MORPHOMETRIC EVALUATION OF THE SPERMATOGENIC PROCESS OF ADULTS WISTAR RATS EXPOSED TO THE 2,4DICLOROPHENOXIACETIC ACID ASSOCIATED TO PICLORAM (TORDON 2,4-D® 64/240 BR)

[Avaliação morfométrica do processo espermatogênico de ratos Wistar adultos expostos ao ácido 2,4diclorofenoxiacético associado ao picloram (TORDON 2,4-D® 64/240 BR)]

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ABSTRACT - Thirty adult male wistar rats were used in five groups. Two groups received oral treatment with aqueous solution of TORDON 2,4-D® 64/240 BR in different concentrations; group O1: 2 ml.L^{-1} (n = 6); group O2: 4 ml.L^{-1} (n = 6). Two groups received topical treatment in the dorsal cervical area, the following concentrations were used, group T1: 16.7 ml.L^{-1} (n = 6), and group T2: 400 ml.L^{-1} (n = 6). A control group was used without the addition of the herbicide (n = 6). At the end of the experiment, the animals were euthanized; the testes were collected and fixed for subsequent histological analysis in light microscope. The T1 group showed increase in albuginea weight ($P < 0.05$). The animals of the O2 group showed significant reduction ($P < 0.05$) of the height of seminiferous epithelium. The animals of the O1 and T2 groups did not present harmful effects on the spermatogenic morphometry.

Keywords: spermatogenesis, 2,4-dichlorophenoxiacetic acid (2,4D), picloram, testicle, rat.

RESUMO - Trinta ratos Wistar adultos foram usados em cinco grupos. Dois grupos receberam tratamento por via oral com solução aquosa de 2,4-D Tordon® 64/240 BR, em diferentes concentrações, grupo O1: 2 ml.L^{-1} (n = 6), grupo de O2: 4 ml.L^{-1} (n = 6). Dois grupos receberam tratamento tópico na região cervical dorsal, foram usadas as seguintes concentrações, grupo T1: 16,7 ml.L^{-1} (n = 6), e um grupo T2: 400 ml.L^{-1} (n = 6). Um grupo controle foi utilizado sem a adição do herbicida (n = 6). No fim do experimento, os animais foram eutanaziados, os testículos foram removidos e fixados para posterior análise histológica em microscópio de luz. Os animais do grupo T1 apresentaram aumento no peso da albugínea ($P < 0,05$). Os animais do grupo de O2 mostraram redução significativa ($P < 0,05$) da altura do epitélio seminífero. Os animais dos grupos O1 e T2 não apresentaram efeitos nocivos sobre a morfometria espermatogênica.

Palavras-Chave: espermatogênese, ácido 2,4-diclorofenoxiacético (2,4D), picloram, testículo, rato.

INTRODUCTION

Among the various factors that can contribute to impair spermatogenic process can be included the effects of many environmental pollutants (Dallinga et al., 2002). Specifically, occupational exposures to environmental pollutants have been linked to serious reproductive disorders, directly affecting the spermatogenic process (Lerda and Rizzi, 1991; Oakes et al., 2002).

Tordon® has a similar formulation, in terms of active compounds, to the herbicide White Agent, used extensively in the Vietnam War (Oakes et al., 2002). Currently, this herbicide is widely used to eradicate weeds in agriculture. Oakes et al. (2002), reported a severe decrease in testicular mass with shrinkage of seminiferous tubules, with the use of Tordon 75D® in adult rats, without interference, however, on the serum levels of testosterone.

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The main component of Tordon® is the 2,4Dichlorophenoxyacetic acid (2,4-D). Pharmacokinetic profile of 2,4-D is well defined in humans. Five volunteers male ingested a single dose of 5 mg/kg of 2,4-D with no detectable clinical effects; moreover, essentially all 2,4-D is absorbed in the human gastrointestinal tract (Sauerhoff et al., 1977).

According to Lachapelle et al. (2007), 2,4-dichlorophenoxyacetic acid blocks *in vitro* meiotic maturation and is therefore a potential environmental endocrine disrupter with reproductive effects. Testicular atrophy has been reported in rats after chronic exposure to 300 mg/kg per day of 2.4D in the diet (Charles et al., 1996).

