



## BIOMEDICAL SCIENCES

# Maternal and developmental toxicity after exposure to formulation of chlorothalonil and thiophanate-methyl during organogenesis in rats

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**Abstract:** Chlorothalonil and thiophanate-methyl are fungicides widely used in agriculture. The aim of this study was to assess maternal toxicity and embryotoxic potential of exposure to chlorothalonil and thiophanate-methyl during organogenesis period in rats. Pregnant rats were divided into four groups: control and exposed to 400 (CT400), 800 (CT800) and 1200 mg<sup>-1</sup>kg bw<sup>-1</sup> day (CT1200) of commercial formulation constituted of 200 g of thiophanate-methyl kg<sup>-1</sup> and 500 g of chlorothalonil kg<sup>-1</sup> by gavage, from 6<sup>th</sup> to 15<sup>th</sup> gestational day. Maternal toxicity, liver, kidney and placenta histology, reproductive performance, and external, skeletal and visceral malformations of fetuses were evaluated. Maternal liver weight was decreased in CT1200 group and focal necrosis and microvesicular steatosis, inflammatory infiltrate and hepatocytes with pyknotic nucleus were observed in CT800 and CT1200 groups. Reproductive performance was similar among groups. The percentage of fetuses small for pregnancy age was increase in CT400 and CT800 groups. Moreover, incidence of skeletal anomalies was increased in the three groups exposed to fungicides. Chlorothalonil and thiophanate-methyl exposure showed affect the prenatal development and induce maternal toxicity.

**Key words:** developmental toxicity, embryo, fetus, malformations, pesticide, rat.

## INTRODUCTION

Increasing demands for food and consequently the need for increased crop productivity have led to widespread worldwide use of pesticides (Handford et al. 2015). The agricultural benefits generated by the use of these chemicals are usually accompanied by impacts to environmental, human and animal health, depending of exposure levels (Nasrala Neto et al. 2014, Furlan & Kreutzweiser 2015, Kim et al. 2017, Cuevas et al. 2018, Hyland et al. 2018).

Pre- and perinatal period are particularly susceptible to environmental xenobiotics exposure, especially to pesticides. Several studies suggest association between gestational

exposure to pesticides and embriotoxicity, low birth weight, congenital malformations, miscarriage, stillbirths (Chrisman et al. 2016, García et al. 2017, Toichuev et al. 2017, Yu et al. 2017), impairment in the neurodevelopment and behavior (Woskie et al. 2017, Laporte et al. 2018, Philippat et al. 2018) and increase of the risk of childhood leukemia (Hyland et al. 2018).

Most toxicity studies evaluate effects of the exposure of alone pesticides, but no in mixtures, as they are commonly used. Chlorothalonil and thiophanate-methyl are two active ingredients widely used alone or mixed in a commercial formulation containing 200 g of thiophanate-methyl kg<sup>-1</sup> and 500 g of chlorothalonil kg<sup>-1</sup> for

the elimination and prevention of anthracnose in various agricultural crops (Tomé et al. 2017).

Fungicide formulation constituted of the chlorothalonil and thiophanate-methyl is widely used in agriculture. However, studies about toxicity of the compounds in mixture are scarce and addressed to immune system or ecotoxicity (Weis et al. 2019, Tschoeke et al. 2019) but no to reproductive system.

Chlorothalonil (2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile) is a broad-spectrum, non-systemic, organochlorine fungicide, commonly used to control fungal foliar diseases in agriculture and horticulture (Van Scoy & Tjeerdema 2014). Moreover, this fungicide can be used as an active biocide applied in antifouling paints (Readman 2006). Its fungicide activity is attributed to the inactivation of cell sulfhydryl enzymes, such as NADPH oxidase (Sherrard et al. 2003). Moreover, it also causes depletion of glutathione, an endogenous antioxidant involved in the detoxification of xenobiotics in many organisms (Rosner et al. 1996, Meyer 2008). Experimental studies show that the exposure to 200, 400 or 600 mg<sup>-1</sup> kg<sup>-1</sup> day of chlorothalonil isolated during gestation days (GD) 1 to 6 or organogenesis (GD 6–15) can cause reproductive disorders, especially developmental toxicity (De Castro et al. 2001, Farag et al. 2006). Moreover, chlorothalonil was detected in maternal (97.1%) and cord (93.9%) serum in humans, indicating transfer of some portion of the maternal dose to the fetus (Barr et al. 2010).

Thiophanate-methyl [dimethyl ((1,2-phenylene) bis(iminocarbonthioyl)) bis(carbamate)] is a systemic benzimidazole fungicide affecting the cell division mechanism by inhibiting fungal DNA synthesis (Seiler 1975). This fungicide is applied to control diseases caused by ascomycota fungal in vegetables, at pre- and post-harvest (Ye et al. 2008). Toxicity of

this fungicide can be associated to its interaction with human serum albumin and generation of reactive oxygen species (Saquib et al. 2010). Thiophanate-methyl is metabolized into benzimidazole compounds, including the well-documented reproductive toxicant carbendazim (Douch 1973), and may act as weak endocrine disruptor (Maranghi et al. 2003).

Therefore, the aim of the present study was to assess maternal toxicity and evaluate the embryotoxic potential of chlorothalonil and thiophanate-methyl during organogenesis period in Wistar rats.

## MATERIALS AND METHODS

### Animals

Healthy male (75 days old, n = 10) and female (75 days old, n = 40) Wistar rats, supplied by the Central Vivarium of UNOESTE – Universidade do Oeste Paulista –, were housed in the Vivarium of Experimentation at the UNOESTE. During the experiment, animals were allocated into polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1 °C) and lighting conditions (12L, 12D photoperiod). Rat chow and filtered tap water were provided *ad libitum*. The experimental protocol was approved by the Ethics Committee for Use of Animals at the UNOESTE (Protocol # 2242-CEUA).

