i.p. injection of $CuCl_2$ (0, 1 ml/10g body weight) showed a significant teratogenic effect in some ossification centers, delayed ossification of bones and decrease in fetal weight [15].

Copper developmental toxicity studied in developmental stages of salmon fish causing delayed hatching, larval deformities as yolk sac abnormalities, craniofacial alterations and deformed heads and spinal cords [16].

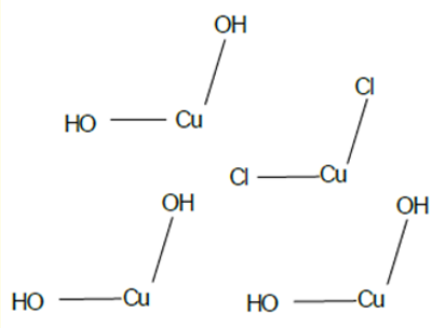
Aim of the Study

The rationale of this study to investigate the teratogenicity of copper oxychloride in female albino rats.

Material and Method

Fungicide: ACROBAT CU 73.2%WP (copper oxychloride) or (COC)

Light green wettable powder (WP) with slight chlorine odor and kindly obtained from Central Agricultural Pesticide Laboratory, Ad Doki, Giza, Giza Governorate.



Figure

Laboratory animals

39 Mature females and 16 mature males albino rats obtained from Experimental Unit in the Faculty of Pharmacy, Mansoura University; Animals weighed about 250 ±10 gm and were obviously healthy then grouped and housed in plastic cages with soft wood shavings as a bedding material that changed adequately to ensures a low level of ammonia and to keep animals clean and dry.

Animals adapted for about 2 weeks and maintained on a balanced ration before the experiment also standard laboratory pelleted diet and water given *ad libitum* throughout the experiment and Light cycles of 12 hours light to12 hours dark seemed to be adequate in order to promote rodents' breeding.

Calculation of LD_{50} of Cu oxychloride

The LD_{50} of COC was calculated according to the Up and Down Procedure (UDP) that proposed by [17] and revised and modified through [18] and accepted as a method for calculation of LD_{50} through AOT 425 statistical program and the estimated LD_{50} was 1470 mg/kg. n.b 15 female albino rats were used in this side experiment.

Determination of zero day of pregnancy

Polygamous or harem mating were applied whereas rats are continually polyestrous (estrous cycle is about 4 - 5-days) and estrus remain for 12 - 24 hours, Females checked daily by vaginal smear examination on wet glass slide for stage of the estrous cycle (proestrus, estrus, metestrus and diestrus) and only females in late proestrus or early estrus were mated with males through pairing of three females with one male (females introduced to males not the vice versa) [19].

On the next each morning females checked by vaginal smear examination until presence of sperms or copulation plug and once females were plug or sperm positive, they were weighed and removed to a new cage and the date designated to be the zero day of pregnancy (GD 0).

Experimental design for prenatal toxicity study of cu oxychloride on developing fetuses

Twenty four synchronized pregnant females were separated into three groups with eight for each whereas the duration of exposure designated to be from day 6^{th} to day 15^{th} of pregnancy daily orally by stomach tube, the first group received 0.5 ml distilled water and used as control, the second group administered 1/10 LD_{50} of COC equivalent to 147 mg/kg Bw., the third group gavaged1/20 LD_{50} of COC equivalent to 73.5 mg/kg Bw, the pregnant females were weighed and kept under observation daily until the day 20^{th} of gestation (the day of sacrifice) (See table 1).

Group	No. of Pregnant rats	Treatment	Oral dosage mg/kg B.wt	Duration of exposure during pregnancy	Sacrificing
I	8	D.W	0.5 ml/rat	6^{th} to 15^{th}	20^{th}
II	8	Cu oxychloride	147	6^{th} to 15^{th}	20^{th}
III	8	Cu oxychloride	73.5	6^{th} to 15^{th}	20^{th}

Table 1: *The experimental design summery for prenatal developmental toxicity of cu oxychloride.*

External Examination

After pregnant dams euthanized with overdose of thiopental Na, the thoracic and abdominal cavities were opened surgically. Pregnancies confirmed by uterine examination, then the uteri were removed and uterine contents as resorption or implantation sites (corpora lutea numbers correspond to implants number), dead and live fetuses were recorded. The fetuses then pulled out weighed and euthanized with hypothermia then carefully evaluated externally from head to tail for any abnormalities, the crown rump length and breadth of each fetus also were recorded and finally half of fetuses injected with Bouin's solution intraperitoneally for visceral and histopathological examination and the other half preserved in ethyl alcohol 95% for skeletal examination.

Visceral examination by Wilson's technique

Soft tissue evaluation by Wilson's Technique involve fixation and decalcification of fetuses in Bouin's solution then placed in supine position, pinned to a paraffin block and a ventral midline incision was applied with a scalpel blade and scissor from the umbilicus to the genital tubercle caudally and to the neck cranially, thoracic viscera as diaphragm and lungs examined for herniation and abnormal lobulation (normally five lobes with three on right, one on left and one intermediate) respectively, the heart examined carefully and sectioned transversally at the ventricles for detection of any abnormal thickening in the ventricular wall then the abdominal viscera examined as the four liver lobes checked for abnormal shape and fusion, also stomach, spleen, small and large intestines were examined. The kidneys examined also for their size and location (the left kidney located more caudal than the right) and each kidney was cut transversely for any renal pelvis dilatation.

Craniofacial region serially cross sectioned (five sections) with a razor blade, the first cut was made as a horizontal section from the mouth to the ears for the tongue and palate exposure, the second cut was made as a longitudinal section just anterior to the eye slits for the nasal passages exposure, the third cut was made through the eyes for cerebral hemispheres, lateral ventricles and eyes structure visualization, the fourth cut was made just anterior to the ear flaps for cerebral hemispheres, cerebellum, lateral and third ventricles and brainstem exposure and finally fifth posterior section made inferior to the ear flaps for cerebellum and surrounding structures observation and all sections examined by the dissecting microscope.

