



## Maternal and fetal effects after inhalation of the herbicide flumetralin

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**ABSTRACT.** Farm workers at Brazilian tobacco plantations are frequently exposed to toxic chemicals and eventually became contaminated with these products. Flumetralin is a inhibitor of axillary bud growth on tobacco and the effects of gestational exposure should be investigated since many pesticides cross the placental barrier and cause birth defects. The aim of this study was to investigate maternal and fetal effects caused by inhalation of Flumetralin. Pregnant Swiss mice inhaled Flumetralin for 10 or 20 minutes on the seventh day of pregnancy. On the 18<sup>th</sup> day, animals were euthanized and subjected to laparotomy for removal of the uterus and embryos. The uterus was weighed and the embryos were examined. Fetuses of both treatments showed visceral changes in the uterus, kidney and liver. Skeletal abnormalities included hydrocephalus and incomplete skull ossification in both groups. In addition, the treatment of 20 minutes exposure caused anomalies in the occipital bone and the 13<sup>rd</sup> rib besides internal bleeding. There was reduction in maternal weight gain and impaired intrauterine development of the fetus. The weight of heart, liver, kidneys and testicles of fetuses were significantly decreased. Inhalation of flumetralin proved to be potentially teratogenic in both treatments, with greater damage in the group treated for 20 minutes.

**Keywords:** flumetralin, reproductive performance, birth defects, pregnancy, mice.

## Efeitos maternos e fetais após inalação do herbicida flumetralin

**RESUMO.** Trabalhadores agrícolas nas plantações de tabaco no Brasil são frequentemente expostos a produtos químicos tóxicos e, eventualmente, são contaminados com esses produtos. O Flumetralin é um inibidor de crescimento de gemas axilares em tabaco e os efeitos de sua exposição no decorrer da gestação devem ser investigados, uma vez que muitos agrotóxicos atravessam a barreira placentária e podem causar prejuízo ao desenvolvimento embrionário. O objetivo deste estudo foi investigar os possíveis efeitos maternos e fetais da inalação de Flumetralin. Camundongos Swiss prenhes inalaram Flumetralin por 10 ou 20 minutos no sétimo dia de gestação. No 18<sup>o</sup> dia, os animais foram eutanasiados e submetidos a laparotomia para coleta do útero e dos embriões. O útero foi pesado e os embriões foram avaliados. Fetos de camundongos em ambos os tratamentos apresentaram alterações viscerais no útero, rins e fígado. Com relação a anomalias esqueléticas, ambos mostraram hidrocefalia e ossificação incompleta do crânio. Além destes, o tratamento por 20 minutos de exposição apresentou alteração no osso occipital, na 13<sup>o</sup> costela e hemorragia interna. Houve diminuição no ganho de peso materno e diminuição do desenvolvimento intrauterino dos fetos. O peso do coração, fígado, rins e testículos dos fetos foram estatisticamente diminuídos. A inalação de Flumetralina mostrou ser potencialmente teratogênica em ambos os tratamentos, com danos maiores no tratado por 20 minutos.

**Palavras-chave:** flumetralin, performance reprodutiva, malformações, prenhez, camundongos.

### Introduction

The use of pesticides in agriculture has been currently widely discussed for various reasons, including the contamination of the rural worker, who handles the product and is exposed and may suffer the consequences (AL-GUBORY, 2014). The main health problems reported occur in the skin, eyes and mucous tissue (DAS et al., 2001). One likely route of exposure to contamination of the population living in rural areas is inhalation, since

many pesticides are sprayed on different crops, including tobacco. Inhalation of toxic substances throughout the gestational period has been indicated by some studies as a factor that increases the chance of birth defects, including central nervous system abnormalities (SINHA et al., 2004) and limb defects (LU; KENNEDY, 1986).

Flumetralin, N-(2-chloro-6-fluorobenzyl)-N-ethyl- $\alpha$ - $\alpha$ -trifluoro-2,6-dinitro-p-toluidine, an anti-budding agent, is applied topically on tobacco

crops to suppress axillary bud growth. It is the active component of the class of herbicides known as growth-regulators. Chemically, it is a dinitroaniline derivative. It is highly persistent in environments, and is classified as a toxicological class I (extremely toxic) compound (ALVARES et al., 2010).

In the Brazilian tobacco plantations, during the preparation and spraying of herbicidal suspensions, farm workers were exposed to toxic chemicals and eventually became contaminated with high levels of these products. Unfortunately, there are few studies on the Flumetralin toxicity and none of them assesses the induction of anomalies in embryo-fetal development. Flumetralin residues were found in tobacco leaves one year after the application of the pesticide (JOHNSON; CONNELL, 2001) being suspected to cause endocrine disruption, developmental abnormalities and genetic disorders in humans (KOCAMAN; BUCAK, 2015; DEARFIELD et al., 1999). Those facts make evident the risks imposed to a rural population.

