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COMPARAÇÃO DOS EFEITOS DO DIURON FORMULADO E TÉCNICO NO UROTÉLIO E NOS PARÂMETROS REPRODUTIVOS DE RATOS WISTAR MACHOS

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Patologia.

Orientadora: Maria Luiza Cotrim Sartor de Oliveira
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Palavras-chave: Bexiga ; Diuron produto formulado; Diuron produto técnico; Microscopia eletrônica de varredura; Parametrôs espermáticos.

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RESUMO

Ratos Wistar expostos por dois anos ao diuron (3-(3,4-diclorofenil)-1,1-dimetilureia) produto técnico (PT) à 2500ppm pela ração desenvolveram alta incidência de tumores uroteliais, sendo esse herbicida classificado pela U.S. Environmental Protection Agency como "provável cancerígeno para o homem". Durante a última década, nosso laboratório tem realizado estudos sobre a toxicidade urotelial do diuron PT fornecido pela ração a ratos Wistar machos. Porém, o diuron na forma de produto formulado (PF) ou comercial, composto por 50% de diuron PT e 50% de ingredientes inertes, não tem sido avaliado em ratos. Em lagartos *P. sicula* machos expostos ao diuron PF à 50% na concentração de 1100ppm na ração ou na água de beber, durante 3 semanas, houve toxicidade direta no sistema reprodutivo com alteração na espermatogênese, morfologia do epidídimo e diminuição dos níveis plasmáticos dos hormônios sexuais, sendo os efeitos mais severos nos grupos que receberam diuron na água de beber. O presente estudo objetivou comparar o potencial tóxico do diuron PF ou PT à 1250ppm oferecido pela ração ou água de beber no urotélio e nos parâmetros reprodutivos de ratos. Cinquenta ratos Wistar machos de seis semanas de idade foram distribuídos em cinco grupos (ração basal, ração com PF, água de beber com PF, ração com PT, água potável com PT) e expostos ao diuron à 1250ppm por 13 semanas. Na eutanásia foram avaliados a morfologia, concentração, motilidade e viabilidade dos espermatozoides. Os rins, fígado, baço, testículos e epidídimos foram retirados e pesados; os rins e testículos processados para histologia. A bexiga foi seccionada ao meio e processada para análise histológica e para microscopia eletrônica de varredura (MEV). Todos os grupos expostos ao diuron tiveram diminuição do peso corpóreo superior a 10%, comparados ao controle, indicando que a máxima dose tolerada (MDT) foi excedida. O consumo de ração e de água foi menor em todos os grupos expostos ao diuron. Não houve diferença nos pesos absolutos e relativos de órgãos entre os grupos. Em todos os grupos expostos ao diuron, os achados microscópicos incluíram hiperplasia urotelial simples (HS) na pelve renal e na bexiga à MEV. Diuron PF e PT induziram alterações na morfologia do esperma e diminuição da motilidade, concentração e viabilidade dos espermatozoides. Esse estudo mostrou que o diuron é nocivo ao urotélio e aos parâmetros reprodutivos de ratos Wistar machos, independentemente de estar na forma técnica (PT) ou formulada (PF) ou se administrado pela ração ou pela água de beber.

Palavras chaves: Diuron produto técnico, diuron produto formulado, bexiga, parâmetros reprodutivos, microscopia eletrônica de varredura, citotoxicidade urotelial

ABSTRACT

Wistar rats exposed during two years to 2500ppm dietary diuron technical product (TP) (3-(3,4-dichlorophenyl)-1,1-dimethylurea) developed high incidence of urothelial tumors, being classified by the U.S. Environmental Protection Agency as a ‘‘known/likely human carcinogen’’. During the last decades our laboratory conducted studies on the urothelial toxicity of the diuron (TP) provided through diet to male Wistar rats. The diuron formulated product (FP) composed by 50% diuron TP and 50% inert ingredients, has not been evaluated in rats. The diuron (FP) was harmful to reproductive system of male *P. sicula* lizards exposed to 1100ppm during 3 weeks; the toxic effects were more prominent in the groups receiving diuron FP in drinking water. This study aimed to compare the potential toxicity of diuron TP or FP 1250ppm offered through diet or drinking water on the urothelium and reproductive parameters of rats. Fifty six-week old Wistar male rats were allocated to five groups (basal diet, FP diet, FP drinking water, TP diet, TP drinking water) and exposed to 1250ppm diuron for 13 weeks. At the end, body weight and weight of kidneys, liver, spleen, testes and epididymis, histopathology of the kidneys and testes and sperm parameters were evaluated. Urinary bladders were bisected and processed for histologic and scanning electron microscopic (SEM) analyses. At the end of the study, all groups exposed to diuron had decreased body weight gain compared to control greater than 10%, indicating that for this experiment the dose exceeded the maximum tolerated dose (MTD). Food and drinking water consumption was lower in the groups exposed to diuron. There were no differences in the absolute and relative organ weights among the groups. In all groups exposed to diuron, microscopic findings included simple urothelial hyperplasia (SH) in the kidney pelvis and in the urinary bladder. By SEM, the urinary bladder had superficial cytotoxicity in all groups exposed to diuron. Diuron FP and TP induced changes in the sperm morphology and concentration, and decreased sperm motility and viability. This study showed that diuron FP as well as diuron TP is harmful to the urothelium and to the reproductive parameters of male Wistar rats, regardless of whether in a diet or drinking water.

Key word: Diuron technical product, diuron formulated product, urinary bladder, reproductive parameters, scanning electron microscopy, urothelial cytotoxicity

SUMÁRIO

REVISÃO DE LITERATURA

Exposição ambiental e desenvolvimento de cancêr de bexiga

O câncer urotelial de bexiga é um dos tumores malignos mais comuns em humanos, com elevada incidência e alta mortalidade. Em 2012, havia aproximadamente 430.000 novos casos de câncer de bexiga em todo o mundo, e a doença representava mais de 165.000 mortes (Torre et al., 2015). No Brasil, as taxas em 2016 foram de aproximadamente 9.670 casos novos, sendo 7.200 em homens e 2.470 em mulheres (INCA, 2016).

A relação entre o desenvolvimento de câncer de bexiga e a exposição à substâncias químicas foi descrita inicialmente pelo médico alemão Rehn em 1895, após documentar incidência crescente de tumores uroteliais em trabalhadores da indústria de corantes à base de anilinas. Mais tarde, foi constatado que o fator de risco para o desenvolvimento do câncer era a amina aromática 2-naftilamina, uma das substâncias corantes utilizadas nessas indústrias (Johansson & Cohen, 1997).

Atualmente, sabe-se que o estilo de vida, fatores ambientais e exposições ocupacionais são as condições de maior risco para o desenvolvimento do carcinoma urotelial (Crawford, 2008; Burger et al., 2013; Czerniak et al., 2016). A exposição a derivados do tabaco, aminas aromáticas, agrotóxicos ou outros compostos, têm associação com alterações da bexiga até o desenvolvimento de câncer (Johansson & Cohen, 1997; Brennan, et al., 2000; Siemiatycki et al., 2004). Outras possíveis condições que podem levar a doença são a exposição à contaminantes presentes na água, alimentação e susceptibilidade genética (Pelucchi et al., 2006; Burger et al., 2013).

Segundo Oliveira et al., (2006) a bexiga de roedores é semelhante a dos seres humanos e pode auxiliar no entendimento do processo cancerígeno. Ademais, o uso de roedores como modelo de carcinogênese urotelial têm vantagens para abordar hipóteses específicas sobre os mecanismos subjacentes a esta doença.

Exposição ambiental e alterações de parâmetros reprodutivos

Os produtos químicos ou antropogênicos, liberados no meio ambiente, denominados contaminantes emergentes, podem agir como desreguladores endócrinos, causando efeitos adversos à saúde, alterando ou interrompendo o sistema hormonal e influenciando na síntese, secreção, transporte, ligação ou na eliminação de hormônios naturais do corpo (USEPA, 1997; Janex-Habibi et al., 2009; WHO, 2012; USEPA, 2017; Solecki et al., 2017). Uma gama de produtos químicos foi identificada como desreguladores endócrinos incluindo hormônios (naturais e sintéticos), agrotóxicos e plastificantes (Diamanti-Kandarakis et al., 2009). O declínio na concentração e na qualidade do esperma tem relação com contaminantes emergentes do ambiente e com o estilo de vida (Skakkebaek et al., 2006; Foster et al., 2008). No caso dos agrotóxicos, a contaminação do meio ambiente ocorre como resultado de aplicações diretas, ou indiretas através da lixiviação, escoamento e deposição seca/úmida (CCME, 1999).