Spermatogenesis is a complex and well organized process, which can be divided into three phases based on morphological and functional considerations: (1) proliferative phase, in which cells undergo rapid and successive mitotic divisions, (2) meiotic phase, in which genetic material is duplicated and undergoes genetic recombination, and (3) stage of differentiation in which the spermatids undergo considerable change, becoming highly specialized cells and structurally equipped to reach and fertilize eggs (Russell et al., 1990).

Quantitative data of populations from different generations of spermatogenic cell lineage, as well as data for tubular diameter, epithelial thickness and volumetric proportions of the different components of testicular parenchyma are decisive for the estimation of sperm production, and the evaluation of external effects on the spermatogenic process. According to Linder et al. (1992) methodologies involving testicular histology comprise the most sensitive and reliable protocols for the detection of toxic effects on sperm production. Creasy (1997) describes the testicular histology as a standard procedure for testing of pesticides.

MATERIAL AND METHODS

Animals

Thirty wistar rats, 60 days old, weighing around 320 grams, from the vivarium of the Nutrition and Health Department of Federal University of Viçosa were used. The animals were divided into control group, topical and orally treated with the 2,4 dichlorofenoxyacetic acid associated with picloram (Tordon 2,4-D® 64/240 BR) with composition of 240g/l 2,4-dichlorophenoxiacetic acid and e 64 g/l picloram.

Experimental design

Aqueous solutions of Tordon® were obtained with the dilutions below. These solutions were offered *ad libitum* and the daily consumption determined by the level differences in the containers. O1 group: 2 $ml.L^{-1}$, orally (n = 6); O2 Group: 4 $ml.L^{-1}$ (n = 6). Two groups received daily topical treatment in the dorsal cervical area, 0.5 ml of solution were used in the following concentrations: T1 Group: 16.7 $ml.L^{-1}$ (n = 6); T2 Group: 400 $ml.L^{-1}$ (n = 6). A control group (n = 6) was used without addition of herbicide and receiving 0.5ml of saline solution in the dorsal cervical area.

Euthanasia and histology

The body weight was monitored weekly and after 39 days all animals were euthanized with overdose of sodium thiopental (Thionembutal). Animals were immediately dissected, and by cannulation of the aorta, were perfused initially with saline and then with fixative solution of 2.5% glutaraldehyde in phosphate buffer 0.1 $mol.L^{-1}$, pH 7.4. The testes were collected, body and gonads weight were measured. Based on body and testicular weights it was calculated the gonadosomatic index (GSI) from the following formula: GSI = GW / BW; where GW = gonad weight and BW = body weight.

One of the testes was dissected to measure the weight of albuginea and the contralateral testis was fragmented, reattached in 2.5% glutaraldehyde in sodium phosphate buffer 0.1 $mol.L^{-1}$, pH 7.4 for 24 hours under refrigeration. The testicular fragments were then dehydrated in increasing concentrations of alcohol (50°, 70°, 80°, 90°, 95° and 100° GL) with exchanges every thirty minutes. After dehydration, the fragments were embedded in glycol methacrylate (Historesin Leica) and subsequently sectioned at 4μm thickness on a rotary microtome, equipped with glass knives. The sections were stained with 1% toluidine blue-sodium borate, mounted with Entellan (Merck) and examined under a light microscope.

Volumetric proportion of the testicular components

By projecting a grid with 100 intersections (points) at 400X magnification, recording 10 fields randomly for each animal the matching points were counted: seminiferous tubules and interstitial tissue. The interstitial components also were counted: Leydig cells, cells and fibers of connective tissue, blood and lymph vessels. The volume (μL) of each testicular component was estimated from its percentage occupied in testis and the testicular volume. As the density of the testicle is around 1

(1.03 to 1.04; França, 1991), the testis weight was considered equal to its volume.