The possible mechanisms by which environmental pollutants exert negative impact on the development involve pathways including disruption of hormonal signaling systems and epigenetic gene regulation, as well as induction of oxidative stress due to generation of reactive oxygen species – ROS (AL-GUBORY, 2014)

In the current investigation, the toxic and teratogenic effects of Flumetralin were evaluated in mice fetuses. The results of this research will contribute to understanding the need for safe use of Flumetralin in the cultivation of tobacco.

## Material and methods

### Animals

Male and female Swiss mice (*Mus musculus*), weighing on average 30g, were obtained from the Central Animal House, State University of Londrina. Animals were housed in polypropylene cages with wood shavings as bedding, kept under controlled temperature (22°C), 12:12 hour light:dark cycle and free access to food and tap water. Pregnancy was identified by the presence of a vaginal plug, and the day of its identification was designated as day 0. All experiments were performed in accordance with the NIH Principles of Laboratory Animal Care.

### Exposure group

Eighty pregnant females were divided into four groups of twenty animals each, two experimental and two control groups. The treated animals were

whole body exposed to vaporized Flumetralin in saline (0.9% NaCl), at 1.25 ppm, the same as that sprayed on tobacco crops, using glass chamber of 20Lt. We performed whole-body exposure because, in Brazil, many growers do not use personal protective equipment (OLIVEIRA-SILVA et al., 2001). One experimental group was exposed to the herbicide for 10 min. and the other for 20 min., on the seventh day of pregnancy, 2.5 days after implantation of the blastocyst, a critical moment in the post-implantation development, at which the placenta is not yet mature (ROSSANT; CROSS, 2001). Animals were exposed only once, considering manufacturer instructions (ALVARES et al., 2010). The exposure time was set according to growers, who reported that the application of Flumetralin takes 10 to 20 minutes. The control groups inhaled the saline and were otherwise treated identically to the Flumetralin-treated groups.

### Maternal and fetal evaluation

Pregnant females were weighed on the first and eighteenth experimental days of treatment and the weight variation within this period was calculated. Animals were euthanized on the 18<sup>th</sup> day of pregnancy and immediately subjected to laparotomy, to record dead and live fetuses, gravid uterus weight and the implantation sites, using the Salewski method (SALEWSKI, 1964). The percentage of post-implantation loss (number of implantations minus the number of live fetuses x 100 / number of implantation) and the rate of fetal viability (number of fetuses x 100 / number of implantations) were calculated. All fetuses were weighed, examined for macroscopic external malformations and measured for crown-rump dimensions. Half of the fetuses was fixed in Bodian's solution and dissected for subsequent visceral examination according to the Wilson method (WILSON, 1965). The other half of the litter was stained with Alizarin red by the technique of Staples and Schnell for skeletal examination (STAPLES; SCHNELL, 1964; KIM et al., 2004). Data are presented as the number of fetuses per litter which presented the variation or abnormality.

### Statistical analysis

The normal distribution of data was analyzed by the Student's t-test and the qualitative data rate by Fisher test using the GraphPad Prism program. The significant level was 5%.

This study was approved by the Ethics Committee for Animal Research of the State University of Londrina under number 10036.2012.10.

## Results and discussion

No maternal deaths were induced by Flumetralin. The 20-minute treatment, there was a significant difference in maternal weight gain compared to control group. There was no difference in fetal viability and post-implantation loss. The reproductive performance of mice treated with Flumetralin is summarized in Table 1.

Individual weight and length of fetuses developing in mice exposed to Flumetralin for 20 min. were significantly lower than those in the mice of either the control group or the group exposed for 10 minute. Data from external, visceral and skeletal examination are presented in Table 2.

The external abnormalities observed included limb malformation, micrognathia, gastroschisis and open eyes, although with no significant difference between the groups.

Morphological changes in internal organs were found in fetuses of mice treated with Flumetralin for 10 and 20 minutes. Fetuses showed alterations, with significant difference, in uterus, kidneys, and liver in individuals treated with Flumetralin for 10 and 20 minutes. These abnormalities were: slightly tilted uterus, kidneys of abnormal size and shape and livers with lobes altered in size and shape. Also,

adhesion of internal organs and internal hemorrhage in the thorax, abdomen, brain and neck were verified in fetuses of dams exposed for 20 minutes.

Skeletal examination showed skull malformations in fetuses of the 20 min. treatment group; among these, the number with deformed occipital bone was statistically significant compared to the control group.