Skeletal examination

After fetuses were dehydrated in ethyl Alcohol 95% for about 2 weeks, fetuses were skinned and eviscerated through a ventral midline incision at the level of the umbilicus followed by staining either through single staining technique (for ossified tissue staining only) or double staining technique (for both ossified and cartilaginous tissues staining).

For Single staining technique eviscerated and skinned fetuses were placed in KOH 2% solution for about 24 - 36h for soft tissue clearance then stained with alizarin red (S) stain (0.1g alizarin red (S)) in the working stock solution (7 ml KOH 4% +60 ml Glycerin + DW up to 300 ml) for 24h then fetuses washed in working stock solution alone for another 24h then put in glycerin different concentration (20, 50, 80, 100%) for 48h for each concentration and kept in pure glycerin 100% until evaluation and photography.

For the double staining technique eviscerated and skinned fetuses were placed in alcian blue stain solution (15 mg alcian blue + 80 ml ethanol 95% + 20 ml pure glacial acetic acid) for 24h then placed in ethanol 95% for another 24 h then stained with alizarin red (S) stain in KOH 2% stock solution (25 mg of alizarin + liter of KOH 2%) for 24 - 36h then fetuses removed and washed in the working stock solution for another 24h then put in glycerin different concentration (20, 50, 80, 100%) for 48h for each concentration and kept in pure glycerin 100% until evaluation and photography.

Transmission electron microscope examination

Liver samples with 1 x 2 mm thickness were taken and Fixed in 5% glutaraldehyde solution immediately after animal dissection for 24 - 48h. Then specimens washed in cacodylate buffer (PH 7.2) for 4 times and for 20 minutes each time followed by fixation in 1% O_4S_4 for 2 hours then washed in the same buffer for four times again. Dehydration then applied in ascending manner with different alcohol concentrations (30 - 50 - 70 - 90 and 100% for 2 hours in each concentration then embedded in epon araldite mixture, the embedded blocks then cut by ultramicrotome in 0.5 - 1 µ thickness and then ultrathin sections using Leica AG ultramicrotome made with 500 - 700 A thickness and then contrasted in lead citrate and uranyl acetate and examined by JEM 100 CXII electron microscope and photographed by XR- 41digital camera [20].

Results

External examination findings

Fetal body weight

Estimation of the mean fetal body weight showed that there was a significant decrease in maternally treated groups (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) in dose dependent manner in respect to the control group and the results observed in table 2 and figure 1.

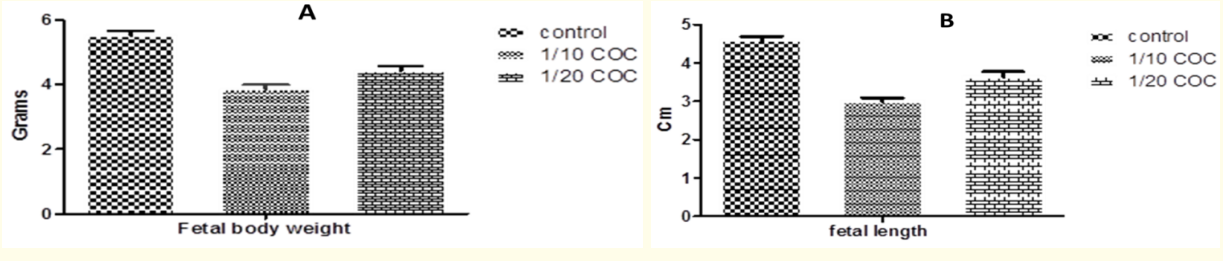


Figure 1: *Showing A: the mean body weight (Gm) of maternally treated fetuses with different doses of COC (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) orally from 6th - 15th days of pregnancy daily in comparison to the control group and B: the mean length (Cm) of maternally treated fetuses with different doses of COC (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw.) orally from 6th - 15th days of pregnancy daily in comparison to the control group.*

Group	Fetal B.wt (g)	Fetal crown-rump length (cm)
Control	$5.5^a \pm 0.16$	$4.58^a \pm 0.11$
1/10 LD_{50} of COC mg/kg B.wt	$3.85^b \pm 0.14$	$3.00^b \pm 0.09$
1/20 LD_{50} of COC mg/kg B.wt	$4.42^c \pm 0.15$	$3.63^c \pm 0.14$

Table 2: *Fetal body weight and crown rump length of maternally treated fetuses with different doses of cu oxychloride orally from 6th - 15th days of pregnancy (Mean ± SE).*

a, b, c: Different letters are significantly different between groups at P ≤ 0.05.

Fetal crown-rump length

Estimation of the mean fetal crown-rump length showed that there was a significant decrease in in treated groups (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) in dose dependent manner in respect to the control group and results observed in table 2 and figure 1.

Embryonic death (resorption and stillbirth) rate

Early embryonic death (resorption) either partial or complete resorption and stillbirth (late embryonic death) were recorded and results showed an increased rates of embryonic death maternally treated groups (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) in dose dependent manner in respect to the control group.

The rate of partial resorption in pregnant females treated with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 25% for both doses groups while complete resorption observed only in the dose group147 mg/kg Bw. with rate of 12.5% of the total examined pregnant uteri.

The rate of still birth of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 14.9% and 5.5% of the total number of fetuses respectively. The results observed in table 3 and figure 2.