The tubular diameter and seminiferous epithelium height were obtained from the measurement randomly from 20 cross sections of seminiferous tubules that showed the outline circular as possible. These measures were performed using a micrometer attached to a light microscope at 150X increase for the measurement of tubular diameter and 600X to measure the height of the seminiferous epithelium. The total length of seminiferous tubules (TL) per testis and per gram of testis was expressed in meters; it was estimated from knowledge of the volume occupied by seminiferous tubules and tubular diameter obtained for each animal. The following formula was used: $TL = TVS / \pi R^2$; where TVS = Total volume of seminiferous tubules, and πR^2 = Cross sectional area of seminiferous tubules (R = tubular diameter / 2).

Calculation of the volume of Leydig cells was done by counting, with a grid with 100 intersections (points) at 1000X magnification, a thousand points over the cytoplasm and the nucleus of Leydig cells per animal. Then it was calculated the proportion (%) between nucleus and cytoplasm. In another step, the nuclear diameter of Leydig cells was obtained using image analysis program Image Pro Plus associated with the light microscope at 1000X magnification. The diameter of 10 nuclei of Leydig cells were measured in each animal. From those data and applying the formulas described below, it was calculated the nuclear volume, volume of the cytoplasm and, consequently, the volume of each Leydig cell. These values were expressed in cubic micrometers (μm^3):

Nuclear volume = $4/3\pi R^3$; where R = nuclear radius;

Cytoplasmic volume = % cytoplasm x nuclear volume / % nucleus;

Cell volume = nuclear volume + cytoplasmic volume.

By volumetric proportion (%) in the testis and the total volume (ml) occupied by Leydig cells in testis was possible to calculate the number of Leydig cells per testis and per gram of testis in each animal.

Statistical analysis

For statistical analysis, data were submitted to analysis of variance (PROC ANOVA; SAS, 2002), applying the Tukey's test for means comparison. Correlations among variables were performed by using Pearson's correlation (PROC CORR). Evaluation of mean and standard error of mean were done by using descriptive statistics. Significant level adopted was $\alpha = 0.05$.

RESULTS

The biometric parameters (body weight, testicular weight, parenquimous weight, GSI and albuginea weight) are showed on the Table 1. Body weight, testicular weight, parenquimous weight and GSI did not show statistical differences ($P > 0.05$) among the treatments. The T1 group showed increase in albuginea weight ($P < 0.05$).

Table 1 – Biometric parameters of adult Wistar rats exposed to 2,4dichlophenoxiacetic associated to picloram (mean ± standard error of mean).

	BW (g)	TW (g)	TPW (g)	GSI	AW (mg)
Control	389.9 ± 17.9[a]	2.7 ± 0.2[a]	2.6 ± 0.2[a]	0.69 ± 0.05[a]	81.3 ± 5.3[b]
O1	396.9 ± 14.2[a]	2.8 ± 0.1[a]	2.7 ± 0.1[a]	0.71 ± 0.02[a]	82.3 ± 2.4[b]
O2	337.2 ± 26.1[a]	2.6 ± 0.1[a]	2.6 ± 0.1[a]	0.80 ± 0.05[a]	78.3 ± 1.7[b]
T1	379.1 ± 20.0[a]	2.8 ± 0.1[a]	2.7 ± 0.1[a]	0.76 ± 0.05[a]	100.7 ± 3.0[a]
T2	390.0 ± 21.7[a]	3.0 ± 0.1[a]	3.0 ± 0.1[a]	0.73 ± 0.03[a]	77.7 ± 3.5[b]
P-value	0.2660	0.5401	0.5703	0.3869	0.0003

Values in the same column followed by different letters differ by Tukey's test ($P < 0.05$). BW = Body weight; TW = Testicular weight; TPW = Testicular parenquimous weight; GSI = Gonadosomatic index; AW = Albuginea weight. O1: 2 $ml.L^{-1}$, orally; O2: 4 $ml.L^{-1}$, orally; T1: 16.7 $ml.L^{-1}$, topically in the dorsal cervical area; T2: 400 $ml.L^{-1}$, topically in the dorsal cervical area; Control group: saline solution, topically, on the dorsal cervical region.

Table 2 – Volumetric proportion and volume of tubular and interstitial compartments of adult Wistar rats exposed to 2,4dichlophenoxiacetic associated to picloram (mean ± standard error of mean).