### Experimental design and exposure

Two female rats were paired to one male fertile rat in a separate cage over night from 5.00 pm to 8.00 am in the following day (Madu 2015). After the mating period, males and females were separated and vaginal smears were examined for the estrous phase and presence of spermatozoa to determine whether copulation had occurred. The first positive finding was defined as

gestational day zero (GD 0) and pregnant females were weighed and caged individually.

Pregnant rats were distributed into four experimental groups. Three groups of dams received Cerconil WP® (formulation constituted of 200 g of thiophanate-methyl  $\text{kg}^{-1}$  and 500 g of chlorothalonil  $\text{kg}^{-1}$ , Iharabras S.A. Chemical Industries, Brazil) at doses of 400 (CT400 group), 800 (CT800 group) or 1200 mg/kg bw/day (CT1200 group). Group four was the control, where dams received only the vehicle (saline solution 0.09%).

The fungicide was diluted and administered at a volume of 0.25mL/100 g of body weight. The choice of the exposure dose was based on studies of De Castro et al. (2001) and Ben Amara et al. (2014), considering median lethal dose ( $\text{LD}_{50}$ ) for rat is  $> 5,000 \text{ mg kg}^{-1} \text{ bw}$  (USEPA 2005), and  $> 10,000 \text{ mg kg}^{-1} \text{ bw}$  (USEPA 1999) for thiophanate-methyl and chlorothalonil, respectively.

The exposure schedule (Figure 1) involved oral administration (gavage) of the fungicides during organogenesis period which is from GD 6 to 15, characterized by an extremely rapid development and consequently vulnerable to malformations. According to Organisation of Economic Cooperation and Development (OECD)

guidelines 414 (OECD 2001), shortly before caesarean section, the pregnant rats exposed during organogenesis must be killed, for the uterine contents examination, and evaluation of the fetuses for soft tissue and skeletal changes.

### Maternal toxicity

Pregnant females were weighed every four days and had their daily intake estimated (in grams) and water (in milliliter). In addition, clinical signs of toxicity and mortality were observed (Christian 2001). On GD20, the females were anesthetized by sodium thiopental ( $100 \text{ mg kg}^{-1} \text{ bw}$ , ip.), killed and submitted to laparotomy.

Maternal liver, kidney, spleen, heart, and lung were collected, weighed and macroscopically inspected for evaluation of the integrity of external structure and presence of edema, necrosis and hemorrhagic foci ( $n = 8/\text{group}$ ). Placenta, liver and kidneys ( $n = 8/\text{group}$ ) were fixed in buffered formalin (10%). The pieces were embedded in paraffin wax and sectioned at  $5\mu\text{m}$  (three nonconsecutive cross-sections per animal). The sections were stained with hematoxylin and eosin (HE) and subjected to histochemical techniques (Periodic Acid Schiff

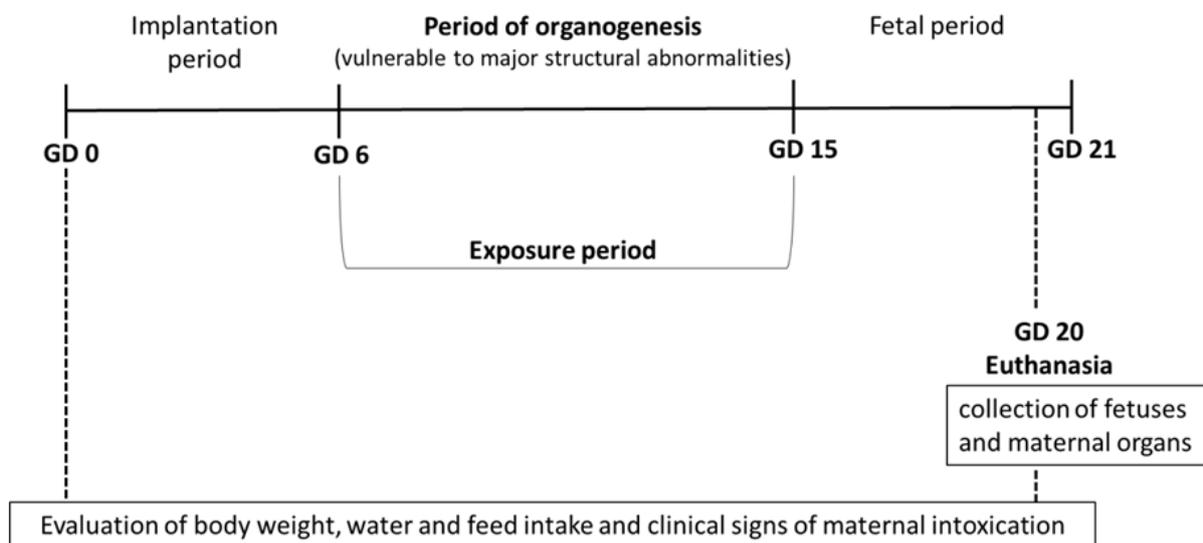


Figure 1. Experimental design. GD = gestational day.

– PAS for views of the inclusions of carbohydrate and Masson's trichrome to visualize the collagen fibers) and examined by light microscopy.

The presence of vacuolar degeneration in the tubules, fibrosis, inflammation, congestion, hemorrhage and vascular and glomerular changes were evaluated in the kidneys. For the liver, presence of inflammation, fibrosis, vacuolar degeneration of hepatocytes, necrosis of hepatocytes, hemorrhage, congestion, endothelial lesion and vascular alterations were considered.

Placental morphometry and identification of regions (labyrinth and spongiotrophoblast) were performed according to Lemos et al. (2014), using a graticulate containing 100 points. Photomicrographs of transverse sections from placenta (10 random fields of the labyrinth and spongiotrophoblast per animal) were captured by microscope (Leica DM2500, 400X magnification), coupled to the digital camera and a microcomputer containing the Leica Q-win software 3 for Windows™.