Os agrotóxicos podem agir como agonistas ou antagonistas de receptores hormonais como os receptores de estrógenos (ER) ou de andrógenos (AR), alterando a ação de hormônios essenciais para equilíbrio do organismo e, consequentemente, os processos hormônios-dependentes, como o comportamento e a reprodução (Le Blanc et al., 1997).

Herbicida diuron

O diuron (3-(3,4-dichlorofenil)-1,1-dimetilureia) é um herbicida derivado da ureia, amplamente utilizado no meio agrícola. Apresenta amplo espectro de ação e é utilizado em aplicações pré e pós-emergentes para o controle de plantas daninhas nas culturas de algodão, cana de açúcar e, alfafa, entre outras (Roque & Melo, 2000; USEPA, 2003; APVMA, 2005). É facilmente absorvido pelas raízes das plantas invasoras e tem como função primordial inibir a fotossíntese, bloqueando a produção de oxigênio (Iyer, 2002; APVMA, 2005).

Nos anos de 2015, a produção de soja, milho e cana-de-açúcar foram as culturas predominantes no Brasil. Os três cultivos agrícolas ocupam aproximadamente 76% de toda a área plantada no país e

foram os que mais consumiram agrotóxicos (Pignati et al., 2017). Os herbicidas representaram total de 111.858 toneladas e são os agrotóxicos mais utilizados no Brasil (SINDIGEV, 2016).

Com a produção intensificada e a utilização de herbicidas como primordial componente nesses cultivos, existe grande preocupação em relação aos potenciais impactos ao meio ambiente e a saúde do homem. De lenta degradação e de alta persistência, o diuron é considerado um poluente biologicamente ativo no solo, sedimentos e na água (Field et al., 2003; Giacomazzi & Cochet, 2004).

Os principais produtos de degradação ambiental do diuron são a 3,4- dicloroanilina (DCA), a 3-(3,4-diclorofenil)-1-metilureia (DCPMU) e a 3,4-diclorofenilureia (DCPU), que exibem alta toxidade (Giacomazzi & Cochet, 2004).

O diuron é vendido para uso comercial na agricultura como produto formulado (PF) na concentração de 50% e 80% de produto técnico e ingredientes inertes. A maioria dos estudos experimentais utiliza o produto técnico (PT) diuron 98%, considerado sua forma pura. Estudos experimentais em animais utilizando o diuron produto formulado (PF) ou produto comercial são raros. Os defensivos agrícolas ou agrotóxicos encontrados no mercado como PF contêm um ou mais ingredientes inertes ou adjuvantes, que são aditivos, solventes, estabilizadores e surfactantes adicionados a produtos técnicos (PT) para aumentar sua eficácia (Foy, 1987). Estes ingredientes têm a capacidade de solubilizar a substância ativa, aumentando sua penetração no organismo alvo, alterando as características físico-químicas da mistura para melhor eficiência, distribuição e redução das derivas (Foy, 1987; Otero et al., 2003; Krogh et al., 2003). Os ingredientes inertes ou adjuvantes podem ser adicionados ao herbicida durante a produção do produto comercial ou adicionados à solução de pulverização como mistura. A concentração do ingrediente ativo do produto técnico no PF varia conforme o fabricante, sendo comercializado em formulações não-aquosas concentradas, concentrados emulsificantes, granulados e sprays (WSSA, 1994).

O etilenoglicol, a caulinita ou caolim e alquilfenóis são algumas das substâncias incluídas como "ingredientes inertes" no diuron PF (Cox, 2003; Xie et al., 2005; Felicio, 2017). O etilenoglicol provoca riscos para a saúde que incluem irritação da garganta e do trato respiratório superior, toxicidade renal e aumento da incidência de malformações fetais (USEPA, 1999). Camundongos fêmeas grávidas expostas

a 0, 150, 1000 ou 2500 mg/m3 de etilenoglicol aerossolizado durante 6 horas por dia mostraram diminuições significativas no número de fetos vivos, que apresentaram alta incidência de malformações externas, viscerais e esqueléticas em exposições maiores ou iguais a 1000 mg/m3 (Tyl et al., 1995). Já a exposição ocupacional à caulinita, um argilo-mineral de alumínio hidratado, aumentou o risco para câncer de pulmão (Szadkowska-Stanczyk, 2001).

Em ratos, os principais alvos de toxicidade do diuron são o sistema hematopoiético e a mucosa urotelial (USEPA, 1997; Iyer, 2002). Em animais como peixes machos Tilápia do Nilo da espécie *Oreochromis niloticus* (Pereira et al., 2015) e lagartos machos da espécie *Podarcis sicula* (Cardone et al., 2008) são descritas alterações nos parâmetros reprodutivos.

Diuron produto técnico (PT) e toxicidade no sistema hematopoiético

O diuron exerceu efeitos hemotóxicos e no baço de ratas Sprague-Dawley tratadas à 250, 500 e 1000 mg/kg pela ração por 14 meses. Foi observado aumento dos pesos relativos e da pigmentação (hemossiderina) na polpa vermelha do baço. Ainda, houve aumento da formação de metahemoglobina, ocasionando anemia secundária e aumento dos eritrócitos anormais, além de diminuição na concentração de hemoglobina e redução no número de glóbulos vermelhos saudáveis (Wang et al., 1993).

Ratos Wistar machos expostos ao diuron nas concentrações de 1250 e 2500ppm pela ração por 14, 28 ou 90 dias apresentaram sinais gerais de toxicidade como redução de ingestão de alimentos, aumento dos pesos relativos do baço, rins e fígado, e níveis séricos elevados de alanina aminotransferase (ALT), albumina, proteína total, creatinina e ureia nos dias 28 e 90 dias de tratamento; a concentração de 125ppm representou o NOAEL para esses efeitos. O estudo indicou toxicidade orgão-específica no baço, com severa depleção de células brancas, diminuição do número de linfócitos T CD4 (+), hematopoiese extramedular aumentada e deposição de hemossiderina na polpa vermelha (Domingues et al., 2011). O aumento do peso do baço e a esplenomegalia são reconhecidos como efeitos adversos induzidos por diuron em ratos (USEPA, 2003; APVMA, 2005; Cardoso et al., 2013; Fava et al., 2015)

Diuron produto técnico (PT) e carcinogênese urotelial

Evidências experimentais indicam que o diuron é cancerígeno para a bexiga de ratos. Estudo com ratos Wistar de ambos os sexos alimentados com o diuron nas concentrações de 0, 25, 250 e 2500ppm por dois anos mostrou que a dose mais elevada de 2500ppm (111mg/kg/day) induziu a formação de papilomas e carcinomas uroteliais na bexiga e na pelve renal dos ratos; a concentração de 250ppm provocou aumento da incidência de hiperplasia urotelial (USEPA,1987, 2003; Iyer, 2002; APVMA, 2011). Por esse fato, a Environmental Protection Agency classificou o diuron como "provável cancerígeno para o homem" (USEPA, 1997). Devido aos seus efeitos adversos, em alguns países da Europa como a Dinamarca, Alemanha, Suécia e o Reino Unido sua utilização foi banida ou restrita (APVMA, 2011).

A carcinogênese urotelial em ratos ocorre por meio de uma sequência de alterações morfológicas que começam com hiperplasia simples e progridem para hiperplasia papilar e nodular, papilomas e, em última instância, neoplasias (Cohen et al., 2002). A hiperplasia simples é considerada uma condição predisponente para a carcinogênese da bexiga e tem relação direta com a dose e duração do estímulo carcinogênico (Cohen, 1998).

O modo de ação (MoA) pelo qual o diuron e seus metabólitos induzem lesões uroteliais não está relacionado com alterações da composição urinária (da Rocha et al., 2010) e nem com genotoxicidade (Nascimento et al., 2006). Os eventos-chaves envolvidos no desenvolvimento da carcinogênese urotelial incluem ativação metabólica, particularmente N-(3,4-dichlorofenil)uréia (DCPU) e 4,5-dichloro-2-hidroxifenil uréia (2-OHDCPU), citotoxicidade, degeneração celular, esfoliação e necrose seguidas de hiperplasia regenerativa e eventualmente formação de tumores (da Rocha et al., 2010, 2012, 2014; Fava et al., 2015).