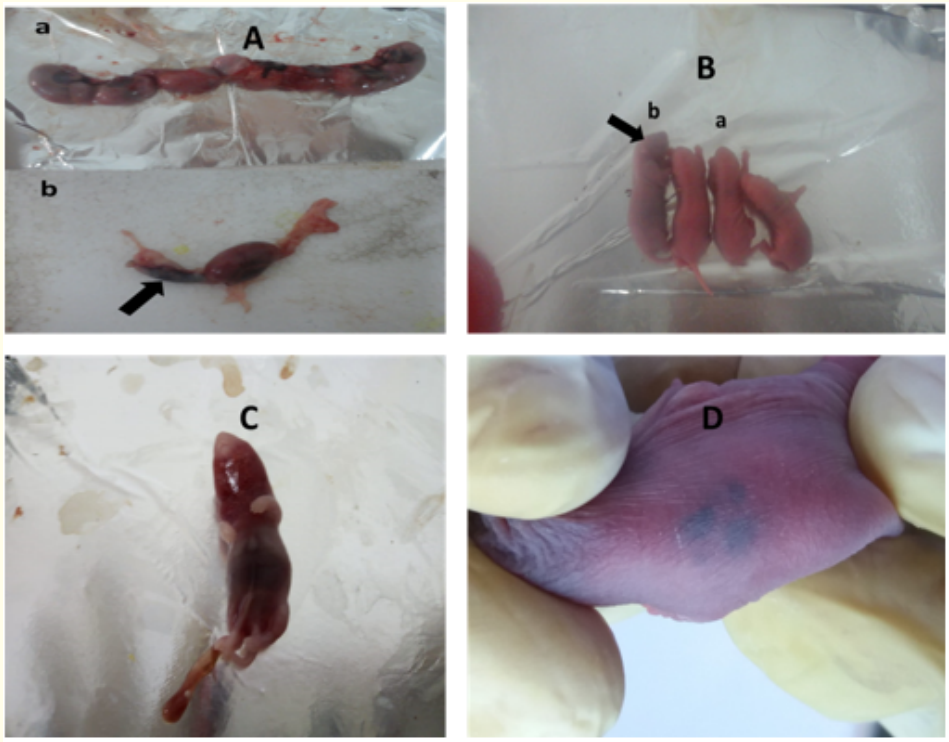


Figure 2: *Showing A: (a) uterus of control pregnant rat (b) uterus of pregnant rat 1/10 LD_{50} of COC displayed partial resorption, B: (a) three control fetuses and (b) maternally treated stillbirth fetus with 1/10 LD_{50} of COC, C: maternally treated fetus with 1/10 LD_{50} of COC displayed sever hematoma in the neck with swelling and ecchymosis throughout the body and D: maternally treated feus with 1/20 LD_{50} COC displayed SC hemorrhage and ecchymosis in the back.*

Group	Total number of pregnant rats	Total number of fetuses	Total number of examined fetuses	Embryonic death			External malformations of examined fetuses				Visceral malformations of examined fetuses					
				Resorption in pregnant dams		Still birth of fetuses	S/C hemorrhage	Hydrocephalus	Malformed limbs	Opened eyes	Cerebral hemorrhage	Cleft palate	Myocardial thickening	Malformed kidneys	Lung lubes fusion	Misshaped livers
				Partial N (%)	Complete											
Control	8	62	31	0	0	0	1 (3.2%)	0	0	0	0	0	0	0	0	0
1/10 LD_{50} of COC	8	47	23	2 (25%)	1 (12.5%)	7 (14.9%)	9 (39.1%)	3 (13.0%)	7 (30.4%)	2 (8.7%)	5 (21.7%)	3 (13%)	6 (26.1%)	4 (17.4%)	5 (21.7%)	4 (17.4%)
1/20 LD_{50} of COC	8	55	27	2 (25%)	0	3 (5.5%)	5 (18.5%)	1 (1.8%)	3 (5.5%)	0	2 (3.6%)	0	2 (3.6%)	1 (1.8%)	1 (1.8%)	1 (1.8%)

Table 3: *Showed rate of embryonic death, External and Visceral malformations of maternally treated fetuses with different doses of COC.*

External malformations

Fetuses of the treated dams with (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) displayed several external anomalies and abnormal features in dose dependent manner in respect to the control group as subcutaneous hemorrhage or hematoma, hydrocephalus, internal hemorrhage and anomalies of the limbs and also high rate of external malformations observed in the higher dose.

The rate of subcutaneous hemorrhage of maternally controlled fetuses was about 3.2% of the total examined fetuses while maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 39.1% and 18.5% total number of examined fetuses respectively.

The rate of hydrocephalus of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 13% and 1.8% total number of examined fetuses respectively.

The rate of malformed limbs of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 30.4% and 5.5% total number of examined fetuses respectively.

The rate of opened eyes malformation of maternally treated fetuses with 147 mg/kg Bw., and 73.5 mg/kg Bw. COC was about 8.7% and 0% total number of examined fetuses respectively. The results observed in table 3 and figure 2.

Visceral malformations

Maternally treated fetuses with (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw., and 73.5 mg/kg Bw. respectively) exhibited different visceral malformations in a dose dependent manner when compared to the control group as cerebral hemorrhage, dilatation of the ventricles, cleft palate, thickening in the myocardium, abnormal shaped and positioned kidneys besides defect in shape and lobulation of livers and lungs respectively.

The rate of cerebral hemorrhage of maternally treated fetuses with 147 mg/kg Bw., and 73.5 mg/kg Bw. COC was about 21.7% and 3.6% total number of examined fetuses respectively.

The rate of cleft palate of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 13% and 0% total number of examined fetuses respectively.

The rate of myocardial thickening of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 26.1% and 3.6% total number of examined fetuses respectively.

The rate of malformed kidneys of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 17.4% and 1.8% total number of examined fetuses respectively.

The rate of lung lubes fusion of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 21.7% and 1.8% total number of examined fetuses respectively.

The rate of misshaped livers of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 17.4% and 1.8% total number of examined fetuses respectively. The results observed in table 3 and figure 3.

