

ORIGINAL

Effects of cypermethrin on cytokeratin 8/18 and androgen receptor expression in the adult mouse Sertoli cell



Hector Rodríguez^{a,*}, Hector Jara^a, Sergio Legua^a, Danitza Campos^a, Jorge Morales^a, Omar Espinoza-Navarro^b

^a *Laboratory of Immunocytochemistry, Program of Anatomy and Developmental Biology, School of Medicine, University of Chile, Chile*

^b *Departamento de Biología, Universidad de Tarapacá, Chile*

Received 20 November 2015; accepted 1 October 2016

Available online 6 January 2017

KEYWORDS

Cypermethrin;
Cytokeratin 8/18;
Androgen receptor;
Sertoli cell;
Spermatogenesis

Abstract

Background: With the explosive population growth an increased use of land for cultivation purposes and the usage of biotechnologies in agriculture—such as pesticides—respond to the need for more efficient systems. However, improper application of pesticides has a negative effect on the environment, on exposed animals and on humans. Cypermethrin, a synthetic pyrethroid, is an insecticide with low risk to human and animal health and with broad insecticidal activity against a large number of pests. Studies in humans and animals show morphological and functional alterations in different organs exposed to cypermethrin. Pyrethroids are chemicals with structural similarity to pyrethrins and possess increased toxicity to insects over mammals. **Objective:** This research analyzes the variations of the state of differentiation of Sertoli cells and androgen receptor expression in testes of healthy adult mice exposed to cypermethrin.

Material and method: Mice were divided into three groups: control 1 (untreated), control 2 (inoculated intraperitoneally with 0.1 ml of vegetable oil), and the experimental group 3 (inoculated with 1/5 of the lethal dose 50 (LD₅₀ = 485 mg/kg) of cypermethrin).

Results: Cypermethrin exerts acute and chronic effects on Sertoli cells in the testis of the adult mouse. These effects are manifested by the significant increase in epithelial height and the dedifferentiation of Sertoli cells evidenced through the presence of the Ck 8/18-type intermediate filament—a characteristic of differentiating cells—especially considering the functional cyclicity of the testicular compartment.

* Corresponding author.

E-mail address: hrodrigu@med.uchile.cl (H. Rodríguez).

PALABRAS CLAVE

Cipermetrina;
Citoqueratina 8/18;
Receptor de
andrógeno;
Célula de Sertoli;
Espermatogenesis

Conclusions: Cypermethrin significantly affects the structure and function of Sertoli cells through the cytoskeleton and the state of maturation.

© 2016 Asociación Española de Andrología, Medicina Sexual y Reproductiva. Published by Elsevier España, S.L.U. All rights reserved.

Efectos de cipermetrina sobre la expresión de citoqueratinas 8/18 y el receptor de andrógenos en la célula de sertoli del ratón adulto

Resumen

Antecedentes: Con el crecimiento explosivo de la población, un mayor uso de la tierra con fines de cultivo y el uso de las biotecnologías en la agricultura—como pesticidas—responden a la necesidad de sistemas más eficientes. Sin embargo, la aplicación inadecuada de pesticidas tiene un efecto negativo sobre el medio ambiente, en los animales expuestos y en los seres humanos. La cipermetrina, un piretroide sintético, es un insecticida de bajo riesgo para la salud humana y animal, y con una amplia actividad insecticida frente a un gran número de plagas. Los estudios en seres humanos y animales muestran alteraciones morfológicas y funcionales en diferentes órganos expuestos a la cipermetrina. Los piretroides son sustancias químicas con estructura muy similar a las piretrinas, a menudo más tóxicas para insectos que para mamíferos. **Objetivo:** La presente investigación analiza las variaciones del estado de diferenciación de las células de Sertoli y de la expresión del receptor de andrógeno en testículos de ratones adultos sanos expuestos experimentalmente a cipermetrina.

Material y método: Los animales fueron distribuidos en 3 grupos: control 1 (n= 3) sin tratamiento, control 2 (n = 15) inoculados con 0,1 ml de aceite vegetal vía intraperitoneal, y el grupo 3 y experimental (n = 15) inoculados con 1/5 de la dosis letal 50 (LD₅₀ = 485 mg/kg) de cipermetrina.