A avaliação do perfil global da expressão gênica de células uroteliais da bexiga urinária de ratos expostos por 7 dias ou 20 semanas a diferentes concentrações do diuron administrado pela ração mostrou uma evidente separação entre os grupos de altas concentrações (1250 e 2500ppm) dos de baixas concentrações (125ppm e 500ppm) (Ihlaseh et al., 2011; 2014). Este perfil dose-resposta ocorreu

também na incidência de lesões morfológicas, conforme demonstrado por estudo de 20 semanas (Cardoso et al., 2013).

Diuron à 2500ppm administrado pela ração aumentou a incidência de hiperplasia urotelial simples (HS) e o índice de proliferação celular a partir de 15 semanas de exposição (Nascimento et al., 2006; da Rocha et al., 2010; Cardoso et al., 2013; Fava et al., 2015). Ainda, após 20 semanas, estudos de dose-resposta mostraram que a concentração de 1250ppm (55mg/kg/dia) induziu as mesmas alterações transcricionais (Ihlaseh et al., 2011) e morfológicas (Ihlaseh et al., 2011; Cardoso et al. , 2013) detectadas na de 2500ppm.

O potencial promotor do diuron na carcinogênese mamária e urinária foi investigado em camundondos Swiss fêmeas expostos a dois carcinógenos iniciadores o DMBA (7,12-dimetilbenz (a) antraceno) e o BBN N-butil-N- (4-hidroxibutil) nitrosamina administrados por gavagem por seis semanas e o diuron a 1250 e 2500 pela ração até a 20^{a} semana. Evidenciou-se incidência aumentada de hiperplasia urotelial simples na bexiga de todos os grupos, dois carcinomas uroteliais no grupo iniciado (DMBA e BBN) e expostos ao diuron 2500ppm. O resultados indicaram que o diuron é promotor para a bexiga, mas não para a glândula mamária (Moura et al., 2010).

Diuron produto técnico (PT) e alterações nos parâmetros reprodutivos

Os herbicidas derivados da ureia podem atuar em receptores hormonais e afetar os níveis de hormônios pituitários que atuam nos sistemas reprodutivos masculinos e femininos (Cook et al., 1993; Iyer, 2002). De fato, em experimentos *in vitro* o diuron e outros herbicidas da família fenilureia mostraram capacidade de se ligar a receptores de andrógenos, alterando a função normal do organismo (Bauer et al., 1998).

Em ratos expostos durante 10 semanas ao isoproturon, um herbicida da familia fenilureia, detectou-se efeitos testiculares como redução dos túbulos seminíferos, diminuição do número de espermatozoides e da porcentagem de espermatozoides móveis, e aumento da porcentagem de espermatozoides morfológicamente anormais (Sarkar et al., 1997). A redução na porcentagem de

espermatozoides móveis pode estar associada a anormalidades estruturais ou metabólicas derivadas de função testicular alterada ou fatores anti motilidade no plasma seminal (WHO, 2010).

Em peixes *Oreochromis niloticus* (Tilápia do Nilo) machos a exposição ao diuron (PT) ou aos seus metabólitos 3,4-dicloroanilina (DCA), 3,4-dichlorophenylurea (DCPU) e 3,4-diclorofenil-N-metilureia (DCPMU) provocou diminuições de níveis de testosterona e de 11-cetotestosterona sérica, do índice gonadossomático, do diâmetro dos túbulos seminíferos e das porcentagens médias de células germinativas, caracterizando aquelas substâncias como anti-androgênicas, nocivas ao sistema reprodutivo desses animais (Pereira et al., 2015).

Parâmetros endócrinos e de biomarcadores enzimáticos foram estudados *in vitro*, no fígado, cérebro e brânquias de tilápias do Nilo (*Oreochromis niloticus*) expostas ao diuron PT, seus metabólitos (DCPMU, DCPU e DCA) e alquilfenóis (substâncias inertes utilizadas também na formulação do herbicida diuron), em diferentes concentrações, por 7 dias. Estes compostos em associação ou não, induziram alterações nos parâmetros analisados, confirmando que podem ser considerados desreguladores endócrinos (Felicio, 2017).

Em estudo de toxicidade reprodutiva com o diuron, ratos Wistar expostos a 125 ou 250 mg/kg durante 30 dias não mostraram diferenças em relação ao grupo controle nas concentrações plasmáticas de testosterona, produção diária de espermatozóides, reservas de esperma no epidídimo e morfologia dos epermatozoides. No entanto, o número de fetos nas ninhadas de ratos expostos ao diuron foi menor ($p<0,05$) do que das ninhadas de ratos do grupo controle (Fernandes et al., 2007).

Para avaliar se o diuron altera a puberdade ou vulnerabilidade à carcinogênese mamária, ratas Sprague-Dawley prenhes receberam ração basal ou ração com diuron a 500, 750 e 1250 ppm a partir do dia 12 de gestação até o final da lactação. Após o desmame, a prole feminina continuou a receber ração basal ou ração com diuron PT até os dias pós-natal (PND) 51, 75, 226 a 233. Não houve diferenças entre os grupos com relação a abertura vaginal, ciclo estral, e morfologia da mama. No entanto, foram evidenciadas reduções no peso do ovário e do corpo lúteo em ratas tratadas com diuron 1250ppm no PND 75 (Grassi et al., 2011).

O diuron, fornecido pela água de beber nas concentrações de 2500, 5000, 10000, 20000 ou 40000μg/L durante 48 horas ou 96 horas, induziu efeitos teratogênicos, levando a malformações no desenvolvimento da cabeça, corpo e cauda e aumento significativo da mortalidade do embrião-larva do peixe rodaballo (*Psetta maxima*). Os organismos que sobreviveram sofreram diminuição significativa no sucesso da incubação, malformações na cabeça, corpo e cauda e edema pericárdico. Os efeitos observados foram mais severos nos grupos de embriões-larva expostos à altas doses (Mhadhbi & Beiras, 2012).

Diuron produto formulado (PF) e parâmetros reprodutivos

Ratas Wistar prenhes expostas ao diuron PF (80% de diuron PT adicionado a 20% de ingredientes inertes) por gavagem do 6º ao 15º dia de gestação apresentaram aumento significante de fetos malformados nas doses de 250 e 500 mg/kg/dia de exposição, foi também observado diminuição do peso fetal médio à dose de 500 mg/kg/dia (Khera et al., 1979).

Em lagartos *Podarcis sicula* machos o diuron PF (50% de diuron TP adicionado a 50% de ingredientes inertes) alterou a espermatogênese, morfologia do epidídimo e secreção de esteroides sexuais plasmáticos, indicando toxicidade reprodutiva masculina direta. Os animais foram expostos pela água e terrários contaminados com diuron PF. As alterações ocorreram em todos os grupos tratados com diuron PF, contudo as lesões foram de maior magnitude nos lagartos expostos em água e terrários ou água, alimentos e terrarios contaminados com diuron (Cardone et al., 2008).

A maioria dos estudos experimentais utilizam o diuron produto técnico (PT) e são raros os estudos com produtos formulados (PF) ou comerciais para determinar efeitos adversos. Não se sabe se os ingredientes inertes contidos no diuron PF poderiam potencializar seus efeitos no urotélio e nos parâmetros reprodutivos em ratos. Assim, desenvolvemos o presente estudo com objetivo de comparar o potencial tóxico do diuron PF e diuron PT na concentração de 1250ppm oferecido pela ração ou água de beber, no urotélio e nos parâmetros reprodutivos de ratos Wistar machos durante o período de 13 semanas (90 dias), que corresponde à duração de um estudo subcrônico convencional em roedores (OECD, 1998).

REFERÊNCIAS

APVMA. Australian Pesticides Veterinary Medicine Authority. Review of the mammalian toxicology and metabolism/toxicokinetics of diuron**.** human health assessment. Canberra: APVMA; 2011.

APVMA. Australian Pesticides Veterinary Medicine Authority. The reconsid-eration of approvals of the active constituent diuron, registrations of products containing diuron and their associated labels. Canberra: APVMA; 2005. p. 2-40.

Bauer ERS, Meyer HHD, Stahlschmidt-Allner P, Sauerwein H. Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. Analyst. 1998;123:2485-7.

Brennan P, Bogillot O, Cordier S, Greiser E, Schill W, Vineis P, et al. Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case–control studies. Int J Cancer. 2000;86:289-94.

Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol. 2013;63:234-41.

Canadian Council of Ministers of the Environment. Canadian water quality guidelines for the protection of aquatic life: Linuron. In: Canadian environmental quality guidelines. Winnipeg: CCME; 1999.
Cardone A, Comitato R, Angeline F. Spermatogenesis, epididymis morphology and plasma sex steroid secretion in the male lizard Podarcis siculaexposed to diuron. Environ Res. 2008;108:214-23.

