Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v38i1.29079

Maternal and fetal toxicity of Wistar rats exposed to herbicide metolachlor

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ABSTRACT. Metolachlor is a selective pre-emergent herbicide widely used in agriculture to control weeds. The aim of this study was to evaluate the possible effects of metolachlor on reproductive performance of adult rats, as well as its teratogenic potential when administered during the period of organogenesis. Pregnant adult female rats were allocated into 4 experimental groups (n = 10 group^{-1}), that received 0 (control); 150 (TA); 300 (TB); or 1000 mg kg^{-1} bw day^{-1} (TC) of metolachlor, by gavage, from the 6th to 15th gestational day (GD). There is reduction in the weight gain of the animals from TB and TC groups compared to the control group. Liver and placenta weights were reduced in TB and TC groups, respectively, while the percentage of post-implantation loss was increased in the TC group. There were no external malformations in either rat of the control or treated groups. However, an increased incidence of skeletal anomalies and visceral anomalies (especially in the urogenital system) was observed in TC group. These results demonstrate that exposure of pregnant rats to metolachlor can lead to signs of general toxicity, late embryonic losses and congenital anomalies.

Keywords: pesticide, reproduction, teratogenicity, fetus.

Toxicidade materna e fetal de ratas Wistar expostas ao herbicida metolacloro

RESUMO. O metolacloro é um herbicida seletivo de pré-emergência, amplamente usado na agricultura para controlar ervas daninhas. O objetivo deste estudo foi avaliar os possíveis efeitos do metolacloro sobre o desempenho reprodutivo de ratas adultas, bem como o seu potencial teratogênico quando administrado durante o período da organogênese. Ratas adultas prenhes foram divididas em quatro grupos experimentais (n = 10 grupo^{-1}), que receberam 0 (controle); 150 (TA); 300 (TB); ou 1.000 mg kg^{-1} dia^{-1} (TC) do metolacloro, via gavagem, do sexto ao 15º dia de gestação (DG). Foi observada redução no ganho de peso dos animais dos grupos TB e TC em comparação ao grupo controle. Os pesos do fígado e placenta foram reduzidos nos grupos TB e TC, respectivamente, enquanto que a percentagem de perda pós-implantação foi aumentada no grupo TC. Não foram observadas malformações externas nos ratos dos grupos controle e tratados. No entanto, foi observado aumento da incidência de anomalias esqueléticas e anomalias viscerais (especialmente no sistema urogenital) no grupo TC. Estes resultados demonstram que a exposição de ratas prenhes ao metolacloro pode levar a sinais de toxicidade geral, perdas embrionárias tardias e anomalias congênitas.

Palavras-chave: praguicida, reprodução, teratogênese, feto.

Introduction

Several studies suggest association between environmental contamination and impact on human and animal health. Pesticides, plasticizers, disinfection by-products, heavy metals and persistent organic pollutants have been related to carcinogenesis, chronic disease, endocrine disruption and congenital anomalies/malformations (Bull et al., 2011; Li & Ko, 2012; Nieuwenhuijsen, Dadvand, Grellier, Martinez, & Vrijheid, 2013; Magliano, Loh, Harding, Botton, & Shaw, 2014; Vested, Giwercman, Bonde, & Toft, 2014). The pesticides stand out in this scenario, considering their wide use in agriculture and consequential exposure of the general public and farm workers (Thulstrup & Bonde, 2006). Metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(methooxyprop-2-yl) acetamide) is a selective pre-emergent herbicide, widely used to control weeds in corn, cotton, beans, sugarcane, soybeans and ornamental plants (U.S. EPA, 1995).

A monitoring study of groundwater in the United States (1993-2011) showed that metolachlor was one of the most frequently detected pesticides (Toccalino, Gilliom, Lindsey, & Rupert, 2014). A

study of Papadakis et al. (2015) detected metolachlor in high concentrations in 29% of the samples collected in rivers and lakes of Greece.

The general population can be widely exposed to metolacholor via consumption of contaminated food or drinking water (U.S. EPA, 1995). However, farmer exposure occurs in higher doses. Studies show that occupational exposure to pesticide can increase risk for reproductive impairment, e.g. infertility, embryonic and/or fetal death, and congenital malformations (Carmichael et al., 2014; Melgarejo et al., 2015).