Resultados: Se observó que la cipermetrina tiene efectos agudos y crónicos sobre las células de Sertoli en el testículo de ratón adulto. Estos efectos se demuestran por el aumento significativo de la altura epitelial, como también por una desdiferenciación de las células de Sertoli a través de la presencia de los filamentos intermedios tipo CK8/18, característico de células en diferenciación, más aun considerando la ciclicidad funcional del compartimiento testicular.

Conclusiones: La cipermetrina afecta significativamente a la estructura y la funcionalidad de las células de Sertoli, a través del citoesqueleto y el estado de maduración.

© 2016 Asociación Española de Andrología, Medicina Sexual y Reproductiva. Publicado por Elsevier España, S.L.U. Todos los derechos reservados.

Introduction

Exposure to pesticides can have acute, chronic and long-term effects on people, animals and the environment.¹ Cypermethrin is a type II pyrethroid widely used in the management of livestock and the production of primary agricultural products (cotton, cereals, vegetables and fruits), as well as a controlling agent for vectors of infectious diseases in public health.² Research in animal models show that pyrethroids exert a significant adverse impact on organs and systems such as liver, brain, immune and reproductive systems.^{3–6}

In the reproductive system of mice, cypermethrin decreases fertility, reduces the number of implantation sites and viable fetuses in females crossed with males previously exposed to cypermethrin,⁷ while in males cypermethrin significantly reduces testosterone levels by inhibiting testicular steroidogenesis, thus deteriorating the normal spermatogenesis.⁸

Sertoli cells (SCs) are the supporting and nourishing cells for male germ cells.⁹ Changes in their general and nuclear morphology might be associated with absent or weak expression of the androgen receptor (AR) during puberty.¹⁰ Testes with total absence of AR expression show alterations in their development and function. The lack of androgen receptors in Sertoli cells affects the production and secretion of testosterone in Leydig cells, as well as the normal spermatogenesis.^{11,12}

The number of SCs determines the size of the adult testis and daily sperm production; this relationship is established because each Sertoli cell sets the number of germ cells that it can sustain. The variation of these parameters provides a clear correlation with the number of functional somatic cells.⁹

Once the individual reaches reproductive maturity, SCs experience a radical change in their morphology and function: from an immature proliferative (fetal) state to a non-proliferative mature (adult) state. In laboratory

animals, evidence indicates an important role of thyroid hormone (T3) in the maturation of SCs. T3 interacts with androgens and perhaps FSH, and both induce the expression of androgen receptor (AR) on the Sertoli cell, making them responsive to androgens and assuming support functions during spermatogenesis.¹¹

Ck-8/18 corresponds to a type of intermediate filament in epithelial cells, including the Sertoli cell. Human Ck-8/18 is a strong marker of cell immaturity, and in adult testis it is used to identify seminiferous tubules where Sertoli cells are immature or have changes in their differentiation process.¹³ When induced by experimental means (i.e. heat stress), Ck-8/18 expression is evidenced by immunohistochemistry 10 days post-induction, with a marked increase in its expression on days 15–30.^{14,15} It is even possible that the optimum conditions are generated entirely to start the process of Sertoli cell proliferation.¹⁶

Therefore, in this research the quality of Sertoli cells (SCs) is studied with the use of morphological and functional markers (Ck-8/18 and AR, respectively) after an experimental intoxication with cypermethrin to identify possible mechanisms of action of cypermethrin on the mouse testis.

Materials and methods

Biological and chemical material

Thirty four male CF1 mice (2.5–3 months old, 28–40 g body weight) were used, maintained under standard feeding conditions, with 12:12 hour cycle of light and darkness in the animal facility of the University of Chile. The selected chemical material, i.e. cypermethrin, was obtained from ANASAC (92.5% (w/w) purity)^{5,6} and suspended in sunflower vegetable oil (Belmont, Chile). During the experimental period, animals were kept as recommended by the Bioethics Committee of the School of Medicine, University of Chile.

Experimental design

Animals were divided into the following groups: control 1 ($n=4$, untreated), control 2 ($n=15$, inoculated intraperitoneally with 0.1 ml vegetable oil), and the experimental group ($n=15$, inoculated intraperitoneally with 1/5 of the LD₅₀ (485 mg/kg b. w.) of cypermethrin suspended in 0.1 ml vegetable oil. The testes of 3 mice from each group were extracted at 1 (including 4 animals in the control group without any treatment), 8.6, 17.2, 25.8 and 34.4 days post treatment, after euthanasia according to the protocols of the National Institute of Health¹⁷ and AVMA.¹⁸ These periods coincide with the time needed for metabolism, elimination and retention of cypermethrin in adipose tissue, with a half life of 9, 12 and 18 days, respectively.