Cardoso AP, Ihlaseh Catalano SM, Rocha MS, Nascimento e Pontes MG, Camargo JL, Oliveira ML. Dose-response of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] in the urothelial mucosa of Wistar rats. Toxicology. 2013;312:1-5.

Cohen SM, Arnold LL, Uzvolgyi E, Cano M, St John M, Yamamoto S, et al. Possible role of dimethylarsinous acid in dimethylarsinic acid-induced urothelial toxity and regeneration in the rat. Chem Res Toxicol. 2002;15:1150-7.

Cohen SM. Urinary bladder carcinogenesis. Toxicol Pathol. 1998;26:121-7.

Cook JC, Mullin LS, Frame SR, Biegel LB. Investigation of a mechanism for Leydig cell tumorigenesis by linuron in rats. Toxicol Appl Pharmacol. 1993;119:195-204.

Cox C. Herbicide factsheet diuron. J Pesticide Reform. 2003;23:12-20.

Crawford JM. The origins of bladder cancer. Lab Invest. 2008;88:686-93.

Czerniak B, Dinney C, McConkey D. Origins of bladder cancer. Annu Rev Pathol. 2016;23;11:149-74.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: endocrine society scientific statement. Endocr Rev. 2009;30:293-342.

Domingues A, Barbisan LF, Martins PR, Spinardi-Barbisan AL. Diuron exposure induces systemic and organ-specific toxicity following acute and sub-chronic exposure in male Wistar rats. Environ Toxicol Pharmacol. 2011;31:387-96.

Fava RM, Cardoso AP, Rocha MS, Nascimento e Pontes MG, Camargo JLV, Oliveira MLCS. Evaluation of early changes induced by diuron in the rat urinary bladder using different processing methods for scanning electron microscopy. Toxicology. 2015;33:100-6.

Felício AA. Efeitos bioquímicos e estrogênicos do N-(3,4-diclorofenil) -N, N-dimetilureia (diuron) e seus metabólitos, isoladamente ou emassociação com alquilfenóis em tilápias do Nilo (Oreochromis niloticus) [tese]. São José do Rio Preto: Universidade Estadual Paulista; 2017.

Fernandes GS, Arena AC, Fernandez CDB, Mercadante A, Barbisan LF, Kempinas WF. Reproductive effects in male rats exposed to diuron. Reprod Toxicol. 2007;23:106-12.

Field JA, Reed RJ, Sawyer TE, Griffith SM, Wigington PJ. Diuron occurance and distribution in soil and surface and ground ware associated with grass seed production. J Environ Qual. 2003;32:171-9.

Foster WG, Neal MS, Han MS, Dominguez MM. Environmental contaminants and human infertility: hypothesis or cause for concern? J Toxicol Environ Health B. 2008;11:162-76.

Foy CL. Adjuvants: terminology, classification, and mode of action. In: Chow PNP, Grant CA, Hinshalwood AM, Simundson E, editors. Adjuvants and agrochemicals. Boca Raton: CRC Press; 1987. p. 1-15.

Giacomazzi S, Cochet N. Environmental impact of diuron transformation: a review. Chemosphere. 2004;56:1021-32.

Grassi TF, Guerra MT, Perobelli JE, Toledo FC, Silva DS, Kempinas WG, et al. Assessment of female reproductive endpoints in sprague–dawley rats developmentally exposed to diuron: potential ovary toxicity. Birth Defects Res. 2011;92:478-86.

Ihlaseh SM, Bailey KA, Hester SD, Jones C, Ren H, Cardoso AP, et al. Transcriptional profile of diuron-induced toxicity on the urinary bladder of male Wistar rats to inform mode of action. Toxicol Sci. 2011;122:330-8.

Ihlaseh-Catalano SM, Bailey K, Cardoso APF, Ren H, Fry RC, Camargo JLV, et al. Dose and temporal effects on gene expression profiles of urothelial cells from rats exposed to diuron. Toxicology. 2014;325:21-30.

INCA. Instituto Nacional do Câncer. Brasil: Portal do Instituto Nacional do Câncer [Internet]. Rio de Janeiro: INCA; 2018 [citado 20 Jan 2018). Disponível em: http://www.inca.gov.br/estimativa/2016/estimativa-2016-v11.pdf.

Iyer P. Evidence on the development and reproductive toxicity of diuron. Draft. Reproductive and Sacramento (CA): Cancer Hazard Assessment Section. Office of Environmental Health Hazard Assessment; 2002. 43 p.

Janex-habib M, Huyard A, Esperanza M, Bruchet A. Reduction of endocrine disruptor emissions in the environment: The benefit of wastewater treatment. Water Res. 2009;43:1565-76.

Johansson SL, Cohen SM. Epidemiology and etiology of bladder cancer. Semin Surg Oncol. 1997;13:291–8.

Khera KS, Whalen C, Trivett G, Angers G. Teratogenicity studies on pesticidal formulations of dimethoate, diuron and lindane in rats. Environ Contam Toxicol. 1979;22:522-9.

Krogh KA, Halling-Sørensen B, Mogensen BB, Vejrup KV. Environmental properties of non-ionic surfactant adjuvants in pesticides: a review**.** Chemosphere. 2003;50:871-901.

Le Blanc GA, Bain LJ, Wilson VS. Pesticides: multiple mechanisms of demasculinization. Mol Cell Endocrinol. 1997;126:1-5.

Mhadhbi L, Beiras L. Acute toxicity of seven selected pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the marine fish (Turbot, Psetta maxima). Water Air Soil Pollut. 2012;223:5917-30.

Moura NA, Grassi TF, Rodrigues MAM, Barbisan LF. Potential effects of the herbicide Diuron on mammary and urinary bladder two-stage carcinogenesis in a female Swiss mouse model. Arch Toxicol. 2010;84:165-73.

Nascimento MG, Oliveira MLCS, Lima AS, Camargo JL. Effects of diuron [3-(3, 4-dichlorophenyl)-1, 1-dimethylurea] on the urinary bladder mucosa of male Wistar rats. Toxicology. 2006;224:66-7.

OECD. Organization for EconomicCo-operation and Development. Test No. 408: repeated dose 90-day oral toxicity study in rodents. In: OECD Guideline for the Testing of Chemicals, Section 4. Paris: OECD; 1998. p. 1-10.

Oliveira PA, Colaço A, De la Cruz PLF, Lopes C. Experimental bladder carcinogenesis rodent models. Exp Oncol. 2006;28:2-11.

Otero RR, Grande BC, Estévez MA, Periago EL, Gándara JS. Procedure for measurement of soils inputs of plant-protection agents washed off through vineyard canopy by rainfall. J Agric Food Chem. 2003;51:5041-6.

Pelucci C, Bosetti C, Negri E, Malvezzi M, La Vecchia C. Mechanisms of disease: the epidemiology of bladder câncer. Nat Clin Pract Urol. 2006;3:327-40.

Pereira TSB, Boscolo CNP, Silva DGH, Batlouni SR, Schlenk D, Almeida EA. Anti-androgenic activities of diuron and its metabolites in male Nile tilapia (*Oreochromis niloticus*). Aquatic Toxicol. 2015;164:10-5.

Pignati WA, Souza e Lima FAN, Lara SS, Correa MLM, Barbosa JR, Leão LHC, et al. Spatial distribution of pesticide use in Brazil: a strategy for health surveillance. Ciênc Saúde Colet. 2017;22:3281-93.

Rocha MS, Arnold LL, Oliveira ML, Catalano SM, Cardoso AP, Pontes MG, et al. Diuron induced rat urinary bladder carcinogenisis: mode of action and human relevance evaluations using the international programme on chemical safety framework. Crit Rev Toxicol. 2014;44:393–406.

Rocha MS, Arnold LL, Pennington KL, Muirhead D, Dodmane PR, Anwar MM, et al. Diuron-induced rat bladder epithelial cytotoxicity. Toxicol Sci. 2012;130:281-8.

Rocha MS, Nascimento MG, Cardoso APF, Lima PLA, Zelandi EA, Camargo JLV, et al. Cytotoxicity and regenerative proliferation as the mode of action for diuron-induced urothelial carcinogenesis in the rat. Toxicol Sci. 2010;113:37–44.

Roque MRA, Melo IS. Isolamento e caracterização de bactérias degradadoras do herbicida diuron. Sci Agric. 2000;57:723-72.

