UNIVERSIDADE ESTADUAL DE CAMPINAS

Arielle Cristina Arena

PARÂMETROS REPRODUTIVOS MASCULINOS E FERTILIDADE DE RATOS ADULTOS EXPOSTOS AO INSETICIDA FENVALERATO

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

Campinas 2006

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Ar33p

Arena, Arielle Cristina
Parâmetros reprodutivos masculinos e fertilidade de ratos adultos expostos ao inseticida fenvalerato / Arielle Cristina Arena -- Campinas, SP: [s.n.], 2006.

Orientadora: Wilma De Grava Kempinas.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Fenvarelato. 2. Fertilidade. 3. Rato. I. Kempinas, Wilma De Grava. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(scs/ib)

Título em inglês: Male reproductive parameters and fertility of adult rats exposed to fenvalerate.
Palavras-chave em inglês: Fenvalerate; Fertility; Rat.
Área de concentração: Biologia Celular.
Titulação: Doutor em Biologia Celular e Estrutural.
Banca examinadora: Wilma De Grava Kempinas, Maria Martha Bernardi, Oduvaldo Câmara Marques Pereira, Paulo Dalsenter, Teresa Lúcia Lamano de Carvalho.
Data da defesa: 11/10/2006.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 11 de outubro de 2006.

BANCA EXAMINADORA

Profa. Dra. Wilma De Grava Kempinas (Orientadora) — Assinatura

Prof. Dr. Oduvaldo Câmara Marques Pereira — Assinatura

Profa. Dra. Teresa Lucia Lamano Carvalho — Assinatura

Prof. Dr. Paulo Roberto Dalsenter — Assinatura

Profa. Dra. Maria Martha Bernardi — Assinatura

Profa. Dra. Daniela Cristina Ceccatto Gerardin — Assinatura

Prof. Dr. Sérgio Luís Felisbino — Assinatura

Prof. Dr. Antonio Francisco Godinho — Assinatura

"Não deixe que a saudade sufoque,
que a rotina acomode,
que o medo impeça de tentar.

Desconfie do destino e acredite em você.

Gaste mais horas realizando que sonhando,
fazendo que planejando,
vivendo que esperando.

Porque, embora
quem quase morre esteja vivo,
quem quase vive já morreu."

Luis Fernando Veríssimo

Dedico este trabalho...

-À Deus*, por sempre me acompanhar, me dando coragem e saúde para enfrentar a vida. É maravilhoso Senhor ter tão pouco a pedir e tanto a agradecer.*

-Aos meus pais, José Maria e Edna, *que me deram a vida e me ensinaram a vivê-la com coragem e dignidade. Muito obrigada pelo amor, compreensão, incentivo e exemplo de vida.*

-Aos meus avós, José Maria e Maria (in memoriam) e Alcides e Gilda, *por todo amor e experiência a mim transmitidos.*

-À minha tia Schirleide Arena (in memoriam), *porque mesmo ausente, está sempre presente em minha vida.*

-Ao meu noivo, Hamilton, *por estar sempre presente em todos os momentos que precisei, me apoiando, me incentivando, me ouvindo... Muito obrigada pelo amor e atenção que sempre me dedicou.*

-A todos os meus familiares, *pelo apoio em todos os momentos de minha vida.*

Agradecimentos especiais

-À minha orientadora, Profa. Dra. Wilma De Grava Kempinas, *pelo exemplo de pessoa e profissional. Aprendi muito com ela durante todos esses anos. Guardarei para sempre todos os seus ensinamentos. Muito obrigada pela confiança e amizade.*

"Se deres um peixe a um homem, ele se alimentará uma só vez. Mas se lhe ensinares a pescar, ele se alimentará por toda a vida."

Kuan Tzu

Agradecimentos

-À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pelo apoio financeiro, fundamental para a realização deste trabalho;

-À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro concedido no início deste trabalho;

-À Sumitomo Chemical pelo fornecimento do fenvalerato técnico;

-Ao Programa de Pós-graduação em Biologia Celular e Estrutural da UNICAMP, pela oportunidade concedida para a realização do Doutorado;

-À Liliam A.S. Panagio, secretária da pós-graduação em Biologia Celular e Estrutural, IB, UNICAMP, por estar sempre pronta a ajudar no que for preciso, e pela sua amizade;

-Aos professores do Departamento de Biologia Celular, IB, UNICAMP, pelos ensinamentos e pela contribuição em minha formação científica;

-À Dra. Denise Z. Bissacot pelo auxílio, atenção, ensinamentos e amizade;

-À Profa. Dra. Eunice Oba, por toda a atenção e por ter auxiliado nas dosagens hormonais;

-Ao Departamento de Morfologia, IB, UNESP de Botucatu, por ter possibilitado a realização deste trabalho;

-Aos professores do Departamento de Morfologia, por todos os ensinamentos ao longo desses anos;

-Ao técnico do Laboratório de Embriologia do Departamento de Morfologia, IB, UNESP, Botucatu, José Eduardo, por sempre estar pronto a ajudar e principalmente por sua amizade;

-Aos funcionários do Departamento de Morfologia, pela colaboração;

-À secretária do Departamento de Morfologia, IB, UNESP, Botucatu, Luciana, por toda sua colaboração e amizade;

-Aos funcionários do Centro de Microscopia Eletrônica da UNESP, Botucatu, pelo auxílio nas análises de microscopia eletrônica;

-Aos Prof. Dr. Áureo Tatsumi Yamada, Profa. Dra. Carla B.C. Buzatto e Profa. Dra. Maria Cristina C.G. Marcondes pelas sugestões e por fazerem parte de minha banca de qualificação;

-Ao Prof. Dr. Oduvaldo C.M. Pereira, por toda sua ajuda e sugestões neste trabalho, e mais do que tudo, pela imensa contribuição em minha formação científica;

-À Profa. Dra. Maria Martha Bernardi, Prof. Dr. Paulo Roberto Dalsenter, Prof. Dr. Sergio Luis Felisbino e Profa. Dra Teresa Lucia Lamano Carvalho, pelas valiosas contribuições dadas para este trabalho;

-Ao Dr. Pedro Esponda, do Centro de Investigaciones Biológicas de Madrid, pela acolhida em seu laboratório, pelos ensinamentos e amizade;

-Aos amigos do laboratório de Biologia da Reprodução e do Desenvolvimento, Ana Paula, Azize, Carla, Daniela Fossato, Davi, Elaine, Fabíola, Glaura, Gustavo, Juliana, Marina, Raquel e Thaiane, pela amizade, colaboração e principalmente pelos momentos maravilhosos que passamos juntos. Agradeço em especial a Carla, Daniela, Elaine e Glaura, amigas sempre prontas a ajudar e a me ouvir nos momentos que precisei;

-À minha amiga Gisleine, por toda a ajuda que me deu neste trabalho, mas acima de tudo, por sua amizade verdadeira;

-Aos meus amigos, Fabiana, Sandro, Daniela Gerardin, Bianca, Danielle, Newton, Fernanda, Adela, Íris, Aitor, Amanda, Justulin, Gisele, Camilla, Luíza, Aline, Cristiano, Juliana, pela amizade que compartilhamos ao longo desses anos;

-A todos que de alguma forma contribuíram para a realização deste trabalho.

Muito obrigada!!!!

Sumário

RESUMO

O fenvalerato é um inseticida piretróide sintético amplamente utilizado na agricultura para o controle de pragas. Embora seja considerado de baixa toxicidade para os mamíferos, trabalhos têm demonstrado que certos piretróides podem apresentar atividade estrogênica e atuar como desreguladores endócrinos, acarretando disfunções reprodutivas importantes no sexo masculino. Está documentado na literatura que a exposição de ratas prenhes ao fenvalerato reduziu os níveis plasmáticos de testosterona e os pesos da vesícula seminal e do ducto deferente dos filhotes machos na idade adulta, além de alterações no comportamento sexual desses animais. Também foi observado que ratos adultos expostos à formulação de fenvalerato, por inalação, exibiram uma redução significativa no peso dos testículos e na contagem espermática no epidídimo. Até o momento pouco se sabe sobre os mecanismos pelos quais o fenvalerato exerce sua ação na reprodução, assim, o objetivo do presente trabalho foi investigar a atividade estrogênica e os efeitos do inseticida piretróide fenvalerato sobre o sistema reprodutor masculino e fertilidade de ratos machos adultos. Para tanto, ratos machos adultos (90 dias de idade) receberam durante 30 dias consecutivos, por gavage (via oral), 40 mg/kg/dia de fenvalerato (grau técnico; 96,8% de pureza). O grupo controle recebeu apenas o veículo (óleo de milho), segundo o mesmo protocolo experimental. No final do tratamento, foram avaliados os seguintes parâmetros: peso corporal; peso absoluto de órgãos da reprodução, fígado e rins; níveis plasmáticos de testosterona; contagem de células germinativas no testículo e no epidídimo; morfologia espermática; estudo da fertilidade através de cruzamentos naturais e inseminação artificial *in utero*; contagem de espermatozóides ejaculados no útero; avaliação do comportamento sexual; análises do testículo e epidídimo em nível de microscopia óptica e eletrônica e avaliação da possível atividade estrogênica de diferentes doses do fenvalerato (0,4; 1,0; 4,0; 8,0 e 40 mg/kg) através do teste uterotrófico. A quantificação de resíduos de fenvalerato por Cromatografia Líquida de Alta

Precisão (HPLC) em órgãos reprodutores e vitais e análises de proteínas espermáticas e epididimárias também foram realizadas. Os resultados foram comparados pelos testes “t” de Student e Mann-Whitney, dependendo da natureza da distribuição dos dados, enquanto os resultados do teste uterotrófico comparados pela ANOVA seguida pelo teste de Tukey. Os resultados da quantificação de fenvalerato revelaram que o piretróide foi retido em órgãos reprodutores (testículo e epidídimo) e vitais (cérebro e fígado). O tratamento com fenvalerato reduziu os pesos absolutos do testículo e do epidídimo. Além disso, o tratamento não provocou diminuição nos níveis plasmáticos de testosterona. Verificou-se também que os ratos tratados apresentaram redução na produção espermática no testículo e no número de espermatozóides no epidídimo. No entanto, não foi observado comprometimento na fertilidade desses machos quando acasalados com fêmeas controles. As análises morfológicas do testículo e epidídimo assim como as análises de proteínas espermáticas e epididimárias não mostraram alterações. Além disso, o fenvalerato, nas doses testadas, não apresentou atividade estrogênica *in vivo*. Concluiu-se que o fenvalerato, nestas condições experimentais, foi retido em órgãos reprodutores e vitais. O fenvalerato foi espermatotóxico, visto que reduziu tanto a produção quanto as reservas espermáticas dos animais tratados. No entanto, apesar dessa alteração, a fertilidade dos animais tratados não foi comprometida, uma vez que o rato tem uma grande eficiência reprodutiva, diferentemente do que acontece com o ser humano.

ABSTRACT

Fenvalerate is a synthetic pyrethroid insecticide widely used in agriculture to control a variety of insects. Although it is considered to be of low acute toxicity to mammals, studies have showed that pyrethroids can have estrogenic activity and can act as endocrine disruptors, causing important reproductive impairment in males. It is documented in the literature that the exposure of pregnant rats to fenvalerate decreased plasma testosterone levels and weights of seminal vesicle and vas deferens in male pups during adult life, besides alterations in their sexual behavior. It was also observed that adult rats exposed to formulated fenvalerate, by inhalation, exhibited a significant reduction in the testis weight and epididymal sperm count. Little is known about the mechanisms by which fenvalerate exerts its action on reproduction; thus, the objective of the present study was to investigate the estrogenic activity and the effects of fenvalerate on the reproductive system and fertility of adult male rats. For this, adult male rats (aged 90 days) received, for 30 consecutive days, by oral gavage, 40 mg/kg/day of fenvalerate (technical grade; 96.8% purity). The control group received only the vehicle (corn oil), in the same experimental conditions. At the end of the treatment, the following parameters were analyzed: body weight; absolute weight of reproductive organs, liver and kidneys; plasma testosterone levels; germ cell count in the testis and epididymis; sperm morphology; fertility tests by natural matings and artificial insemination *in utero*; ejaculated sperm counts in uterus; sexual behavior; analysis of testis and epididymis at the optical and electron microscopic levels, and evaluation of possible estrogenic activity of different doses (0.4; 1.0; 4.0; 8.0 and 40 mg/kg) of fenvalerate by the uterotrophic test. Fenvalerate residues were quantified using High Performance Liquid Chromatography (HPLC) in reproductive and vital organs; sperm and epididymal protein were also realized. The results were compared by Student-t and Mann-Whitney tests, according to the characteristics of each variable, while the results of the uterotrophic test were compared by ANOVA followed by the Tukey test. The results of fenvalerate quantification revealed that

the pyrethroid was retained in reproductive (testis and epididymis) and vital organs (brain and liver). The treatment with fenvalerate decreased the absolute weights of testis and epididymis. Furthermore, the treatment did not provoke reduction of plasma testosterone levels. It was also verified that the treated rats presented a reduction in daily sperm production and in epididymal sperm number. The fertility tests did not reveal differences related to the treatment. The results of the fenvalerate quantification revealed high concentrations of insecticide residues in the epididymis, testis, brain and liver. The histopathology of the testis and epididymis as well as analysis of sperm and epididymal proteins did not show alterations. Moreover, fenvalerate, at the tested doses, did not present estrogenic activity *in vivo*. It was concluded that fenvalerate, in these experimental conditions, was retained in reproductive organs and was spermatotoxic, since it reduced sperm production and storage, but this alteration was not sufficient to compromise fertility by virtue of the high reproductive efficiency of rodents in contrast with humans.

INTRODUÇÃO

O comprometimento da função reprodutiva de seres humanos e de espécies animais tem sido motivo de especial preocupação nos últimos anos. Muitos fatores podem interferir com os componentes e com a função reprodutiva e ocasionar infertilidade e outras alterações funcionais e estruturais. Doenças, fatores psicológicos, estresse, variações hormonais e exposição a substâncias químicas são alguns dos fatores que contribuem para o surgimento de distúrbios no sistema reprodutor masculino (Neubert e Chahoud, 1995). Efeitos induzidos por substâncias químicas podem ocorrer pela interação direta da substância com componentes do sistema reprodutor ou indiretamente pela interferência na regulação endócrina, uma vez que o desenvolvimento e manutenção do sistema reprodutivo é particularmente dependente de uma série de interações hormonais (Whitley et al., 1994; Neubert e Chahoud, 1995). Sendo assim, qualquer tóxico que atue desordenando a interação coordenada do eixo hipotálamo – hipófise – gônada, responsável pela regulação endócrina, pode levar a anormalidades reprodutivas (Sokol, 1997).

Para considerarmos a importância dos efeitos de substâncias tóxicas sobre o sistema reprodutor masculino, torna-se necessário entender seu aspecto geral.

1. Aspectos gerais da morfologia e fisiologia do sistema reprodutor masculino

O sistema reprodutor masculino da maioria dos mamíferos é composto por testículos (gônadas), epidídimos, ductos deferentes, glândulas sexuais e órgão copulador (Figura 1).

Cada testículo pode ser, funcionalmente e anatomicamente, dividido em duas partes: tecido intersticial e túbulos seminíferos, responsáveis pela esteroidogênese e pela espermatogênese, respectivamente (Rodrigues e

Favaretto, 1999). Os túbulos seminíferos são constituídos pelo epitélio seminífero, células de Sertoli e tecido peritubular e, em animais adultos, ou seja, animais sexualmente maduros, possuem ainda uma ou duas gerações de espermatogônias, espermatócitos e espermátides (Figura 2). A espermatogênese é um processo elaborado pelo qual células-tronco espermatogoniais tornam-se células haplóides altamente diferenciadas, os espermatozóides (Clermont, 1972). O tecido intersticial fica entre os túbulos seminíferos e possui vasos sanguíneos e linfáticos, nervos e as células intersticiais ou de Leydig responsáveis pela produção de andrógenos, principalmente testosterona, e fonte para uma variedade de outros esteróides (Russell et al., 1990). A função testicular é regulada por uma série de relações entre o hipotálamo, a hipófise, os hormônios testiculares e o compartimento germinativo (Sokol, 1997).

Nos mamíferos em geral, os epidídimos são divididos anatomicamente em três regiões conhecidas como cabeça (com o segmento proximal), corpo e cauda (Reid e Cleland, 1957). Estas porções são subdivididas histologicamente em zonas que são designadas de acordo com a altura do epitélio e a distribuição e quantidade dos tipos celulares. Seu epitélio pseudoestratificado ciliado possui seis tipos celulares: basais, principais, estreitas, halos, claras e apicais (Hermo e Robaire, 2002). Já se sabe que em todas as espécies que estão sendo estudadas é necessário que o espermatozóide passe pela região proximal do epidídimo para que ocorra o processo de maturação espermática (capacidade para motilidade, reconhecimento e penetração pela zona pelúcida do oócito) (Jones, 1999). Há evidências de que este processo de maturação ocorra pela ação de proteínas do epidídimo produzidas e secretadas sob controle de andrógenos (Orgebin-Crist e Jahad, 1978).

A vesícula seminal consiste de um ducto único muito dilatado e enovelado. Este ducto é revestido por um epitélio pseudo-estratificado pregueado, constituído por células epiteliais secretoras e células basais. A camada muscular lisa que reveste o órgão é constituída por duas lâminas: uma interna, de fibras circulares, e outra externa, de fibras longitudinais. A luz é ocupada pelo produto de secreção,

de aspecto hialino (Hayward et al., 1996a,b).

A próstata é formada por um conjunto de glândulas tubuloalveolares ramificadas, cujos ductos desembocam na uretra prostática. O epitélio é colunar simples formado por células secretoras, basais e neuroendócrinas. Ela é envolta por uma cápsula fibroelástica rica em músculo liso, que envia septos para o interior da glândula. No homem, este órgão é compacto (alobular) apresentando três zonas: central, periférica e de transição. No rato, a próstata é dividida em quatro pares de lóbulos definidos como próstata anterior, dorsal, ventral e lateral (Roy-Burman et al., 2004).

Os produtos de secreção da vesícula seminal e próstata contribuem para a nutrição e suporte dos espermatozóides fora do trato genital masculino. As funções destas glândulas são dependentes de estímulo androgênico como a testosterona, que atua diretamente nos órgãos sexuais acessórios masculinos (Mann, 1974).