Testis processing

Testes were fixed in Bouin's alcoholic solution for 8 h. Subsequently, they were embedded in paraffin (melting point 56–58 °C) and subjected to standard histological techniques (fixation, dehydration in alcohols, paraffin embedding, cutting, staining/IHC and assembly). Five micron thick tissue

sections (LEICA LEITZ 1512 microtome) were then mounted on silanized slides (Star Frost, USA).

Morphometric analysis

After Mayer's hematoxylin-PAS staining, field micrographs were obtained on an OLYMPUS CX31 microscope (under 100× objective) with a digital camera and quantitative software Mshot Digital Imaging System v9.3 (Guang Zhou Micro-shot Technology Co., China), always considering three histological sections per animal. In epithelial tissues, the height of the luminal cells (epithelial height is measured from the base to the top of the tubular compartments, in micrometers) represents the intensity of their activity according to their physiological status and their association with other cells. Changes in testis from digitized images were assessed in the areas of spermatogenesis and perimeters of the seminiferous tubules (areas and perimeters were measured following the contours of the seminiferous tubules in cross sections, in micrometers). Both variables were expressed in micrometers.

Immunohistochemistry

Immunohistochemistry was carried out using specific anti-Ck 8/18 and anti-AR antibodies, according to the protocols recommended by the respective manufacturer:

1. Cytokeratin 8/18 (Ck-8/18) and specific antibody (clone 5D3, Thermo Fisher Scientific Co., LabVision Cat # MS-743-R7). Sertoli cells were analyzed using specific anti-Ck-8/18 monoclonal antibodies. The evaluation was done by quantifying the number of positive Sertoli cells with cytoplasmic brown color per total seminiferous tubules (tubular index).
2. Androgen receptor protein (Androgen Receptor AB-1, mouse monoclonal antibody, clone AR 44, Thermo Fisher Scientific Cat. # MS-443-B0). The evaluation was performed by quantifying the number of positive Sertoli cells with a dark brown nucleus per total seminiferous tubules (tubular index).

Both antibodies were revealed through the DAB/HRP system, which generates a dark brown precipitate after positive reaction. IHC assays were performed with three repetitions, and in each section at least 30 cross-sectional seminiferous tubules (positive cells/total tubules) were evaluated.

Statistical analysis

Data were analyzed in Excel 2007 worksheets (Microsoft, USA). The differences between the means for each variable among groups were analyzed by analysis of variance with post hoc tests (ANOVA, Kruskal–Wallis test and Dunn's test, considering $p < 0.05$) using the statistical software Stat-Graph.

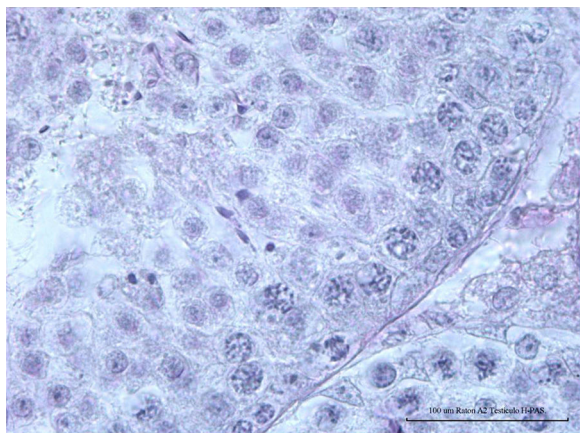


Figure 1 Cross section of a seminiferous tubule where healthy and integral tubular compartment is observed. In this compartment there is a normal cellularity and spermatogenesis (adult control A2; H-PAS, 100 $\times$).