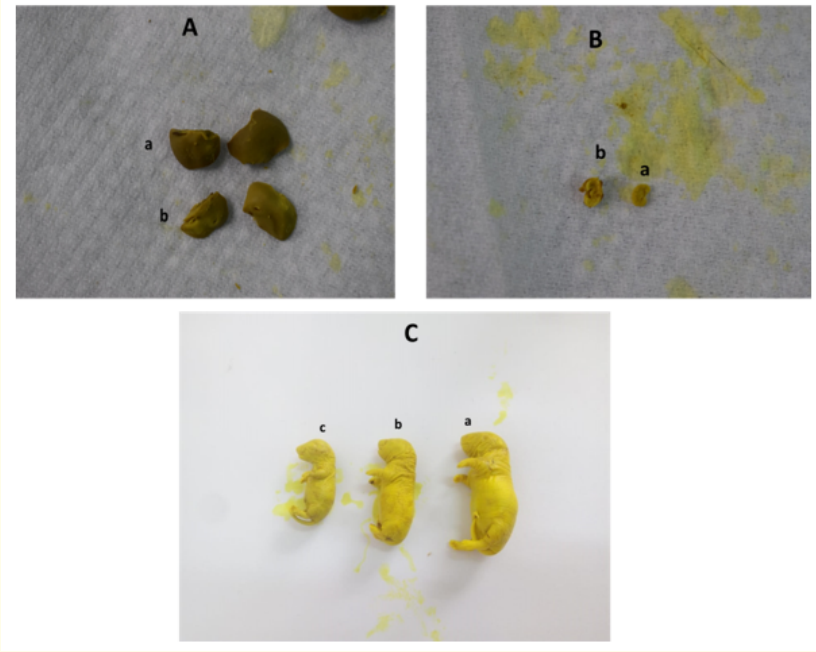


Figure 3: *Showing A: (a) liver lobes (right and left lateral) of maternally controlled fetus (b) liver lobes (right and left lateral) of maternally treated fetus with 1/10 LD_{50} of COC displayed misshaped and atrophy of the liver lobes, B: (a) kidney of maternally controlled fetus (b) kidney of maternally treated fetus with 1/20 LD_{50} of COC displayed dilatation of the renal pelvis and C: decrease in the crown rump length in a dose dependent manner (a) normal maternally controlled fetus (b) maternally treated fetus with 1/20 LD_{50} of COC (c) maternally treated fetus with 1/10 LD_{50} of COC.*

Skeletal malformations

Maternally treated fetuses with (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively) showed numerous skeletal malformations in a dose dependent manner when compared to the control such defects could be grouped as axial and peripheral skeletal defects, axial skeletal defects as delayed or malformed ossification of bone of the skull (interparietal, exoccipital, supraoccipital, parietal, frontal and nasal bones) or abnormal rib cages, sternbrae and vertebral columns besides peripheral skeletal defects as delayed or misshapen ossifications of the fore and hind limbs (scapular, humeral, radial, pelvic, femoral, tibial and phalangeal bones).

The results showed an increased rate of skeletal malformations in the treated groups in a dose dependent manner (1/10 LD_{50} and 1/20 LD_{50} of COC equivalent to 147 mg/kg Bw. and 73.5 mg/kg Bw. respectively when compared to the control group.

Craniofacial malformations

The rate of delayed ossification of the skull bone plates of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 75% and 32.1% total number of examined fetuses respectively.

The rate of misshaped skull of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 16.7% and 3.6% total number of examined fetuses respectively.

Rib cage and vertebral column malformations

The rate of delayed ossification of sternbrae of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 45.8% and 10.7% total number of examined fetuses respectively.

The rate of absence of sternbrae of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 25% and 7.1% total number of examined fetuses respectively.

The rate of delayed ossification of vertebrae of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 41.7% and 10.7% total number of examined fetuses respectively.

The rate of defect in the rib cage of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 25% and 14.3% total number of examined fetuses respectively.

Appendicular skeleton malformations

The rate of delayed ossification of fore limb bones of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 54.2% and 25% total number of examined fetuses respectively.

The rate of delayed ossification of hind limb bones of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 62.5% and 14.3% total number of examined fetuses respectively.

The rate of delayed ossification and absence of the phalanges of maternally treated fetuses with 147 mg/kg Bw. and 73.5 mg/kg Bw. COC was about 79.2% and 17.9% total number of examined fetuses respectively. The results observed in table 4 and figure 4 and 5.