Sarkar SN, Majumdar AC, Chattopadhyay SK. Effect of isoproturon on male reproductive system: clinical, histological and histoenzyonological studies in rats. Indian J Exp Biol. 1997;35:133-8.

Siemiatycki J, Richardson L, Straif K, Latreille B, Lakhani R, Campbell S, et al. Listing occupational carcinogens. Environ Health Perspect. 2004;112:1447-59.

SINVEG. Sindicato Nacional da Indústria de Produtos para Defesa Vegetal. Banco de dados [Internet]. São Paulo: SINVEG; 2016 [citado 15 Jan 17]. Disponível em: http://sindiveg.org.br/importacoes-de-defensivos-agricolas-tem-aumento-no-primeiro-semestre-de-2016.

Skakkebaek NE, Jorgensen N, Main KN, Meyts ER, Leffers H, Andersson A, et al. Is human fecundity declining?. Int J Androl. 2006;29:2-12.

Solecki R, Kortenkamp A, Bergman A, Chahoud I, Degen HG, Dietrich D, et al. Scientific principles for the identification of endocrine-disrupting chemicals: a consensus statement. Arch Toxicol. 2017;91:1001-6.

Szadkowska-Stanczyk I, Szymczak W. Nested case-control study of lung cancer among pulp and paper workers in exposures to dusts. Am J Ind Med. 2001;39:547-56.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65:87-108.

Tyl RW, Ballantyne B, Fisher LC, Fait DL, Dood DE, Klonne DR, et al. Evaluation of the developmental toxicity of ethylene glycol aerosol in Cd-1 mice by nose-only exposure. Fundam Appl. Toxicol. 1995;27:49-62.

USEPA. U. S. Environmental Protection Agency. Carcinogenicity peer review of diuron: office of prevention, pesticides and toxic substances. Cincinnati: USEPA;1997. 22 p. Memorandum.

USEPA. U. S. Environmental Protection Agency. Technology transfer network. Air toxics website: Ethylene glycol [Internet]. Cincinnati: USEPA; 1999 [citado 15 01 18]. Disponível em:www.epa.gov/ttn/atw/hlthef/ethy-gly.html#ref7.

USEPA. U. S. Environmental Protection Agency. U. S. Environmental Protection Agency. Registration eligibility decision for diuron. List A. Case 0046. Washington: Office of Prevention, Pesticides and Toxic Substances; 2003.

USEPA. U. S. Environmental Protection Agency. What is endocrine disruption [Internet]. Washington: USEPA; 2017 [citado 26 Jan 2018]. Disponível em: https://www.epa.gov/endocrine-disruption/what-endocrine-disruption.

Wang SW, Chu CY, Hsu JD, Wang CJ. Haemotoxic effect of phenylurea herbicides in rats: role of haemoglobin-adduct formation in splenic toxicity. Food Chem Toxicol. 1993;31:285-95.

WHO. World Health Organization. Laboratory manual for the examination of human semen and processing of human semen and sperm-cervical mucus interaction. 5th ed. Geneva WHO Press; 2010. 286 p.

WHO-UNEP. World Health Organization. United Nations Environment Programme. In: Bergman A, Heindel JJ, Jobling S, Kidd KA, Zoeller RT, editors. State of the science of endocrine disrupting chemicals [Internet]. Geneva: WHO; 2012 [citado 26 Jan 2018]. Disponível em: http://www.who.int/ceh/publications/endocrine/en/index.html.

WSSA. Weed Science Society of America. Herbicide Handbook. 7th ed. Champaign: Weed Science Society of America; 1994. 313 p.

Xie LT, Thrippleton K, Irwin MA, Siemering GS, Mekebri A, Crane D, et al. Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay. Toxicol Sci. 2005;87:391-8.

COMPARISON OF FORMULATED AND TECHNICAL DIURON EFFECTS ON THE UROTHELIUM AND REPRODUCTIVE PARAMETERS OF MALE WISTAR RATS

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ABSTRACT

Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) is a herbicide widely used in Brazil. Wistar rats exposed for two years to 2500ppm dietary technical product (TP) of diuron developed a high incidence of urothelial tumors of the urinary bladder and kidney pelvis, greater in males compared to females, and it was classified by the U.S. Environmental Protection Agency as a ‘‘known/likely human carcinogen’’. Studies of the urothelial toxicity by diuron (TP) provided through the diet to male Wistar rats have established the dose response and mode of action. The dietary influence of the diuron-formulated product (FP), composed of 50% TP and 50% inert ingredients or adjuvants, has not been evaluated in rats. The present study was designed to compare the potential toxicity of diuron FP or TP at 1250ppm offered through diet or drinking water on the urothelium and reproductive parameters of rats. Fifty six-week old male Wistar rats were randomly allocated to five groups (GI: Basal diet, GII: FP diet, GIII: FP drinking water, GIV: TP diet, GV: TP drinking water) for 13 weeks. Diuron concentration was 1250ppm for groups GII – GV. Body, kidney, testis and epididymis weights, kidney and testis histopathology, and sperm parameters were evaluated. Urinary bladders were bisected and processed for histologic and scanning electron microscopic (SEM) analyses. At the end of the study, all groups exposed to diuron had decreased body weight gain compared to control greater than 10%, indicating that for this experiment the dose exceeded the maximum tolerated dose (MTD). Food consumption was lower in the groups exposed to FP and TP diuron through diet. Water consumption was lower in all diuron groups, mainly in those exposed through the drinking water. There were no differences in the absolute and relative organ weights among the groups. In all groups exposed to diuron, microscopic findings included simple urothelial hyperplasia (SH) in the kidney pelvis (GII 5/8, GIII 7/10, GIV 8/10, and GV 9/10, respectively) and in the urinary bladder. By SEM, the urinary bladder had superficial cytotoxicity in all groups exposed to diuron. Diuron FP and TP induced changes in the sperm morphology and concentration, and decreased sperm motility and viability. This study showed that diuron FP as well as diuron TP is harmful to the urothelium and to the reproductive parameters of male Wistar rats, regardless of whether in a diet or drinking water.

Key word: Diuron technical product, diuron formulated product, urinary bladder, sperm parameters, scanning electron microscopy, cytotoxicity

INTRODUCTION

Diuron [3-(3,4-Dichlorophenyl)-1,1-dimethylurea] is a herbicide belonging to the phenylamide family and subclass of phenylureas that is efficient in controlling weeds amongst cotton, sugarcane, soybean, citrus fruit, coffee and other agricultural crops (Giacomazzi & Cochet, 2004; Oturan et al., 2008). It is absorbed in the weed roots and prevents oxygen production by photosynthesis inhibition (APVMA, 2005). Following applications to the soil, diuron has been shown to undergo run-off to rivers and lakes (Lamoree et al., 2002; Gooddy et al., 2002; Matallo et al., 2003). The U.S. Geological Survey 2000 identified diuron in about 20% of United States rivers being a widespread water contaminant. In soil, diuron has a half-life of about 90 days (USDA, 1995), but due to its physicochemical properties it is considered an active biological pollutant and can remain in the environment for long times when applied in agriculture (Field et al., 2003; Giacomazzi & Cochet, 2004). For these reasons, its potential toxicity in humans, animals and the environment raise concern, requiring a range of studies to evaluate its toxic potential and to evaluate the dose response and mode of action of any toxicities that are identified.

The diuron formulated product (FP) consists of inert ingredients or adjuvants (substances that include additives, solvents, stabilizers and surfactants) added to diuron TP which ‘’modifies and aids the action of the main ingredient’’ (Foy, 1987). Basically, these inert ingredients or adjuvants are used to optimize the action of the active compound, increasing the penetration in the target organism, solubilizing the active substances, altering the chemical and physical characteristics of the mixture for better application efficiency, distribution and drift reduction in agricultural crops (Otero et al., 2003; Krogh et al., 2003). Studies using diuron FP are not common, but 1250ppm diuron FP is the substance used on agricultural crops which farmers, animals and environment are exposed.