Metolachlor is metabolized to metolachlor mercapturic acid conjugates and excreted in the urine (Driskell & Hill, 1997). Its metabolite was detected in a large proportion of urine samples from farm families (Curwin, Hein, Barr, & Striley, 2010) and pre-season applicators (Hines et al., 2003), and associated with reproductive injuries, especially in semen quality (Swan et al., 2003).

There are few studies about reproductive toxicology of metolachlor using model animals. A study of Greenlee, Elli and Berg (2004) showed that *in vitro* incubation of embryos with metolachlor alone or in combination with other pesticides increases pre-implantation embryonic loss in mice, with consequent reduction in blastocyst development rates. This study related that the injury can occur at low concentrations, similar to occupational exposure and/or via drinking contaminated water. Impairment in embryonic development from chicken, frog *Xenopus laevis* and Pacific oyster also has been related (Osano, Admiraal, & Otieno, 2002; Mai et al., 2014; Várnagy et al., 2003). However, *in vivo* studies about the fetal development effects of metolachlor in a mammalian model have not been found until the moment.

The aim of this study was to evaluate the effects of the herbicide metolachlor on reproductive performance of adult rats and its teratogenic potential (skeletal and visceral development) when administered during the period of organogenesis.

Material and Methods

Animals

Male (75 days old, n = 10) and female (75 days old, n = 37) Wistar rats, supplied by the Central Vivarium of Unoeste, Universidade do Oeste Paulista, São Paulo State, were housed in the Vivarium of Experimentation at the Unoeste. During the experiment, animals were allocated into polypropylene cages (43 x 30 x 15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1ºC) ad 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 # 1595-CEUA).

Experimental design and treatment

Female rats were mated to male rats by placing 2 females in a cage with 1 male and breeding was conducted in the dark period of the cycle. After the mating period, males and females were separated and vaginal smears were examined for the 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 (n = 10 each group) were distributed into four experimental groups. Three groups of dams received Metolachlor (commercial formulation of Dual Gold; Syngenta AG; São Paulo, Brazil), via oral (gavage) at doses of 150 (TA), 300 (TB) or 1000 mg kg^{-1} bw day^{-1} (TC), from GD 6 to 15. Group 4 was the control, where dams received only the vehicle (deionized water) following the same procedure. Pregnant females were weighed three times per week and had their daily food (in grams) and water (in milliliters) intake estimated.

Reproductive performance and fetus analysis

On GD20, the females were anesthetized by sodium thiopental (i.p.), humanely killed and submitted to laparotomy for collection of uterus and ovaries to determine the fertility potential (implantation sites/corpora lutea x 100), uterus weight with fetuses, number of live fetuses, fetal and placental weight, sex ratio (number of male fetuses/number of female fetuses x 100), and rates of pre-implantation (number of corpora lutea - number of implantations/number of corpora lutea x 100) and post-implantation (number of implantations - number of live fetuses/number of implantations x 100) loss.

The fetal weights were classified according to Soulimane-Mokhtari et al. (2005). 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. Half the fetuses of each dam were fixed in Bodian fluid and serial sections were prepared, according to Wilson (1965) for visceral examination. The remaining fetuses were prepared for examination of the skeletons by the staining procedure described by Staples and Schnell (1964).

Histological analysis

Maternal liver, kidneys, spleen, heart and lungs were collected, macroscopically inspected and weighed. Placenta, liver and kidneys were fixed in buffered formalin (10%). The pieces were embedded in paraffin wax and sectioned at 5μm. The sections were stained with hematoxylin and eosin (HE) and examined by light microscopy.

Statistical analysis

For comparison of the maternal reproductive and fetal development parameters Anova test with *a poteriori* Dunnett test or nonparametric Kruskal-Wallis test with *a posteriori* Dunn test was performed, according to the characteristics of each variable. Fisher's exact test was applied to compare the proportion data. For all the tests, the limit of significance established was $p < 0.05$, using InStat 3.0 software.

Results

The rats from TB and TC groups gained less ($p < 0.05$) body weight than the control rats over the course of the gestation (Figure 1A). The body weight of the TC group was significantly lower ($p < 0.05$) than control group on GD 12 and GD 20 (final body weight), but not in GD 16 ($p = 0.07$) (Figure 1B).