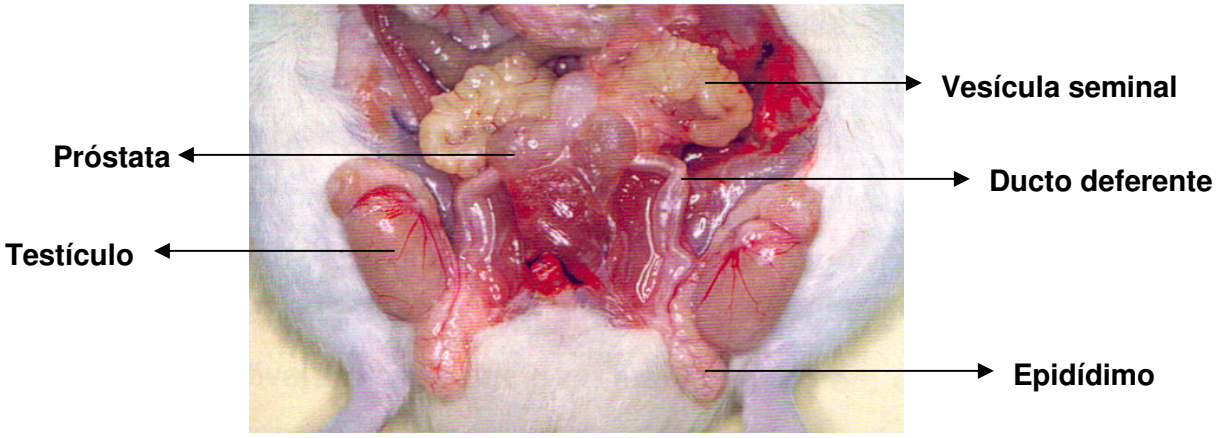


Figura 1. Órgãos reprodutores de rato macho Wistar

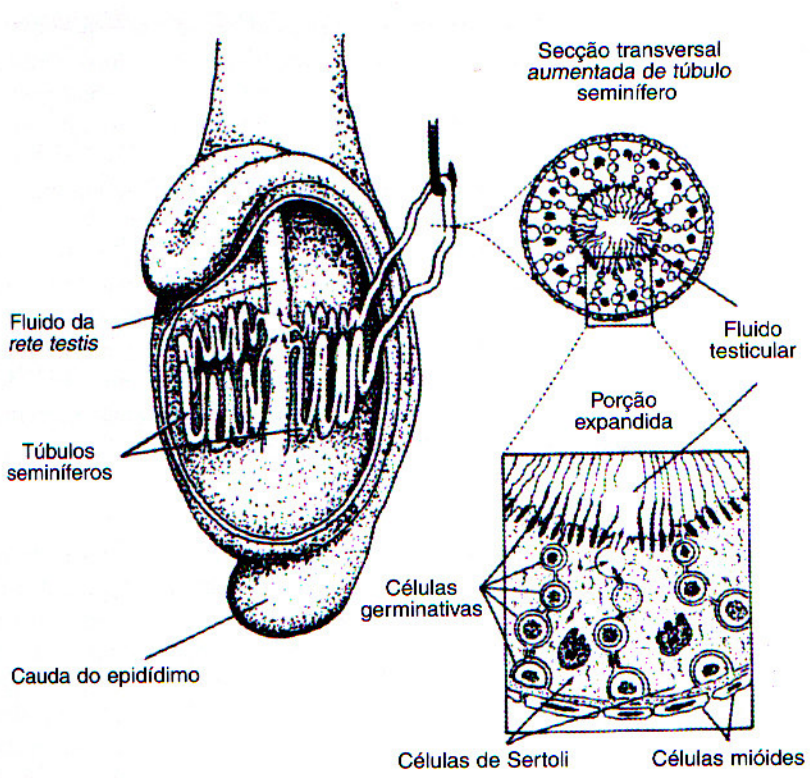


Figura 2. Representação esquemática da estrutura do testículo.
Fonte: Hafez, E.S.E., Hafez, B., 2004. Reprodução Animal. Manole, p.105.

1.1 Desreguladores Endócrinos

No início de 1947, nos Estados Unidos, uma correlação entre a contaminação ambiental e o aparecimento de doenças e alterações reprodutivas na população humana e em animais começou a preocupar a opinião pública (Tarin, 1972). A partir destas evidências iniciou-se uma série de debates entre a comunidade científica de diversos países e as agências regulatórias internacionais (United States Environmental Protection Agency -US EPA, Organization for Economic Co-operation and Development -OECD, Food and Drug Administration - FDA) a respeito dos efeitos adversos que podem resultar da exposição a um

grupo de químicos que têm o potencial de alterar o funcionamento normal do sistema endócrino de animais e humanos. Entre estas alterações está um significante declínio na qualidade e quantidade do sêmen humano (Auger et al., 1995; Irvine et al., 1996; Van Waeleghem et al., 1996). Ao mesmo tempo, a incidência de hipospadia e criptorquidia vem crescendo consideravelmente (Cour-Palais, 1966; Czeizel, 1985; Matlai e Beral, 1985; Yucesan et al., 1993), assim como o câncer testicular, o qual é agora a malignidade mais comum encontrada em homens jovens (Nethersell et al., 1984; Boyle et al., 1987; Pike et al., 1987; Forman e Moller, 1994).

Em paralelo com o crescente debate sobre a qualidade espermática e desordens do trato urogenital na espécie humana, vários pesquisadores têm se preocupado em avaliar disfunções reprodutivas em espécies animais selvagens (gastrópodos, répteis, peixes, aves e mamíferos). R. Carson, no seu livro “Silent Spring”, publicado em 1962, já havia apontado problemas reprodutivos sérios em aves, e sugeriu que pesticidas seriam os agentes causadores. Além disso, nos anos 80 T. Colborn e colaboradores, trabalhando para o World Wildlife Fund, descobriu que 16 espécies de predadores de diferentes classes de Vertebrados, que vivem na área dos Grandes Lagos, nos Estados Unidos, apresentavam também sérios distúrbios reprodutivos. Este trabalho abriu a discussão a respeito dos poluentes que têm propriedades parecidas com hormônios, e que, portanto, agem como tal no organismo.

Os agentes químicos que possuem propriedades hormonais são conhecidos como desreguladores endócrinos ou xenohormônios, e são definidos como “agentes exógenos que interferem nos processos de produção, liberação, transporte, metabolismo, ligação, ação ou eliminação de hormônios naturais do organismo, responsáveis pela manutenção da homeostase e regulação dos processos de desenvolvimento” (Kavlock et al., 1996).

A interferência com a produção ou ação de hormônios esteroidais pode ocasionar diversos prejuízos para o trato reprodutor masculino. Os desreguladores endócrinos podem apresentar similaridades estruturais com os hormônios

esteroidais endógenos, e se ligarem aos seus receptores citoplasmáticos (Neubert, 1997). Assim, em investigações laboratoriais esses compostos apresentaram atividades que mimetizam ou antagonizam aquelas dos hormônios sexuais esteroidais, e que, portanto tornam esses compostos fortes candidatos a agentes causadores da deteriorização da qualidade espermática e de desordens do trato reprodutivo masculino (Sharpe e Skakkebaek, 1993). Diversos contaminantes químicos ambientais, tais como inseticidas, herbicidas, fungicidas, plastificantes, entre outros, já foram identificados como tendo atividades anti-androgênica e/ou estrogênica, atuando assim como desreguladores endócrinos (Kelce et al., 1997; Gray et al., 1999; Parks et al., 2000; Jarfelt et al., 2005).

1.2 Inseticidas Piretróides

Nas últimas décadas houve um crescimento significativo na utilização de pesticidas no combate a pragas da lavoura, assim como no combate de endo e ectoparasitas na área veterinária (Ecobichon, 1996). Apesar de seus benefícios no aumento da produtividade agropecuária, o uso dos pesticidas também passou a representar um sério risco ao ambiente e à saúde humana e animal.

Os inseticidas piretróides são uma classe de compostos orgânicos natural e sintético amplamente utilizados no mundo, tanto em áreas agrícolas quanto no controle de insetos domésticos. Embora sejam considerados de baixa toxicidade para o homem, em comparação a outras classes de inseticidas, a exposição à piretróides tem sido associada a efeitos reprodutivos agudos, bem como a alterações crônicas no desenvolvimento (He, 1994; Miyamoto et al., 1995; Tanenbaum et al., 1998).

As piretrinas naturais são ésteres de extrato seco de flores de *Chrysanthemum cinerariaefolium*. Modificações físico-químicas nas estruturas das piretrinas melhoram sua estabilidade e propriedades, originando os inseticidas piretróides sintéticos (Soderlund et al., 2002). Várias formulações de inseticidas

freqüentemente combinam piretrinas e piretróides com outros químicos, conhecidos como sinergistas, para aumentar a potência e a persistência no ambiente. Baseados na estrutura química e nos sinais e sintomas de toxicidade em animais, os piretróides sintéticos podem ser divididos em duas classes: piretróides do Tipo I, como o aletrim, e do Tipo II, como a cipermetrina, a deltametrina e o fenvalerato. Os piretróides do Tipo I, os quais não contêm um grupo ciano na posição alfa carboxil, prolongam a abertura de canais de sódio nas membranas nervosas, produzindo descargas neuronais repetidas, acarretando em uma hiper-excitabilidade em animais intoxicados. Os piretróides do Tipo II, que possuem um grupamento ciano em sua molécula, promovem um prolongamento ainda maior na abertura de canais de sódio levando a despolarização da membrana e bloqueio da condução nervosa em axônios sensoriais e motores e provocando disparos repetidos em fibras musculares (IPCS, 1990; Ecobichon, 1996; Barlow et al., 2001). Além de seus efeitos sobre os canais de sódio, os piretróides do Tipo II também podem atuar através de outros mecanismos, como a inibição da Ca^{+2}, Mg^{+2} ATPase, aumentando os níveis intracelulares de cálcio e a liberação de neurotransmissores, e a ação inibitória sobre os receptores do ácido gama-aminobutírico (GABA) (IPCS, 1990; Ecobichon, 1996; Clark, 1997; Forshaw et al., 2000).

O fenvalerato [(RS)-α-ciano-3-fenoxibenzil (RS)-2-(cloro-fenil)-3-metilbutirato] (Figura 3) é um inseticida piretróide do Tipo II desenvolvido para substituir outros grupos de inseticidas, devido ao melhoramento em sua potência inseticida (Who, 1990). O fenvalerato é uma mistura de 4 isômeros ópticos, sendo sua fórmula molecular $C_{25}H_{22}ClNO_3$. Apresenta-se sob a forma de um líquido amarelado viscoso, o qual possui um odor característico. Possui boa estabilidade química, resistindo à degradação pelo calor e pela luz. O fenvalerato tem sido muito utilizado no combate de pragas de diversas culturas como algodão, arroz, café, feijão, milho, batata, soja, tomate, trigo, como também para o controle de insetos domésticos. São produzidas aproximadamente 1000 toneladas de fenvalerato por ano. A Organização Mundial da Saúde classifica o fenvalerato

como um produto moderadamente tóxico (IPCS, 1990). Este inseticida é moderadamente persistente no solo por causa de sua baixa solubilidade em água e seu alto coeficiente de partição octanol-água (Adelsbach e Tjeerdema, 2003). Além disso, o fenvalerato tem o potencial de se acumular em sedimentos aquáticos e na biota. A população humana está exposta a este inseticida ocupacionalmente e também através do consumo de alimentos que contenham resíduos do piretróide. Quantidades significativas de resíduos de fenvalerato têm sido detectadas em frutas e vegetais (Patel et al., 1990; Boyer et al., 1992). Em um estudo realizado nos E.U.A., maças foram tratadas com um concentrado emulsificado a 30% de fenvalerato, por 4 vezes, em uma taxa de 0,67kg de ingrediente ativo/ha. Os níveis residuais encontrados foram de 2,2 mg/kg em maças inteiras, 7,3 mg/kg na casca e 0,03 mg/kg em maças descascadas seis semanas após a última aplicação (FAO/Who, 1980).

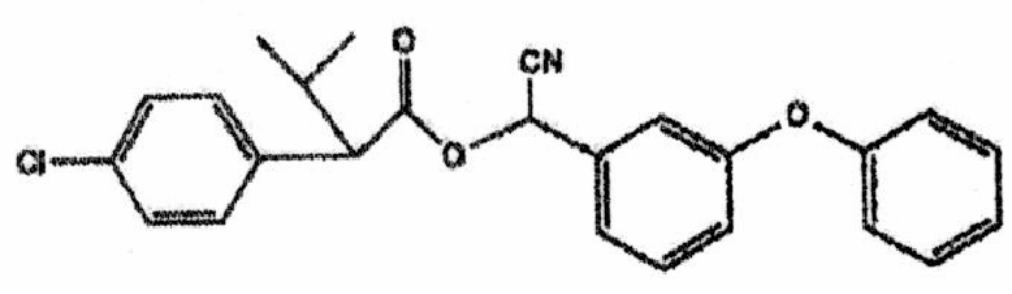


Figura 3. Estrutura química do fenvalerato

O fenvalerato pode ser absorvido através do trato gastrointestinal, da pele e por inalação (IPCS, 1990). Em animais experimentais, este inseticida é rapidamente hidrolisado, sendo o ácido fenvalérico seu maior metabólito (Kaneko et al., 1981). O modo como o organismo metaboliza este inseticida inclui dissolução de pontes de ésteres pela ação da esterase e oxidação de várias

partes da molécula, além da indução de enzimas microssomais hepáticas (Who, 1990). O valor da DL_{50} (Dose letal para 50% de um grupo de animais) do fenvalerato varia consideravelmente entre 82 – 3200mg/kg/peso corporal, dependendo da espécie animal, via de exposição e excipiente (Fenvalerate – Environmental Health Criteria 95/ World Health Organization, 1990). Segundo o International Program on Chemical Safety – Fenvalerate, publicada pela WHO em julho de 1996, a DL_{50} via oral para ratos é de aproximadamente 400 mg/kg. No entanto, de acordo com um estudo realizado por uma equipe de pesquisadores da Sumitomo Chemical Company (Japão), no qual o fenvalerato foi administrado para ratos, por via oral, diluído em óleo de milho, a DL_{50} para ratos machos é igual a 100 mg/Kg/dia (Kunimatsu et al., 2002).

Mandal et al. (1996) estudaram a cinética do fenvalerato em cabras após aplicação dérmica de 100 ml de uma solução 0,25% (peso/volume). O maior nível residual sanguíneo foi detectado 2 horas após a aplicação (3,67 mg/l), que foi decrescendo gradualmente, sendo que 72 horas após a aplicação do produto foi detectado o menor valor residual (0,24 mg/l). Nesses mesmos animais, quatro dias após a aplicação, as maiores concentrações de fenvalerato foram encontradas na glândula adrenal, bíceps, omento, fígado, rim e cérebro, respectivamente. Bissacot e Vassilieff (1997) detectaram a presença dos inseticidas piretróides cipermetrina, deltametrina e flumetrina, em amostras de sangue e leite de 40 vacas que foram previamente tratadas por aplicação dérmica com única dose terapêutica desses piretróides. Observou-se que os resíduos estiveram presentes no organismo dos animais até o 28º dia após as aplicações dos produtos.

Porém, são poucos os trabalhos que relatam a cinética dos piretróides no organismo. Croucher et al. (1985), em estudo com vacas lactantes que receberam juntamente com a dieta doses orais de cipermetrina, mostraram que as maiores vias de excreção desse piretróide foram a urina e as fezes e, em menor quantidade, o leite. A persistência de piretróides no sangue e leite também foi relatada por Mandal et al. (1995) em cabras lactantes que receberam, por via intravenosa, doses únicas de cipermetrina (57 mg/kg) e fenvalerato (45 mg/kg).

Além dos efeitos clássicos atribuídos aos inseticidas piretróides, tais como neurotoxicidade, estudos indicam que o fenvalerato pode ser genotóxico para células somáticas e germinativas de animais e humanos (Chatterjee et al., 1982; Pati e Bhunya, 1989; Puig et al., 1989). Além do efeito genotóxico, este piretróide tem sido apontado recentemente como indutor de estresse oxidativo em tecidos animais (Gupta et al., 1999; Giray et al., 2001; Prasanthi, 2001; Prasanthi et al., 2005).

1.3 Piretróides e Reprodução

Estudos têm demonstrado que a exposição a inseticidas piretróides está associada com efeitos reprodutivos agudos, podendo também produzir efeitos crônicos e alterações no desenvolvimento (He, 1994; Miyamoto et al., 1995; Garey e Wolff, 1998; Landrigan et al., 1998; Tanenbaum et al., 1998). No entanto, esses estudos ainda são poucos e, além disso, as condições experimentais são diferentes, especialmente no que diz respeito à pureza e origem dos compostos utilizados para o tratamento dos animais. Foi documentado que ratos expostos a cipermetrina, por 12 semanas, mostraram uma redução no número de espermatozóides e na produção espermática diária (Elbetieha et al., 2001). Abd El-Aziz et al. (1994) demonstraram que a administração oral de 1,0 e 2,0 mg/kg de deltametrina, para ratos adultos, durante 65 dias, provocou efeitos adversos em diversos parâmetros reprodutivos, tais como redução nos níveis plasmáticos de testosterona e no número de espermatozóides.

Moniz et al. (1999) demonstraram que a exposição de ratas a 10 mg/kg de fenvalerato no 18° dia de gestação e do 1° ao 5° dia de lactação reduziu significativamente os níveis plasmáticos de testosterona e os pesos da vesícula seminal e do ducto deferente dos descendentes machos na idade adulta. Foram observadas também alterações no comportamento sexual desses animais, indicando assim a interferência do fenvalerato na fisiologia reprodutiva de ratos machos. Em experimento desenvolvido em nosso laboratório, a administração de

fenvalerato técnico (96% de pureza), na dose de 40 mg/kg, à ratas do 12° dia de prenhez até o final da lactação, foi transferido pela placenta e pelo leite materno, provocando efeitos tardios no desenvolvimento reprodutivo da prole masculina (Nassr, 2005).

Mani et al. (2002), estudando a exposição, por inalação, de ratos adultos a uma formulação contendo fenvalerato, durante três meses, relataram diminuição do peso dos testículos, da contagem espermática no epidídimo, da motilidade espermática e da atividade de enzimas testiculares para biossíntese de testosterona nos animais tratados. Da mesma forma, um grupo de pesquisadores chineses, que administraram fenvalerato para ratos adultos, por 15 e 30 dias, relataram diminuição da produção espermática, e correlacionaram esses dados com depleção androgênica e danos sobre as células de Sertoli e epitélio germinativo (Hu et al., 2002). Evidenciou-se também que a exposição ocupacional ao fenvalerato pode afetar a qualidade do sêmen de trabalhadores, especialmente a contagem e a motilidade espermática (Tan et al., 2002). Esses dados sugerem que o fenvalerato apresenta toxicidade reprodutiva masculina.