### **Reproductive performance**

Uterus and ovaries (control (n = 7) and exposed groups (n = 6)) were collected to determine the fertility potential (implantation sites/corpora lutea×100), uterus weight with fetuses, number of live fetuses, fetal and placental weight, sex ratio (number of male fetuses/number of female fetuses×100), and rates of pre-implantation (number of corpora lutea – number of implantations/number of corpora lutea×100) and post-implantations (number of implantations – number of live fetuses/number of implantations×100) loss.

### **Fetal and placenta analysis**

The mean fetal weight (g) of the control group was used for classification of fetus as adequate for pregnancy age (APA), small for pregnancy age

(SPA) or large for pregnancy age (LPA). Fetuses whose weights did not diverge more than + 1.7 standard deviations (SDs) from the control group mean were classified as APA. Those whose weights were at least 1.7 SDs greater than the control group mean were classified as LPA. Those whose weights were at least 1.7 SD lower than the control group mean were classified as SPA (Soulimane-Mokhtari et al. 2005).

The placental index was determined by the relation between the quotient of placental weight and fetal weight (in grams). The placental diameter, thickness and volume were obtained as described by Del Nero et al. (2002). Fetuses were examined for gross external malformations, with detailed analysis of the eyes, mouth, ear implantation, cranial conformation, fore and hindlimbs, integrity of the abdominal wall, tail and anal drilling.

One half the fetuses of each dam were fixed in Bodian fluid and serial sections were prepared for visceral examination (eyes, palate, brain and ear, thorax, abdomen and genitourinary tract), according to Wilson (1965).

The remaining fetuses were prepared for examination of the skeletons by method described by Staples & Schnell (1964). The fetuses were fixed in alcohol, carefully eviscerated, diaphanized with potassium hydroxide solution (1% KOH), stained with alizarin red S and stored in glycerin until the moment of analysis in stereomicroscope. In skeletal analysis, changes in position, shape, bone calcification and absence of bones were evaluated. Ossification points (sternabra, proximal and distal phalanges, metacarpals, metatarsals and caudal vertebrae) were observed and counted, according to the method proposed by Aliverti et al. (1979).

### **Statistical analysis**

For comparison of the maternal reproductive and fetal development parameters ANOVA

with *a posteriori* Tukey test or nonparametric Kruskal-Wallis test with *a posteriori* Dunn test were performed. Fisher's exact test was applied to compare the proportion data. A Kolmogorov-Smirnov test was applied to test for normal distributions before the statistical analyses. Differences were considered significant when  $p < 0.05$ .

## RESULTS

### Maternal toxicity

The control group did not show any clinical signs of toxicity. Only one rat from CT800 group showed piloerection, while another had change in pattern of ambulation. In the CT1200 group, one rat showed vaginal blood loss. These signs of toxicity were observed only during exposure. No remarkable changes in behavior were observed in dams from control or exposure groups. There was no significant difference ( $p > 0.05$ ) in the percentage of weight gain (Table I) and daily consumption of water and food (data not shown) of pregnant rats among experimental groups.

The weights of kidneys, heart, lung and spleen of the dams were similar among the experimental groups. However, the weight of the liver was decreased in the CT1200 group in comparison to the control group (Table I).

Liver histology is shown in Figure 2. Histological analysis of the liver of rats showed areas of focal necrosis, inflammatory infiltrate (Figure 2d) and presence of hepatocytes with pyknotic nucleus (condensed chromatin, highly eosinophilic), suggestive of apoptosis (Figure 2e) in CT800 and CT1200 groups. Furthermore, focal area with microvesicular steatosis was observed in the same groups (Figure 2c). The histochemical analysis by Masson trichrome stain indicated positive reaction of the same intensity around the portal triad among the experimental groups. However, in the groups exposed to fungicides was observed areas collagen deposition between hepatocytes (Figure 2g). Moreover, higher intensity staining positive PAS (Figure 2i) was observed in the three exposed groups.

The cortical and medullary region of the kidney was normal and showed similar

**Table I. Weight gain and weight of maternal organs of the rats from control and exposed to chlorothalonil and thiophanate-methyl groups.**

Parameter	Control	CT400	CT800	CT1200
<sup>1</sup> Weight gain (%)	54.80 (50.16 – 57.22)	44.69 (39.33 – 50.19)	31.31 (11.61 – 80.37)	22.90 (2.88 – 44.69)
<sup>2</sup> Liver (g)	14.48 ± 1.11a	12.38 ± 0.91ab	12.57 ± 2.80ab	11.54 ± 2.65b
<sup>2</sup> Kidney (right) (mg)	996.25 ± 102.81	863.33 ± 80.91	931.43 ± 100.40	942.50 ± 77.04
<sup>2</sup> Kidney (left) (mg)	945.00 ± 79.64	851.67 ± 86.12	928.57 ± 108.54	937.50 ± 89.08
<sup>2</sup> Heart (mg)	805.00 ± 105.02	748.33 ± 43.55	817.14 ± 54.07	770.00 ± 204.73
<sup>2</sup> Lung (g)	1.54 ± 0.17	1.55 ± 0.13	1.70 ± 0.34	1.66 ± 0.35
<sup>2</sup> Spleen (mg)	581.25 ± 63.79	540.00 ± 82.95	505.71 ± 81.82	515.00 ± 155.47

<sup>1</sup>Values expressed as median (Q1-Q3). <sup>2</sup>Values expressed as mean ± SD. ANOVA with *a posteriori* Tukey test. Different letters indicate statistically significant difference ( $p < 0.05$ ). Exposed groups to chlorothalonil and thiophanate-methyl at doses of 400 (CT400), 800 (CT800) or 1200 mg -1kg bw -1 day (CT1200).

morphology among the four experimental groups. The histochemical analysis of kidney indicated positive reaction of the same intensity among the experimental groups.