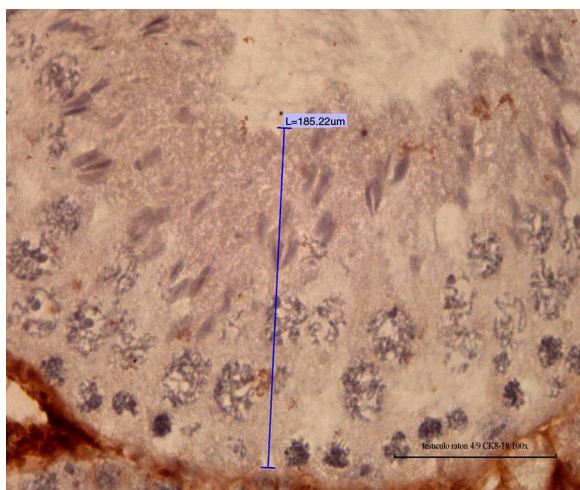


Figure 2 Cross section of a seminiferous tubule. The height (length measurement, L) of normal mouse seminiferous epithelium belonging to control group (L; 100 $\times$ L 185.22 μ m) is noted.

Results

Normal spermatogenesis: control group animals

As depicted in Fig. 1, the seminiferous tubules of adult mice possess a tubular compartment exhibiting the cell population that represents the full spermatogenic process, including the concepts of spermatogenic stages (12 stages in mouse, with specific cell associations by tubules), seminiferous epithelium cycle (length: 8.6 days) and wave (space in the seminiferous tubules for 4 cycles) in a normal mouse. Fig. 2 (animal from control group; hematoxylin–PAS staining) corresponds to a cross section of a seminiferous tubule showing a seminiferous epithelium in normal spermatogenesis process, including Sertoli cells and germline cells (spermatogonia, pachytenes, and spermatids).

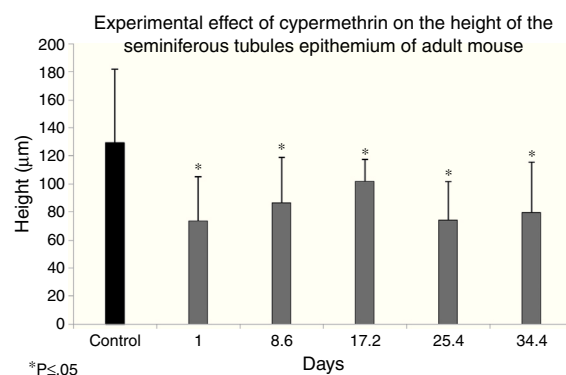


Figure 3 Effect of cypermethrin on the differentiation state of the seminiferous epithelium (Sertoli cells and spermatogenic cells) through epithelial height measurement. The graph is constructed using means and standard deviations.

Morphometric analysis

Fig. 3 shows the distribution of means and standard deviations of the measurements of seminiferous epithelium height from control and experimental groups, according to the experimental design (treatment with 1/5 LD_{50} = 485 mg/kg of cypermethrin in 0.1 ml of vegetable oil, intraperitoneally). Hence, the difference of means and standard deviations between the control group and each of the experimental groups were statistically significant according to the ANOVA analysis of variance ($p < 0.05$). This suggests that cypermethrin may exert a significant effect on the organization of

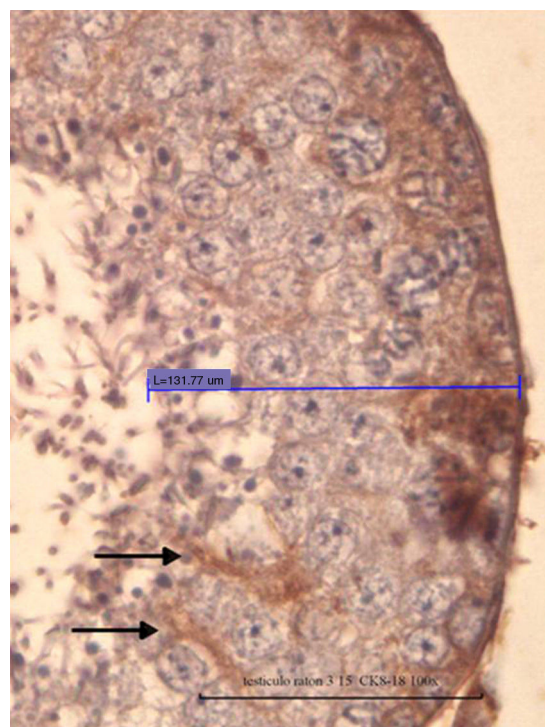


Figure 4 Cross section of a seminiferous tubule shown. Height (measuring length, L) of the seminiferous epithelium of normal mouse and DAB/HRP reaction specific for Sertoli cells positive for CK 8/18 IHC is observed with intense brown 100 $\times$).