	Tubular comp. (%)	Interstitial comp. (%)	Tubular comp. vol. (ml)	Interstitial comp. vol. (ml)
Control	92.0 ± 1.4	8.0 ± 1.4	2.4 ± 0.2	0.21 ± 0.03
O1	91.6 ± 0.9	9.4 ± 0.9	2.5 ± 0.1	0.26 ± 0.03
O2	89.8 ± 1.7	10.2 ± 1.7	2.3 ± 0.1	0.25 ± 0.03
T1	91.4 ± 1.1	8.6 ± 1.1	2.5 ± 0.1	0.24 ± 0.03
T2	92.6 ± 0.3	7.4 ± 0.3	2.5 ± 0.1	0.20 ± 0.01
P-value	0.4920	0.4920	0.5005	0.5767

Vol. = Volume; Comp. = Compartment. O1: 2 $ml.L^{-1}$, orally; O2: 4 $ml.L^{-1}$, orally; T1: 16.7 $ml.L^{-1}$, topically in the dorsal cervical area; T2: 400 $ml.L^{-1}$, topically in the dorsal cervical area; Control group: saline solution, topically, on the dorsal cervical region.

The values of the proportion (%) of seminiferous tubules and interstitial space are showed on the Table 2. There were no statistical differences among the treatments ($P > 0.05$).

Seminiferous tubules occupied, on average, 91.3% of the testicular parenquimous and the interstitial space 8.7%. With the volumetric proportion values, it was obtained the volume of each compartment (Table 2). There was no difference among the treatments ($P > 0.05$).

Volumetric proportion of the interstitial compartments (Leydig cells, cells and fibers of connective tissue, blood and lymph vessels) are presented on the Table 3, as well the values of the volume of interstitial compartments, there were no statistical differences among treatments ($P > 0.05$).

Table 3 – Volumetric proportion among the interstitial components, and volume of interstitial components of adult Wistar rats exposed to 2,4dichlophenoxiacetic associated to picloram (mean ± standard error of mean).

	LS (%)	BV (%)	CT (%)	LC (%)	LS vol. (µl)	BV vol. (µl)	CT vol. (µl)	LC vol. (µl)
Control	40.3 ± 4.0	6.3 ± 2.0	42.3 ± 2.7	11.1 ± 2.1	87.4 ± 23.4	11.9 ± 4.2	86.7 ± 13.1	20.3 ± 2.9
O1	39.3 ± 4.2	5.8 ± 1.2	48.6 ± 4.8	6.3 ± 1.5	106.3 ± 24.7	15.9 ± 4.1	118.3 ± 4.3	16.3 ± 4.0
O2	34.2 ± 3.8	4.9 ± 1.7	50.1 ± 3.6	10.9 ± 1.6	91.4 ± 19.6	15.0 ± 6.7	122.2 ± 8.9	25.5 ± 2.4
T1	41.6 ± 2.0	8.3 ± 5.1	41.4 ± 3.1	8.7 ± 2.2	98.6 ± 13.5	26.8 ± 18.4	93.9 ± 8.4	18.1 ± 4.5
T2	36.9 ± 2.4	3.9 ± 1.3	51.9 ± 2.9	7.3 ± 1.1	75.5 ± 7.2	7.4 ± 2.4	106.0 ± 9.9	14.6 ± 2.1
P-value	0.5671	0.8183	0.1614	0.7641	0.8180	0.6608	0.0555	0.2015

LS = Lymphatic space; BV = Blood vessels; CT = Connective tissue; LC = Leydig cell; vol. = volume. O1: 2 $ml.L^{-1}$, orally; O2: 4 $ml.L^{-1}$, orally; T1: 16.7 $ml.L^{-1}$, topically in the dorsal cervical area; T2: 400 $ml.L^{-1}$, topically in the dorsal cervical area; Control group: saline solution, topically, on the dorsal cervical region.

Means of tubular diameters, height of seminiferous epithelium and total length of seminiferous tubules are showed on Table 4. Tubular diameters, total length of seminiferous and total length of seminiferous per gram of testicle did not present statistical differences among treatments ($P > 0.05$). Nevertheless, O2 group showed height of seminiferous epithelium smaller than control group (Figure 1; $P < 0.05$).