Os pesticidas piretróides sintéticos vêm sendo apontados como possíveis desreguladores endócrinos (US EPA, 1997). Recentemente, vários estudos *in vitro* e *in vivo* têm investigado as atividades estrogênicas e anti-androgênicas de diversos piretróides. Alguns estudos demonstraram que o fenvalerato, entre outros piretróides, possui atividade estrogênica (Eil e Nissula, 1990; Garey e Wolff, 1998; Go et al., 1999; Chen et al., 2002) ou anti-androgênica *in vitro* (Xu et al., 2006). Em contrapartida, outros pesquisadores concluíram que os piretróides, em baixas doses, não possuem efeitos estrogênicos nem anti-androgênicos *in vitro* (Gaido et al., 1997; Nishihara et al., 2000; Saito et al., 2000a,b; Sumida et al., 2001a,b). Recentemente, Kunimatsu et al. (2002) testando as doses de 20, 40 ou 80 mg/kg/dia de fenvalerato em ratos, não encontraram atividades hormonais *in vivo*.

2. JUSTIFICATIVA E RELEVÂNCIA DO TEMA

Os desreguladores endócrinos, dentre eles os inseticidas piretróides, constituem uma classe de compostos com possíveis atividades estrogênicas, que podem causar disfunções reprodutivas importantes no sexo masculino. Tem crescido o interesse, por parte de vários países, em se estudar esses agentes químicos, introduzidos ou disseminados pelo homem no ambiente, pois existem evidências de que a qualidade espermática humana e em outros animais tem diminuído, paralelamente ao aumento de problemas do trato reprodutor masculino.

Embora existam inúmeras evidências a respeito da neurotoxicidade do fenvalerato, pouco é conhecido sobre sua toxicidade reprodutiva. Adicionalmente, os trabalhos existentes sobre a ação deste inseticida sobre a reprodução são discrepantes, sendo necessária a realização de mais pesquisas sobre as possíveis propriedades hormonais destes compostos e o seu impacto na saúde reprodutiva humana.

Assim, o tema do presente projeto, além de atual, tem grande aplicabilidade, pois tem aumentado a preocupação com os danos que o homem vem causando ao ambiente, e um dos aspectos influenciados por esse fato é a reprodução dos organismos, responsável pela manutenção das espécies.

3. OBJETIVO

Avaliar a potencial atividade estrogênica e os efeitos do inseticida piretróide fenvalerato no sistema reprodutor e fertilidade de ratos machos adultos.

4. CAPÍTULO

Este trabalho deu origem ao artigo **"Reproductive toxicity of the pyrethroid insecticide fenvalerate on adult male rats"** que foi submetido para o periódico "Toxicology".

Reproductive toxicity of the pyrethroid insecticide fenvalerate on adult male rats

Arielle Cristina Arena[a], Carla Dal Bianco Fernandez[a], Elaine Manoela Porto[a], Denise Zuccari Bissacot[b], Oduvaldo Câmara Marques Pereira[c], Wilma De Grava Kempinas[d*]

[a]Department of Cell Biology, Institute of Biology, State University of Campinas, Campinas, SP, Brazil. [b]Center for Toxicological Assistance (CEATOX), Institute of Biosciences, São Paulo State University, Botucatu, SP, Brazil, [c]Department of Pharmacology, Institute of Biosciences, São Paulo State University, Botucatu, SP, Brazil, [d]Department of Morphology, Institute of Biosciences, São Paulo State University, Botucatu, SP, Brazil.

*Corresponding author:

Wilma De Grava Kempinas
Departamento de Morfologia
Instituto de Biociências – UNESP
Caixa Postal 510
18618-000, Botucatu, SP, Brazil
Tel: + 55 14 3811 6264 ext. 104; Fax: + 55 14 3811 6264
E-mail address: kempinas@ibb.unesp.br

Running Title: Reproductive toxicity of the fenvalerate in male rats

Reproductive toxicity of the pyrethroid insecticide fenvalerate on adult male rats

Abstract

Fenvalerate is a pyrethroid insecticide widely used to control a wide range of pests. However, little is known about its direct reproductive toxicity. The objective of this work was to investigate the potential estrogenic activity and the reproductive and fertility effect of fenvalerate on adult male rats. Adult animals were treated, for 30 consecutive days, with 40 mg/kg/day of fenvalerate or corn oil (vehicle), by gavage (oral rote). Fenvalerate, at the doses tested (0.4; 1.0; 4.0; 8.0 and 40mg/kg), was not able to produce estrogenic activity *in vivo*, as assessed by uterotrophic assay. Exposure to fenvalerate was toxic to the testis and epididymis, as shown by the decrease in the absolute weights and sperm counts of the testis and epididymis. Fenvalerate residues, measured by HPLC, were found in the testis and epididymis of treated animals. While the sperm counts were reduced, the fertility and sexual behavior were similar in both groups. It was concluded that fenvalerate, in these experimental conditions, was not able to produce estrogenic activity *in vivo* at the tested doses. Furthermore, fenvalerate was retained in reproductive organs and was spermatotoxic, since it reduced sperm production and storage. However, this alteration was not sufficient to compromise fertility by virtue of the high reproductive efficiency of rodents in contrast with human.

Key words: Endocrine disruptors; Pyrethroids; Fenvalerate; Sperm production; Reproductive toxicity; Male rats.

1. Introduction

There is evidence, as yet unconfirmed, that sperm quality in humans and other animals has diminished (Jégou et al., 1999; Paumgartten, 2003), in parallel with increase of problems of the male reproductive tract such as testicular cancer, cryptorquidism, and hypospadia. It is speculated that these problems are due to environmental modifications presented over the last 50 years, during which considerable changes have occurred in the physical, chemical, biological and socio-cultural contexts.

Several chemical substances present in environmental pollution have the capacity to act as hormones, which can affect the natural hormones in the body, interfering in important biological processes (Bhatt, 2000). For this reason are known as endocrine disruptors (Paumgartten, 2003). Endocrine disruptor is an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and development processes (Kavlock et al., 1996).

Synthetic pyrethroids are among the most common pesticides in current use worldwide (Naumman, 1990), yet little has been done to assess their potential hormonal activities. These pyrethroids are relatively safe due to a high insect/mammalian toxicity ratio (Elliot and Janes, 1978), although pyrethroid exposure have been associated with acute reproductive effects and also may produce chronic and developmental impairments (He, 1994; Miyamoto et al., 1995; Landrigan et al., 1998). Human exposure may occur occupationally during insecticide application, or through pyrethroid residues such as those detected in cow milk, bread, fruits, vegetables and carpeting (Who, 1990; Landrigan et al., 1998).

Fenvalerate, a member of the type II synthetic pyrethroid family, is widely used in agricultural and other domestic applications (Adelsbach and Tjeerdema, 2003). Some recent reports indicate that pyrethroids, such as fenvalerate, are

linked to endocrine disruption, subsequently leading to reproductive dysfunction (Lifeng et al., 2006) and have aroused worldwide concern. However, only a few studies have been conducted on the reproductive effects of fenvalerate. In addition, the available experimental data are contradictory, in part due to the different origins and purities of fenvalerate utilized in experiments and to the different substances employed as vehicle of administration to animals. Abd El-Aziz et al. (1994) showed that oral administration of 1.0 and 2.0 mg/kg of deltamethrin, to adult rats, for 65 days, provoked adverse effects in several reproductive parameters, such as decrease in plasma testosterone levels and sperm counts. It was also reported that rats exposed to cypermethrin, for 12 weeks, exhibited a reduction in sperm count and daily sperm production (Elbetieha et al., 2001). It was also demonstrated that perinatal exposure to 10 mg/kg of fenvalerate decreased testosterone levels and weights of the seminal vesicle and vas deferens (Moniz et al., 1999). Mani et al. (2002) also reported that treatment of adult male rats with different doses of formulated fenvalerate, by inhalation, five days a week, for 3 months, was able to reduce testis weight, epididymal sperm counts and testicular enzymes activities for testosterone biosynthesis. Recently, He et al. (2004) reported that fenvalerate is able to disrupt endocrine function by altering FSH-stimulated steroidogenesis.

Several studies have shown that certain compounds of the pyrethroid family possess estrogenic properties and may function as xenoestrogens. Some synthetic pyrethroids, such as sumithrin, fenvalerate and alethrin, are able to elicit *in vitro* estrogenic responses (Eil and Nissula, 1990; Garey and Wolff, 1998; Go et al., 1999). However, Kunimatsu et al. (2002) demonstrated that treatment with different doses (20, 40 or 80 mg/kg/day) of fenvalerate in rats, did not show hormonal response *in vivo*. On the other hand, Moniz et al. (2005) attributed an anti-estrogenic effect to fenvalerate, after perinatal treatment in female rats. Because study on endocrine disruption is an emerging field, studies are needed to enhance knowledge of endocrine disruptors.

In the context of the few conflicting studies on the effects of fenvalerate on

the reproductive system, the objective of the present work was to evaluate a potential estrogenic activity and the reproductive and fertility effect of fenvalerate on adult male rats.

2. Materials and methods

Animals

Adult male (n=135) and female (n=100) Wistar rats were supplied by Central Biotherium of the São Paulo State University and maintained in polypropylene cages (43x30x15 cm) with bedding of wood shavings, 2 animals/cage, under controlled temperature (±23°C), with a constant 12 h light-dark cycle and free access to food and water. The animals used in this study were maintained in accordance with Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by the Biosciences Institute/UNESP Ethical Committee for Animal Research (Protocol number: 094/03).

Experimental groups

Adult male rats (aged 90 days) were treated for 30 consecutive days (once a day) by gavage (oral route) with technical grade fenvalerate (purity 96.8%; Sumitomo Chemical, Japan) at the 40 mg/kg/day dose, at a volume of 2 ml/kg/day. According to Kunimatsu et al. (2002), who utilized fenvalerate from the same precedence in their experiments, dissolved in corn oil, this dose corresponds to 2/5 of the dose lethal to male rats (LD50 = 100mg/kg). The chemical was dissolved in corn oil (vehicle) before administration. Control animals received only vehicle. The rats were weighed on alternate days.

Uterotrophic assay

For the uterotrophic assay were used immature female rats (21±1days old, n=6), as normally used in this type of study (Ashby et al., 1997; Odum et al., 1997;

kang et al., 2000; Andrade et al., 2002). The fenvalerate was given daily for 3 consecutive days by oral gavage. Five dose levels of fenvalerate (0.4; 1.0; 4.0; 8.0 and 40 mg/kg/day) were used to assess possible estrogenic activity. The vehicle (corn oil) was administered as a negative control while estradiol benzoate (β-Estradiol 3-Benzoate, Sigma; 0.4 mg/kg/day) was used as a positive control for estrogenicity.

Twenty-four hours after the final dose, animals were weighed and anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Uteri were excised, trimmed free of fat, pierced, and blotted to remove fluid. The body of the uterus was cut just above its junction with the cervix and the junction of the uterine horns with the ovaries (Odum et al., 1997). Wet uterus weights were determined and expressed as relative weights (wet uterus weight/body wt x 100).

Body weight and weight of male reproductive organs, liver and kidneys

At the end of the treatment, 8 or 9 rats per group were weighed and killed by decapitation. The left testis and epididymis, vas deferens, ventral prostate, seminal vesicle (without the coagulating gland and full of secretion), liver and kidneys were removed and their absolute weight determined.

Testosterone levels

For determination of plasma testosterone levels, blood collected from the cervical vessels ruptured by decapitation was collected between 9:00 and 11:30 h, in a heparinized tube and then centrifuged in a refrigerated apparatus at 2400 rpm for 20 minutes at 3.5^{o}C, for separation of plasma, which was frozen at – 20ºC until the moment of the hormonal determination. Plasma testosterone levels were determined by double-antibody radioimmunoassay technique utilizing Kit Coat-A-Count® Total Testosterone (DPC, Los Angeles, CA, USA). Intra- and interanalysis variations were 1.75% and 20%, respectively.

Daily sperm production per testis, sperm counts and transit time in the epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) in the testis and sperm in the caput/corpus and caudal epididymidis portions were counted as described previously (Robb et al., 1978), with adaptations described as follows: the right testis, decapsulated and weighed soon after collection, was homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a dilution of 10 times, a sample was transferred to Newbauer chambers (4 fields per animal), preceding a count of mature spermatids. To calculate daily sperm production per testis (DSP) the number of spermatids at stage 19 was divided by 6.1 days, which is the number of days these spermatids are present in the seminiferous epithelium. Next, the DSP per gram was calculated in order to determine the efficiency of the process (Ashby et al., 2003). In the same manner, caput/corpus and cauda epididymidis portions were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by DSP.

Evaluation of sexual behavior

For this study 10 rats per group were utilized, soon after termination of treatment. For observation of sexual behavior, male rats were placed individually in boxes of polycarbonate crystal, measuring 44x31x16 cm, five minutes before introduction of one adult female in natural estrus (sexually receptive) determined by vaginal smear. The animals were observed in the dark period of the cycle with the aid of red lamps. All sexual behavior tests were conducted performed 2-4 h after the beginning of the dark period. For the next 40 minutes the following parameters were evaluated: latency to the first mount, intromission and ejaculation;

number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions and number of ejaculations (Ahlenius and Larsson, 1984; Ågmo, 1997). The males who did not mount in the initial 10 minutes were considered sexually inactive.

Fertility tests

Natural Mating

In the case of rats that ejaculated during evaluation of sexual behavior, the couples stayed together for an additional four hours, permitting a greater number of ejaculations. The animals that had been deemed inactive were tested again for, at the maximum, five days, during which different females in estrus were placed in the boxes of the males during the dark period of the cycle. At the end of the afternoon males and females were separated and vaginal smears were collected. The day sperm were found in the smear was determined to be day zero of gestation. On the 20th day of gestation the females were killed by decapitation. After collection of the uterus and ovaries the number of corpora lutea, implants, reabsorptions, live fetuses and dead fetuses were determined. From these results the following parameters were calculated: gestation rate, number of pregnant females/number of inseminated females x 100; fertility potential (efficiency of implantation): implantation sites/corpora lutea x 100; rate of pre-implantation loss: number of corpora lutea – number of implantations/number of corpora lutea x 100; rate of post-implantation loss: number of implantations – number of live fetuses/number of implantations x 100; sex ratio: number of male fetuses/number of female fetuses x 100.

In utero artificial insemination

According to the technique of in utero artificial insemination a fixed number of sperm collected in the cauda epididymidis is inseminated directly into the uterus permitting evaluation of sperm quality, without the interference of other factors

such as alterations of the sexual behavior pattern and number of sperm available for ejaculation (Klinefelter, 2002).

Females in natural estrus were paired with sexually experienced, vasectomized males for 1 h. Receptive females (that exhibit lordosis) were selected for insemination. The isolation and preparation of distal cauda sperm for insemination was the same as described previously (Klinefelter et al., 2002). Just prior to the inseminations, 0.25 mg/ml bovine lipoprotein was added to the medium. Sperm were allowed to diffuse after the epididymal tubule was pierced with a n°10 scalpel blade. The dish was allowed to shake gently and, after 5 min of dispersion, an aliquot of sperm was diluted 1:10 with fixative (10% formalin in PBS with 10% sucrose, pH 7.4) and counted using a Newbauer chambers; sperm concentration ranged from 100 to 280 x 10^6/ml. Within 15 min, each uterine horn was injected with a volume containing 5 x 10^6 sperm. One female was inseminated per male. All inseminations were performed while the recipient female was in a surgical plane of sodium pentobarbital anesthesia. The bifurcation of the uterine horns was exposed through a low, midventral incision. Fine curved forceps were used to elevate each horn while the insemination volume was injected through the wall of each horn via an 18-gauge i.v. catheter attached to a 0.5-ml syringe. Each injection site was cauterized immediately upon withdrawal of the needle. When insemination was complete, the abdominal musculature was sutured. Females were killed 20 days later to evaluate fertility as described previously.

Sperm morphology

The cauda sperm suspensions used for the artificial insemination were diluted 1:10 with 10% neutral buffered formalin in Dulbecco's PBS with 5% sucrose, and the spermatozoa were evaluated for individual sperm morphology. Two hundred spermatozoa (heads only or intact sperm) per animal were evaluated for head and/or flagellar defects by phase-contrast microscopy (x200, total magnification) in wet preparations.

Ejaculated sperm counts after natural mating

The procedure was the same as described previously (Kempinas et al., 1998), with some adaptations. Each male was paired with a proestrous virgin female, for 4 h. The females were killed and uterine sperm were enumerated. A fine curved forceps was used to elevate the cervix was ligated. The uterine horns were excised and washed in PBS, transferred to a Petri dish containing 2 ml of warm Medium 199 (Sigma), and opened using small scissors. The dish was shaken gently, thereby allowing the uterine sperm to disperse. The sperm suspension was then transferred to a 15-ml conical tube and homogenized. A sample was diluted 5 times, and sperm were counted using a hemacytometer.

Histopathological analyses

The left testis and epididymis of 5 animals per group were removed and immersed in a fixative mixture of Karnovsky (2.5% glutaraldehyde, 8% paraformaldehyde). The pieces were included in resin and sectioned at 3 µm. The sections were stained with hematotoxilin and eosin (HE), and observed by light microscopy for general histopathological examination.

Electron microscopy analyses

This evaluation was conducted in order to assess possible effects of fenvalerate in the testis and epididymis that could not be revealed by optical microscopy. For the procedure of Transmission Electron Microscopy testis and epididymis fragments were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1M Sorensen phosphate buffer, pH 7.4. The material was postfixed for 2h in the dark in 1% osmium tetroxide in the same buffer, contrasted in block with aqueous solution of 5% uranyl acetate for 2h, dehydrated in acetone, embedded in araldite, and sectioned and stained with a saturated solution of uranyl acetate in

50% alcohol, and lead citrate. Electron micrographs were obtained using a Phillips - CM 100 transmission electron microscope.

Determination of fenvalerate residues

Soon after termination of treatment, 10 rats per group were killed by decapitation, collecting the brain, liver, testes and epididymis to determine fenvalerate residue by high-performance liquid chromatography (Liquid Chromatograph model 480 C, Instrumentos Científicos C.G.Ltda-São Paulo, Brazil). The procedure was the same as described previously by Bissacot and Vassilieff (1997a). Fenvalerate residues were extracted from the samples with chloridric acid and acetonitrile, and cleanup in column chromatographic with Florisil ®, n-hexane and n-hexane-diethyl ether. Quantitative measurements were made by the external standard technique and by measurements of the peak areas of the chromatograms.