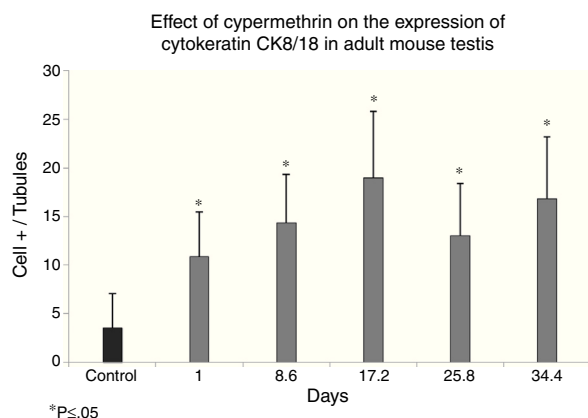


Figure 5 Effect of cypermethrin on the differentiation state of Sertoli cells (cells CK8/18 +) via DAB/HRP specific IHC in mouse testis experimentally exposed to cypermethrin. Results are expressed by the ratio between the number of Sertoli cells with brown cytoplasm per total seminiferous tubules (tubular index). The graph is constructed with means and standard deviations.

the cytoskeleton in Sertoli cells, and that these changes may be important in germ cell phenotype, considering that spermatogenesis occurs surrounded and nourished by Sertoli cells.

Immunohistochemical analysis of Ck-8/18

Fig. 4 shows positive cells for Ck-8/18 identified by the intense brown color in their cytoplasm. The photomicrograph shows a positive reaction in the cytoplasm of the Sertoli cells from a control mouse testis (black arrow). Silhouettes of Sertoli cells showed them as tall cells with lateral branches. In Fig. 5, the bar graph shows the means and standard deviations for control and experimental groups according to the experimental design. Cypermethrin exerts

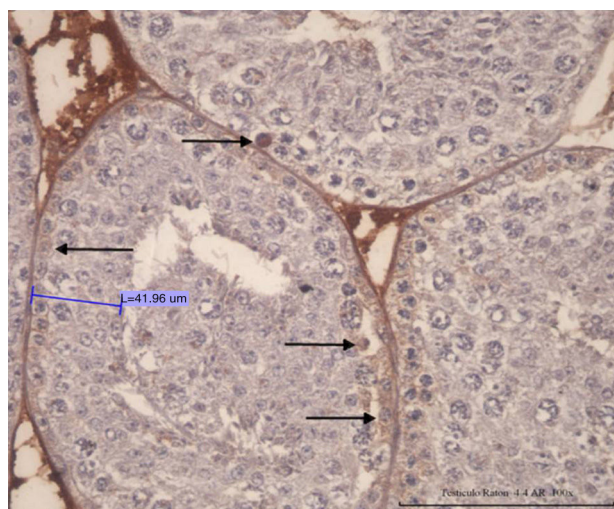


Figure 6 Cross section of a seminiferous tubule. Height (measuring length, L) of the seminiferous epithelium of normal mouse and DAB/HRP reaction specific for RA-positive Sertoli cells observed (black arrows, 100×).

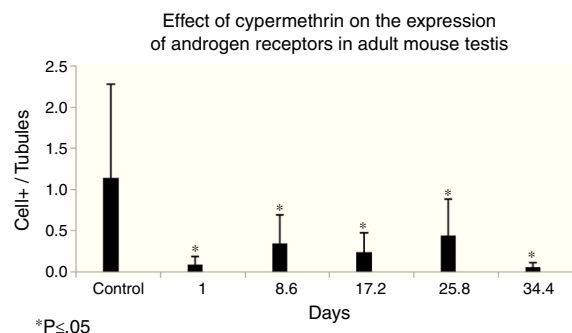


Figure 7 Effect of cypermethrin on the expression of the androgen receptor in adult mouse testis shown by IHC detection of DAB/HRP on specific testis from mice exposed to cypermethrin. Results are expressed by the ratio between the number of Sertoli cells with brown cytoplasm per total seminiferous tubules (tubular index). The graph is constructed with means and standard deviations (* $p < 0.05$).

acute (experimental group 1 day post inoculation), subacute (experimental groups 8.6 and 17.2 days post inoculation), and chronic effects (experimental groups 25.8 and 34.4 days post inoculation). These differences exerted by the action of cypermethrin are statistically significant ($p < 0.05$).