Most experimental studies of diuron have used the technical product (TP) that contains 98% active ingredient. Wistar rats exposed to high concentration (2500ppm) of diuron TP in the feed for 2 years developed high incidences of urothelial tumors, with a greater effect in males than females. No carcinogenic effect was identified in mice (Iyer, 2002). Based on these studies, it was classified by the

U.S Environmental Protection Agency as a "known/likely human carcinogen" using its older cancer guidelines (USEPA, 1997). Its classification based on the USEPA Cancer Guidelines (2005), which incorporates information regarding mode of action and dose response, has not been assessed. In our laboratory, studies with male Wistar rats fed diuron TP at 1250 and 2500ppm for 20 weeks showed increased urothelial simple hyperplasia (SH) and increase cell proliferation (increased labeling index) in the urinary bladder and kidney pelvis. By scanning electron microscopy (SEM) the urinary bladder showed superficial necrosis, exfoliation and simple hyperplasia (Nascimento et al., 2006; da Rocha et al., 2010, 2014; Cardoso et al., 2013; Fava et al., 2015). The mode of action for the urothelial tumors involves metabolism, concentration and excretion of cytotoxic metabolites, cytotoxicity and increased regenerative cell proliferation (da Rocha et al., 2014).

Diuron TP and other herbicides from the phenylurea family have shown *in vitro* ability to bind to androgen receptors (Bauer et al., 1998), but *in vivo* corroboration has not been reported.

In a reproductive toxicity study, evaluation of plasma testosterone concentrations, parameters of daily sperm production, sperm reserves in the epididymis, sperm morphology and measured components of male sexual behavior in Wistar rats exposed to diuron TP at doses 125 or 250 mg/kg per day for 30 days, did not show differences between diuron and control groups. However, the number of fetuses in the litters from diuron-treated rats was slightly less than litters from control rats ($p<0.05$) (Fernandes et al., 2007).

The possible adverse effects on the male reproductive system, Sprague-Dawley pregnant rats received a diuron TP diet of 500 or 750 ppm through the lactation period were evaluated. After weaning, the male offspring received diet containing diuron until the peripubertal age. Male offspring exposed to diuron had reduced body weight, but diuron did not induce significant changes in daily sperm production, morphology, sperm motility, and testosterone levels. Moreover, no histological changes were observed in the reproductive organs (Fernandes et al., 2012).

To assess if diuron TP alters puberty or susceptibility to breast carcinogenesis, pregnant Sprague-Dawley rats received a basal diet or diuron (TP) diet at 500, 750 and 1250 ppm from day 12 to the end of lactation. After weaning, the female offspring continued to receive basal diet or diuron

diet until postnatal day (PND) 51, 75 and 226 to 233. There were no differences between groups in vaginal opening, estrous cycle, breast morphology or carcinogenesis. However, reductions in ovary weight and corpora lutea were identified in rats treated at diuron 1250 ppm at PND 75, showing potentially toxic (Grassi et al., 2011).

In a two-generation reproduction study, CRL: CD rats exposed to diuron (TP) 1750ppm, the F1 and F2 offspring showed decreased body weight, but no effects were observed on testicular weights or histologic changes in testes, epididymis, prostate and seminal vesicle (USEPA 2003, 2004).

Pregnant Wistar rats exposed from sixth to the 15th day of gestation to 80% diuron added to 20% inert ingredients (FP Karmex®) at 125, 250 or 500 mg/kg/day by esophageal intubation, had increase of malformed fetuses in 250 and 500 mg/kg/day and decreased mean fetal weight at the 500 mg/kg /day (Khera et al., 1979).

Diuron FP (50% diuron TP added to 50% inert ingredients) in male lizards *Podarcis sicula* changed spermatogenesis, epididymis morphology and plasma sex steroid secretion, indicating direct male reproductive toxicity. Besides the control group, lizards were divided into three groups placed for 3 weeks in terraria on polluted soil substrate sprayed with 3.75 L/ha of herbicide 50%. Each terrarium was supplemented either water, or food or both water and food contaminated with diuron. All changes observed occurred in all herbicide-treated groups with greater magnitude in lizards exposed in water or water and food contaminated (Cardone et al., 2008).

The present study was designed to compare the potential toxicity on the rat urothelium and reproductive parameters of 1250ppm diuron FP or TP and to compare the effects when administered in the diet or drinking water.

MATERIAL AND METHODS

Experimental outline

This study was approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, SP, Brazil, protocol No. 1181/2016. Fifty six-week old male Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, SP, Brazil). The chemicals used were diuron technical product (CAS No. 30-54-1; Sigma Chemical Co., St Louis, MO, USA, 98% purity) and diuron formulated product (MAPA No. 08895, NORTOX®, Arapongas, PR, Brazil, 50% m/v). They were mixed into a powdered commercial diet (Nuvilab CR1; Nuvital Nutrientes S/A, Colombo, PR, Brazil) or were mixed into drinking water (tap water) at final concentrations of 1250ppm of diuron. To solubilize the diuron in water, three to five drops of Tween® 20 (CAS No. 9005-64; Sigma Chemical Co., St Louis, MO, USA) were added to the substance, homogenized and after water was added.

After two weeks of acclimation, the rats were randomized and allocated to five experimental groups of ten animals each, according to body weights: Group I (GI) Basal diet; Group II (GII) diuron formulated product (FP) in diet; Group III (GIII) diuron formulated product (FP) in drinking water; Group IV (GIV) diuron technical product (TP) in diet; Group V (GV) diuron technical product (TP) in drinking water. The animals were housed in polypropylene cages (five animals/cage) with metallic grill covers and corncob bedding in a room with a targeted temperature of 21 ± 3◦C and relative humidity of 50 ± 20%, on a 12h light/dark cycle, with the lights changing at 7:00 and 19:00. Water and diet were provided *ad libitum* for 13 weeks. Body weights and water and food consumptions were determined at the commencement and at every other week during the experiment (Figure1). The test protocol of 13 weeks (90 days) was chosen as conventional subchronic study in rodents (OECD, 1998).

After treatment for 13 weeks, animals were anesthetized with a mixture of ketamine (80ml, 90 mg/Kg) and xylazine (2%, 10-13 mg/kg) injected intraperitoneally. The spermatozoa were collected from the left vas deferens for sperm analysis between 7:30 and 9:30 AM, one from each group, alternated and non-fasted overnight. The urinary bladder was exposed, inflated by intraluminal injection through urethra with Bouin's fixative, removed quickly and immersed in the same fixative for 4hr. Immediately after, the

animals were euthanized under anesthesia by opening the abdominal cavity and sectioning the inferior vena cava. The right testis, epididymis, seminal vesicle (with seminal secretion), both kidneys, spleen and liver were removed and weighed, then placed in fixative. The right testis was placed in modified Davidson's fixative for 24h (Latendresse at al., 2002; Kittel et al., 2004). The other tissues were fixed in buffered formalin.

Histological and Scanning Electron microscopy (SEM) processing and analyses

After 4hr fixation, the urinary bladders were cut mid-sagittally and rinsed three times in 70% alcohol. One-half of each bladder was cut longitudinally into four segments, which were embedded in paraffin blocks and stained with hematoxylin and eosin (H&E) after sectioning. The urinary bladders of all animals were analyzed by light optical microscopy (*Olympus* Optical *Co*., Ltd, Japan) for histopathology using the diagnostic criteria of Cohen (1983). The other half of the urinary bladders was processed for scanning electron microscopy (SEM) (Haddad et al., 1998) and analyzed in a Scanning Electron Microscope (Carl Zeiss Model EVO LS15, Cambridge UK) at the Department of Physics of UNESP's Bauru School of Sciences, SP, Brazil. The urothelial alterations were classified in five categories according to Cohen et al. (2002). Normal urothelium and slightly altered mucosa are included in Classes 1-3, and altered mucosa is classified in Classes 4 and 5. The LM and SEM analyses were performed blindly.

After 24hr fixation, the right testis was cut into three slices and rinsed three times in 70% alcohol. The middle slice was embedded in paraffin, cut in 5μm thick sections and stained H&E. The seminiferous tubules were analyzed by LM and classified as normal or abnormal including the presence of acidophilic cells, multinucleated cells, spermatids retained in the epithelium, intraepithelial vacuolization, germinal lineage cells in the tubular lumen, or degeneration or depletion of any cell of the tubules according to Foley (2001).

Sperm analyses

All spermatozoa present in the left vas deferens were collected and diluted in 1ml of modified HTF medium (Ham Nutrient F-10 Modify; Cultilab, SP, Brazil) for the evaluation of sperm concentration, motility, vitality and morphology (luminal sperm content in the vas deferens). Sperm

concentration and motility were analyzed in a Makler counting chamber (Sefi-Medical, Haifa, Israel). One hundred spermatozoa/animal were evaluated per 10-µl drop in the five drops analyzed from each sample under a bright field microscope (*Olympus* Optical *Co*., Ltd, Japan) at 100x magnification. The proportion of spermatozoa immotile, motile with progression and motile with progressive movement was recorded. The spermatozoa viability was performed by the eosin-nigrosine method (WHO, 2010). One hundred spermatozoa from each animal were evaluated using a bright field microscope (400x magnification) and were classified as either dead (if they were orange-red in color, indicating that the stain had passed through the membrane) or alive (if they were not stained). The results were expressed as the percentage of live sperm. To evaluate sperm morphology, smears were prepared on histological slides, stained with Papanicolaou stain and observed by LM at a magnification 1000x. Spermatozoa were classified as normal or abnormal (Seed et al., 1996; WHO, 2010). All sperm analyses were performed by blind visual estimation.