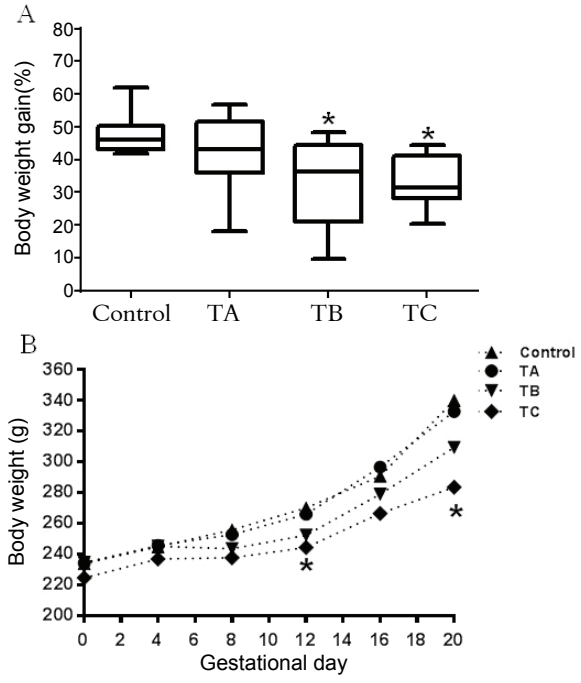

Figure 1. Maternal body weight of rats from control and treated with 150 (TA), 300 (TB) and 1000 mg kg^{-1} bw day^{-1} (TC) of metolachlor groups. A) Body weight gain (%). B) Body weight evolution during gestation (g). In A, values expressed as median (Q1-Q3). Kruskal Wallis test with *a posteriori* Dunn test. In B, values expressed as mean ± S.E.M. Anova with *a posteriori* Dunnett test. *$p < 0.05$, statistically significant difference compared to control group.

The daily water consumption was similar among experimental groups throughout the gestational period and feed intake was significantly reduced ($p < 0.05$) only in the TC group, on GD 10 (data not shown). During the treatment period, clinical signs of toxicity of metolachlor in the groups treated with different doses of the herbicide were not observed.

The maternal weights (absolute and relative) of the kidneys, heart, lungs and spleen were unaffected by metolachlor treatments. However, the absolute and relative weights of the liver were decreased ($p < 0.05$) in rats from TB group in comparison to the control rats. The normal tissue structure of the kidneys and liver was maintained after metolachlor exposure in different doses. Histology analysis showed in the placental disc the presence of the labyrinth area, spongiotrophoblast and trophoblast giant cells in the four experimental groups. Moreover, the labyrinth area was higher than the spongiotrophoblast area. Migration of the trophoblast giant cells in the spongiotrophoblast layer was observed in the metolachlor-treated groups.

Uterus weight with fetuses, number of corpora lutea and implants, pre-implantation loss and number of live fetuses were similar ($p > 0.05$) among experimental groups (Table 1). The total number of resorptions from control and treated groups was not significantly different, however in the TC group there is a tendency of increase ($p = 0.05$) in comparison to the control group. Despite the augmented post-implantation loss ($p < 0.05$) in the rats from the TC group, the fertility potential was unaffected by herbicide exposure (Table 1).

The mean fetal body weight of the rats from treated groups was similar to the control group. However, the percentage of large fetuses for pregnancy age was increased ($p < 0.05$) in the TA group, and consequently the percentage of small fetuses for pregnancy age was decreased ($p < 0.05$) (Table 1). There were no differences in the placental index and sex ratio between treated and control groups, but the placental weight was decreased ($p < 0.05$) in the TC group (Table 1). There were no external malformations in either the control or treated groups. The incidences of skeletal anomalies observed are shown in Table 2. The gestational exposure to metolachlor increased ($p < 0.05$) the incidence of fetuses with absent xiphoid process (TA group), incomplete ossification of sternebra (TB and TC groups), increased distance between hamulus and basisphenoid, absent sternebrae and incomplete ossification of xiphoid process (TC group) (Table 2).

Table 1. Reproductive performance and fetal parameters of the rats from control and treated with 150 (TA), 300 (TB) and 1000mg kg^{-1} bw day^{-1} (TC) of metolachlor groups.