Sperm and cauda protein analysis

Procedures are described in detail in the "2-D Electrophoresis using immobilized pH gradients: principles & methods" (Amersham Pharmacia Biotech, 1998). After termination of treatment, the left cauda epididymis of each male (control and treated groups, 8 animals per group) was removed and frozen at -80ºC. Prior to 2D gel eletroforesis, the cauda epididymis was homogenized in 2 ml of lysis solution consisting of 19.2 g urea, 1.6 g CHAPS and 800 µl pharmalyte 3-10 per 40 ml. Following a centrifugation (10.000 rpm, 5 min, 4^{o}C), the supernatant was removed and added 250 µl of rehydration solution consisting of 12 g urea, 0.5 g CHAPS, 50 µl bromophenol blue, 12.5 µl pharmalyte and 7 mg/2.5 ml DTT per 25 ml. Before this procedure, 125 µl of this solution was transferred to a ceramic strip holder. Isoelectric focusing (8000 V, 2:40 h) was carried out in mini isoelectric focusing gels (Immobiline DryStrip). Molecular weight separation was carried out in

mini 15% acrylamide gels (250 V, 1:30 h). Gels were silver stained using a PlusOne Silver Staining Kit (Amersham Pharmacia). All gel images were analysed using an Image Master System 2-D Elite (Amersham Pharmacia).

Statistical Analysis

Student's *t*-test and Mann-Whitney test were employed. ANOVA with the "a posteriori" Tukey test was utilized for uterotrophic assay. The results were considered significant for $p<0.05$.

3. Results

There was no significant difference among groups in the evolution of body weight during the experimental period (results not shown). In reference to reproductive organs, there was a reduction in the absolute weight of the testis and epididymis in the animals treated with fenvalerate. No differences were observed between groups in the absolute weight of the liver and kidneys. Furthermore, there was no significant difference in plasma testosterone levels among experimental groups (Table 1).

Application of the High Precision Liquid Chromatographic technique revealed the presence of fenvalerate residues in all organs analyzed from treated group. The highest concentrations of fenvalerate were found in the epididymis, followed by the brain, testis and liver, respectively (Table 2). The methodology utilized, whose detection limit is 0.001µg/g (micrograms of fenvalerate per gram of tissue), did not reveal fenvalerate residues in control animals.

Male rats treated with fenvalerate exhibited a significant decrease in the absolute (Figure 1) and relative numbers of spermatids in the testis and sperm in the caput/corpus and cauda of the epididymis, and a significant decrease in DSP (Table 3). In the same manner, there was a significant decrease in DSP/g of testis in the animals treated with fenvalerate. No differences were observed between groups in sperm transit time through the epididymis (Table 3).

Table 4 shows the results of sexual behavior. None of the parameters investigated were significantly different between the control and treated groups. Again, in relation to fertility, there were no significant differences among the experimental groups in any parameters evaluated (Table 5).

The morphological sperm analysis showed similarity between the groups in the percentages of normal and abnormal forms (control =96.5%; treated = 94.5%; n=10) and number of sperm ejaculated (control = $23.27 \times 10^6 \pm 7.84$; treated = $28.09 \times 10^6 \pm 11.62$; n=10).

The results of the uterotrophic assay are shown in Figure 2. The administration of different doses of fenvalerate did not alter the relative uterine weight of immature rats that received the insecticide for 3 days, relative to the control group. Estradiol (positive control) significantly increased relative uterine weight compared with the vehicle-treated group.

A quantitative analysis of cauda epididymal and sperm proteins by SDS-PAGE revealed treatment-related diminutions in only four proteins (Figure 3). The analysis of testis and epididymis at the optical and electron microscopic levels did not reveal morphological or ultrastructural alterations related to the treatment (data not shown).

4. Discussion

Fenvalerate has been listed as one type of endocrine-disrupting chemical (EDC) and has aroused worldwide concern (He et al., 2004). The present study evaluated the effects of the pyrethroid insecticide fenvalerate on the reproductive system and fertility of adult male rats.

The presence of residual concentrations of the pyrethroids in environment due to the use of different formulations may possibly contribute to humans exposure either by inhalation or skin resorption (Class, 1991; Curtis et al., 1998; Zaim et al., 2000). The detection and determination of unchanged insecticide residues in the stomach, intestine, liver, kidney, spleen, lungs, brain and other organs are useful indicators of exposure and help in accessing adverse health effects (Mcleese et al., 1980; Brempong-Yeboah et al., 1983; Mandal et al., 1996). Besides this, the evaluation of residues in the blood (Braun and Stanek, 1982; Bissacot and Vassilieff, 1997b; Ripley et al., 2001) and body fluids (Leng et al., 1996; Shan et al., 1999) gives an indication about the extent of exposure. It is known that fenvalerate is highly lipophilic and may infiltrate adipose tissue, testis and ovarian follicular fluid (Chen et al. 2005). In the present work, the analyses realized by High Performance Liquid Chromatography (HPLC) revealed the presence of fenvalerate residues in reproductive (testis and epididymis) and vital (brain and liver) organs, in which high insecticide concentrations were found in the testis and epididymis, organs were sperm is produced and stored, respectively. Thus, the insecticide residues found in these organs, in special in the epididymis, may have contributed to the reproductive effects discussed ahead.

Changes in absolute or relative reproductive organ weights provide sufficient evidence for initially classifying an agent as a potential male reproductive hazard (Zenick and Clegg, 1989). Absolute weight of the testes is preferentially used to relative weight since in normal adult males testes weight and body weight are independent variables (Robb et al., 1978; Zenick and Clegg, 1989). After the treatment with 40 mg/kg of fenvalerate, for 30 days, a significant decrease was

observed in the absolute weights of testis and epididymis in relation to the control group. These results can be related to the diminution of testis sperm production and sperm storage in the epididymis, as discussed below. Mani et al. (2002) also observed a reduction in the testis weight in adult rats exposed to a fenvalerate formulation by inhalation, for three months.

An important parameter to evaluate the effects of xenobiotics on male reproduction is the number and quality of sperm (Neubert, 1997). A toxic agent can affect the maturation, function and survival of sperm by direct action on the sperm or altering the epididymal function (Zenick et al., 1994). It has been shown that occupational exposure to fenvalerate can affect semen quality, especially sperm count and motility (Tan et al., 2002). In the treated group of the present study there was a reduction in the sperm production and storage in the epididymis. Mani et al. (2002) also observed a reduction in the sperm counts in rats exposed subchronically to formulated fenvalerate by inhalation. In a previous study realized in our laboratory (data not shown) adult rats treated with 40 mg/kg of fenvalerate, for 14 days, presented decrease in the number of sperm in the cauda epididymis. Thus, these results, taken together, suggest that fenvalerate is spermatotoxic.

Some recent studies have been associating occupational exposure to fenvalerate with genotoxic effects in human sperm (Xia et al., 2004). Reduction in the sperm count of occupational workers was related to impairment of the sperm DNA, and may have lead to cell death or induction of mutations (Bian et al., 2004; Lifeng et al., 2006). Furthermore, fenvalerate can induce oxidative stress in various tissues of mammals (Prasanthi, 2001; Prasanthi et al., 2005), a situation that can also contribute to cell death. On the other hand, Lombet et al. (1988) reported that pyrethroids can strongly bind to a non-specific sites of biological membranes. It was also reported that fenvalerate could be localized close to the region of the hydrophobic end of the lipidic chain and disturb the organizational structure of the membrane, becoming more fluid (Sarkar et al., 1993). Based on these considerations, in the present work, it is possible that fenvalerate may have had a direct or indirect genotoxic effect on the sperm, since residues of the insecticide

were found in the testis. In this way, this effect could have resulted in sperm cell death, causing a reduction in sperm production.

Effects on the spermatogenesis can also occur as a result of a general toxicity in the organism. For instance, toxic effects on the liver would be likely to affect normal testicular function (Kim and Wang, 1993). In this respect, fenvalerate was shown to enhance the activity of hepatic lysosomal enzymes (Balbaa et al., 1998; Morisseau et al., 1999).

The endocrine control of reproduction involves a series of interactions of the hypothalamus-hypophysis-gonad axis, constituting a wide variety of target sites for the action of endocrine disruptors (US EPA, 1997). The uterotrophic test is an *in vivo* assay of short duration utilized in the preliminary assessment of (anti) estrogenic substances (Edstac, 1998; Gray, 1998, Baker, 2001). In the present study fenvalerate was not able to produce estrogenic activity *in vivo* at the tested doses, corroborating Kunimatsu et al. (2002). In the same way there were no significant differences in the plasma testosterone levels between the experimental groups, suggesting that the mechanism of action of fenvalerate, in the present experimental conditions, is not mediated by androgenic depletion.

In spite of the reduced sperm count, the treatment with fenvalerate did not compromise the fertility of the rats after natural matings and *in utero* artificial insemination. Moreover, there were no differences between control and treated rats in the sexual behavior or sperm morphology or sperm number ejaculated in the uterus. It is important to emphasize that the reduction in number and quality of sperm is not a direct measure of fertility, unless a very drastic effect had been induced (Neubert, 1997). It is known that in some strains of rats and mice, sperm production can be reduced by 90% without compromising fertility. However, less severe reductions can have dramatic consequences for human males who function nearer the threshold for the number of sperm needed to ensure reproductive competence (Zenick and Clegg, 1989).

Histophatological evaluation is generally accepted as the most sensitive endpoint for detecting adverse effects of chemicals on male reproductive function

(Creasy, 2003). In the present work, the analysis of testis and epididymis at the optical and electron microscopic levels did not reveal apparent injuries related to the treatment.

Moniz et al. (1999) observed that perinatal exposure to fenvalerate disrupt male sexual behavior, suggesting a interference in the male brain sexual differentiation process. However, the treatment with fenvalerate in adult life did not alter the male sexual behavior, dispite the fact that fenvalerate residues were found in the brain, as discussed above.

The sperm are continuously modified during their development (Okabe et al., 1987). The addition and loss of some proteins and glycoproteins, produced by epididymal epithelium, is one of the important alterations that the sperm cell suffers during passage through the epididymis (Vernon et al., 1987). A small number of these proteins are androgen-dependent (Brooks, 1983). The physiological role of these proteins is unknown, but there are indications that their addition or loss is important in the acquisition of sperm motility and fertilization capacity. The use of compounds that alter the production or functionality of these proteins can provoke serious reproductive problems. In this way the protein profile study of sperm of individuals exposed to environmental chemicals is an important tool in current toxicology for evaluation of reproductive alterations. In the present work analysis of sperm and epididymal proteins present in the cauda epididymis through the technique of bidimensional electrophoresis, revealed that the animals treated with fenvalerate presented diminution in only four proteins. However, it was not possible, with the methodology employed in the present work, to determine the function of these altered proteins.

It may be concluded that fenvalerate, dilluted in corn oil, at a dose of 40 mg/kg, administered orally to adult rats for 30 days, was not able to produce estrogenic activity *in vivo* at the tested doses. Furthermore, the fenvalerate was retained in reproductive (in special in the epididymis) and vital organs. Fenvalerate was also spermatotoxic since both production and sperm reserves were reduced in treated animals. Despite this alteration, the fertility of treated animals was not

altered since rats have a high reproductive efficiency in contrast with humans. Additional studies need to be completed to confirm the mechanisms by which fenvalerate exerts its toxic action on sperm.

Acknowledgments

This work was supported by grants from CNPq (National Council for Scientific and Technological Development) and FAPESP (The State of São Paulo Research Foundation, Proc. 02/09455-6 and 04/08627-3). The authors are also grateful to Sumitomo Chemical for supplying technical-grade fenvalerate and to Dr. Eunice Oba of the Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Zootechny, São Paulo State University, for help with testosterone determination.

References

Abd El-Aziz, M.I., Sahlab, A.M., Abd El-Khalik, M., 1994. Influence of diazinon and deltamethrin on reproductive organs fertility of male rats. Dtsch. Tierarztl. Wochenschr. 101(6), 230-232.

Adelsbach, T.L., Tjeerdema, R.S., 2003. Chemistry and fate of fenvalerate and esfenvalerate. Rev. Environ. Contam. Toxicol. 176, 137-54.

Ågmo, A., 1997. Male rat sexual behavior. Brain Res. Protoc. 203-209.

Ahlenius, S., Larsson, K., 1984. Apomorphine and haloperidol-induced effects on male rat sexual behavior: no evidence for actions due to stimulation of central dopamine autoreceptors. Pharmacol. Biochem. Behav. 21(3), 463-466.

Andrade, A.J., Araujo, S., Santana, G.M., Ohi, M., Dalsenter, P.R., 2002. Screening for in vivo (anti)estrogenic and (anti)androgenic activities of technical and formulated deltamethrin. Regul. Toxicol. Pharmacol. 35(3), 379-382.

Ashby, J., Lefevre P.A., Odum, J., Tinwell, H., Kennedy, S.J., Beresford, N., Sumpter, J.P., 1997. Failure to confirm estrogenic activity for benzoic acid and clofibrate: Implications for lists of endocrine-disrupting chemicals. Regul. Toxicol. Pharmacol. 26, 96-101.

Ashby, J., Tinwell, H., Lefevre, P.A., Joiner, R., Haseman, J., 2003. The effect on sperm production in adult Sprague-dawley rats exposed by gavage to bisphenol A between postnatal days 91-97. Toxicol. Sci. 74, 129-138.

Baker, V.A., 2001. Endocrine disrupters – Testing strategies to assess human hazard. Toxicol. In vitro 15, 413-419.

Balbaa, M., Abdelhamid, E.M.E., Bassiouny, K., 1998. Enhancement of lysosomal enzymes by the pyrethroids fenvalerato and trans-cypermethrin. Japan. J. Toxicol. Environ. Health. 44, 83-91.

Bhatt, R.V., 2000. Environmental influence on reproductive health. Int. J. Gynaecol. Obstet. 70, 69-75.

Bian, Q., Xu, L.C., Wang, S.L., Xia, Y.K., Tan, L.F., Chen, J.F., Song, L., Chang, H.C., Wang, X.R., 2004. Study on the relation between occupacional fenvalerate exposure and spermatozoa DNA damage of pesticide factory workers. Occup. Environ. Med. 61, 999-1005.

Bissacot, D.Z., Vassilieff, I., 1997a. HPLC determination of flumethrin, deltamethrin, cypermethrin residues in the milk and blood of lactating dairy cows. J. Anal. Toxicol. 21(5), 397-402.

Bissacot, D.Z., Vassilieff, I., 1997b. Pyrethroid residues in milk and blood of dairy cows following single topical applications. Vet. Hum. Toxicol. 39 (1), 6.

Braun, H.E., Stanek, J., 1982. Application of the AOAC multi-residue method to determination of synthetic pyrethroid residues in celery and animal products. J. Assoc. Off. Anal. Chem. 65, 685.

Brempong-Yeboah C.Y., Saito T., Miyata T., 1983. Injection toxicity of some pyrethroids in the armyworm. J. Pest. Sci. 8, 95.

Brooks, D.E., 1983. Epididymal functions and their hormonal regulation. Aust. J. Biol. Sci., 36, 205-221.

Chen, J., Chen, H., Liu, R., He, J., Song, L., Bian, Q., Xu, L., Zhou, J., Xiao, H.,

Dai, G., Chang, H., Wang, X., 2005. Effects of fenvalerate on progesterone production in cultured rat granulose cells. Reprod. Toxicol. 20, 195-202.

Class, T.J., 1991. Determination of pyrethroids and their degradation products in indoor air and surfaces by HRGC-ECD and HRGC-MS (NCI). HRC-J High Resolut. Chromatogr. 14 (7), 446-450.

Creasy, D.M., 2003. Evaluation of testicular toxicology: A synopsis and discussion of the recommendations proposed by the society of toxicologic pathology. Birth Defects Res. (Part B) Dev. Reprod. Toxicol. 68 (5), 408-415.

Curtis, C.F., Maxwell, C.A., Finch, R. J., Njunwa, K.J., 1998. A comparison of use of a pyrethroid either for house spraying or for bednet treatment against malaria vectors. Trop. Med. Int. Health. 8, 619.

Elbetieha, A., Da'as, S.I., Khamas, W., Darmani, H., 2001. Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. Arch. Environ. Contam. Toxicol. 41(4), 522-528.

Eil, C., Nissula, B.C., 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. J. Steroid. Biochem. 35, 409-414.

Elliot, M., Janes, N.F., 1978. Synthetic pyrethroids – a new class of insecticide. Chem. Soc. Rev. 7, 473-480.

Endocrine Disruptor Screening And Testing Advisory Committee (Edstac), 1998. EPA; 743; R-98;003: Final Report. Washington.

Garey, J., Wolff, M.S., 1998. Estrogenic and antiprogestagenic activities of

pyrethroid insecticides. Bioch. Bioph. Res. Comm. 251, 855-859.

Go, V., Garey, J., Wolff, M.S., Pogo, B.G.T., 1999. Estrogenic potencial of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. Environ. Health Perspect. 107(3), 173-177.

Gray, L.E., 1998. Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicol. Lett. 102-103, 677-680.

He, F., 1994. Synthetic pyrethroids. Toxicology 91(1), 43-49.

He, J., Chen, J., Liu, R., Wang, S., Song, L., Chang, H.C., Wang, X., 2004. Alterations of FSH-stimulated progesterone production and calcium homeostasis in primarily cultured human luteinizing-granulosa cells induced by fenvalerate. Toxicology 203, 61-68.

Jégou, B., Auger, J., Multigner, L., 1999. The saga of the sperm count decrease in humans and wild and farm animals. In: The male gamete: from basic to clinical applications. Chapter 41.Gagnon (Ed.) Cache River Press, pp. 445-454.

Kang, k.S., Kim, H.S., Ryu, D.Y., Che, J.H., Lee, Y.S., 2000. Immature uterotrophic assay is more sensitive than ovariectomized uterotrophic assay for the detection of estrogenicity of p-nonylphenol in Sprague-Dawley rats. Toxicol. Lett., 118, 109-115.

Kavlock, R.J., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ. Health Perspect. 104, 715-740.

Kempinas, W.G., Suarez, J.D., Roberts, N.L., Strader, L., Ferrell, J., Goldman,

J.M., Narotsky, M.G., Perreault, S.D., Evenson, D.P., Ricker, D.D., Klinefelter, G.R., 1998. Fertility of rat epididymal sperm after chemically and surgically induced sympathectomy. Biol. Reprod. 59, 897-904.

Kim, K.W., Wang, Z., 1993. In: L.D. Russell and M.D. Griswold, (Eds), The Sertoli cell. Cache River Press, FL, p.517.

Klinefelter, G.R., 2002. Actions of toxicants on the structure and function of the epididymis. In: B. Robaire, B.T. Hinton, (Eds), The Epididymis – from molecules to clinical pratice. New York: Kluwer Academic/ Plenum Publisher, pp. 353-69.