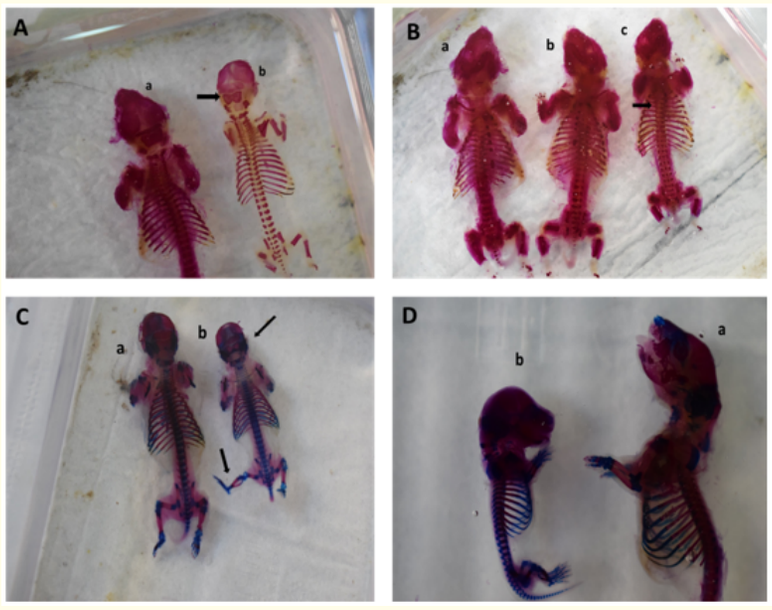


Figure 4: *Showing A: (a) control fetus and (b) maternally treated fetus with 1/10 of LD_{50} of COC from 6^{th} - 15^{th} days of pregnancy daily displayed delayed ossification of the supraoccipital and interparital bone plates, B: (a) control fetus and (b) maternally treated fetus with 1/20 of LD_{50} of COC from 6^{th} - 15^{th} days of pregnancy daily displayed delayed ossification of sternbrae and (c) maternally treated fetus with 1/10 of LD_{50} of COC from 6^{th} - 15^{th} days of pregnancy daily displayed sever delayed ossification and absence of sternbrae, vertebrae and phalanges of the hind limbs, C: (a) control fetus and (b) maternally treated fetus with 1/10 of LD_{50} of COC from 6^{th} - 15^{th} days of pregnancy daily displayed delayed ossification of skull bone plates (exoccipital, supraoccipital and interparital), malpositioned and twisted hind limb (hind limb) and D: (a) control fetus and (b) maternally treated fetus with 1/10 of LD_{50} of COC from 6^{th} - 15^{th} days of pregnancy daily displayed delayed ossification of the skull bone plates (forontal, parital, interparital and supraoccipital), fore limb bones, phalanges and vertebral column.*

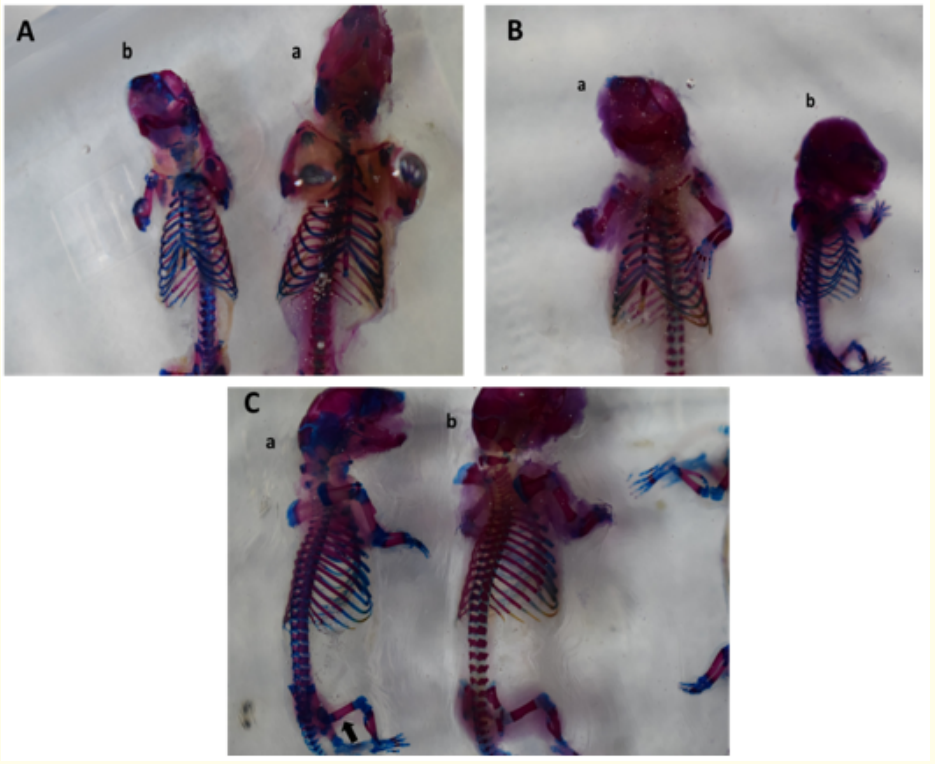


Figure 5: *Showing A: (a) control fetus and (b) maternally treated fetus with 1/20 of LD_{50} of COC from 6th - 15th days of pregnancy daily displayed delayed ossification of the sternbrae and lumber vertebrae, B: (a) control fetus and (b) maternally treated fetus with 1/10 of LD_{50} of COC from 6th - 15th days of pregnancy daily displayed complete delayed ossification of sternbrae and fore limb phalanges and C: (a) control fetus and (b) maternally treated fetus with 1/20 of LD_{50} of COC from 6th - 15th days of pregnancy daily displayed incomplete ossification of the fumer bone with delayed ossification of skull bone plates (exoccipital, supraoccipital and interparital).*

Groups	Total number of pregnant rats	Total number of fetuses	Total number of examined fetuses	Skeletal malformations								
				Axial skeleton malformations						Appendicular skeleton malformations		
				Craniofacial malformations		Rib cage and vertebral column malformations						
				Delayed ossification of the skull bone plates	Misshaped skull	Delayed ossification of sternbrae	Absence of sternbrae	Delayed ossification of vertebrae	Defect in the rib cage	Delayed ossification of fore limb bones	Delayed ossification of hind limb bones	Delayed ossification and absence of the phalanges
Control	8	62	31	1 (3.2%)	0	0	0	0	0	0	0	1 (3.2%)
1/20 LD_{50} of COC	8	47	24	18 (75%)	4 (16.7%)	11 (45.8%)	6 (25%)	10 (41.7%)	6 (25%)	13 (54.2%)	15 (62.5%)	19 (79.2%)
1/20 LD_{50} of COC	8	55	28	9 (32.1%)	1 (3.6%)	3 (10.7%)	2 (7.1%)	3 (10.7%)	4 (14.3%)	7 (25%)	4 (14.3%)	5 (17.9%)

Table 4*: Showed rate of skeletal malformations of maternally treated fetuses with the different doses of COC.*

Transmission electron microscope

The results showed that there was a clear morphological changes in the cellular structure and function of the fetal liver that considered as a hematopoietic organ in the fetal life in a dose dependent manner of maternally treated fetuses with different doses of COC (147 mg/kg Bw., 73.5 mg/kg Bw.) orally from 6th - 15th days of pregnancy daily and the changes were obvious in the higher dose groups in comparison to the control group.