Parameters	Control	TA	TB	TC
[1]Uterus weight+fetuses (g)	69.11 ± 3.47	60.69 ± 6.90	62.25 ± 5.55	54.22 ± 2.90
[1]Corpora lutea	15.12 ± 0.66	15.90 ± 0.83	16.33 ± 0.88	15.44 ± 0.60
[1]Implants	13.87 ± 0.66	13.10 ± 1.01	13.88 ± 0.71	13.88 ± 0.69
[1]Resorptions	0.37 ± 0.8	1.10 ± 0.58	1.11 ± 0.99	2.00 ± 0.60
[1]Live fetuses	13.62 ± 0.75	12.00 ± 1.35	12.77 ± 1.10	11.88 ± 0.63
[2]Fertility potencial (%)	93.0 (87.84-100.00)	87.08 (85.17-92.18)	87.50 (80.00-93.92)	92.85 (86.66-100.00)
[2]Pre-implantation loss (%)	6.69 (0.00-12.15)	12.91 (7.81-14.82)	12.50 (8.33- 20.00)	7.14 (0.00-13.33)
[1]Fetal weight (g)	3.3 ± 0.12	3.50 ± 0.10	3.31 ± 0.05	3.18 ± 0.10
[3]APA fetuses	119/132 (90.15%)	111/119 (93.27%)	115/126 (91.26%)	97/107 (90.65%)
[3]LPA fetuses	0/132 (0%)	8/119 (6.72%)⋆	0/126 (0%)	1/107 (0.93%)
[3]SPA fetuses	13/132 (9.84%)	0/119 (0%)⋆	11/126 (8.73%)	9/107 (8.41%)
[1]Placental weight(g)	0.47 ± 0.02	0.44 ± 0.01	0.44 ± 0.00	0.39 ± 0.01⋆
[1]Placental index	0.14 ± 0.00	0.12 ± 0.00	0.13 ± 0.00	0.12 ± 0.00
[2]Sex ratio (%)	117.14 (81.25-168.75)	108.33 (63.54-136.25)	100.00 (100.00-183.33)	140.00 (75.00-180.00)

APA = Adequate for pregnancy age, LPA = large for pregnancy age, SPA = small for pregnancy age. [1]Values expressed as mean ± S.E.M. Anova with *a posteriori* Dunnett test. [2]Values expressed as median (Q1-Q3). Kruskal Wallis test with *a posteriori* Dunn test. [3]Values are number/total (percent). Fisher's exact test. ⋆$p < 0.05$, statistically significant difference compared to control group.

Table 2. Frequency of fetal skeletal and visceral anomalies of the rats from control and treated with 150 (TA), 300 (TB) and 1000mg kg^{-1} bw day^{-1} (TC) of metolachlor groups.

Parameter	Control	TA	TB	TC
Number of fetuses examined/ number of litter	45/9	47/10	38/9	47/9
Perforated basisphenoid	2 (4.50)	2 (4.25)	1 (2.63)	0
Incomplete ossification of hamulus	2 (4.50)	0	0	0
Absent hamulus	2 (4.50)	0	1 (2.63)	0
Incomplete ossification of interparietal	1 (2.20)	0	0	0
Increased distance between hamulus and basisphenoid	8 (17.8)	13 (27.60)	9 (23.68)	22 (46.8)⋆
Incomplete ossification of sternebra	6 (13.33)	2 (4.25)	26 (68.42)⋆	11 (23.40)⋆
Absent sternebra	18 (40.00)	16 (34.04)	13 (34.21)	34 (72.34)⋆
Bipartite 2nd sternebra	1 (2.2)	0	0	0
Slender manubrium sterni	5 (11.10)	3 (6.38)	2 (5.26)	5 (10.60)
Slender xiphoid process	2 (4.50)	0	0	0
Absent xiphoid process	0	6 (12.76)⋆	1 (2.65)	1 (2.12)
Incomplete ossification of xiphoid process	13 (28.90)	19 (40.4)	9 (23.70)	28 (59.57)⋆
Thickening of the xiphoid process	0	0	1 (2.63)	4 (8.50)
Detached rib	2 (4.5)	2 (4.25)	4 (10.5)	6 (12.76)
Number of fetuses examined/ number of litter	56/9	58/10	53/9	53/9
Total number of fetus with visceral anomalies	0	3 (5.17)	4 (7.54)	10 (18.86)⋆
Testis agenesis	0	1 (1.72)	0	2 (3.77)
Ectopic testis	0	1 (1.72)	0	1 (1.88)
Distended bladder	0	1 (1.72)	4 (7.54)	2 (3.77)
Bladder agenesis	0	0	0	1 (1.88)
Kidney agenesis	0	0	0	1 (1.88)
Ectopic kidney	0	0	0	1 (1.88)
Ureter agenesis	0	0	0	1 (1.88)
Hypoplastic heart	0	0	0	1 (1.88)

Values are number/total (percent). Fisher's exact test. ⋆$p < 0.05$, statistically significant difference compared to control group.