Klinefelter, G.R., Strader, L.F., Suarez, J.D., Roberts, N.L., 2002. Bromochloroacetic acid exerts qualitative effects on rat sperm: implications for a novel biomarker. Toxicol. Sci. 68, 164-173.

Kunimatsu, T., Yamada, T., Ose, K., Sunami, O., Kamita, Y., Okuno, Y., Seki, T., Nakatsuka, I., 2002. Lack of (anti-) androgenic or estrogenic effects of three pyrethroids (esfenvalerate, fenvalerate, and permethrin) in the hershberger and uterotrophic assays. Regul. Toxicol. Pharmacol. 35, 227-237.

Landrigan, P.J., Claudio, L., Mcconnell, R., 1998. In: Lippman, M., (Eds.), Environmental toxicants, VanRhinehold, NY.

Leng, G., Kuhn, K.H., Idel, H., 1996. Biological monitoring of pyrethroid metabolites in urine of pest control operators. Toxicol. Lett. 88 (1-3), 215.

Lifeng, T., Shoulin, W., Junmin, J., Xuezhao, S., Yannan, L., Qianli, W., Longsheng, C., 2006. Effects of fenvalerate exposure on semen quality among occupational workers. Contraception 73(1), 92-96.

Lombet, A., Mourre, C., Lazdunski, M., 1988. Interaction of insecticides of the pyrethroid family with specific binding sites on the voltage-dependent sodium channel from mammalian brain. Brain Res. 459, 44-53.

Mandal, T.K., Chakraborty, A.K., Bhattacharyya, A., Ghosh, R.K., Majumder, S., 1996. The disposition kinetics and residues of fenvalerate in tissues following a single dermal application to black Bengal goats. Vet. Res. Commun. 20 (3), 265.

Mani, U., Islam, F., Prasad, A.K., Kumar, P., Suresh, Kumar, V., Maji, B.K., Dutta, K.K., 2002. Steroidogenic alterations in testes and sera of rats exposed to formulated fenvalerate by inhalation. Hum. Exp. Toxicol. 21 (11), 593-597.

Mcleese, D.W., Metcalfe, C.D., Zitko, V., 1980. Lethality of permethrin, cypermethrin and fenvalerate to salmon, lobster and shrimp. Bull. Environ. Contam. Toxicol. 25, 950.

Miyamoto, J., Kaneko, H., Tsuji, R., Okuno, Y., 1995. Pyrethroids, nerve poisons: how their risks to human health should be assessed. Toxicol. Lett. 82-83, 933-940.

Moniz, A.C., Cruz-Casallas, P.E., Oliveira, C.A., Lucisano, A., Florio, J.C., Nicolau, A.A., Spinosa, H.S., Bernardi, M.M., 1999. Perinatal fenvalerate exposure: behavior and endocrinology changes in male rats. Neurotoxicol. Teratol. 21(5), 611-618.

Moniz, A.C., Cruz-Casallas, P.E., Salzgeber, S.A., Varoli, F.M., Spinosa, H.S., Bernardi, M.M., 2005. Behavioral and endocrine changes induced by perinatal fenvalerate exposure in female rats. Neurotoxicol. Teratol. 27(4), 609-614.

Morisseau, C., Derbel, M., Lane, T.R., Stoutamire, D., Hammock, B.D, 1999. Differential induction of hepatic drug-metabolizing enzymes by fenvaleric acid in male rats. Toxicol. Sci. 52, 148-153.

Naumman, K., 1990. Synthetic pyrethroid insecticides: structures and properties. Chemistry of Plant Protection 4, Springer-Verlag, NY.

Neubert, D., 1997. Vulnerability of the endocrine system to xenobiotic influence. Regul. Toxicol. Pharmacol. 26, 9-29.

Odum, J., Lefevre, P.A., Tittensor, S., Paton, D., Routledge, E.J., Beresford, N.A., Sumpter, J.P., Ashby, J., 1997. The rodent uterotrophic assay: critical protocol features, studies with phenols, and comparison with a yeast estrogenicity assay. Regul. Toxicol. Pharmacol. 25, 176-188.

Okabe, M., Adachi, T., Takada, K., Oda, H., Yagasaki, M., Kohama, Y., Mimura, T., 1987. Capacitacion-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate in vitro. J. Reprod. Immunol. 11, 91-100.

Paumgartten, F.J.R., 2003. Adverse health consequences of environmental exposure to ‘endocrine disruptors’. Annu. Rev. Biomed. Sci. 5, 45-55.

Prasanthi, K., 2001. Investigations in to the mechanism of toxicity of pyrethroids in mammalian system. Thesis. University of Mysore, India, 226.

Prasanthi, K., Muralidhara, Rajini, P.S., 2005. Morphological and biochemical perturbations in rat erythrocytes following in vitro exposure to fenvalerate and its metabolite. Toxicol. In Vitro 19, 449-456.

Ripley, B.D., Ritcey, G.M., Harris, C.R., Denomme, M.A., Brown, P.D., 2001. Pyrethroid insecticide residues on vegetable crops. Pest. Manag. Sci., 57(8), 683-687.

Robb, G.W., Amman, R.P., Killian, G.J., 1978. Daily sperm production and epididymal sperm reserves of puberal and adult rats. J. Reprod. Fertil. 54, 103-107.

Sarkar, S.N., Balasubramanian, S.V., Sikdar, S.K., 1993. Effect of fenvalerate, a pyrethroid insecticide on membrane fluidity. Biochim. Biophys. Acta 1147, 137-142.

Shan, G., Wengatz, I., Stoutamire, D.W., Geeand, S.J., Hammock, B.D., 1999. An enzyme-linked immunosorbent assay for the detection of esfenvalerate metabolites in human urine. Chem. Res. Toxicol. 12 (11), 1033.

Tan, L.F., Wang, S.L., Sun, X.Z., Li, Y.N., Wang, Q.L., Ji, J.M., Chen, L.S., Wang, X.R., 2002. Effects of fenvalerate exposure on the semen quality of occupational workers. Zhonghua Nan Ke Xue 8(4), 273-276.

U.S. Environmental Protection Agency (US EPA), 1997. Epa/630/R-96/012: Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis. Washington.

Vernon, R. B., Muller, C., Eddy, E. M., 1987. Further characterization of a secreted epididymal glycoprotein in mice that binds to sperm tails. J. Androl. 8, 523-535.

World Health Organization (Who) (1990). Fenvalerate. Environmental Health Criteria 95. WHO, Geneva.

Xia, Y., Bian, Q., Xu, L., Cheng, S., Song, L., Liu, J., Wu, W., Wang, S., Wang, X., 2004. Genotoxic effects on human spermatozoa among pesticide factory workers exposed to fenvalerate. Toxicology 203, 49-60.

Zaim, M., Aitio, A., Nakashima, N., 2000. Safety of pyrethroid-treated mosquito nets. Med. Vet. Entomol. 14, 1.

Zenick, H., Clegg, E.D., 1989. Assessment of male reproductive toxicity: A risk assessment approach. In: W. Hayes, (Ed), Principles and Methods of Toxicology, Raven Press, New York, pp. 275-309.

Zenick, H., Clegg, E.D., Perreault, S.D., Klinefelter, G.R., Gray, L.E., 1994. Assessment of male reproductive toxicity: a risk assessment approach. In: Principles and methods of toxicology. 3. ed. New York: Raven, pp. 937-988.

Table 1

Final body weight, plasma testosterone levels and absolute weight (g and mg) of male reproductive organs, liver and kidneys from controls and group treated with 40 mg/kg/day of fenvalerate for 30 days.

	Experimental groups	
	Control (n=8)	**Treated (n=9)**
Final body weight (g)	492±16.60	473±14.60
Plasma testosterone levels	142.46±34.19	117.37±23.09
Testis (g)	1.91±0.06	1.64 ± 0.05*
Epididymis (mg)	704±23.75	631 ± 13.99*
Vas deferens (mg)	133±8.81	109±4.23
Ventral prostate (mg)	633±47.65	525±50.11
Seminal vesicle (g)	1.37±0.07	1.46±0.10
Kidney (g)	1.40±0.04	1.38±0.06
Liver (g)	14.03±0.35	13.49±0.63

Values expressed as mean ± S.E.M.

* *p<0.05* (Student's *t*-test).

Table 2

Fenvalerate residues (ppm) in tissues of control and group treated with 40 mg/kg/day of fenvalerate for 30 days.

Tissues	**control**	**fenvalerate**
Liver	ND	0.02±0.01
Testis	ND	0.03±0.02
Brain	ND	0.06±0.02
Epididymis	ND	0.29±0.12

Values expressed as mean± S.E.M., n=10.

Detection limit of the method was 0.001 µg/g.

ND= not detected

Table 3

Daily sperm production per testis (absolute and relative), relative number of sperm in the testis and in the caput/corpus and cauda epididymidis, and sperm transit time in the epididymis from controls and group treated with 40 mg/kg/day of fenvalerate for 30 days.

	Experimental groups	
	Control (n=7)	**Treated (n=9)**
Daily sperm production (x 10^6/testis/day)	37.24 ± 2.08	25.72 ± 1.49*
Daily sperm production (x 10^6/g/testis/day)	20.63 ± 0.82	16.13 ± 0.78*
Relative sperm count in the testis (x 10^6/g/organ)	127.00 ± 6.20	98.43 ± 4.75*
Relative sperm count in the caput/corpus epididymidis (x 10^6/g)	362.07 ± 15.96	244.12 ± 11.17*
Relative sperm count in the cauda epididymidis (x 10^6/g)	728.72 ± 54.00	502.89 ± 26.52*
Sperm transit time in the caput/corpus (days)	3.61±0.34	3.16±0.20
Sperm transit time in the cauda (days)	5.64±0.40	5.17±0.32

Values expressed as mean ± S.E.M.

* *p<0.05* (Student's *t*-test).

Table 4

Percentage (%) of male rats that mounted during a 10 min session and other parameters of sexual behavior investigated in control and treated groups. The latencies are shown in seconds.

PARAMETERS	N	CONTROL	N	TREATED
% of males that showed mount	10	100%	8	80%
Latency to the 1st mount	10	72.00 ± 19.75	8	58.75 ± 16.72
Latency to 1st intromission	10	136.60 ± 46.69	8	77.75 ± 20.44
N° of intromissions	10	12.30 ± 2.05	8	9.88 ± 1.30
Latency to 1st ejaculation	10	892.30 ± 168.20	8	513.75 ± 83.75
Latency to 1st intromission post-ejaculation	9	369.77 ± 28.17	8	365.13 ± 42.15
N° of intromissions post-ejaculation	9	6.80 ± 1.67	8	6.87 ± 0.58
N° of ejaculations	8	1.75 ± 0.25	8	1.88 ± 0.13

Values expressed as mean ± S.E.M.

N = Number of animals that showed the respective behavior.

No significant difference was found by Student's *t*-test (*p>0.05*).

Table 5

Fertility tests results from the controls and group treated with 40 mg/kg/day of fenvalerate (n=10) after natural mating (NM) and artificial insemination (AI).

		Experimental groups	
		Control	**Treated**
Body weight of dams (g)	**NM**	370.35±12.30	341.43±6.73
	AI	334.20±19.20	333.58±9.89
Uterus weight with fetuses (g)	**NM**	70.67±3.79	71.28 ± 3.56
	AI	48.98±6.43	46.29±6.39
Number of corpora lutea	**NM**	14.10±0.67	14.00 ± 0.36
	AI	12.00±0.93	12.38±0.59
Number of implants	**NM**	13.70±0.67	13.00±0.42
	AI	9.83±1.30	9.50±1.34
Number of reabsorptions	**NM**	0.50±0.22	0.44±0.18
	AI	0.17±0.17	0.38±0.26
Number of live fetuses	**NM**	13.20±0.63	12.00±0.72
	AI	9.67±1.23	9.13±1.27
Fetuses weight (g)	**NM**	3.39±0.10	3.52±0.09
	AI	3.02±0.13	2.98±1.05
Gestation rate (%)	**NM**	100	90
	AI	75	89
[1]Sex ratio (%)	**NM**	108.33 (85.70-182.00)	75 (50.00-200.00)
	AI	61.90 (51.80-76.70)	83 (58.30-150.00)
[1]Fertility potential (%)	**NM**	100.00 (100.00-100.00)	100.00 (92.80-100.00)
	AI	90.45 (74.10-97.70)	88.78 (70.30-100.00)
[1]Pre-implantation loss (%)	**NM**	0.00 (0.00-0.00)	0.00 (0.00-7.10)
	AI	9.55 (2.30-25.90)	11.21 (0.00-29.70)
[1]Post-implantation loss (%)	**NM**	0.00 (0.00-4.20)	7.69 (0.00-7.70)
	AI	0.00 (0.00-0.00)	0.00 (0.00-2.80)

Values expressed as mean ± SEM. [1] Values are expressed as median (Q_1-Q_3), *p>0.05*; Mann-Whitney test. No significant difference was found by Student's *t*-test (*p>0.05*).

Figure Legends

Figure 1. Sperm counts in the testis and caput/corpus and cauda epididymidis of adult male rats, control (n=7) and treated (n=9) with fenvalerate for 30 days. Values expressed as mean ± S.E.M., *$p<0.05$ (Student's *t*-test).

Figure 2. Relative uterus weight (mg/100g of body weight) of immature female rats treated for 3 days with fenvalerate (0.4, 1.0, 4.0, 8.0 and 40 mg/kg/day), 0.4 mg/kg of estradiol benzoate (EB) and vehicle (C). Values expressed as mean ± S.E.M., with n=6 rats per group. ^{a,b} Different letters indicate groups that differ statistically. $p<0.05$ (ANOVA – Tukey).

Figure 3. Silver stained two-dimensional gel profiles of proteins from the cauda epididymidis of a control animal. The proteins surrounded by circles are diminished in the gels of the rats treated with fenvalerate. y-axis= molecular weight (X 10^{-3}); x-axis= pI range.

Figure 1

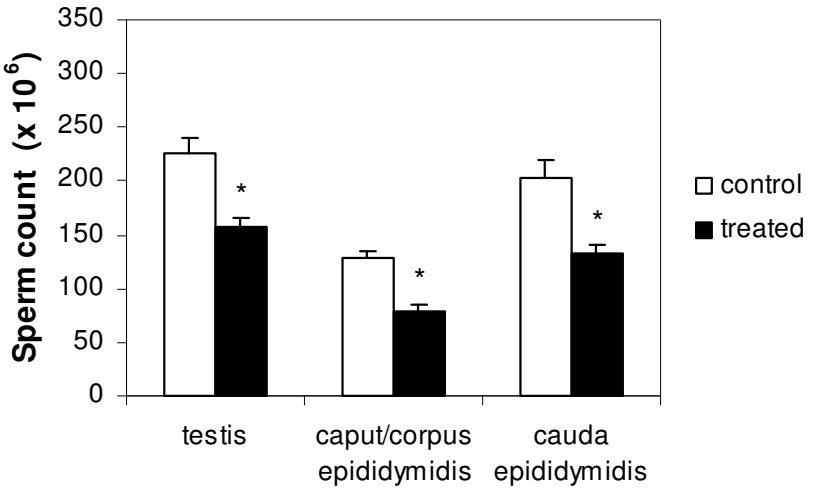


Figure 2

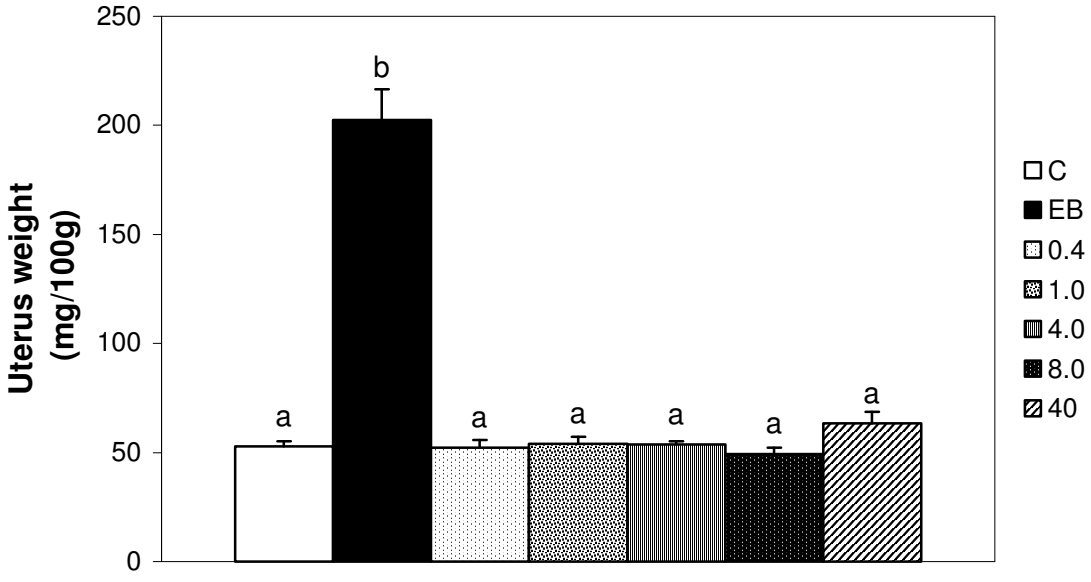
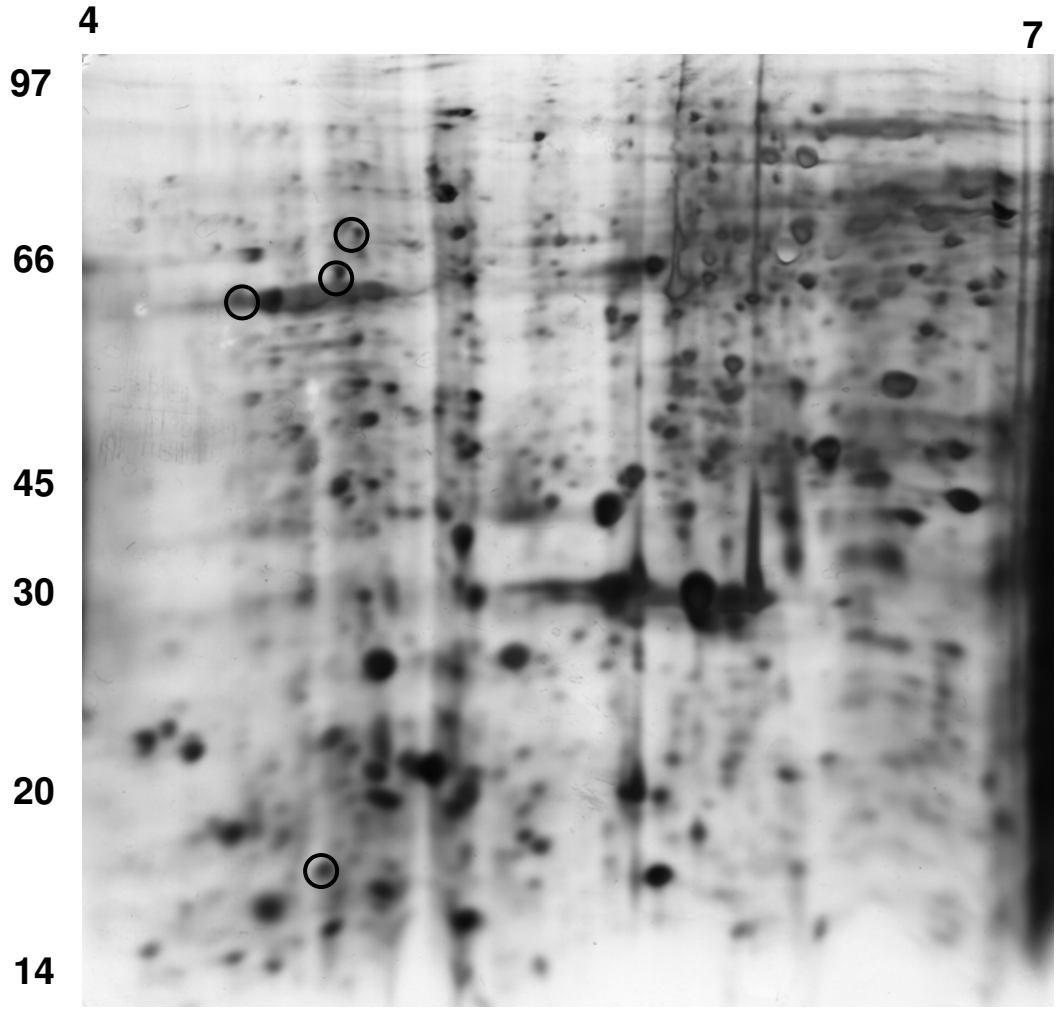


Figure 3



Anexo

Fotomicrografias (Figuras 4 a 6) e eletronmicrografias (Figuras 7 a 12) que ilustram a histologia e a ultraestrutura de testículos e epidídimos dos ratos do experimento que, por não mostrarem diferenças entre os grupos controle e tratado, não foram introduzidas no artigo científico submetido à revista Toxicology.