Maternally treated fetuses with different doses of COC showed a significant increase in the hematopoietic stem cells in a dose dependent manner as monoblast and lymphoblast besides large number of abnormal shaped megakaryocytes that showed variable sized and shaped nuclei. The results observed in figure 6.

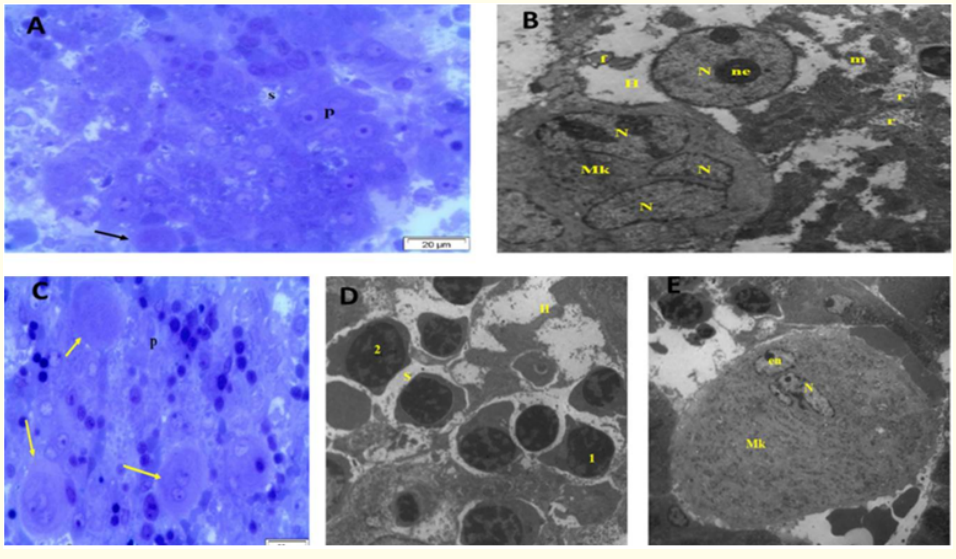


Figure 6: *Showing A: Light micrograph of liver of maternally controlled fetus showing the hepatic lobule formed by the hepatic cell plats with presence of blood sinusoid in between, the hepatocytes having large vesicular nucleus containing prominent nucleolus and faintly stained and vacuolated cytoplasm. The sinusoids (s) contain deeply stained blood cells and megakaryocytic cells (arrow). T.B. Stain. B: T.E. micrograph of hepatic tissue of maternally controlled fetus displaying presence of lymphoblast and megakaryocyte (Mk) containing abundant cytoplasm and multiple nucleus (N), the hepatic cells (H) having large vesicular nucleus (N) contain two nucleolus (ne) and their cytoplasm contain cell organelles such as free ribosomes (r) mitochondria (m) and fat globules (f). C: Light micrograph of hepatic tissue of maternally treated fetus with 1/10 of LD_{50} of COC from 6th - 15th days of pregnancy daily showing the hepatic cells (H) arranged in plates (p) or cords with presence of blood spaces or sinusoids (S) in between filled large numbers of hematopoietic stem cells as monoblast, lymphoblast and megakaryocytes (arrow), the hepatocytes having vesicular nucleus with appearance of the nucleolus in few cells and their cytoplasm mostly faintly stained or vacuolated. T.B. Stain. D: T.E. micrograph of hepatic tissue of maternally treated fetus with 1/10 of LD_{50} of COC from 6th - 15th days of pregnancy daily displaying the hepatic cells (H) and the sinusoids or blood spaces (S) filled with numerous numbers of hematopoietic stem cells mostly monoblast (1) and lymphoblast (2).*
E: T.E. micrograph of hepatic tissue of maternally treated fetus with 1/20 of LD_{50} of COC from 6th - 15th days of pregnancy daily displaying the hepatic sinusoids with large number of lymphoblast and monoblast besides megakaryocytes (MK) that contain numerous variable shape and size nuclei (N) with prominent electron dens nucleolus (en) and the cytoplasm contain small electron dens granules and few cell organelles. 15th days of pregnancy daily displayed incomplete ossification of the fumer bone with delayed ossification of skull bone plates (exoccipital, supraoccipital and interparital).

Discussion

The results showed that there was a significant decrease in the mean fetal body weight and crown-rump length in all treated groups with different doses of COC (147 and 73.5 mg/kg Bw.) in a dose dependent manner in respect to the control group.

The results also showed that there was a significant increase in embryonic death (resorption and still birth), external malformations (subcutaneous hemorrhage or hematoma, hydrocephalus, internal hemorrhage and anomalies of the limbs), visceral malformations (cerebral hemorrhage, dilatation of the ventricles, cleft palate, thickening in the myocardium, abnormal shaped and positioned kidneys besides defect in shape and lobulation of livers and lungs respectively) and skeletal malformations (axial and peripheral skeletal defects) in the treated groups with different doses of COC (147 and 73.5 mg/kg Bw.) in a dose dependent manner in respect to the control group.

Pesticides transported from the placenta to the fetuses with the fetal circulation and exert their teratogenic effect due to ill developed biotransformation and enzymatic activity, antioxidant system and presence of high rate of lipids that are susceptible to peroxidation by the toxic unchanged pesticides [21].

Pesticides can cross the placental barrier then enter to fetal circulation so exposure to pesticides specially the period of organogenesis beginning with the neurons development till hardening of the cleft palate (6 - 15 days) of pregnancy in rats that considered as a critical period in the fetal development with high risk of fetal malformations induction [22].