The ratio between nucleus and cytoplasm of the Leydig cells in this experiment was, on average, 67.7% and 32.3%, respectively, there was no difference ($P > 0.05$) among the treatments. Nuclear diameter, volume, number of Leydig cell per testicle an per gram of testicle did not presented statistical differences among the treatments ($P > 0.05$; Table 5).

Table 4 – Seminiferous epithelium height, tubular diameter, and total length of seminiferous tubules of adult Wistar rats exposed to 2,4dichlophenoxiacetic associated to picloram (mean ± standard error of mean).

	SEH (µm)	TD (µm)	TLST (m)	TLST (m)/g T
Control	82.1 ± 4.2[a]	299.1 ± 6.2[a]	34.0 ± 2.2[a]	12.7 ± 0.4[a]
O1	71.3 ± 2.1[ab]	289.8 ± 3.9[a]	37.2 ± 0.7[a]	13.4 ± 0.4[a]
O2	67.7 ± 2.7[b]	285.0 ± 6.5[a]	36.2 ± 2.2[a]	13.7 ± 0.6[a]
T1	69.9 ± 1.2[ab]	297.3 ± 7.2[a]	36.2 ± 1.2[a]	12.8 ± 0.6[a]
T2	70.2 ± 4.2[ab]	302.5 ± 11.3[a]	36.2 ± 3.2[a]	12.8 ± 1.0[a]
P-value	0.0254	0.4649	0.8600	0.7342

Values in the same column followed by different letters differ by Tukey's test ($P < 0.05$); SEH = Seminiferous epithelium height; TD = Tubular diameter; TLST = Total length of seminiferous tubules; T = Testis. O1: 2 $ml.L^{-1}$, orally; O2: 4 $ml.L^{-1}$, orally; T1: 16.7 $ml.L^{-1}$, topically in the dorsal cervical area; T2: 400 $ml.L^{-1}$, topically in the dorsal cervical area; Control group: saline solution, topically, on the dorsal cervical region.

Table 5 – Nuclear diameter, volume of Leydig cells, and number of Leydig cells per testis and per gram of testis of adult Wistar rats exposed to 2,4dichlophenoxiacetic associated to picloram (mean ± standard error of mean):

	ND (µm)	NV ($µm^3$)	CV ($µm^3$)	TV ($µm^3$)	N° LC/T ($x10^7$)	N° LC/g T ($x10^7$)
Control	7.5 ± 0.2	225.9 ± 17.2	489.9 ± 66.1	715.8 ± 75.7	1.5 ± 0.2	1.1 ± 0.1
O1	7.2 ± 0.4	205.1 ± 32.0	462.4 ± 59.7	667.5 ± 89.8	1.3 ± 0.4	0.9 ± 0.3
O2	7.2 ± 0.2	193.5 ± 15.4	394.7 ± 44.2	588.2 ± 58.6	2.3 ± 0.4	1.8 ± 0.3
T1	7.1 ± 0.2	186.5 ± 15.3	390.1 ± 51.3	576.6 ± 66.2	1.7 ± 0.5	1.2 ± 0.4
T2	7.6 ± 0.1	233.0 ± 11.5	492.7 ± 36.0	725.7 ± 43.0	1.0 ± 0.2	0.7 ± 0.1
P-value	0.3610	0.3978	0.4694	0.4076	0.1502	0.0811

ND = Nuclear diameter; NV = Nuclear volume; CV = Cytoplasm volume; TV = Total volume; LC = Leydig cell; T = Testis. O1: 2 $ml.L^{-1}$, orally; O2: 4 $ml.L^{-1}$, orally; T1: 16.7 $ml.L^{-1}$, topically in the dorsal cervical area; T2: 400 $ml.L^{-1}$, topically in the dorsal cervical area; Control group: saline solution, topically, on the dorsal cervical region.

DISCUSSION

According to Oakes et al. (2002), rats treated with high dosages of Tordon 75D® initially lost body weight, but in the end of 9 weeks, their gain of weight (+4.1%) was similar to the control animals (+6,1%). Besides of that, testicular weight of rats treated with high dosage of Tordon 75D® was not affected during the first 4 weeks, however, in the end of 9 weeks, testicular weight was significantly reduced (-24%) as well as the GSI (-29%). GSI found in this experiment was 0.7%, but there was no difference among treatments ($P > 0.05$).