Figura 4. Fotomicrografias de cortes transversais de testículos, corados com HE, de animais controles (A-B) e tratados com fenvalerato (C-D). A- estágio XI do ciclo espermatogênico; B- estágio VII do ciclo espermatogênico, destacando o epitélio seminífero com células de Sertoli (s), espermatogônias (g), espermatócitos primários (cit), espermátides arredondadas (seta) e espermatozóides (sp); C- estágio XII do ciclo espermatogênico, evidenciando uma espermátide alongada (seta) e D- estágio VII do ciclo espermatogênico. Barra=20 µm.

Figura 4

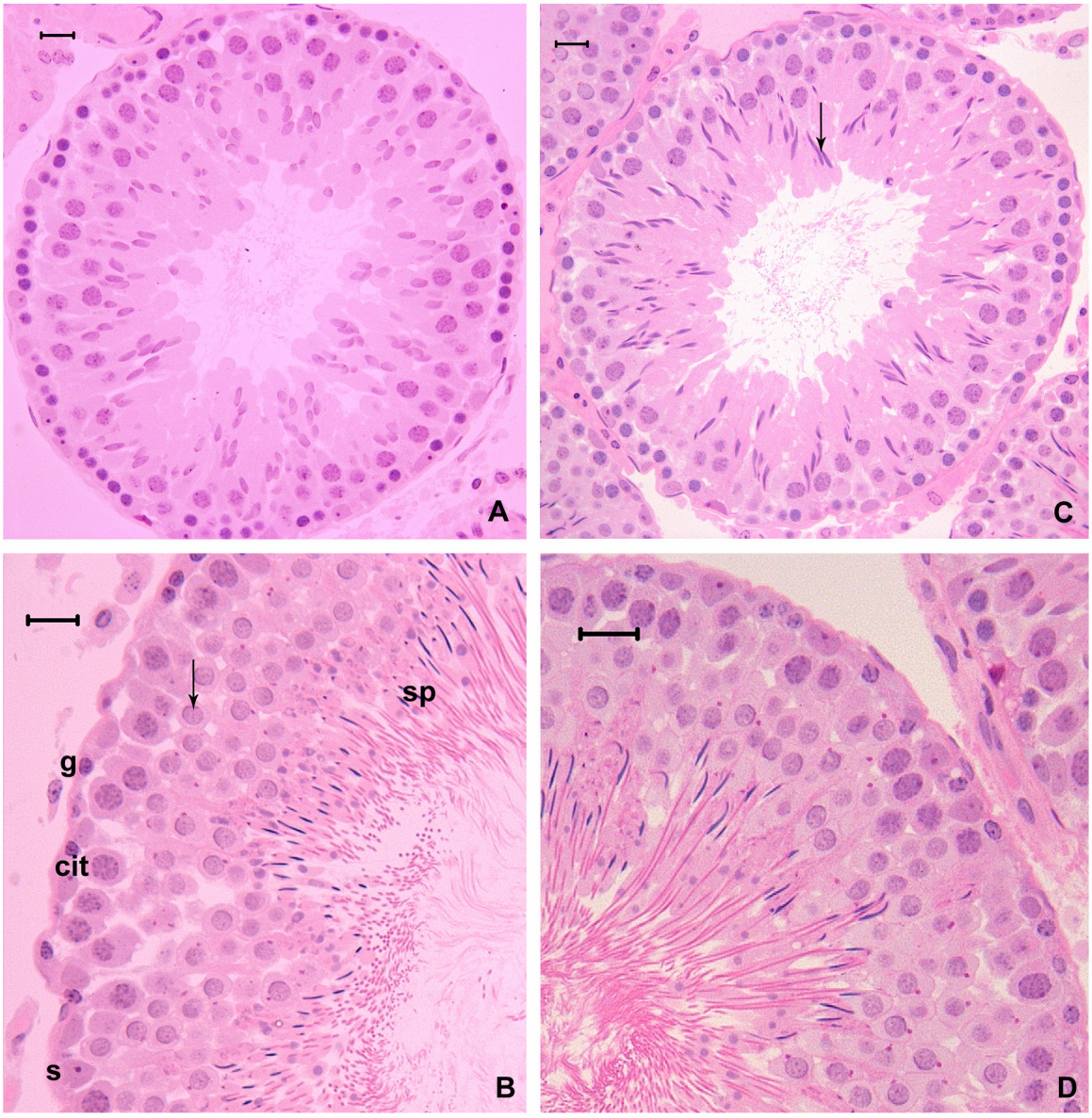


Figura 5. Fotomicrografias de cortes longitudinais da região da cabeça do epidídimo, corado com HE, de animais controles (A-B) e tratados com fenvalerato (C-D), evidenciando, em D, o epitélio (e) com células principais (p), lúmen apresentando espermatozóides (sp) e tecido conjuntivo intersticial (in). A e C, Barra= 50 µm; B e D, Barra=20 µm.

Figura 5

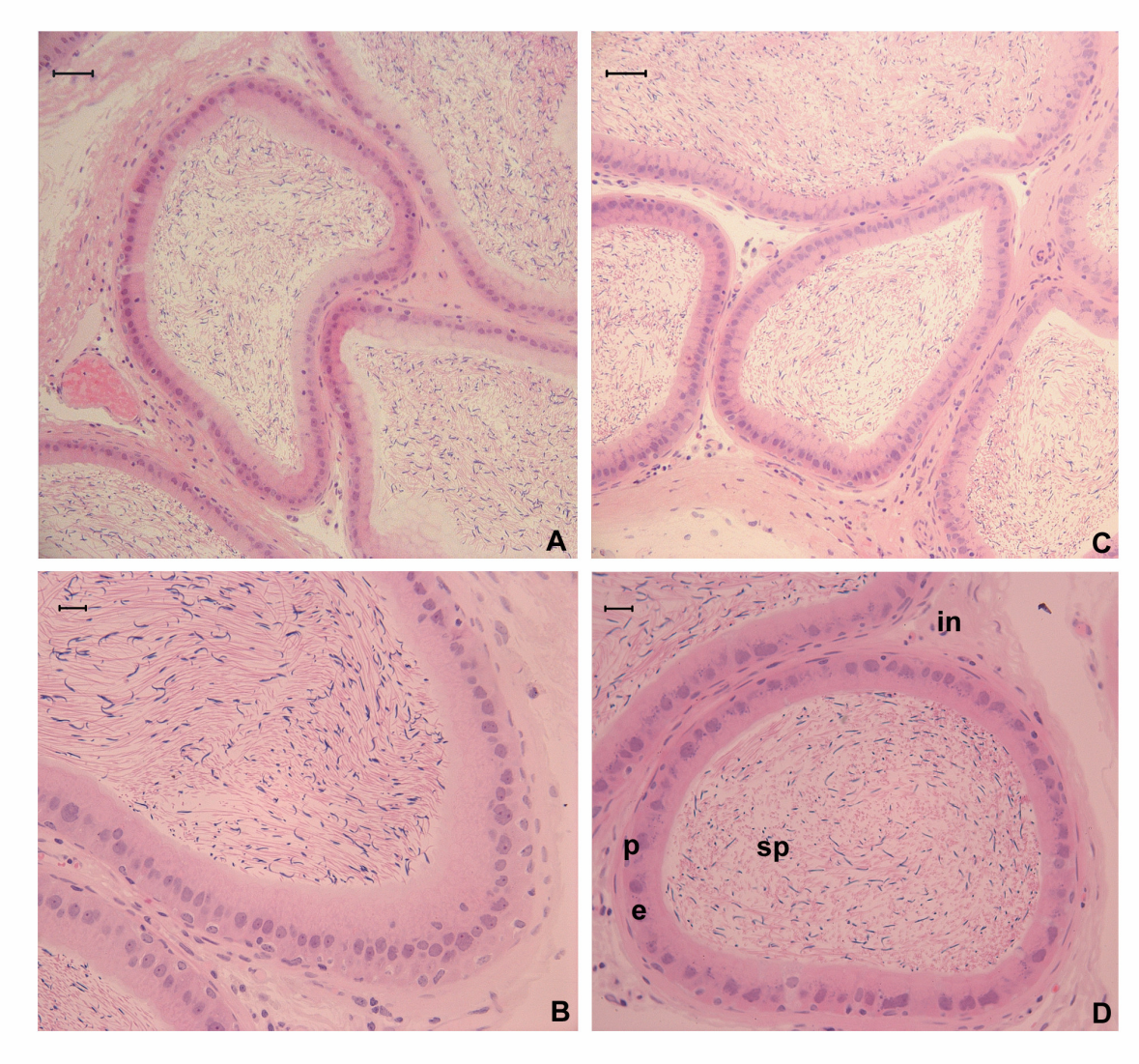


Figura 6. Fotomicrografias de cortes longitudinais da região da cauda do epidídimo, corado com HE, de animais controles (A-B) e tratados com fenvalerato (C-D), destacando, em D, o epitélio (e) com células principais (p) e células claras (c), lúmen apresentando espermatozóides (sp) e tecido conjuntivo intersticial (in). A e C, Barra=50 µm; B e D, Barra=20 µm.

Figura 6

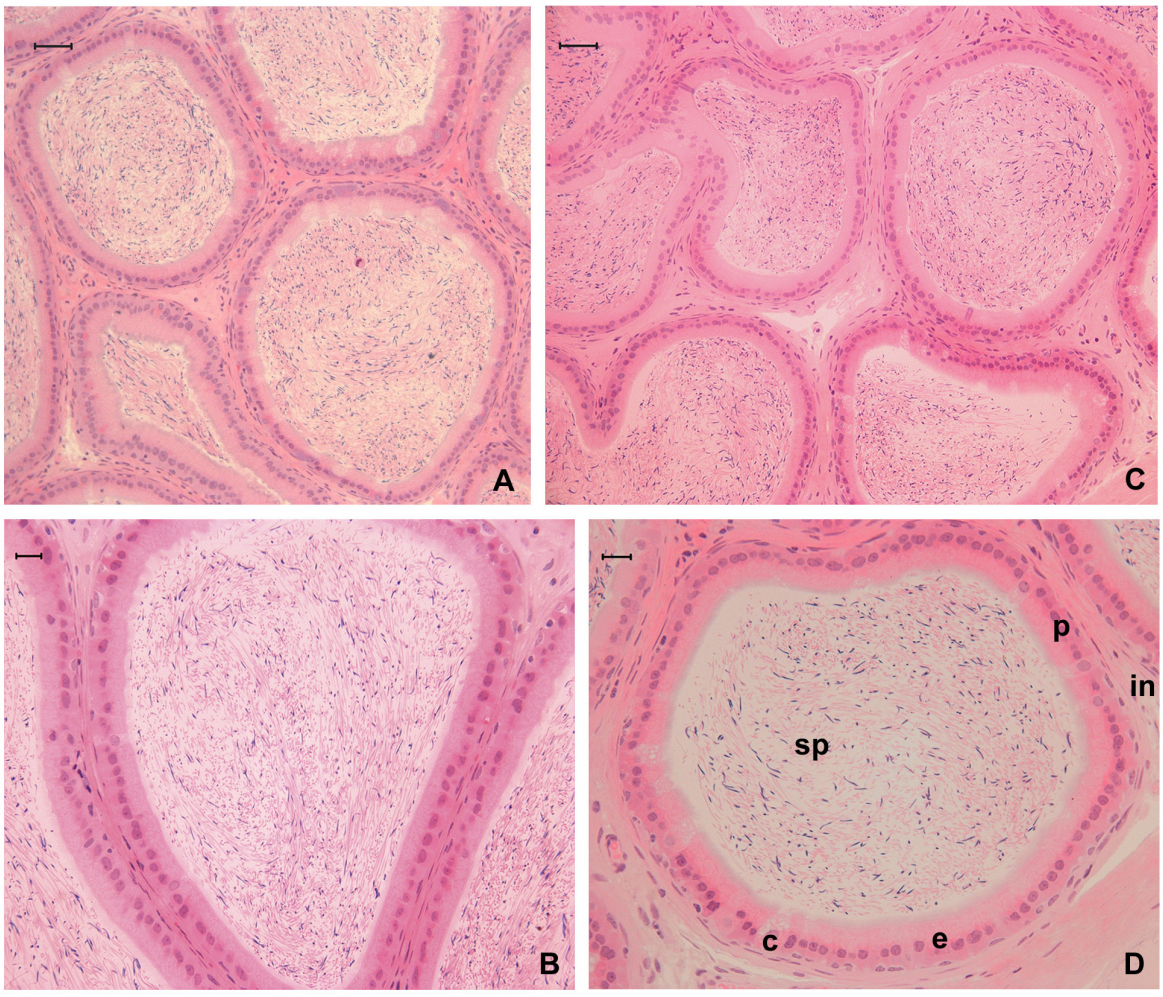


Figura 7. Eletronmicrografias do compartimento basal do epitélio seminífero do testículo, evidenciando núcleo de célula de Sertoli (s). A- animal controle e B- animal tratado com fenvalerato. Barra=1 µm.

Figura 7

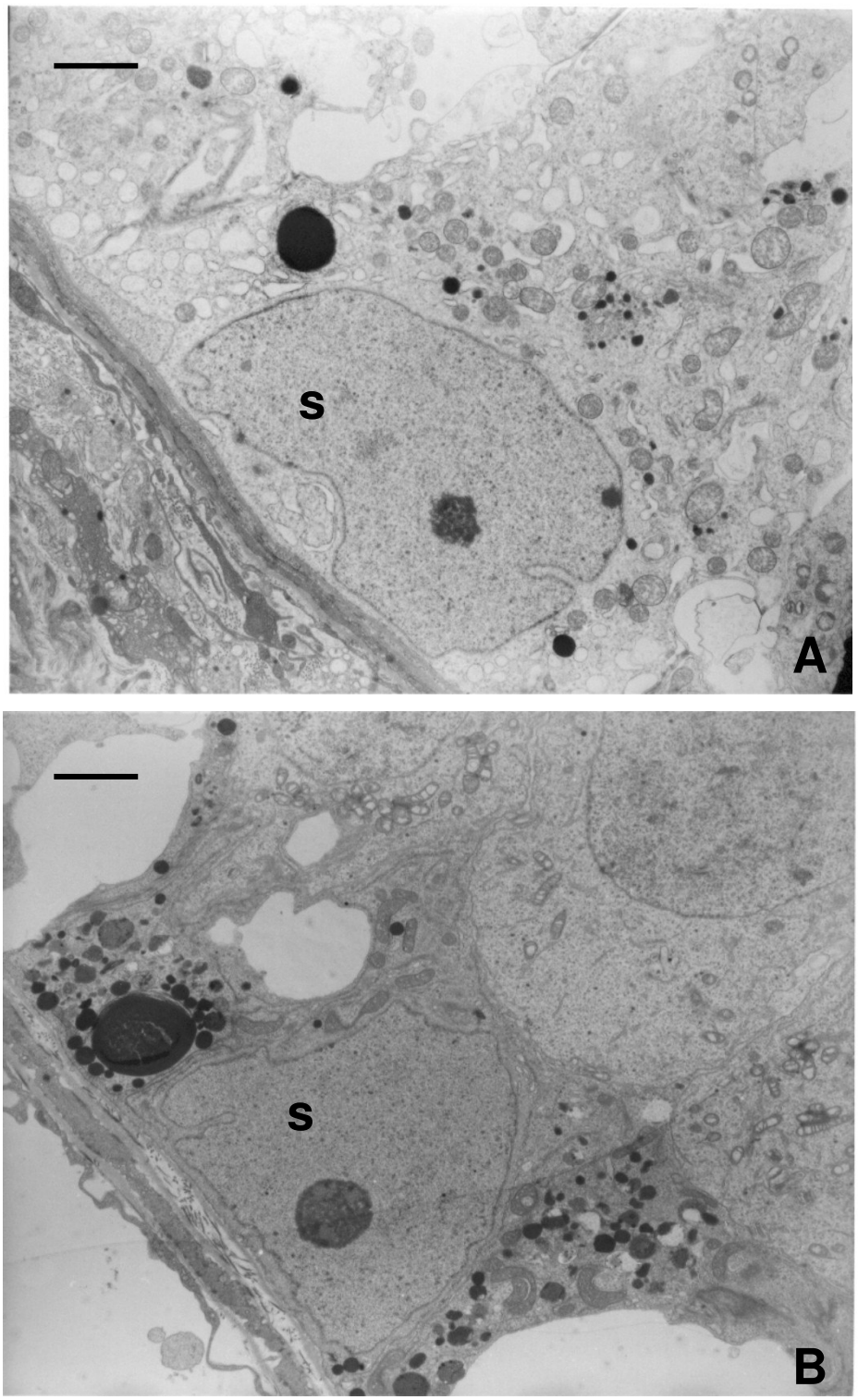


Figura 8. Eletronmicrografias do compartimento basal do epitélio seminífero do testículo, evidenciando núcleo de espermatócito primário (n). A- animal controle (11500X) e B- animal tratado com fenvalerato. Barra=5 µm.