Statistical Analysis

Absolute and relative body weights, food and water consumptions, Absolute and relative organ weights and parametric semen analysis were initially compared by ANOVA among the experimental groups; when a significant ($p< 0.05$) difference was detected, it was followed by the Tukey test. The Fisher exact test was used to analyze the incidences of urinary bladder lesions verified by LM and SEM. For sperm concentration, the non-parametric Kruskall-Wallis test was used, with a posteriori Dunn test, according to the characteristics of each variable. Differences were considered significant at $p < 0.05$.

RESULTS

Final mean body weight and weight gain of diuron groups were significantly decreased ($p<0.05$) compared to GI (Basal diet). The decrease in weight gain compared to control was greater than 10%, indicating that for this experiment the dose exceeded the maximum tolerated dose (MTD). Accordingly, the GII and GIII groups had significant reductions in body weight gain (up to 17 and 19%, respectively) and GIV and GV groups (up to 24 and 23%, respectively) lower than control. During the experiment, the food consumption was significantly decreased ($p<0.05$) in the GII (diuron FP in diet) and GIV (diuron TP in diet) groups compared to other groups (GI, GIII and GV). Water consumption was significantly decreased in the GIII and GV (diuron FP and TP in water) groups compared to GI and GII ($p<0.05$). Diuron intake was higher in the GIII and GV groups (diuron in water) compared to GII and GIV groups (diuron in diet) and significantly higher ($p<0.05$) in the GV group (Table 1).

There were no significant differences in the mean absolute and relative organ weights among the groups after 13 weeks of the study (Table 2, 3). Although not significant, the spleen weight was slightly increased in all diuron groups compared to controls. Increased spleen weight and splenomegaly have been previously recognized as a diuron-induced effect in the hematopoietic system (USEPA 2003; APVMA: 2005; Cardoso et al., 2013; Fava et al., 2015).

There were no significant differences among the groups in the incidence of histopathologic lesions in the urinary bladders, but the in kidneys pelvis simple hyperplasia (SH) was observed in all diuron groups at significantly higher rates compared to control ($p< 0.05$) (Table 4, Figure 2). In addition, there were no significant incidences of lesions in the testis, epididymis and seminal vesicles among the groups. In the testis, minimal foci of depletion, degeneration and intraepithelial vacuolization were identified in seminiferous tubules of all groups, including controls, and were considered normal (data not shown).

By SEM (Table 4, figure 3) significant urinary bladder cytotoxicity was observed in all groups exposed to diuron compared with control ($p<0.05$). The GII and GIII groups (FP) show tended to more severe alterations compared to GIV and GV groups (TP), but not statistically significant.

All diuron groups showed decreases of spermatozoa concentration compared to the GI (Basal diet) ($p<0.05$). The percentages of mobile, live sperm and of normal morphology were decreased significantly in all diuron groups compared to the control ($p<0.05$) (Table 5).

Two animals from the GII group were euthanized and excluded in the first month from the experiment due causes unrelated to treatment; one animal had a spontaneous tumor (lymphoma) and the other had occluded teeth.

DISCUSSION

The current study compared diuron FP and diuron TP at a concentration of diuron itself at 1250ppm, administered through the diet or drinking water for 13 weeks. Simple urothelial hyperplasia in the kidney pelvis and urinary bladder (not statistically significant in the bladder) and SEM ultra-structural changes of necrosis, exfoliation and simple hyperplasia in the urinary bladder, were observed. In addition, slight alterations in sperm parameters were detected, but without histologic changes in the testis or in other male reproductive organs.

Food consumption and water consumption were decreased with diuron treatment in the diet and drinking water, respectively, suggesting that there were some palatability issues. Overall, it appears that FP and TP gave similar results by both routes of administration, suggesting that the inert materials in FP diuron did not affect the toxicokinetics or toxicodynamics of diuron. Whether FP and TP behave the same in other species is unknown.

There were no significant differences between the relative and absolute organs weights of spleen, kidneys, liver, testis, epididymis or seminal vesicle among the groups. Although not statistically significant, the relative weight of the spleen was higher in the groups exposed to diuron compared to controls, consistent with observations in previous long term studies (APVMA, 2005). Nevertheless, it suggests that the slight changes in spleen parameters were most likely related to this systemic toxicity rather than a chemical specific effect.

The 13-week exposure period seemed insufficient to produce statistically significant morphologic alterations in the urinary bladder by LM in the present study. However, we observed significant simple hyperplasia in the kidney pelvis in all groups, indicating that 1250ppm concentration and the exposure time did produce a toxic effect on the urothelial mucosa. Furthermore, cytotoxicity of the urinary bladder urothelium was detected by SEM. The structure and function of the rodent lower urinary tract is remarkably similar to humans (Oyasu, 1995). It extends from the kidney pelvis through the ureters, urinary bladder, and into the urethra (Squire, 1998), and is lined

with the urothelium. Agents affecting one part of lower urinary tract usually affect all parts, but often to a different extent.

Hyperplasia in the urinary bladder and kidney pelvis of male Wistar rats has been demonstrated at diuron TP concentrations of 500, 1250 and 2500ppm administered for 20 weeks (Nascimento et al., 2006; da Rocha et al., 2010; Ihlaseh et al., 2011; Cardoso et al., 2013; Fava et al., 2015). In a short-term study (1, 3 and 7 days), male Wistar rats fed diuron TP 2500ppm showed swollen cells by SEM as the initial change caused by diuron cytotoxicity (da Rocha et al., 2012). In the present study, changes in the urinary bladder were detected by SEM in all diuron groups, regardless of the form (FP or TP) or route of exposure (diet or drinking water). Although, the SEM alterations appeared somewhat more intense in GIII (diuron FP in drinking water), the SEM classifications were statistically similar between groups.

No significant morphological changes were seen in the testis by light microscopy**.** Our results are consistent with the findings of Fernandes et al. (2007) in a 30-day study of reproductive toxicity of diuron TP, with no morphological alterations observed in the reproductive organs, but the number of fetuses in the litters from diuron-treated rats was slightly less than litters from control rats. Besides we did not observed any morphological changes in the testis or other male reproductive organs, we did detected small but consistent and statistically significant changes in sperm parameters in all diuron-treated groups. This occurred with a dose that in the present study produced a decreased body weight gain greater than 10% in all diuron-treated groups, which indicates that it was above MTD. Previous studies from our laboratory using concentrations of diuron up to 2500ppm also exceeded the MTD (Nascimento et al., 2006; da Rocha et al., 2010; Ihlaseh et al., 2011; Fava et al., 2015). It is known that excessive toxicity of a test chemical may compromise the usefulness of study and or the quality of the data generated (OECD, 2002, 2010).

However, due to its physicochemical properties, diuron can remain in the environment for a long time and thus is considered a biologically active pollutant requiring in appropriate risk assessment and appropriate measures to reduce exposure as much as possible (Field et al., 2003; Giacomazzi and Cochet, 2004).

In summary, this study indicated that 1250ppm diuron is toxic to the urothelium and sperm parameters of male Wistar rats, regardless of whether administered as TP or FP or administered in the diet or drinking water. The changes on various sperm parameters but without histologic alteration on the testis or other male reproductive organs and could be related at a dose that was in excess of the MTD in this study.

CONFLICT OF INTEREST STATEMENT

The employment affiliation of each author is shown on the cover page. All the authors were associated with academic institutions when the paper was prepared. After the study was executed, one of the authors – Merielen Garcia Nascimento e Pontes – has become employee of Syngenta private company. None of the authors have appeared before regulatory agencies or participate in legal proceedings concerned with safety of diuron.

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REFERENCES

Australian Pesticides and Veterinary Medicine Authority (APVMA), 2005. The Reconsideration-of Approvals of the Active constituent diuron, registrations of products containing diuron and their associated labels. Technol. Assess. Rep. 2, 40.

Bauer, E.R.S., Meyer, H.H.D., Stahlschmidt-Allner, P., Sauerwein, H., 1998. Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. Analyst. 123, 2485-2487.