A statistically significant increased proportion of a short 13<sup>th</sup> rib and dumbbell-shaped sternebra was registered in fetuses of the 20 min. exposure group. Enlargement of the fontanelles in the skull was considered here as hydrocephalus and it invariably occurred together with incomplete skull ossification in fetuses of the treatment groups. Both variables showed significant differences between treated and control groups, since no anomaly was detected in either of the control groups.

Mice fetuses treated with Flumetralin for 20 min. possessed significantly fewer ossification centers in hindlimb phalanges and caudal vertebra. On the other hand, no such differences were seen in the metacarpals, forelimb phalanges, metatarsals or sternebra.

Similar effects were observed in the mean weight of heart, liver, testicles and kidneys, whose values were significantly lower in the 20 min. treated group as shown in Table 3.

**Table 1.** Effect of inhalation of flumetralin on the reproductive performance of mice.

	Control		Treatment	
	10	20	10	20
Exposure time (min.)	10	20	10	20
Number of dams	20	20	20	20
Maternal weight gain (g)	27.2 ± 3.6	28.6 ± 2.4	23.5 ± 2.8	22.7 ± 2.6 <sup>ab</sup>
Gravid uterus weight (g)	19.7 ± 3.0	20.2 ± 2.2	19.3 ± 1.8	19.8 ± 2.8
Number of implantations	15.4 ± 1.4	14.8 ± 1.0	15.0 ± 2.0	13.7 ± 1.6
Number of live fetuses	11.6 ± 0.8	11.3 ± 1.8	11.3 ± 1.2	10.8 ± 1.6
Fetal viability (%)	77.3 ± 12.2	75.8 ± 10.6	77.0 ± 7.2	75.6 ± 10.8
Post-implantation loss (%)	28.6 ± 10.4	28.5 ± 11.2	28.2 ± 11.0	28.3 ± 7.2
Individual fetal weight (g)	1.49 ± 0.08	1.49 ± 0.04	1.50 ± 0.08	0.94 ± 0.06 <sup>ab</sup>
Litter crown-rump length (cm)	2.55 ± 0.14	2.68 ± 0.18	2.64 ± 0.04	2.34 ± 0.24 <sup>ab</sup>

Data are presented as means ± SEM; quantitative comparison of the treatment groups in different exposure times and their respective control groups using Student's t-test. Legend \*p < 0.5, \*\*p < 0.01 and \*\*\*p < 0.001; a = control 10 minute, b = control 20 minute, c = treated 10 minute, d = treated 20 minute.

**Table 2.** External, visceral and skeletal abnormalities observed in fetuses of mice treated with Flumetralin

	Control		Treatment	
	10	20	10	20
Exposure (minutes)	10	20	10	20
External abnormalities (%)				
Limb malformations	0	5.31	6.67	7.06
Micrognathia	2.04	4.25	4.44	4.71
Gastroschisis	0	4.25	2.22	7.06
Visceral abnormalities (%)				
Internal hemorrhage	3.06	5.31	7.78	18.82 <sup>b**</sup>
Alterations in uterus	3.06	4.25	18.88 <sup>a**</sup>	20.00 <sup>b**</sup>
Alterations in heart	2.04	0	5.55	3.53
Alterations in kidney	0	5.31	21.11 <sup>a**</sup>	22.35 <sup>b**</sup>
Alterations in liver	6.12	6.38	31.11 <sup>a**</sup>	35.29 <sup>b**</sup>
Skeletal abnormalities (%)				
Changes in the occipital bone	0	0	2.22	12.94 <sup>b*</sup>
Short 13 <sup>th</sup> rib	0	0	5.55	29.8 <sup>b**</sup>
Hydrocephalus	0	0	21.11 <sup>a**</sup>	29.41 <sup>b**</sup>
Incomplete ossification of the skull	0	0	20.00 <sup>a**</sup>	27.06 <sup>b**</sup>

Data presented as absolute and as percentages, quantitative comparison of the treatment groups in different exposure times and their respective control groups using Student's t-test. Legend \*p < 0.5, \*\*p < 0.01 and \*\*\*p < 0.001; a = control 10, b = control 20, c = 10 treated, d = 20. Data are presented as means ± SEM;