The total number of fetuses with visceral anomalies in the TC group was higher ($p < 0.05$) than control group. There was an increase of these anomalies in the TB group, but it was not statistically significant in comparison to the control group ($p = 0.052$). Most of the visceral anomalies observed were related to the urinary and reproductive systems (Table 2). These anomalies were similar ($p > 0.05$) among the control and treated groups, when considered individually.

Discussion

Despite the extensive use of metolachlor in agriculture, data of its toxicity are scarce. Results from this study showed that exposure to the herbicide during organogenesis period caused impairment in fetal development and maternal toxicity, mainly evidenced by decrease in body weight gain.

Decrease in body weight gain was observed in dams from TB and TC groups. In spite of the alteration in body weight, the food and water consumption was no affected. The maternal LOEL previously reported after oral exposure to metolachlor in rabbits (GD6-18) was 360 mg kg^{-1} bw day^{-1}, based on reduced body weight gain, lacrimation and miosis (U.S. EPA, 1995). In the present study with Wistar rats, clinical signs of toxicity were not observed, but body weight gain, evidence of maternal toxicity, was observed from 300mg kg^{-1} bw day^{-1}. These results contrast studies with Sprague-Dawley and CD rats (oral exposure from GD6-15), which did not observe maternal

toxicity at this dose (NOEL was greater than 360 and 300 mg kg^{-1} bw day^{-1}, respectively) (U.S. EPA, 1995). In both reports, the LOEL was 1000 mg kg^{-1} bw day^{-1}, based on deaths, salivation, lacrimation, convulsions, reduced body weight gain and food consumption.

The reduction in body weight gain of rats from groups exposed to 300 and 1000 mg kg^{-1} bw day^{-1} and the absent significant difference in number of live fetuses and mean fetal weight indicate that in Wistar rats the LOEL was 300 mg kg^{-1} bw day^{-1} and NOEL was 150 mg kg^{-1} bw day^{-1} (maternal toxicity).

Several studies indicate maternal toxicity, with body weight gain reduced by pesticide exposure (Akhtar, Srivastava, & Raizada, 2006; Farag, Karkour, & Okazy, 2006; Kawamura, Kato, Nakaoka, & Fantel, 2014; Sitarek, 2001). A study of Matsumoto, Fujii, Hirose and Ema (2010) observed maternal toxicity after exposure to the pesticide 2-sec-butyl-4,6-dinitrophenol (dinoseb) at doses of 6.52 or 8.50 mg kg^{-1} bw day^{-1}. This toxicity was evidenced by significantly decreased body weight gain, as in this present study. Moreover, the authors observed reduced food consumption during the exposure period (6 to 15th gestational day). Lower body weight gain and decreased food consumption were also observed after gestational exposure to the herbicide triclopyr (100 and 300 mg kg^{-1} bw day^{-1}, exposure during organogenesis) (Carney, Billington, & Barlow, 2007).

Although increase in liver and kidney weights are indicative of toxicity, in the present study a decrease ($p < 0.05$) in absolute and relative weights of liver in the TB group was observed. A study with human liver (HepG2) cells suggests that metolachlor inhibits cell proliferation, affecting progression through the S phase of the cell cycle and entrance into the G2 phase (Lowry, Greiner, Fretheim, Ubben, & Dhanwada, 2013).

Steatosis has been related to exposure to organophosphorus pesticides (Baconi, Bârcă, Manda, Ciobanu, & Bălălău, 2013; Lasram et al., 2014). A study of Al-Eryani et al. (2015) identified 123 chemicals associated with fatty liver. Pesticides and their intermediates corresponded to 44% of these chemicals, and herbicides and fungicides were more frequently associated to this hepatic injury. However, the hepatic structure of the metolachlor-treated groups was similar to control.