### Reproductive performance

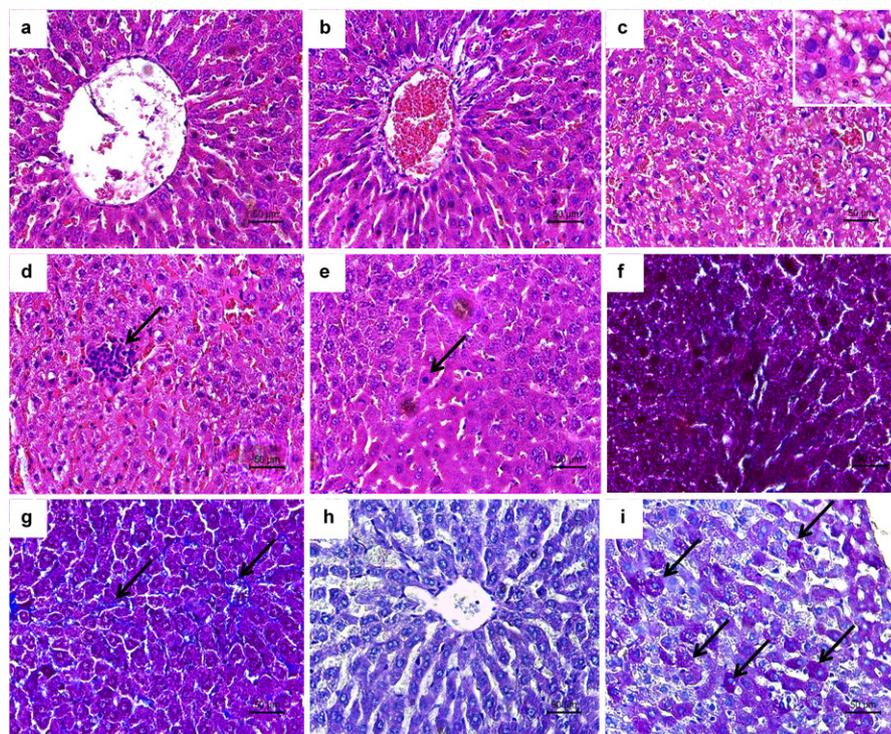
Uterus weight with fetuses, number of corpora lutea and implants, pre-implantation loss, fertility potential and number of live fetuses were similar ( $p > 0.05$ ) among experimental groups (Table II). Post-implantation loss rate was not significantly ( $p > 0.05$ ) affected by exposure to fungicides. However, some rats with great number of late pregnancy loss were observed in CT800 and CT1200 groups (Figure 3a).

### Fetal and placenta analysis

The mean of body weight of fetus was similar ( $p > 0.05$ ) among experimental groups. However, the percentage of fetuses with appropriate size for gestational age (APA) decreased ( $p < 0.05$ ) in CT400 and CT800 groups, when compared to control groups and CT1200. Consequently, the

percentage of small fetuses for gestational age (SPA) increased ( $p < 0.05$ ) in the CT400 group, when compared to the other experimental groups. Moreover, SPA was increased in CT800 group, when compared to the control group. The percentage of large fetuses for gestational age (LPA) was similar among the four groups (Table II). There was no statistically significant difference in the craniocaudal length, sex ratio and absolute and relative anogenital distances among experimental groups (Table II).

There was no significant difference in placental weight, index and volume (Table III). Histological analysis of the placenta revealed a normal morphological structure in the four experimental groups. The labyrinth area was characteristically larger than the spongiotrophoblast area in all groups (Figure 4a, d, g and j). However, the placental morphometry analysis indicated changes in proportion of the components from labyrinth and spongiotrophoblast layers (Table III). There was a decrease ( $p < 0.05$ ) of the wall of the



**Figure 2.** Liver histology. a, b, f and h) Control group. c, d, e, g and i) Groups exposed to chlorothalonil and thiophanate-methyl. c) Presence of focal area with microvesicular steatosis in CT1200 group. d) Presence of inflammatory infiltrate (arrow) in CT800 group. e) Presence of hepatocytes with pyknotic nucleus (condensed chromatin, highly eosinophilic), suggestive of apoptosis (arrow) in CT1200 group. g) Collagen deposition between hepatocytes (arrows) in CT800 group. i) Higher intensity of staining positive PAS (arrows) in CT400 group. a – e: H&E. f and g: Masson's trichrome. h and i: PAS.

**Table II. Reproductive performance and fetal parameters of the rats from control and exposed to chlorothalonil and thiophanate-methyl groups.**

Parameter	Control	CT400	CT800	CT1200
<sup>1</sup> Uterus weight+fetuses (g)	63.41 ± 9.33	63.62 ± 10.36	51.31 ± 12.92	47.54 ± 25.37
<sup>1</sup> Corpora lutea	14.00 ± 1.63	14.67 ± 2.42	13.2 ± 1.64	14.4 ± 2.61
<sup>1</sup> Implants	12.00 ± 1.53	13.33 ± 1.21	11.80 ± 1.48	11.00 ± 1.87
<sup>1</sup> Resorptions	0.14 ± 0.38	0.50 ± 0.84	1.60 ± 1.51	0.40 ± 0.89
<sup>1</sup> Live fetuses	11.86 ± 1.68	12.83 ± 1.17	10.20 ± 0.84	9.40 ± 4.04
<sup>2</sup> Fertility Potencial (%)	92.31 (85.12 – 92.82)	93.09 (92.44 – 93.33)	93.33 (91.67 – 100.00)	68.75 (66.67 – 92.86)
<sup>2</sup> Pre-implantation loss (%)	7.69 (7.18 – 14.88)	6.90 (6.67 – 7.55)	6.67 (0.00 – 8.33)	31.25 (7.14 – 33.33)
<sup>2</sup> Post-implantation loss (%)	0.00 (0.00 – 0.00)	0.00 (0.00 – 6.25)	10.00 (8.33 – 16.67)	0.00 (0.00 – 0.00)
<sup>1</sup> Fetus weight(g)	3.67 ± 0.14	3.35 ± 0.57	3.38 ± 0.77	3.00 ± 1.29
<sup>3</sup> AIP	74/83 (89.16)a	43/77 (55.84)b	32/51 (62.74)b	40/47 (85.11)a
<sup>3</sup> PIP	3/83 (3.61)a	24/77 (31.17)b	13/51 (25.49)c	6/47 (12.76)ac
<sup>3</sup> GIP	6/83 (7.23)	10/77 (12.99)	6/51 (11.76)	1/47 (2.13)
<sup>1</sup> Craniocaudal medium lenght (mm)	36.40 ± 2.16	34.09 ± 1.67	34.43 ± 3.72	31.22 ± 8.14
<sup>2</sup> Sex ratio (%)	100.00 (71.43 – 126.67)	74.11 (58.48 – 121.43)	100.00 (83.33 – 175.00)	100.00 (71.43 – 175.00)
<sup>1</sup> Absolute anogenital distance (mm)				
Male	3.71 ± 0.40	3.51 ± 0.23	3.81 ± 0.44	3.24 ± 0.99
Female	2.19 ± 0.40	2.19 ± 0.18	2.32 ± 0.31	1.93 ± 0.65
<sup>1</sup> Relative anogenital distance (mm/body weight <sup>1/3</sup> )				
Male	2.30 ± 0.25	2.34 ± 0.22	2.51 ± 0.15	3.20 ± 1.65
Female	1.43 ± 0.26	1.50 ± 0.21	1.60 ± 0.21	1.33 ± 0.38