**Table 3.** Effect of inhalation of flumetralin on the viscera of fetuses.

	Control		Treatment	
	Exposure Time (min)			
	10	20	10	20
Fetuses examined (N)	98	94	90	85
Heart/Fetal Weight (mg)	7.60 ± 0.79	7.52 ± 0.76	7.40 ± 0.85	6.3 ± 1.12b**
Liver/Fetal Weight (mg)	50.2 ± 2.18	52.0 ± 4.10	49.45 ± 3.19	44.5 ± 4.3b*
Kidney/Fetal Weight (mg)	5.44 ± 0.25	6.72 ± 0.45	5.10 ± 1.25	4.36 ± 0.66b**
Testicle/Fetal Weight (mg)	0.83 ± 0.19	1.07 ± 0.12	0.96 ± 0.11	0.75 ± 0.065b**

Data are reported as means ± SEM; quantitative comparison of the treatment groups in different exposure times and their respective control groups using Student's t-test. Legend \*p < 0.5, \*\*p < 0.01 and \*\*\*p < 0.001; a = control 10 minute, b = control 20 minute, c = treated 10 minute, d = treated 20 minute.

In this study, the maternal effects and teratogenic potential of Flumetralin were demonstrated, by administration to pregnant mice. In this type of research, it is important to disassociate effects on the fetus from toxic effects on the mother, as the latter can cause abnormal embryonic development (MANSON; KANG, 1994).

Our results indicated that pregnant mice exposed to Flumetralin by inhalation on the 7<sup>th</sup> day of gestation showed no deaths during the experimental period. Moreover, the percentage of post-implantation loss was unaffected by Flumetralin, under the concentration and exposure times tested. The results showed that Flumetralin inhaled for 20 min. reduced maternal weight gain, in agreement to previous findings for Trifluralin treated rats and rabbits (BYRD et al., 1995). Trifluralin is another herbicide used in tobacco plantation. There are no previous reports of toxic effects of Flumetralin on embryonic development of mice.

The retarded development was revealed by the diminished weight and length of fetuses in dams exposed to Flumetralin for 20 minutes. This reduction in weight corroborates previous studies (SCHARDEIN, 2000; BYRD et al., 1990). Mice that inhaled Flumetralin for 20 min. also exhibited reduced mean weight of fetal heart, liver, testicles and kidneys, compared to the control group, which shows that these organs were especially affected by exposure to Flumetralin in the uterus. The liver is responsible for a wide range of metabolic tasks, among them the processing of xenobiotics, so that it is highly sensitive to contaminants present in the environment. The kidneys play a key role in the excretion of extraneous substances (GUYTON; HALL, 2002).

External abnormalities were detected in fetuses of the experimental animals exposed for 10 and 20 min., including deformed limbs, micrognathia, gastroschisis and open eyes. Gastroschisis is a congenital defect of the abdominal wall, not involving the umbilical cord, that allows the protrusion of abdominal viscera outside the body. It results from the incomplete closure of the lateral folds of the body of the embryo. Exposure to

chemicals may be implicated in the etiology of this anomaly (MOORE; PERSAUD, 2004).

The 10 and 20 min. exposure to Flumetralin caused a rise in the incidence of several different morphological abnormalities in the viscera of the fetuses. Generally, uteri, kidneys and liver exhibited alterations in both size and shape.

The diverse abnormalities observed in fetuses were probably the result of teratogenic action of Flumetralin. It is now known that most chemical agents, if they possess physicochemical properties that allow them to pass through membranes, can readily cross the placenta by simple diffusion down a gradient. Each toxic agent thus has a critical dose, beyond which the toxic effect on embryo-fetal development begins to appear (NISHIMURA; TANIMURA, 1976).

Fetuses with hemorrhages were verified in individuals treated for 20 min. with Flumetralin. Possibly the hemorrhaging was a consequence of the abnormal functioning of the affected organs, involving the main blood vessels.

Skeletal analysis showed that, in mice treated with the herbicide for 10 and 20 min., there was a significant number of fetuses with hydrocephalus and, concomitantly, incomplete ossification of the skull; in those exposed for 20 min., there was a significant proportion of fetuses with malformed occipital bones, shorter 13<sup>th</sup> ribs, dumbbell-shaped sternebra and reduced number of ossification centers in hindlimb phalanges and caudal vertebra.

The increased proportion of fetuses showing signs of incomplete ossification, along with reduced fetal weight and length, suggest a correlation between morphological change and retarded growth. This indicates inhibition of embryofetal development caused by Flumetralin, similar to those found in previous research (EMA et al., 2004). The occurrence of fetuses with short 13<sup>th</sup> ribs, hydrocephalus and dumbbell-shaped sternebra could be a sign of teratogenicity of the substance under study.

## Conclusion

Flumetralin inhalation presents teratogenic potential in the experimental model proposed. The

prejudicial effects on mice fetuses cannot be ignored when considering human health. In particular, pregnant tobacco-growers should be considered as a high-risk group.

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