The cylindrical shape of the Sertoli cell is closely related to its function in spermatogenesis. Cypermethrin induces a reduction of the height of the seminiferous epithelium (Sertoli cell in Fig. 3) and participates in the regulation of the expression of cytoskeletal proteins (cytokeratins Ck 8/18, Fig. 5). Both characteristics evaluated here represent signs of cellular dedifferentiation.

Immunohistochemical analysis of AR

The photomicrograph of Fig. 6 specifically shows a positive reaction in the nucleus of Sertoli cells from the testis of mouse in the control group (black arrows). Immunohistochemistry with specific anti androgen receptor (AR) antibody and the DAB-HRP detection system reveals the cells expressing the androgen receptor. Positive cells were observed with an intense brown precipitate in the nuclear area. Fig. 7 shows the means and standard deviations for the control versus the experimental groups. The differences amongst the control group and each one of the experimental groups were significantly significant ($p < 0.05$).

Classically, cypermethrin is recognized as an extremely potent endocrine disruptor. Then, the presence of cypermethrin can alter both the expression of the androgen receptor synthesis machinery as can also act through deregulation (disruption) of the hypothalamic-pituitary-gonadal axis. Such mechanism might intensify the effects on cell dedifferentiation.

Discussion

Some studies have evaluated the central role of androgens in the control of spermatogenesis by acting directly on testicular somatic cells.¹² The lack of androgens or androgen receptors indirectly affects the height of the seminiferous

epithelium or the spermatogenesis since the germline stem cells do not express testosterone receptors.¹⁹

In the present research, a decrease in the height of epithelial cellularity of the seminiferous tubules in the presence of cypermethrin was evidenced. A recent study on the effects of cypermethrin in adult rats showed alterations in the structure of seminiferous tubules and in spermatogenesis. The authors concluded that damage on the male reproductive system may be attributed to an imbalance of circulating testosterone.²⁰

The increased expression of the Ck-8/18 intermediate filaments induced by cypermethrin seen in our immunohistochemical assessment evidences the state of cell immaturity by augmenting the expression of this protein in the cytoskeleton. These results clearly supports the deleterious effect of cypermethrin on androgen receptor expression, thus reducing the total cellularity of the seminiferous epithelium but with an increase in the number of immature cells, as seen by the augmented number of cells expressing the Ck-8/18 protein and inducing its dysfunction via an antiandrogenic effect.

Cytokeratins belong to the protein family of intermediate filaments and constitute an important tool for cancer diagnosis. In the past decade, more than 20 cytokeratins have been identified.²¹ However, the intermediate filaments proteins Ck8 and Ck18, along with Ck19, are associated with the plasma membrane of all simple epithelia as part of the cytoskeleton matrix.²²

Gao et al.²³ described that cypermethrin may lead to gonadal dysgenesis in prepubertal male rats and alter functional mRNA and protein expression in Sertoli cells. Cypermethrin, orally administered (25 mg/kg) in male *Rattus rattus* from post natal day 35 to day 70, decreased testosterone synthesis and its plasma and intratesticular levels by inhibiting both the mRNA synthesis and protein expression of StAR, an essential molecule for androgens synthesis that acts facilitating the entry of cholesterol into the Leydig cell for subsequent steroidogenesis.⁸

Wang et al.⁸ described that the levels of testicular and serum testosterone were significantly reduced in mice treated with cypermethrin. Other researchers showed an increase in germ cell apoptosis directly induced by various endocrine disruptors, including cypermethrin, a phenomenon interpreted as a cytotoxic event.^{24–26} Prolonged exposures to cypermethrin during puberty generate a marked adverse effect on the normal spermatogenic cycle associated with low levels of intratesticular testosterone.

The major hormones that control the development of male germ cells are the gonadotropins FSH and LH. FSH acts via specific G protein-coupled receptors (GPCRs) present in Sertoli cells,²⁷ constituting the primary mediator of androgen action on the control of spermatogenesis and, therefore, on the epithelial height of germ cells.^{25,28} A decrease in the activity of Leydig cells or the lack of androgen receptor expression in Sertoli cells decreases germ cell meiosis, which posteriorly manifests as a decrease in the cellularity or in the height of the seminiferous epithelium.