<sup>1</sup>Values expressed as mean ± SD. ANOVA with *a posteriori* Tukey test. <sup>2</sup> Values expressed as median (Q1-Q3). Kruskal Wallis test with *a posteriori* Dunn test. <sup>3</sup>Values are number/total (%). Different letters indicate statistically significant difference (*p* < 0.05). Exposed groups to chlorothalonil and thiophanate-methyl at doses of 400 (CT400), 800 (CT800) or 1200 mg <sup>-1</sup>kg bw<sup>-1</sup> day (CT1200).

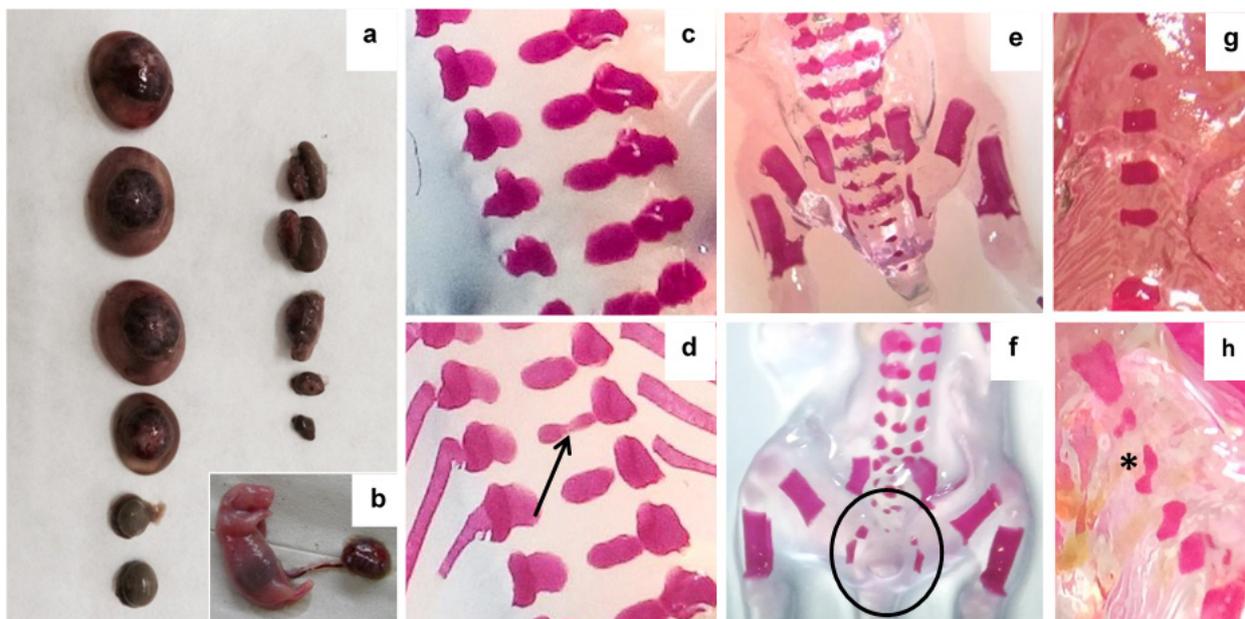
fetal vessels and maternal blood space and a consequential increase ( $p < 0.05$ ) in lumen of the fetal vessels in the labyrinth of the CT800 and CT1200 groups. In CT400 group, the wall of the fetal vessels and lumen of the fetal vessels were increased and decreased in comparison to control group, respectively.

In spongiotrophoblast, there was an increase ( $p < 0.05$ ) in maternal vascularisation and a decrease in mesenchyme in the CT800 and CT1200 groups. The proportion of trophoblast indifferiate cells was increased ( $p < 0.05$ ) in the three groups exposed to fungicides in comparison to control group (Table III).

No difference in positive PAS marking was observed in the different layers of the four experimental groups (Figure 4g and 4j). However, the cells of the syncytial trophoblast were richly stained (Figure 4h and 4l). Histochemical analysis by Masson's Trichrome indicated a

positive reaction of the same intensity between the experimental groups (Figure 4i and 4m).