Albuginea weight usually corresponds to 10% of testicle weight in domestic animals (França and Russell, 1998). In this experiment, albuginea weight was not higher than 4% of testicular weight, but it was higher ($P < 0.05$) in the T1 group (3.6%; 100.7 mg) than control group (3.1%; 81.3 mg). Rats of T1 group were treated with 16.7$ml.L^{-1}$ Tordon 2,4D® solution; this dosage is the same that is used in pulverization procedure. Furthermore, there was correlation of the albuginea weight with testicular weight ($r = 0.54$; $P < 0.01$), testicular parenquimous weight (0.51; $P < 0.01$) and tubular volume ($r = 0.51$; $P < 0.01$); nevertheless, alterations in albuginea weight caused by Tordon® administration have been not reported which difficult interpreting these results.

In a large part of the mammals already investigated, the main constituent element of the interstitial compartment is the Leydig cell, and its amount is very varied among the different species, this variation is not found for other endocrine glands (Paula, 1999). In this study the exposed animals to the Tordon 2.4D® 64/240 BR did not present significant changes in the volumetric proportion of testicular components ($P > 0.05$).

The measure of the tubular diameter is an approach classically used as indicator of the spermatogenic activity in investigations involving the testicular function (Paula et al., 2002). Several factors contribute to the constitution of the tubular

diameter: number of myoid cells layers, size and population of Sertoli cells and spermatogenic cells, and fluid secretion for the Sertoli cells, what determines the tubular lumen (Paula et al., 2002). In the present study, there were no significant differences among treatments for the tubular diameter.

However, the height of seminiferous epithelium was reduced in O2 group when compared to the control ($P < 0.05$). According to Oakes et al. (2002), Tordon 75D® caused a severe decrease in testicular mass with shrinkage of seminiferous tubules of adult rats. Tordon® and in particular its 2,4D component, may have a direct effect on the epithelium of the seminiferous tubules. A possible toxic effect is direct inhibition of DNA synthesis in the testes (Oakes et al., 2002). A study in mice showed that a single oral dose of 200 mg/kg 2,4D reduced DNA synthesis in the testes by 29% (Seiler, 1979).

Leydig cells are the main responsible for the steroids production. However, it is not known the reason of the great variation for the occupied percentage for these cells in testicle. In this study, the larger the nuclear diameter of the Leydig cells greater the tubular diameter ($r = 0.42$; $P < 0.05$) and the tubular percentage ($r = 0.38$; $P < 0.05$). Nevertheless, studies that correlated the structure and the function of Leydig cells in several mammal species showed that variations in the testosterone secretion result more of the individual capacity of this cell in secreting testosterone than differences in the total volume of the same ones in testicle. This capacity is highly associated to the amount of endoplasmic reticulum that is present in these cells (Paula, 1999).

Rats that were treated with 2,3,7,8-tetrachlorodibenzeno-ρ-dioxin (TCDD) showed dose-dependent reduction on the volume of cytoplasm and nucleus of Leydig cells, although the ultra-structural characteristics of these cells have been not altered. These volume alterations were explained by enzymatic and steroidogenic activity reduction of testis. TCDD was an Orange agent contaminant and it was implicated as a Leydig cell toxicant (Johnson et al., 1992). However, TCDD is not a contaminant of 2,4D formulations (Cochrane et al., 1981).

There were no differences ($P > 0.05$) for the number of Leydig cells per testis and per gram of testis among the treatments of this experiment. These results agree with Oakes et al. (2002) that showed that Tordon 75D® did not affect the number of Leydig cells neither the testosterone levels in rats, suggesting that Tordon® treatment had no direct effect on the hypothalamic–pituitary–testicular cell axis.

Another Tordon® component is the picloram. In chronic feeding studies of picloram in rats and mice, performed by National Cancer Institute (NCI), no testicular damage was reported (Robens, 1980). However, a pathologist of NCI reexamined the material of these studies and concluded that most exposed rats and mice had testicular atrophy (Reuber, 1981). Nevertheless, in the present study, no treatment showed considerable variation in the body and testicle weight.

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