In studies with selective knockout for AR in Sertoli cells (SCARKO) mice, a complete blocking of the meiotic process was observed. One study pointed up the critical role of the AR-dependent regulation on the maturation or differentiation of germ cells in the seminiferous epithelium.¹²

Other authors described results such as arrest of the spermatogenesis during meiosis, infertility due to defective spermatogenesis, and hypotestosteronemia.²⁸

In the immunohistochemical analysis of AR expression in the present study, a significant decrease in the number of cells expressing the androgen receptor in both the control and experimental groups was found, probably due to the lipid-based vehicle employed to inoculate the pesticide, which could affect the expression of steroid receptors by mediating cell signaling pathways for transcription and subsequent expression of AR, as well as by interfering with cholesterol entry into the cell for subsequent steroidogenesis.

Willems et al.,¹² on a detailed study comparing SCARKO to control animals, showed that total lack of AR expression in Sertoli cells results in deficient formation of the blood-testis barrier. This research points up to morphological defects with alterations in the process of nuclear maturation accompanied by an aberrant positioning in the expression and localization of molecules related to cell adhesion and interaction and cytoskeletal dynamics.

The immunohistochemical analysis for the expression of AR in the present research found a significant decrease in the number of AR-expressing cells in all experimental groups, which implies that cypermethrin exerts an acute damage on this variable, interpreted as an anti-androgenic effect that possibly generates a cypermethrin-induced cellular dysfunction.

These results indicate that increasing endocrine disruption affects the reproduction of various cell populations by interfering with the expression of androgen receptors in Sertoli cells, leading to deterioration in the normal spermatogenic process and affecting the normal testicular morphology in individuals who have been exposed to endocrine disruptors like cypermethrin.

In recent years, the impact of environmental toxicants on reproduction and spermatogenesis has become urgent to understand in order to put into perspective the decline in male fertility levels. This has provoked a significant impact on other research topics such as the development of new technologies for male contraception that will provide additional tools for controlling population overgrowth and the development of new research lines focused on the development of new anticancer drugs that stop the process of cellular mitosis.

The results presented indicate that cypermethrin exerts an acute, subacute, and chronic effect on seminiferous epithelium height and Ck-8/18 protein expression, which demonstrates the immature cell stage induced by cypermethrin on Sertoli cells. In addition, cypermethrin alters the expression of nuclear androgen receptors in Sertoli cells. Overall, cypermethrin induces altered morphology and dysfunction on testicular tissue.

Ethical disclosures

Protection of human and animal subjects. The authors state that the procedures followed conformed to the ethical standards of the responsible human experimentation committee and in agreement with the World Medical Association and the Declaration of Helsinki.

Confidentiality of data. The authors declare that in this article they are no patient data.

Right to privacy and informed consent. The authors declare that in this article they are no patient data.

Partial funding

Proyecto UTA Mayor N° 4712-13.

Conflict of interest

The authors declare no conflict of interest.