There was no occurrence of gross external malformations and visceral anomalies in the experimental groups. The incidence of skeletal abnormalities was increased ( $p < 0.05$ ) in the exposed groups, when compared to the control group (Table IV and Figure 3c-h). Gestational exposure to fungicides increased ( $p < 0.05$ ) the incidence of fetuses with decreased sternal centers in CT400 and CT1200 groups, when compared to control and CT800 groups. An increased in the incidence of fetuses with xiphoid process absent and malformation of the supraoccipital was observed in CT400 and CT800 groups, respectively, when compared to the other experimental groups. In CT800 group, an increase of frequency of absence of caudal vertebrae was observed, when compared to the control and CT400 group. Other changes such as malformation of the xiphoid process,



**Figure 3. Fetal Changes.** a) Late gestational loss (post-implantation loss), represented by reabsorption points and late gestational loss with presence of placenta and dysmorphic fetus (Groups exposed to chlorothalonil and thiophanate-methyl). b) Normal fetus with its placenta (control group). Fetus from control group with normal vertebrae (c), caudal vertebrae (e) and sternebra (g). Fetus of the groups exposed to fungicides with bipartite vertebral centrum in CT400 (d), absence of caudal vertebrae in CT800 (f), and malformation of sternal centers (butterfly-shaped sternebra (\*) and decreased sternal centers (arrow)) in CT1200 (h).

**Table III. Placental parameters of the rats from control and exposed to chlorothalonil and thiophanate-methyl groups.**

Parameter	Control	CT400	CT800	CT1200
<sup>1</sup> Weight (g)	0.49 ± 0.04	0.41 ± 0.05	0.47 ± 0.13	0.39 ± 0.13
<sup>1</sup> Placental index	0.13 ± 0.01	0.30 ± 0.44	0.14 ± 0.02	0.14 ± 0.04
Labyrinth				
<sup>2</sup> Wall of the fetal vessels	10.00 (8.00 – 13.00)a	14.00 (11.00 – 17.00)b	8.00 (6.00 – 10.00)c	7.00 (5.00 – 12.00)c
<sup>2</sup> Lumen of the fetal vessels	61.00 (54.00 – 66.75)a	49.00 (40.00 – 53.25)b	64.00 (64.00 – 72.00) c	67.50 (61.75 – 74.25) c
<sup>2</sup> Maternal blood space	14.00 (11.00 – 18.75)a	17.00 (14.00 – 21.00)a	7.50 (5.00 – 11.00)b	6.50 (4.00 – 10.00)b
Spongiotrophoblast				
<sup>2</sup> Maternal vascularisation	15.00 (10.00 – 21.00)a	17.00 (12.00 – 22.25)a	24.00 (16.50 – 9.75)b	31.00 (23.00 – 42.00)b
<sup>2</sup> Trophoblast indifferiate cells	5.00 (4.00 – 7.00)a	9.00 (7.00 – 12.00)b	7.50 (5.25 – 10.00)b	8.00 (5.00 – 11.00)b
<sup>2</sup> Trophoblast intermediate cells	16.50 (12.25 – 19.75)	14.50 (12 – 17.25)	15.50 (11.00 – 20.00)	14.00 (12.00 – 19.25)
<sup>2</sup> Trophoblast giant cells	2.00 (1.00 – 4.00)	2.00 (1.00 – 4.00)	2.00 (1.25 – 3.00)	2.00 (1.00 – 3.00)
<sup>2</sup> Syncytial cells	0.00 (0.00 – 0.00) a	0.00 (0.00 – 0.00)a	0.00 (0.00 – 1.00)b	0.00 (0.00 – 1.00)b
<sup>2</sup> Mesenchyme	58.50 (53.25 – 65.00)a	55.00 (50.25 – 60.25)a	49.50 (43.00 – 55.00) b	41.50 (31.00 – 48.50) b

<sup>1</sup>Values expressed as mean ± SD. ANOVA with *a posteriori* Tukey test. <sup>2</sup>Values expressed as median (Q1-Q3). Kruskal Wallis test with *a posteriori* Dunn test. Different letters indicate statistically significant difference (p<0.05). Exposed groups to chlorothalonil and thiophanate-methyl at doses of 400 (CT400), 800 (CT800) or 1200 mg<sup>-1</sup>kg bw<sup>-1</sup> day (CT1200).

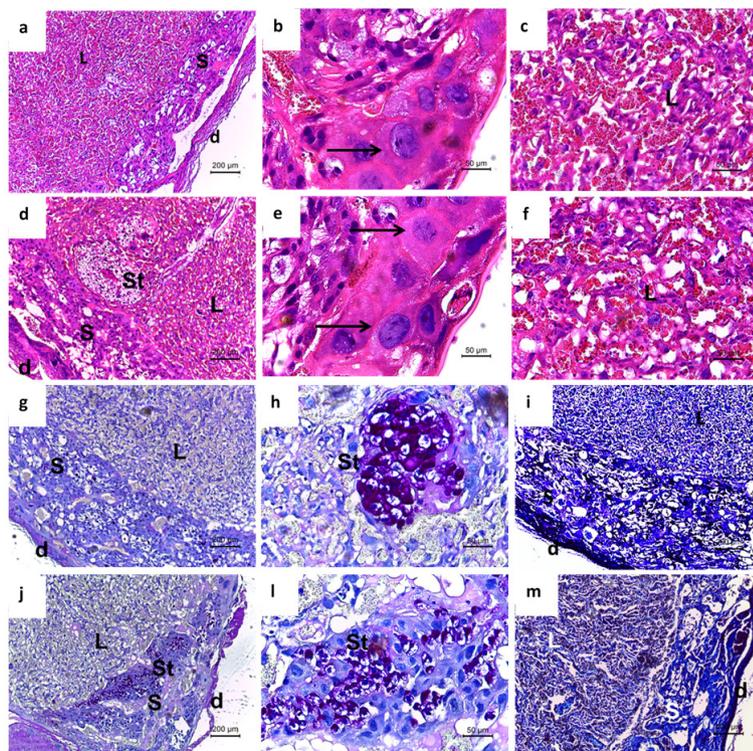
and basisphenoide, hamulus and manubrium, bipartite vertebral centrum and incomplete ossification of the skull were also identified, but there were no significant difference among the experimental groups (Table IV).