Such results agreed with [23] who carried a teratogenic study on hamster after exposure to 2.13 mg/kg BWt copper sulphate intravenously and results showed that a significant increase in the thoracic wall hernias, spina bifida, microphthalmia and encephaloceles malformations.

Copper considered as essential element and biologically active transition heavy metal that excessive exposure and accumulation of copper resulted in toxicity and even inherited anomalies as Wilson's disease, hemolytic anemia, basal ganglia degeneration and hepatic cirrhosis [24].

Teratogenicity of some pesticides may occur due to excessive ROS and highly reactive metabolites production via the cytochrome system together with poorly developed antioxidant defense system in embryo with oxidation of the cellular macromolecule as proteins, lipids and DNA forming DNA adducts with DNA fragmentation through Fenton reaction resulting in hydroxyl, ROS radicals, oxidative stress causing increase in the levels of SOD, malondialdehyde and glutathione-S-transferase especially in liver tissues [25].

Excessive ROS generation as hydroxyl radical, hydrogen peroxide and singlet oxygen can compromise the cellular lipids, DNA and proteins integrity [26].

Reduction in the maternal body weights after exposure to pesticides usually accompanied by decrease in fetal weights and lengths, Fluid retention in the fetal brains, delayed and incomplete ossification of the skeleton besides elevation of the resorptions rates either partial or complete and growth retardation also incomplete ossification may also occur due to effect of the pesticides on calcium metabolism with disruption in calcium hemostasis and bone morphometry [22].

Maternal toxicity leads to acute phase response induction and disturbances in the maternal metabolism with disruption of nutrients in the fetal circulation that usually supplied through the maternal side [27].

Most of the fetal malformations particularly the skeletal anomalies associated with delayed growth and development that occurred secondary to the maternal toxicity that considered as the sole nutrients, oxygen and electrolyte source with detoxification of the metabolic wastes and their elimination or primarily through the direct effect on the fetuses [28]. Other studies found that reduction in the fetal weight may be due to extracellular liquid resorption [29].

The decrease fetal numbers and embryonic death in treated groups may be because of the decrease of oval production and incomplete placental formation besides DNA damage either partial or complete and transcription inhibition in the highly active fetal cells respectively due to maternal toxicity, partial damage in the DNA resulted in malformations while complete damage resulted in embryonic death [30].

Visceral abnormalities as ventricular dilatation and abnormal shaped liver and kidney may be attributed to lack of the placental transfusion nutrients as amino acid, arginine and defective fetal metabolism [27].

Several hemorrhagic spots or subcutaneous hemorrhage had been observed and cause may be due to the platelet dysfunction that may occurred due to the oxidative stress in case of cu oxychloride and also be due to the hepatoxicity and liver injuries induced by pesticides with subsequent disturbances in the circulating coagulation factors and defect in the coagulation cascade [31].

Excess copper may result in significant reduction in the zinc level that is necessary in the bone growth and development and the whole ossification process resulted in Limb displacements and defective tail in rat fetuses [29,32].

The ultrastructural microscopical examination of embryonic liver that considered as hematopoietic organ in the fetal life revealed that was a clear morphological change in the cellular structure and function of maternally treated fetuses with different doses of COC (147 and 73.5 mg/kg Bw.) in a dose dependent manner in respect to the control group.

For maternally treated fetuses with different doses of COC there was a significant increase in the hematopoietic stem cells in a dose dependent manner as monoblast and lymphoblast besides large number of abnormal shaped megakaryocytes.

Hepatic hematopoiesis and megakaryocytosis occurred physiologically in rat fetuses from the 11th day of pregnancy and reach the peak at the 13th day then reduced again at the end of pregnancy but when liver showed a severe extramedullary hematopoiesis at the end of gestation this may indicate severe fetal anemia and that necessity increase in the hepatic erythroblastosis HSC congestion in hepatocytes and sinusoidal hypertension [33] also such results confirmed with increase in the rate of subcutaneous hemorrhage due to the platelet dysfunction produced from abnormal shaped megakaryocytes that may occurred due to the oxidative stress in case of cu oxychloride [31,34,35].

Conclusion

The current study revealed the potential teratogenicity of COC fungicide in a dose dependent manner in female albino rats besides a clear increase in the hematopoietic stem cells (HSC) proliferation occluding the liver blood spaces as lymphoblasts, monoblasts with dysfunction of the megakaryocytes that might indicate a severe fetal anemia confirmed through fetal subcutaneous hemorrhage and hematoma.

Ethical Committee

All procedures of this current study were agreed with the ethical guidelines of Faculty of Veterinary Medicine, Mansoura University.

Acknowledgment

To Dr. Amr Elbawady for his aid in obtaining of the fungicide acrobat copper from Central Agricultural Pesticide Laboratory, Ad Doki, Giza, Giza Governorate beside analysis of the fungicide in HPLC before usage to confirm the active principle level.

Conflict of Interest

All authors have no conflict of interest and there are no financial support.