References

1. Ferrer A. Pesticide poisoning. *Anales del Sistema Sanitario de Navarra*. 2003;26 Suppl. 1:155–71.
2. Pesticides news (website). Cypermethrin – a synthetic pyrethroid. Pesticide Action Network UK. 1995. In: <http://www.pan-uk.org/pestnews/Actives/cypermethrin.htm>
3. Manna S, Bhattacharyya D, Mandal TK, Das S. Repeated dose toxicity of alfa-cypermethrin in rats. *J Vet Sci*. 2004;5:241–5.
4. Wielgomas B, Krechniak J. Effect of α -Cypermethrin and chlorpyrifos in a 28-days study on free radical parameters and cholinesterase activity in wistar rats. *Pol J Environ Stud*. 2007;16:91–5.
5. Jimenez L, Quilodran J, Miranda JP, Rodriguez H. Efecto de dosis única intraperitoneal de cipermetrina en la corteza cerebral somatosensorial de ratones CF-1. *Int J Morphol*. 2008;26:19–26.
6. Rodríguez H, Silva I, Jiménez L, Sánchez C, Espinoza-Navarro O, Boarelli P, et al. Presencia cualitativa y distribución de caveolina 1 (cav-1) en la celularidad de estadios del ciclo de la espermatogénesis. *Rev Int Androl*. 2009;7:92–7.
7. Elbetieha A, Da'as SI, Khamas W, Darmani H. Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and parameters in the male rats. *Arch Environ Contam Toxicol*. 2001;41:522–8.
8. Wang H, Wang Q, Zhao X, Liu P, Meng X, Yu T, et al. Cypermethrin exposure during puberty disrupts testosterone synthesis via downregulating StAR in mouse testes. *Arch Toxicol*. 2010;84:53–61.
9. Amann P, Schanbacher B. Physiology of male reproduction. *J Anim Sci*. 1983;57:380–403.
10. Sharpe R, McKinnell C, Kivlin C, Fisher J. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*. 2003;125:769–84.
11. Chang CH, Chen Y, Yeh S, Xu Q, Wang R, Guillou F, et al. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in sertoli cells. *Endocrinology*. 2007;32:96–106.
12. Willems A, Batlouni S, Esnal A, Swinnen J, Saunders P, Sharpe R, et al. Selective ablation of the androgen receptor in mouse sertoli cell affects sertoli cell maturation, barrier formation and cytoskeletal development. *PLoS One*. 2010;5:e14168.
13. Luetjens CM, Gromoll J, Engelhardt M, Eckardstein S, Bergmann S, Nieschlag E, et al. Manifestation of Y-chromosomal deletions in the human testis: a morphometrical and immunohistochemical evaluation. *Hum Reprod*. 2002;17:2258–66.
14. Zhang X, Zhang Z, Jin X, Wei P, Hu X, Chen M, et al. Dedifferentiation of adult monkey Sertoli cells through activation of extracellularly regulated kinase 1/2 induced by heat treatment. *Endocrinology*. 2005;147:1237–45.
15. Zhang X, Zhang Z, Guo S, Yang W, Zhang Z, Yuan J, et al. Activation of extracellular signal-related kinases 1 and 2 in Sertoli cells in experimentally cryptorchid rhesus monkeys. *Asian J Androl*. 2006;8:263–72.
16. Hayrabedian S, Todorova K, Pashova S, Mollova M, Fernández N. Sertoli cell quiescence – new insights. *Am J Reprod Immunol*. 2012;68:451–5.
17. NIH Office of Animal Care and Use. Guidelines for euthanasia of rodents using carbon dioxide; 2013. <http://oacu.od.nih.gov/ARAC/documents/Rodent.Euthanasia.Adult.pdf>
18. American Veterinary Medical Association (AVMA). AVMA guidelines for the euthanasia of animals; 2013. Edition ISBN 978-1-882691-21-0.
19. Bremner WJ, Millar MR, Sharpe R, Saunders PT. Immunohistochemical localization of androgen receptor in the rat testis: evidence for stage-dependent expression and regulation by androgen. *Endocrinology*. 1994;135:1227–34.
20. Li Yan Fang, Chen PAN, Jin Xia HU, Jing LI, Li Chun XU. Effects of cypermethrin on male reproductive system in adult rats. *Biomed Environ Sci*. 2013;26:201–8.
21. Barak V, Goike H, Panaretakis WK, Einarsson R. Clinical utility of cytokeratins as tumor markers. *Clin Biochem*. 2004;37:529–40.
22. Chou CF, Smith AJ, Bishr Omary M. Characterization and dynamics of O-linked glycosylation of human cytokeratin 8 and 18. *J Biol Chem*. 1992;267:3901–6.
23. Gao L, Li X, Cai D. Study on the mechanism of di-2-ethylhexyl phthalate and cypermethrin inducing gonadal dysgenesis in the prepubertal male rats. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*. 2014;32:195–201.
24. McClusky LM, Jager C, Bornman MS. Stage-related increase in the proportion of apoptotic germ cells and altered frequencies of stages in the spermatogenic cycle following gestational: lactational, and direct exposure of male rats to p-nonylphenol. *Toxicol Sci*. 2007;95:249–56.
25. Pérez Z, Cristina P. Efectos de la cipermetrina sobre la celularidad y morfometría del testículo de ratón adulto sano: Modelo animal. Tesis (Médico Veterinario). Santiago, Chile: Universidad Santo Tomás, Escuela de Medicina Veterinaria; 2009, 63 p.
26. Saradha B, Vaithinathan S, Mathur PP. Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas-FasL and mitochondria-dependent pathway. *Toxicology*. 2009;255:131–9.
27. Plant TM, Marshall GR. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. *Endoc Rev*. 2001;22:764–86.
28. Verhoeven G, Willems A, Denolet E, Swinnen V, Gendt K. Androgen and spermatogenesis: lessons from transgenic mouse model. *Philos Trans R Soc B*. 2010;365:1537–56.