The number of phalanges of the right fore limb was decreased (p < 0.05) in the CT400 group, when compared to the other experimental groups. While the number of phalanges of the left fore limb was decreased (p < 0.05) in the CT400 group, when compared to control and

CT800 groups. The number of metatarsals was decreased (p < 0.05) in the CT800 group, when compared with the control group (Table IV).

## DISCUSSION

Despite the widespread use of the fungicide formulation consisting of the chlorothalonil and thiophanate-methyl in agriculture, the toxicity data of the compounds alone or in combination



**Figure 4. Placenta histology.** a – c and g – m) Control group. d – f and j – l) Groups exposed to chlorothalonil and thiophanate-methyl. L – labyrinth, S – spongiotrophoblast, d – decidua, St – syncytial trophoblast, giant trophoblastic cells (arrow). a – f: H&E. i – m: Masson's trichrome. g-h and j-l: PAS.

are scarce, especially about reproduction endpoints. This is the first study that correlates the exposure to formulation combined of these fungicides with maternal toxicity and prenatal development. Moreover, chlorothalonil and thiophanate-methyl had molecular weights of 265.91 and 342.4 g mol<sup>-1</sup>, respectively. Chemicals with molecular weight less than 600 may transmigrate from mother to fetus through the placenta (Mirkin 1973).

The absence of significant impair in maternal weight gain was consistent with live fetuses number. When considering as indicators of systemic maternal toxicity, the parameters of water consumption and ration, body weight, piloerection, deambulation, diarrhea and mortality, it is verified that the exposure to fungicides caused low general toxicity at the highest exposure doses. Since these effects caused were punctual and manifested in few animals. Nevertheless, moderate maternal toxicity was observed after fungicides exposure,

when parameters of hepatic toxicity were evaluated.

Study of Farag et al. (2006) reported signs of maternal toxicity (weakness and reduction in the activity) after exposure to 400 and 600 mg<sup>-1</sup> kg<sup>-1</sup>day of chlorothalonil during organogenesis (GD 6–15) in mice. Moreover, the authors observed maternal weight gain reduced, without changes in feed consumption. Traina et al. (1998) observed reduction of maternal weight gain and of daily food consumption after exposure pre-(GD 2-5) and peri-implantation (GD 6-9) to 650 mg<sup>-1</sup> kg<sup>-1</sup>day of thiophanate-methyl.

The liver is an important toxicological target, so several studies indicate hepatic impairment after exposure to pesticides (Paolini et al. 1999, Buono et al. 2007, Braeuning et al. 2018). In the present study, the maternal liver weight was reduced in the animals exposed to the highest dose, which corroborates the histopathological findings (i.e. focal necrosis, inflammatory infiltrate, presence of hepatocytes with picnotic

**Table IV. Frequency of fetal skeletal anomalies and points of ossification of the rats from control and exposed to chlorothalonil and thiophanate-methyl groups.**

Parameter	Control	CT400	CT800	CT1200
<sup>1</sup> Number of fetuses examined/ number of litter	41/5 (12.19%)a	38/25 (65.79%)b	25/16 (64.00%)b	21/13 (61.90%)b
Malformation of sternal centers	4 (9.76%)a	21 (55.26%)b	6 (24.00%)a	12 (57.14%)b
Xiphoid process absent	1 (2.44%)a	4 (10.53%)a	5 (20.00%)b	1 (4.76%)a
Malformation of the supraoccipital	0a	14 (36.84%)b	3 (12.00%)a	0a
Absence of caudal vertebrae	0a	0a	4 (16.00%)bc	0ac
Malformation of the xiphoid process	1 (2.44%)	6 (15.79%)	0a	2 (9.52%)a
Absence of hamulus	0	1 (2.63%)	1 (4.00%)	0
Bipartite vertebral centrum	0	1 (2.63%)	0	0
Absence of manubrium	0	0	3 (12.00%)	0
Malformation of the manubrium	0	0	1 (4.00%)	0
Incomplete ossification of the skull	0	0	1 (4.00%)	0
Four caudal vertebrae	0	0	2 (8.00%)	0
Malformation of the basisphenoide	0	0	0	1 (4.76%)
Points of ossification				
<sup>2</sup> Phalanges of the fore limb (right)	5.44 ± 0.95a	4.34 ± 1.46b	5.68 ± 0.75a	5.28 ± 0.96a
<sup>2</sup> Phalanges of the fore limb (left)	5.22 ± 1.13a	4.37 ± 1.50bc	5.64 ± 0.86a	5.28 ± 0.96ac
<sup>2</sup> Metatarsals (right)	4.00 ± 0.00a	3.97 ± 0.16ac	3.88 ± 0.33bc	4.00 ± 0.00ac
<sup>2</sup> Metatarsals (left)	4.00 ± 0.00a	3.97 ± 0.16ac	3.88 ± 0.33bc	4.00 ± 0.00ac
<sup>2</sup> Metacarpals (right)	3.71 ± 0.46	3.68 ± 0.47	3.76 ± 0.44	3.86 ± 0.36
<sup>2</sup> Metacarpals (left)	3.73 ± 0.45	3.66 ± 0.48	3.76 ± 0.44	3.86 ± 0.36
<sup>2</sup> Sternebrae	3.93 ± 0.34	3.74 ± 0.50	3.52 ± 0.92	3.76 ± 0.54
<sup>2</sup> Caudal vertebrae	3.15 ± 0.42a	2.63 ± 0.59bc	2.52 ± 1.19ac	2.86 ± 0.48ac

<sup>1</sup>Values expressed in number of fetuses with changes (percentage). Fisher exact test. <sup>2</sup>Values expressed as mean ± SD. ANOVA with *a posteriori* Tukey test. Different letters indicate statistically significant difference ( $p < 0.05$ ). Exposed groups to chlorothalonil and thiophanate-methyl at doses of 400 (CT400), 800 (CT800) or 1200 mg<sup>-1</sup>kg bw<sup>-1</sup> day (CT1200).

nucleus, suggestive of apoptosis, and area of microvesicular steatosis). In contrast, increase in the absolute weight of liver after exposure to chlorothalonil ( $600 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}$ ) was observed by Farag et al. (2006).