Figura 8

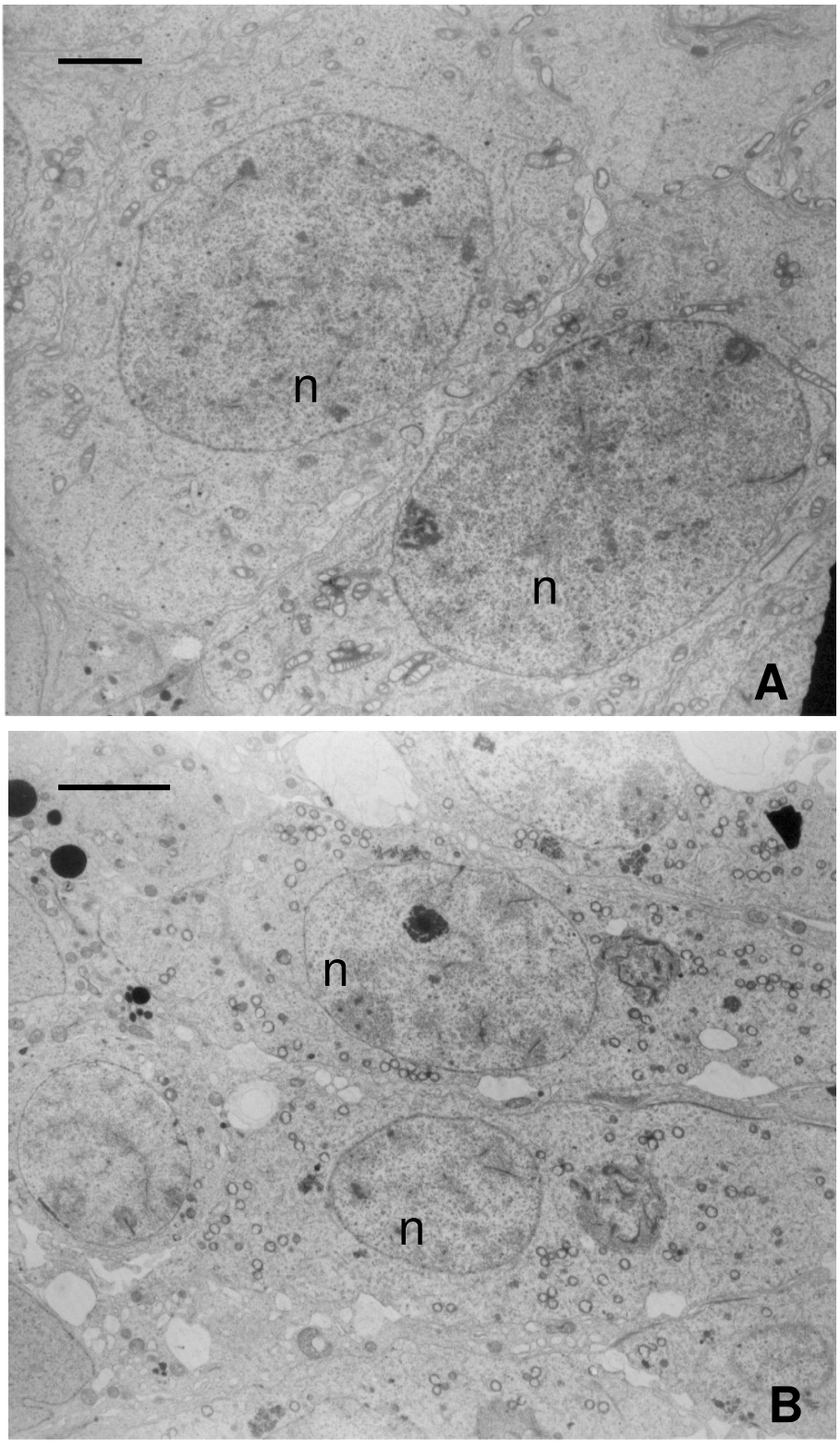


Figura 9. Eletronmicrografias do compartimento adluminal do epitélio seminífero do testículo, evidenciando núcleo de espermátide alongada (n). A- animal controle e B- animal tratado com fenvalerato. Barra=5 µm.

Figura 9

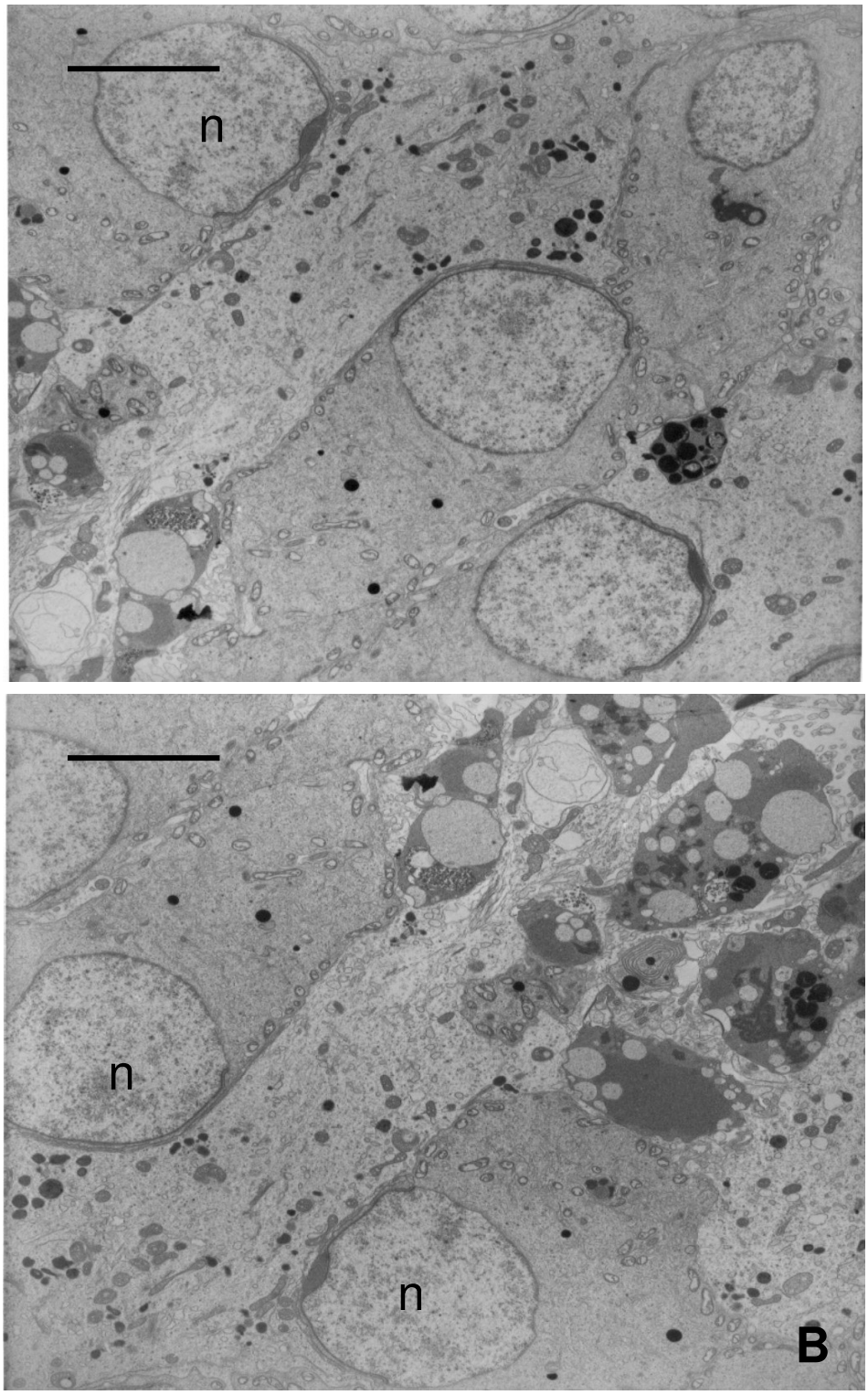


Figura 10. Eletronmicrografias das regiões da cabeça (A) e cauda (B) do epidídimo de animais controles, evidenciando as células principais (p). n= núcleos, e= estereocílios, corte transversal de espermatozóide no lúmen (seta). Barra=5 µm.

Figura 10

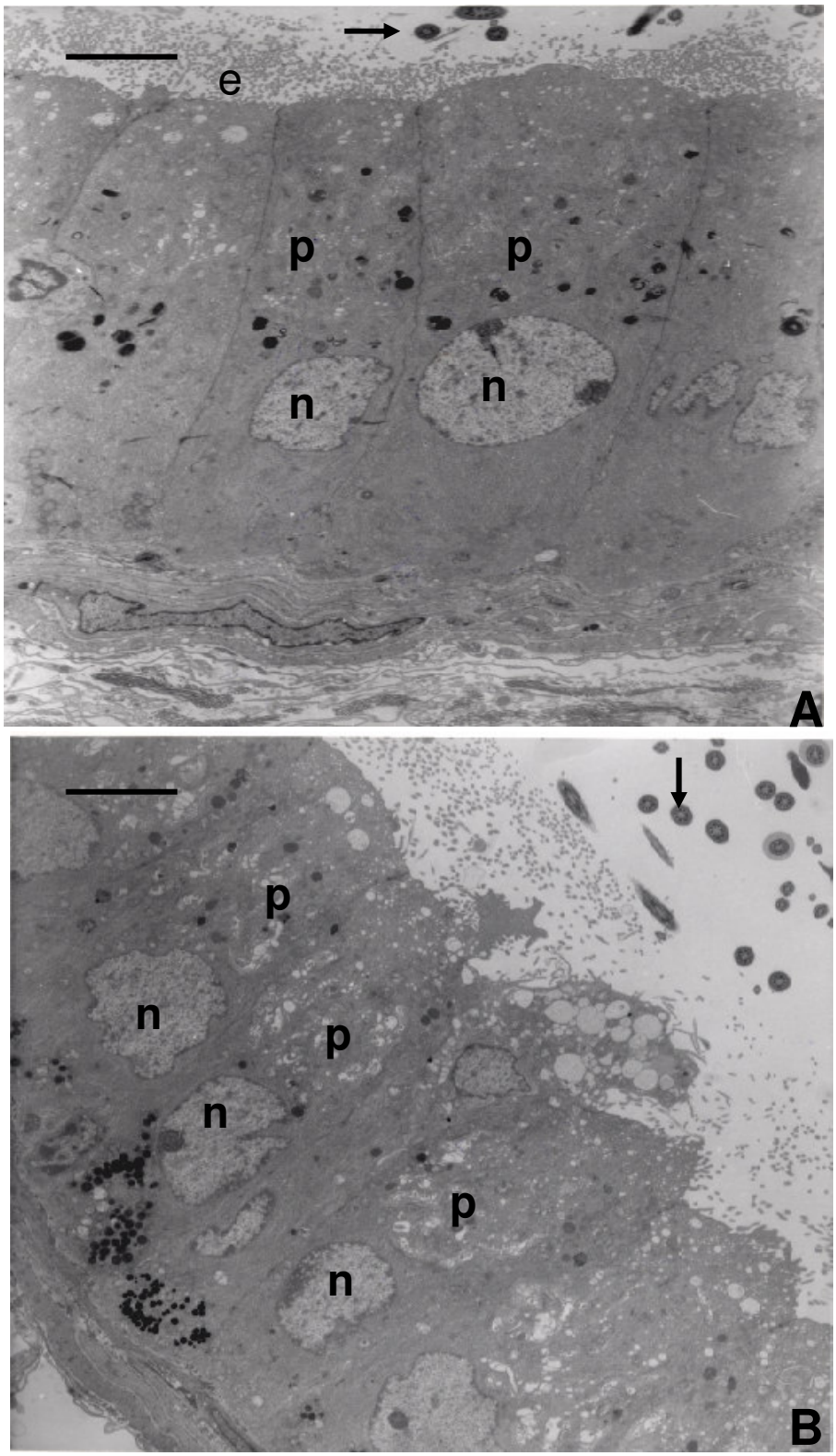


Figura 11. Eletronmicrografias da região da cabeça do epidídimo de animais controles, evidenciando uma célula apical (a) em A e uma célula basal (b) em B. n= núcleo, p= células principais, e= estereocílios. Barra=5 µm.

Figura 11

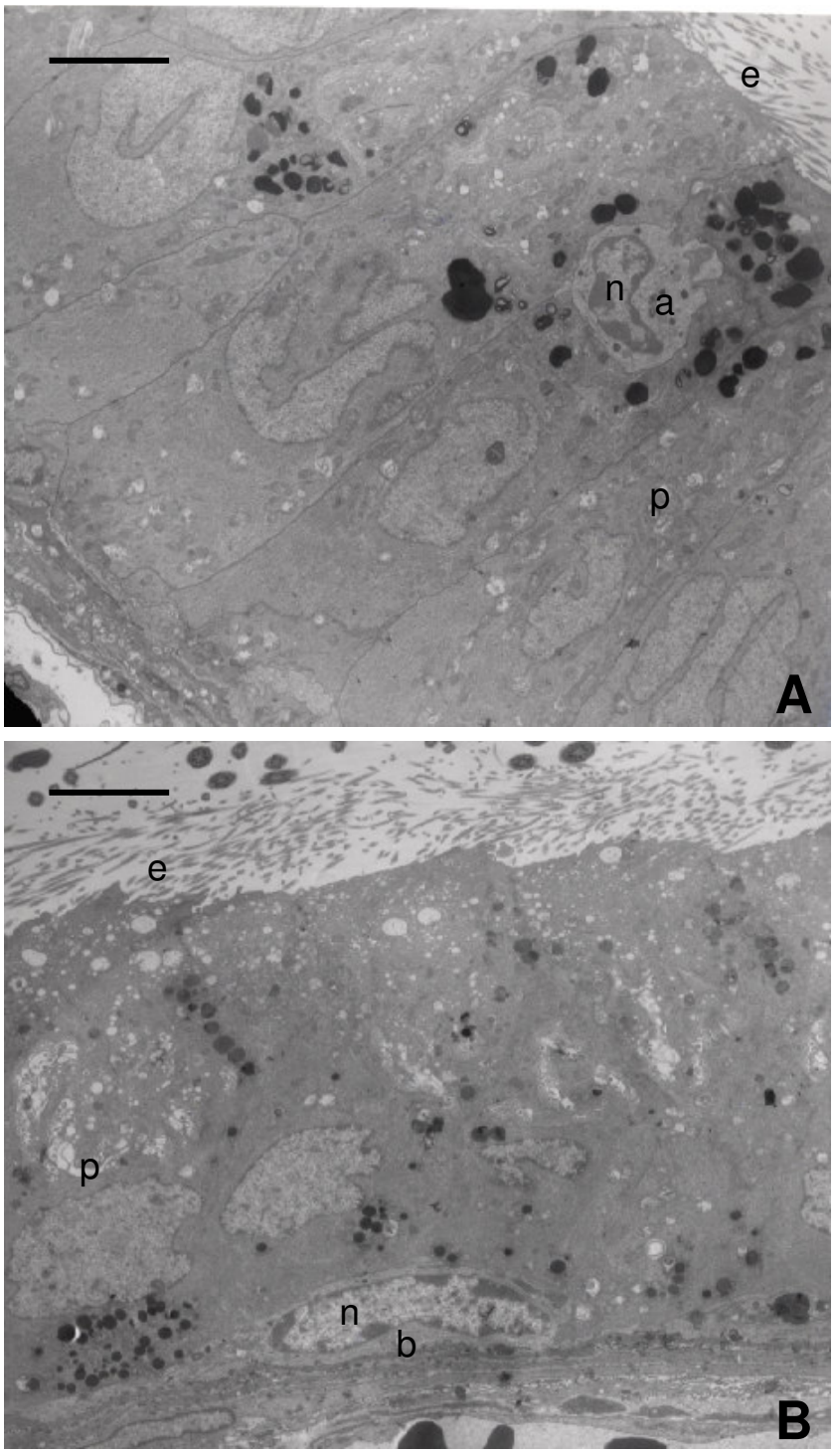
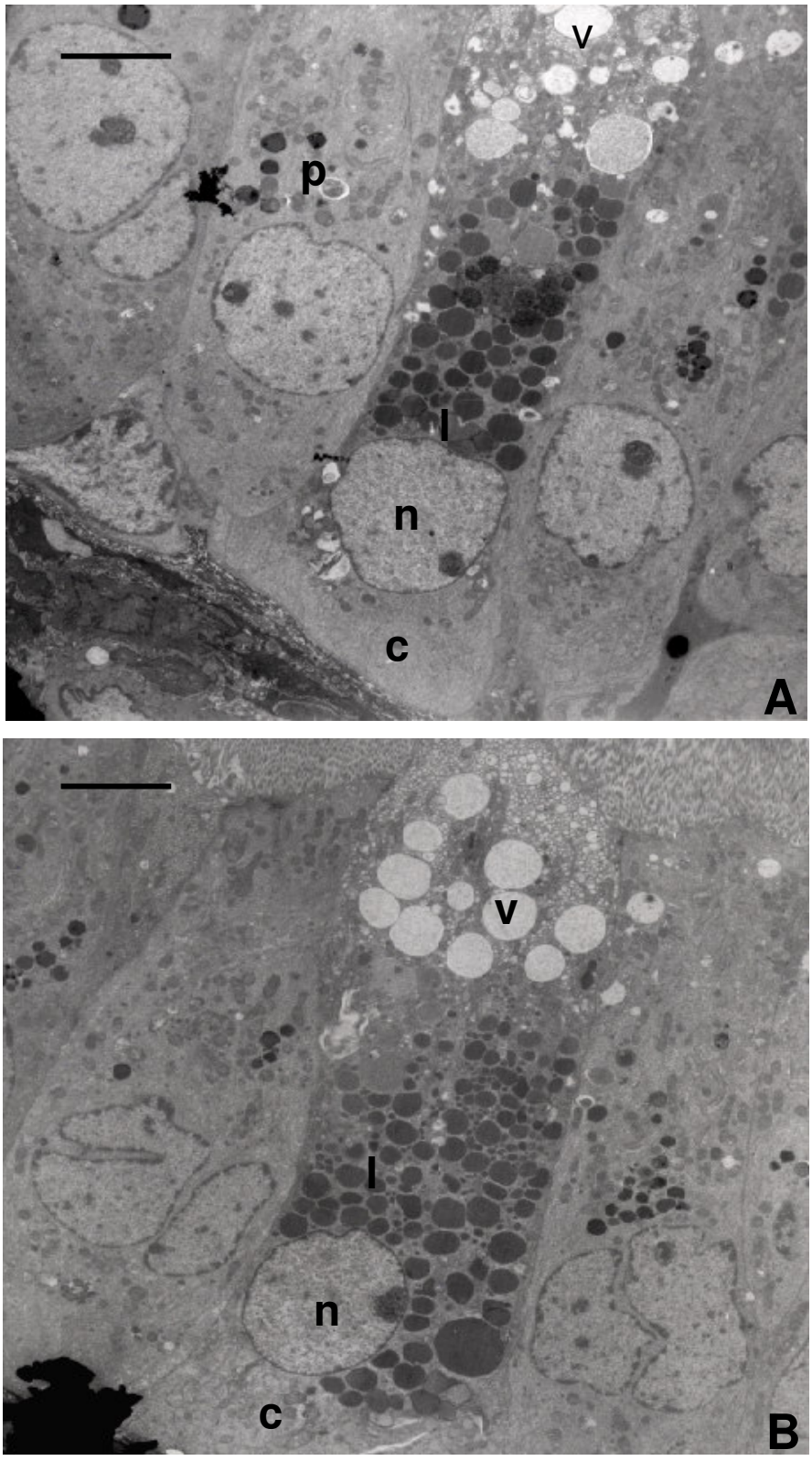


Figura 12. Eletronmicrografias da região da cauda do epidídimo de animais controles, evidenciando células claras (c). Notar a presença de grandes vacúolos (v) na região apical e de numerosos lisossomos. n= núcleo, p= células principais. Barra=5 µm.