Bibliography

1. Anwar WA. "Biomarkers of human exposure to pesticides". *Environmental Health Perspectives* 105.4 (1997): 801-806.
2. Naibur BE., *et al*. "Toxic Effects of Copper-Based and Synthetic Organic Pesticides on Activated Sludge". *CLEAN-Soil, Air, Water* 40.1 (2012): 39-44.
3. Remor AP., *et al*. "Occupational exposure of farm workers to pesticides: biochemical parameters and evaluation of genotoxicity". *Environment International* 35.2 (2009): 273-278.
4. Husak VV. "Copper and copper-containing pesticides: metabolism, toxicity and oxidative stress". *Journal of Vasyl Stefanyk Precarpathian National University* 2.1 (2015): 39-51.
5. Illing HPA. "Is working in greenhouses healthy? Evidence concerning the toxic risks that might affect greenhouse workers". *Occupational Medicine* 47.5 (1997): 281-293.
6. Sevcikova M., *et al*. "Biochemical, haematological and oxidative stress responses of common carp (Cyprinus carpio L.) after sub-chronic exposure to copper". *Veterinární Medicína* 61.1 (2016).: 35-50.
7. Babaei H., *et al*. "The effects of copper toxicity on histopathological and morphometrical changes of the rat testes". *Asian Pacific Journal of Tropical Biomedicine* 2.3 (2012): S1615-S1619.
8. Shepard TH and Lemire RL. "Catalog of teratogenic agents eleven edition (Volume 83)". Baltimore: Johns Hopkins University Press (2004).
9. Uriu-Adams JY., *et al*. "Influence of copper on early development: prenatal and postnatal considerations". *Biofactors* 36.2 (2010): 136-152.
10. Pagano G., *et al*. "Factors affecting R6 fungicide toxicity on sea urchin fertilization and early development: roles of exposure routes and mixture components". *Human and Experimental Toxicology* 20.8 (2001): 404-411.
11. Checiu I., *et al*. "Ultrastructural modifications induced by copper upon early chick embryos". *Annals of West University of Timisoara, Series of Biology* 5.6 (2003): 95-103.
12. Checiu I., *et al*. "Teratogenic effects of copper upon mice preimplantation embryos (in vitro and in vivo) experimental investigations". *Annals of West University of Timisoara, Series of Biology* 4 (2001): 58-69.
13. Ferm VH and Hanlon DP. "Toxicity of copper salts in hamster embryonic development". *Biology of Reproduction* 11.1 (1974): 97-101.
14. Checiu I., *et al*. "Teratogenic effects of copper upon early postimplantational mouse embryos-in vitro experimental investigation". *Annals of West University of Timisoara: Series of Biology* 11 (2008): 51-56.
15. Checiu M., *et al*. "The effect of acute maternal CuCl2 intoxication upon mouse fetal skeleton". *Mental* 81.10 (2004): 8-10.
16. Mahrosh U., *et al*. "Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (Salmo salar)". *Journal of Hazardous Materials* 280 (2014): 331-339.
17. Bruce RD. "An Up-and-Down Procedure for Acute Toxicity Testing". *Fundamental and Applied Toxicology* 5.1 (1985): 151-157.
18. Organisation for Economic Co-operation and Development OECD. "Acute Oral Toxicity (OECD Test Guideline 425) Statistical Programme (AOT 425 StatPgm)". Version: 1.0 (2001).

19. Hood RD. "Developmental and reproductive toxicology: a practical approach". CRC press (2016).

20. Bozzola J and Russell L. "Electron microscopy principles and techniques for biologists". Jones and Bartlitt publishers (1991).

21. Waliszewski SM., *et al*. "Carry-over of persistent organochlorine pesticides through placenta to fetus". *Salud Pública de México* 42.5 (2000): 384-390.

22. Gabr GA., *et al*. "Teratogenic Effects in rat foetuses subjected to the concurrent in utero exposure to emamectin benzoate insecticide". *Pakistan Journal of Biological Sciences* 18.7 (2015): 333-340.

23. Mason RW., *et al*. "Teratogenicity of combinations of sodium dichromate, sodium arsenate and copper sulphate in the rat". *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 93.2 (1989): 407-411.

24. Harris ZL and Gitlin JD. "Genetic and molecular basis for copper toxicity". *The American Journal of Clinical Nutrition* 63.5 (1996): 836S-841S.

25. Lushchak VI. "Environmentally induced oxidative stress in aquatic animals". *Aquatic Toxicology* 101.1 (2011): 13-30.

26. Rikans LE and Hornbrook KR. "Lipid peroxidation, antioxidant protection and aging". *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1362.2-3 (1997): 116-127.

27. Aboubakr M., *et al*. "Embryotoxic and teratogenic effects of norfloxacin in pregnant female albino rats". *Advances in Pharmacological Sciences* (2014): 924706.

28. Mink PJ., et al. "Potential effects of chlorpyrifos on fetal growth outcomes: implications for risk assessment". Journal of Toxicology and Environmental Health Part B 15.4 (2012): 281-316.

29. Shabbir A., *et al*. "Evaluation of developmental toxicity of guaifenesin using pregnant female rats". *Indian Journal of Pharmacology* 48.3 (2016): 264.-269.

30. Corbett JW., *et al*. "Inhibition of clinically relevant mutant variants of HIV-1 by quinazolinone non-nucleoside reverse transcriptase inhibitors". *Journal of Medicinal Chemistry* 43.10 (2000): 2019-2030.

31. Kopec AK and Luyendyk JP. "Coagulation in liver toxicity and disease: role of hepatocyte tissue factor". *Thrombosis Research* 133.1 (2014): S57-S59.

32. El-Hak HNG and Mobarak YM. "The ameliorative impacts of curcumin on copper oxychloride-induced hepatotoxicity in rats". *The Journal of Basic and Applied Zoology* 79.1 (2018): 44.

33. Lemos PVRB., *et al*. "Hepatic damage in newborns from female rats exposed to the pesticide derivative ethylenethiourea". *Acta Cirurgica Brasileira* 27.12 (2012): 897-904.

34. Ludwig S and Lavelle J. "Resuscitation-pediatric basic and advanced life support". Textbook of Pediatric Emergency Medicine. 6th edition. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams and Wilkins, (2010): 1-31.

35. Elalfy MM ., et al. "Mahmoud HA, Abomosallam M, Hamed MF and Sleem F. "Maternal Toxicity and Ultrastructural Changes of Copper Oxy-Chloride in Pregnant Female Albino Rats". *International Journal of Zoology and Animal Biology* (2019): 2-5.

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