Some studies (Paolini et al. 1999, Buono et al. 2007) indicate that thiophanate-methyl may lead to hepatic morphological alterations, glycogen depletion and hepatocellular apoptosis. In addition, thiophanate-methyl may change hepatic metabolism of substances administered concomitantly, which may interfere on the toxicity caused by the commercial formulation. It has been reported that chlorothalonil can cause increased lipid peroxidation and oxidative damage in DNA of liver cell (Lodovici et al. 1997, Suzuki et al. 1997).

Despite the absence of impact on the weight and histology of the kidneys observed in this study, Farag et al. (2006) observed increase in the absolute weight of this organ after exposure to  $600 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}$  of chlorothalonil (GD 6–15) in mice. In addition, Wilkinson & Killen (1996) reported that chronic exposure of rodents to chlorothalonil can cause nephrotoxicity and renal tubular hyperplasia.

The absence of significant difference in the number of corpora lutea among the experimental groups suggests that the maternal hormonal environment was adequate for the beginning of the gestational process. Even after exposure to fungicides, there was no significant disturbance of this process, which led to the successful implantation, indicated by the absence of significant changes in the number of implants and resorptions and in the rate of pre-implantation loss. In spite post-implantation loss rate to be not significantly affected by exposure to fungicides, some dams (CT800 and CT1200 groups) had late gestational loss of all fetuses implanted. This result demonstrated relevant impact on embryo-fetal development.

Absence of changes in resorption rate and pre-implantation loss was observed after exposure to thiophanate-methyl at GD 6-9 (Traina et al. 1998). In the other hand, study of Farag et al. (2006) showed increase in dead fetuses and early resorptions after exposure to chlorothalonil alone.

Maternal exposure did not alter the reproductive performance of rats. The CT800 group had a lower number of live fetuses than the other experimental groups, but with no significant statistical difference. The same occurred with the fertility potential in the CT1200 group.

There was no impact of the exposure on the mean weight and craniocaudal length of the fetuses, corroborating absence of impact on length of the embryo (GD12) after exposure peri- (GD 2-5) and peri-implantation (GD 6-9) to thiophanate-methyl (Traina et al. 1998). However, when considering the percentages of classification of fetuses in adequate, small or large for gestational age, there is a significant difference among experimental groups in this study. This demonstrates that the mean fetal weight alone may not be the best indicative of intrauterine growth restriction, as reported by Sinzato et al. (2012). The observed difference between mean fetal weight and weight adequacy at gestational age may be related to the difference in size between male and female fetuses and the very variability of litter size.

In the present study, there was no change in placental weight, index and volume, which could generally be indicative of absence of blood flow compromise between the uterus and the placenta (Chahoud et al. 1999, Lang et al. 2003). However, the more specific analysis of the morphometry of the layers and constituents of the placenta indicated an impact on the placental vascularization.

The placental labyrinth is the site of oxygen and nutrient exchange between the mother and the fetus. In the present study, there was a decrease of the wall of the fetal vessels and maternal blood space in labyrinth area in the groups exposed to two higher doses of fungicides. Study of Guo et al. (2019) showed reduction in blood sinusoid area in the labyrinth layer associated to growth restriction in fetuses whose mothers were exposed to pesticide fenvalerate (20 mg kg<sup>-1</sup>) at the late gestational stage (GD13 - 17). In current study, the significant ( $p < 0.05$ ) impact on fetal growth was observed in CT800 group (SPA – 25.49%), but not in CT1200 (SPA – 12.76%) in comparison to control group (3.61%). Despite this, we observed a relevant numeric difference between CT1200 and control group.

In CT400 group, despite the increase of wall of the fetal vessels in labyrinth layer, the frequency of fetus SPA (31.17%) was increased. The causes of intrauterine growth restriction are multiple (i.e., maternal, fetal, placental and environmental factors) and involving complex mechanisms, which difficult the understanding of this pathophysiology (Sankaran & Kyle 2009).

The increase in maternal vascularisation and trophoblastic indifferentiate cells in spongiotrophoblast of CT800 and CT1200 groups may be indicative of placental compensatory mechanism. This is important for maintenance of the basic functionalities of the placenta and mainly for dilution of toxic molecules (pesticide) at the cellular level (Levario-Carrillo et al. 2004).

Although the organogenesis (6th to 15th gestational day in rats) represents the window of exposure to teratogens the most susceptible to appearance of morphological alterations (Dencker & Eriksson 1998), no external and visceral malformations were observed in the exposed groups. However, there was a significant increase in the total incidence of fetuses

with skeletal abnormalities after exposure to the fungicides. The end of the organogenesis period corresponds to the development of the ossification process in rats (Fritz & Hess 1970). As this is an important indicator of fetal maturity, this alteration can be considered a relevant impact to prenatal development.

Reduced ossification in fetuses may be correlated to change in calcitonin level and in calcium metabolism or lower levels of calcium and magnesium ion (El Ghareeb et al. 2015). The fungicide thiophanate-methyl can induce bone resorption and reduced serum calcium and phosphorus in rats (USEPA 2009). Thus, the decrease of these minerals, constituents in bone development, might have led to impact to the ossification process.

## CONCLUSION

The experimental exposure to the combination of fungicides methyl thiophanate and chlorothalonil caused changes in embryo-fetal development in the three doses studied, especially on the ossification process. Despite the lack of impact on reproductive performance, fungicides caused moderate toxicity to maternal general health in the two highest doses. Thus, future studies that evaluate the reproductive and developmental toxicity of these agrochemicals at levels of real exposure, in women farmer and population that live near agricultural land are necessary and important.

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