Figura 12



Conclusões finais

Concluiu-se que o fenvalerato, diluído em óleo de milho, na dose de 40 mg/kg, administrado oralmente para ratos adultos durante 30 dias consecutivos, não foi estrogênico nas doses testadas *in vivo*. Além disso, foi retido em órgãos reprodutivos e vitais, em especial no epidídimo, importante órgão envolvido no transporte, maturação e estocagem de espermatozóides. O fenvalerato foi também espermatotóxico, visto que reduziu tanto a produção quanto as reservas espermáticas dos animais tratados, sem alterar a fertilidade, uma vez que o rato tem uma grande eficiência reprodutiva, diferentemente do que acontece com o homem.

Referências da Introdução

Abd El-Aziz, M.I., Sahlab, A.M., Abd El-Khalik, M., 1994. Influence of diazinon and deltamethrin on reproductive organs fertility of male rats. Dtsch. Tierarztl. Wochenschr. 101(6), 230-232.

Adelsbach, T.L., Tjeerdema, R.S., 2003. Chemistry and fate of fenvalerate and esfenvalerate. Rev. Environ. Contam. Toxicol. 176, 137-54.

Auger, J., Kunstmann, J.M., Czyglik, F., Jouannet, P., 1995. Decline in semen quality among fertile men in Paris during the past 20 years. N. Engl. J. Med. 332, 281-285.

Barlow, S.M., Sullivan, F.M., Lines, J., 2001. Risk assessment of the use of deltamethrin on bednets for the prevention of malaria. Food Chem. Toxicol. 39, 407-442.

Bissacot, D.Z., Vassilieff, I., 1997. HPLC determination of flumethrin, deltamethrin, cypermethrin residues in the milk and blood of lactating dairy cows. J. Anal. Toxicol. 21(5), 397-402.

Boyer, A.C., Lee, P.W., Potter, J.C., 1992. Characterization of fenvalerate residues in dairy cattle and poultry. J. Agric. Food Chem. 40, 914-918.

Boyle, P., Kaye, S.B., Robertson, A.G., 1987. Changes in testicular cancer in Scotland. Eur. J. Cancer Clin. Oncol. 23, 827-830.

Carson, R., 1962. Silent Spring. Boston University Press, Boston, Massachusetts., 400 p.

Chatterjee, K.K., Talukder, G., Sharma, A., 1982. Effects of synthetic pyrethroids on mammalian chromosomes. I. Sumicidin Mutat. Res. 105, 101-106.

Chen, H., Xiao, J., Hu, G., Zhou, J., Xiao, H., Wang, X., 2002. Estrogenicity of organophosphorus and pyrethroid pesticides. J. Toxicol. Environ. Health A 65, 1419-1435.

Clark, J.M., 1997. Insecticides as tools in probing vital receptors and enzymes in excitable membranes. Pestic. Biochem. Physiol. 57, 235-254.

Clermont, Y., 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev. 52, 198-236.

Colborn, T., Dumanoski, D., Myers, J.P., 1996. Our Stolen Future. New York: Benguin Books, 336 p.

Cour-Palais, I.J., 1966. Spontaneous descent of the testicle. Lancet 1 (7452),1403-1405.

Croucher, A., Hutson, D.H., Stoydin, G., 1985. Excretion and residues of the pyrethroid insecticide cypermethrin in lactating cows. Pestic. Sci. 16, 287-301.

Czeizel, A., 1985. Increasing trends in congenital malformations of male external genitalia. Lancet 1 (8426), 462-463.

Ecobichon, D.J., 1996. Toxic effects of pesticides. In: C. D. Klaassen, Casarett and Doull's Toxicology. The basic science of poisons. New York: McGraw-Hill, pp. 643-689.

Eil, C., Nissula, B.C., 1990. The binding properties of pyrethroids to human skin

fibroblast androgen receptors and to sex hormone binding globulin. J. Steroid. Biochem. 35, 409-414.

Elbetieha, A., Da'as, S.I., Khamas, W., Darmani, H., 2001. Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. Arch. Environ. Contam. Toxicol. 41(4), 522-528.

Fao/Who, 1980. 1979 Evaluations of some pesticide residues in food, Rome, Food and Agriculture Organization of the United Nations, 299-353 (FAO Plant Production and Protection Paper 20 Sup.).

Forman, D., Moller, H., 1994. Testicular cancer. Cancer Surv., 19-20, 323-341.

Forshaw, P.J., Lister, T., Ray, D.E., 2000. The role of voltage-gated chloride channels in type II pyrethroid insecticide poisoning. Toxicol. Appl. Pharmacol. 163, 1-8.

Gaido, K.W., Leonard, L.S., Lovell, S., Gould, J.C., Babai, D., Portier, C.J., Mcdonnell, D.P., 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol. Appl. Pharmacol. 143, 205-212.

Garey, J., Wolff, M.S., 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Bioch. Bioph. Res. Comm. 251, 855-859.

Giray, B., Gurbay, A., Hincal, F., 2001. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E and allopurinol. Toxicol. Lett. 118, 139-146.

Go, V., Garey, J., Wolff, M.S., Pogo, B.G.T., 1999. Estrogenic potencial of certain

pyrethroid compounds in the MCF-7 human breast carcinoma cell line. Environ. Health Persp. 107(3), 173-177.

Gray, L.E.J., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R.L., Ostby, J., 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and Ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rats. Toxicol. Ind. Health 15, 94-118.

Gupta, A., Nigam, D., Gupta, A., Shukla, G.S., Agarwal, A.K., 1999. Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. J. Appl. Toxicol. 19, 67-72.

Hayward, S.W., Baskin, L.S., Haughney, P.C., Cunha, A.R., Foster, B.A., Dahiya, R., Prins, G.S., Cunha, G.R., 1996a. Epithelial developmental in the rat ventral prostate, anterior prostate and seminal vesicle. Acta Anat. (Basel) 155, 81-93.

Hayward, S.W., Baskin, L.S., Haughney, P.C., Foster, B.A., Cunha, A.R., Dahiya, R., Prins, G.S., Cunha, G.R., 1996b. Stromal developmental in the ventral prostate, anterior prostate and seminal vesicle of the rat. Acta Anat. (Basel) 155, 94-103.

He, F., 1994. Synthetic pyrethroids. Toxicology 91(1), 43-49.

Hermo, L., Robaire, B., 2002. Epididymal cell types and their functions. In: B. Robaire, B.T. Hinton, (Eds), The epididymis – from molecules to clinical practice. KA/PP, New York, pp. 81-102.

Hu, J.Y., Wang, S.L., Zhao, R.C., Yang, J., Chen, J.H., Song, L., Wang, X.R., 2002. Effects of fenvalerate on reproductive and endocrine systems of male rats. Zhonghua Nan Ke Xue 8 (1), 18-21.

IPCS – International Program on Chemical Safety, 1990. Environmental health criteria 95. Fenvalerate. Geneva; World Health Organization.

Irvine, D.S., 1996. Declining sperm quality: a review of facts and hypotheses. Bailliere's Clin. Obstet. Gynecol. 11, 655-671.

Jarfelt, K., Dalgaard, M., Hass, U., Borch, J., Jacobsen, H., Ladefoged, O., 2005. Antiandrogenic effects in male rats perinatally exposed to a mixture of di-(2-ethylhexyl) phthalate and di-(2- ethylhexyl) adipate. Reprod. Toxicol. 19, 505-515.

Jones, R.C., 1999. To store or mature spermatozoa? The primary role of the epididymis. Int. J. Androl. 22, 57-67.

Kaneko, H., Ohkawa, H., Miyamaoto, J., 1981. Comparative metabolism of fenvalerate and the {2S, αS} isomer in rats and mice. Pestic. Sci. 6, 317-326.

Kavlock, R.J., 1996, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ. Health. Perspect. 104, 715-740.

Kelce, W.R., Lambright, C.R., Gray, L.E., Roberts, K.P., 1997, Vinclozolin and p' p'-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. Toxicol. Appl. Pharmacol. 142, 192-200.

Kunimatsu, T., Yamada, T., Ose, K., Sunami, O., Kamita, Y., Okuno, Y., Seki, T., Nakatsuka, I., 2002. Lack of (anti-) androgenic or estrogenic effects of three pyrethroids (esfenvalerate, fenvalerate, and permethrin) in the hershberger and uterotrophic assays. Regul. Toxicol. Pharmacol. 35, 227-237.

Landrigan, P.J., Claudio, L., Mcconnell, R., 1998. In: M. Lippman, (Eds), Environmental toxicants, VanRhinehold, NY.

Mandal, T.K., Chakraborty, A.K., Bhattacharya, A., 1995. Disposition kinetics of cypermethrin and fenvalerate in black Bengal goats. Pestic. Sci. 45, 215-219.

Mandal, T.K., Chakraborty, A.K., Bhattacharya, A., Ghosh, R.K., Majumder, S., 1996. The disposition kinetics and residues of fenvalerate in tissues following a single dermal application to black Bengal goats. Vet. Res. Commun. 20 (3), 265.

Mani, U., Islam, F., Prasad, A.K., Kumar, P., Suresh Kumar, V., Maji, B.K., Dutta, K.K., 2002. Steroidogenic alterations in testes and sera of rats exposed to formulated fenvalerate by inhalation. Hum. Exp. Toxicol. 21 (11), 593-597.

Mann, T., 1974. Secretory function of the prostate, seminal vesicle and other male acessory organs of reproduction. J.Reprod. Fert. 37, 179-188.

Matlai, P., Beral, V., 1985. Trends in congenital malformations of external genitalia. Lancet 1 (8420), 108.

Miyamoto, J., Kaneko, H., Tsuji, R., Okuno, Y., 1995. Pyrethroids, nerve poisons: how their risks to human health should be assessed. Toxicol. Lett. 82-83, 933-940.

Moniz, A.C., Cruz-Casallas, P.E., Oliveira, C.A., Lucisano, A., Florio, J.C., Nicolau, A.A., Spinosa, H.S., Bernardi, M.M., 1999. Perinatal fenvalerate exposure: behavior and endocrinology changes in male rats. Neurotoxicol. Teratol. 21(5), 611-618.

Nassr, A. C. C., 2005. Desenvolvimento reprodutivo de ratos machos expostos ao fenvalerato in utero e lactação. 61p. Dissertação (Mestrado). Universidade Estadual de Campinas, Campinas.

Nethersell, A.B., Drake, L.K., Sikora, K., 1984. The increasing incidence of testicular cancer in East Anglia. Br. J. Cancer 50, 377-380.

Neubert, D., Chahoud, I., 1995. Possible consequences of pre- or early postnatal exposure to substances with estrogenic or androgenic properties. Endocrin. Chemic. Environ. 3, 24-52.

Neubert, D., 1997. Vulnerability of the endocrine system to xenobiotic influence. Regul. Toxicol. Pharmacol. 26, 9-29.

Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., Utsumi, H., 2000. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. J. Health Sci. 46, 282-298.

Orgebin-Crist, M.C., Jahad, N., 1978. The maturation of rabbit epididymal spermatozoa in organ culture: inhibition by antiandrogens and inhibitors of ribonucleic acid and protein synthesis. Endocrinology 103, 46-53.

Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J., Gray, L.E.J., 2000. The plasticizer di-ethylhexyl phthalate induces malformations by decreasing fetal testosterone synyhesis during sexual

differentiation in the male rat. Toxicol. Sci., 58, 339-349.

Patel, P.S., Shah, P.G., Patel, B.K., Patel, T.R., 1990. Residues of fenvalerate in pigeon pea. J. Food Sci. Technol. 27, 317-318.

Pati, P.C., Bhunya, S.P., 1989. Cytogenetic effects of fenvalerate in mammalian in vivo test system. Mutat. Res., 222 (3), 149-154.

Pike, M.C., Chilvers, C.E., Bobrow, L.G., 1987. Classification of testicular cancer in incidence and mortality statistics. Br. J. Cancer 56, 83-85.

Prasanthi K., 2001, Investigations in to the mechanism of toxicity of pyrethroids in mammalian system. Thesis. University of Mysore, India, 226.

Prasanthi, K., Muralidhara, Rajini, PS., 2005. Morphological and biochemical perturbations in rat erythrocytes following in vitro exposure to fenvalerate and its metabolite. Toxicol. In Vitro 19, 449-456.

Puig, M., Carbonell, E., Xamena, N., Creus, A., Marcos, R., 1989. Analysis of cytogenetic damage induced in cultured human lymphocytes by the pyrethroid insecticides cypermethrin and fenvalerate. Mutagenesis 4, 72-74.

Reid, B.L., Cleland, K.W., 1957. The structure function of the epididymis. I- The histology of the rat epididymis. Aust. J. Zool. 5(3), 223-251.

Rodrigues, J.A., Favaretto, A.L.V., 1999. Sistema reprodutor. In: M.M. Aires, Fisiologia. Guanabara Koogan, Rio de Janeiro, 877-917.

Roy-Burman, P., Wu, H., Powell, W.C., Hagenkord, J., Cohen, M.B., 2004. Genetically defined mouse models that mimic natural aspects of human

prostate cancer developmental. Endocr. Relat. Cancer 11, 225-324.

Russell, L.D., Ettlin, R.A., Sinhahikin, A.T., Clegg, E.D., 1990. Histological and histopathological evaluation of the testis. Cache River Press, Clearwater.

Saito, K., Sumida, N., Ohe, N., Tomigahara, Y., Isobe, N., Nakatsuka, I., Kaneko, H., 2000a. Evaluation of agonistic and antagonistic effects of several chemicals, mainly pyrethroid insecticides, using three in vitro assays with human estrogen, androgen or progesterone receptor mediated mechanisms. Toxicologist 54, 274.

Saito, K., Tomigahara, Y., Ohe, N., Isobe, N., Nakatsuka, I., Kaneko, H., 2000b. Lack of significant estrogenic or antiandrogenic activity of pyrethroid insecticides in three in vitro assays based on classic estrogen receptor mediated mechanisms. Toxicol. Sci. 57, 54-60.

Sharpe, R.M., Skakkebaek, N.E., 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 35, 1392-1395.

Soderlund, D.M., Clark, J.M., Sheets, L.P., Mullin, L.S., Piccirillo, V.J., Sargent, D., Stevens, J.T., Weiner, M.L., 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 1, 171 (1), 3-59.

Sokol, R.Z., 1997. The hypothalamic – pituitary – gonadal axis as a target for toxicants. In: Sipes, G., McQueen, C.A., Gandolfi, A.J., Comprehensive toxicology, Cambridge University Press, Cambridge, pp. 87-98.

Sumida, K., Ooe, N., Nagahori, H., Saito, K., Isobe, N., Kaneko, H., Nakatsuka, I., 2001a. An in vitro reporter gene assay method incorporating metabolic activation with human and rat S9 or liver microsomes. Biochem. Biophys. Res.

Commun. 280, 85-91.

Sumida, K., Saito, K., Ooe, N., Isobe, N., Kaneko, H., Nakatsuka, I., 2001b. Evaluation of in vitro methods for detecting the effects of various chemicals on the human progesterone receptor, with a focus on pyrethroid insecticides. Toxicol. Lett. 118, 147-155.

Tan, L.F., Wang, S.L., Sun, X.Z., Li, Y.N., Wang, Q.L., Ji, J.M., Chen, L.S., Wang, X.R., 2002. Effects of fenvalerate exposure on the semen quality of occupational workers. Zhonghua Nan Ke Xue 8(4), 273-276.

Tanenbaum, D.M., Wang, Y., Williams, S.P., Sigler, P.B., 1998. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proc. Natl. Acad. Sci. USA 95, 5998-6003.

Tarin, D., 1972. Tissue interactions and the maintenance of histological structure in adults. In: D. Tarin, (Eds), Tissue Interactions in Carcinogenesis. Academic Press, London, 81-94.

U.S. Environmental Protection Agency (US EPA), 1997. Epa/630/R-96/012: Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis. Washington.

Van Waeleghem, K., De Clercq, N., Vermeulen, L., Schoonjane, F., Comhaire, F., 1996. Deterioration of sperm quality in young healthy Belgian men. Hum. Reprod. 11, 325-329.

Whitley, R., Meikle, A.W., Watts, N.B., 1994. Endocrinology. In: E.R. Ashwood, C.A. Burtis, Tietz textbook of clinical chemistry. Philadelphia: W. B. Saunders Company, 1645-1660.

World Health Organization (Who), 1990. d-Phenothrin. Environmental Health Criteria. Geneva.

Xu, L.C., Sun, H., Chen, J.F., Bian, Q., Song, L., Wang, X.R., 2006. Androgen receptor activities of p, p'-DDE, fenvalerate and phoxim detected by androgen receptor reporter gene assay. Toxicol. Lett. 160, 151-157.

Yucesan, S., Dindar, H., Olcay, I., Okur, H., Kilicasian, S., Ergoren, Y., Tuysuz, C., Koca, M., Civilo, B., Sen, I., 1993. Prevalence of congenital abnormalities in Turkish school children. Eur. J. Epidemiol. 9, 373-380.