In spite of the reduced placental weight in the TC group, the placenta index was not affected by herbicide exposure. Thus, lesions associated with decrease placental weight such as mitotic inhibition, apoptosis and degeneration of the trophoblastic cells were not found. Nevertheless, migration of giant cells into spongiotrophoblast layer was observed in the metolachlor-treated groups. A study of Levario-Carrillo et al. (2004) showed that the organophosphate pesticide methyl parathion caused several histology injuries, degeneration of the trophoblastic giant cells, vascular congestion in the labyrinth, fibrosis and hemorrhage in the decidua.

The toxicity of the embryo-fetal development was more evident in the higher dose of metolachlor. In this group, a trend of increased resorption sites ($p = 0.05$), with consequential increase in post-implantation loss rate was observed. Nevertheless, the maternal exposure did not alter the number of live fetuses, mean of fetal weight or sex ratio evaluated at gestational day 20.

In CD rats, the LOEL for developmental toxicity is 1000 mg kg^{-1} bw day^{-1} (via gavage on gestation days 6-15), based on reduced mean fetal body weight, reduced number of implantations, decreased litter size and slight increase in resorptions resulting in an increase in post-implantation loss (U.S. EPA, 1995).

There were not gross external malformations in fetuses sired by dams exposed to metolachlor. However, there was an increase in the incidence of fetuses with skeletal and visceral anomalies following exposure to herbicide.

Ossification of the skeleton is considered an important indicator of maturity and in rats starts around the 15-16th GD (Fritz & Hess, 1970). The majority of the skeletal anomalies observed in metolachlor-exposed fetuses consisted of absent xiphoid process (150 mg kg^{-1} bw day^{-1}), incomplete ossification of sternebra (300 and 1000 mg kg^{-1} bw day^{-1}), absent sternebra, incomplete ossification of xiphoid process and increased distance between hamulus and basisphenoid (1000 mg kg^{-1} bw day^{-1}).

Despite the fact that variations in the ossification of the sternum of rats are common, about 95 to 100% of the sternebra is ossified at birth (Fritz & Hess, 1970). In the current study, high percentages of fetuses from the TC group (1000 mg kg^{-1} bw day^{-1}) had delay and/or anomalies in ossification (72.34% for absent sternebra and 23.40% for incomplete ossification of sternebra). In rat fetuses, at one day before birth, the incomplete ossification may range from 1-7% for sternebrae 1-4 and 6, and up to 35% for sternebra 5 (Fritz & Hess, 1970). However, the high rate of absent of sternebra in the TC group indicates impairment in the fetal ossification after exposure to metolachlor. Beyond the skeletal changes, metolachlor also led to an increase of visceral anomalies in the TC group, especially in the urinary and reproductive systems. There was a slight increase of these anomalies in the

TB group, but it was not statistically significant in comparison to the control group ($p = 0.052$).

Studies with other species also showed developmental toxicity caused by metolachlor and its metabolites. Studies of Mai et al. (2012; 2013) indicated that exposure of Pacific oyster (*Crassostrea gigas*) to environmentally relevant concentrations of metolachlor may induce spermiotoxicity and embryotoxicity, caused by DNA damage and changes in expression of several genes, especially in the early life stages.

Chicken development also may be affected by metolachlor (Dual Gold 960EC) alone or in combination with cadmium sulphate. After injection into the air-chamber (day zero of incubation), embryo mortality increased and development disorders such as oedema, shortening of the beak mandible, incorrect posture of the feet and the neck were observed (Szabó, Budai, Lehel, & Kormos, 2011).

Teratogenesis and maternal toxicity were observed in the TC group. Nevertheless this does not imply that the former arises as a secondary consequence (Iyer, Gammon, Gee, & Pfeifer, 1999). In the TB group, significant increase in body weight gain also was observed, but the development was slightly affected.

Conclusion

Metolachlor given orally to rats on GD6-15, was embyotoxic and teratogenic at a dose of 1000 mg kg^{-1} bw day^{-1} and maternally toxic at doses equal to and higher than 300 mg kg^{-1} bw day^{-1}. It is of note that toxicity was found at doses that are much higher than those to which humans are usually exposed (acceptable daily intake is 0.15 mg kg^{-1}) (U.S. EPA, 1991). Moreover, occupational exposure to metolachlor, particularly for applicators and farmers, may be substantially higher. These results indicate the importance of identifying the exposure levels in the workplace, especially for women of reproductive age.

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Received on September 2, 2015.
Accepted on February 17, 2016.