Cardone, A., Comitato, R., Angeline, F., 2008. Spermatogenesis, epididymis morphology and plasma sex steroid secretion in the male lizard *Podarcis sicula* exposed to diuron. Environml Res. 108, 214–223.

Cardoso, A.P., Ihlaseh Catalano, S.M., da Rocha, M.S., Nascimento e Pontes, M.G, de Camargo, J.L, de Oliveira, M.L. 2013. Dose-response of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] in the urothelial mucosa of Wistar rats. Toxicology. 312, 1-5.

Cohen, S.M. Pathology of experimental bladder cancer in rodents. 1983, in: Cohen S.M., Bryan G.T. (Eds.), The Pathology Bladder Cancer. Boca Raton, CRC Press, v. 2, pp. 1-40.

Cohen, S.M., Arnold, L.L., Uzvolgyi, E., Cano, M., St John, M., Yamamoto, S., Lu, X., Le, X.C., 2002. Possible role of dimethylarsinous acid in dimethylarsinic acidinduced urothelial toxity and regeneration in the rat. Chem. Res. Toxicol. 15, 1150–1157.

da Rocha, M.S., Nascimento, M.G., Cardoso, A.P., de Lima, P.L., Zelandi, E.A., de Camargo,J.L., de Oliveira, M.L. 2010. Cytotoxicity and regenerative proliferation as the mode of action for diuron-induced urothelial carcinogenesis in the rat. Toxicol. Sci. 113, 37–44.

da Rocha, M.S., Arnold, L.L., Pennington, K.L., Muirhead, D., Dodmane, P.R., Anwar, M. M., Battalora, M., de Camargo, J.L., Cohen, S.M., 2012. Diuron-induced rat bladder epithelial cytotoxicity. Toxicol. Sci. 130, 281–288.

da Rocha, M.S., Arnold, L.L., de Oliveira, M.L., Catalano, S.M., Cardoso, A.P., Pontes, M. G., Ferrucio, B., Dodmane, P.R., Cohen, S.M., de Camargo, J.L., 2014. Diuron induced rat urinary bladder carcinogenisis: mode of action and human relevance evaluations using the international programme on chemical safety framework. Crit. Rev. Toxicol. 44, 393–406.

Fava, R.M., Cardoso, A. P., da Rocha, M.S., Nascimento e Pontes, M.G., de Camargo, J.L.V., de Oliveira, M.L.C.S., 2015. Evaluation of early changes induced by diuron in the rat urinary bladder using different processing methods for scanning electron microscopy. Toxicology. 33, 100-6.

Fernandes, G.S., Arena, A.C., Fernandez, C.D.B., Mercadante, A., Barbisan, L.F., Kempinas, W.F., 2007. Reproductive effects in male rats exposed to diuron. Reprod. Toxicol. 23, 106–112.

Fernandes, G.S., Favareto, A.P.A., Fernandez, C.D.B., Bellentani, F.F., Arena, A.C., Grassi, T.F., Kempinas, W.F., Barbisan, L.F. 2012. Effects of diuron on male rat reproductive organs: a developmental and postnatal study. J Toxicol Environ Health 75, 1059–1069.

Field, J.A., Reed, R.J., Sawyer, T.E., Griffith, S.M., Wigington, P.J., 2003. Diuron occurance and distribution in soil and surface and ground ware associated with grass seed production. J. Environ. Qual. 32, 171-179.

Foley, G.L. 2001. Overview of male reproductive pathology. Toxicol Pathol. 29, 49–63.

Foy, C.L., 1987. Adjuvants: terminology, classification, and mode of action, in: Chow, P.N.P., Grant, C.A., Hinshal-Wood, A.M., Simundson, E. (Eds.), Adjuvants and Agrochemicals. CRC Press, Boca Raton, pp. 1–15.

Giacomazzi, S., Cochet, N., 2004. Environmental impact of diuron transformation: a review. Chemosphere. 56, 1021-1032.

Gooddy, D.C., Chilton, P.J., Harrison, I., 2002. A field study to assess the degradation and transport of diuron and its metabolites in a calcareous soil. Sci. Total Environ. 297, 67–83.

Grassi, T.F., Guerra, M.T., Perobelli, J.E., de Toledo, F.C., da Silva, D.S., Kempinas, W. G., Barbisan, L.F. 2011. Assessment of female reproductive endpoints in sprague–dawley rats developmentally exposed to diuron: potential ovary toxicity. Birth Defects Res. 92, 478–486.

Haddad, A., Sesso, A., Attias, M., Farina, M., Meirelles, M.N., Silveira, M., Benchimol, M., Soares, M.J., Barth, O.M., Machado, R.D., Padrón, T.S., de Souza, W., 1998. Técnicas básicas de microscopia eletrônica aplicadas às ciências biológicas. Sociedade Brasileira de Microscopia e Microanálise, Rio de Janeiro.

Ihlaseh-Catalano, S.M., Bailey, K.A., Hester, S.D., Jones, C., Ren, H., Cardoso, A.P., de Camargo, J.L.V., de Oliveira, M.L.C.S., 2011. Transcriptional profile of diuron-induced toxicity on the urinary bladder of male Wistar rats to inform mode of action. Toxicol. Sci. 122, 330-338.

Iyer, P., 2002. Evidence on the development and reproductive toxicity of diuron. Draft. Reproductive and Cancer Hazard Assessment Section. Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, 43.

Khera, K.S., Whalen, C., Trivett, G., Angers, G., 1979. Teratogenicity studies on pesticidal formulations of dimethoate, diuron and lindane in rats. Environ Contam Toxicol. 22, 522-529.

Kittel, B., Ruehl-Fehlert, C., Morawietz G., Klapwijk J., Elwell M.R., Lenz B., O'Sullivan, M,G., Roth, D.R., Wadsworth, P.F., 2004. Revised guides for organ sampling and trimming in rats and mice - Part 2 - A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol. 55, 413-431.

Krogh, K.A., Halling-Sørensen, B., Mogensen, B.B., Vejrup, K.V., 2003. Environmental properties of non-ionic surfactant adjuvants in pesticides: a review. Chemosphere. 50, 871-901.

Lamoree, M.H., Swart, C.P., Van der Horst, A., Van Hattum, B., 2002. Determination of diuron and the antifouling paint biocide Irgarol 1051 in Dutch marinas and coastal waters. J. Chromatogr. A 970, 183–190.

Latendresse, J.R., Warbrittion, A.R., Jonassen, H, Creasy, D.M., 2002. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. Toxicol Pathol. 30, 524-533.

Matallo, M. B., Luchini, N.C., Gomes M.A.F., Spadotto C.A., Cerdeira A.L., Marin G.C., 2003. Lixiviação dos herbicidas tebuthiuron e diuron em colunas de solo. Pesticidas Rev. Ecotoxicol. Meio Amb. 3, p. 83-90.

Nascimento, M.G., de Oliveira, M.L., Lima, A.S., de Camargo, J.L., 2006. Effects of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] on the urinary bladder of male Wistar rats. Toxicology. 224, 66–73.

OECD. Organization for Economic Co-operation and Development. Test No. 408: repeated dose 90-day oral toxicity study in rodents. In: OECD Guideline for the Testing of Chemicals, Section 4. Paris: OECD; 1998. p. 1-10.

OECD, 2002. Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies. OECD Series on Testing and Assessment No. 35. OECD, Paris http://www.oecd-ilibrary.org/environment/guidance-notes-for-analysis-andevaluation-of-chronic-toxicity-and-carcinogenicity-studies 9789264078499-en

OECD, 2010. Guidance on the Design and Conduct of Chronic Toxicity and Carcinogenicity Studies, Supporting TG 451, 452 and 453. OECD Series on Testing and Assessment No. 116. OECD, Paris http://www.oecd.org/ chemicalsafety/testing/44076587.pdf

Otero, R.R., Grande, B.C., Estévez, M.A., Periago, E.L., Gándara, J.S. 2003. Procedure for measurement of soils inputs of plant-protection agents washed off through vineyard canopy by rainfall. J. Agric. Food Chem. 51, 5041-5046.

Oturan, N., Trajkovska, S., Oturan, M.A., Couderchet, M., Aaron, J.J. 2008. Study of the toxicity of diuron and its metabolites formed in aqueous medium during application of the electrochemical advanced oxidation process "electro-Fenton". Chemosphere 73, 1550-1556.

Oyasu, R., 1995 Epithelial tumours of the lower urinary tract in humans and rodents. Food Chem. Toxicol. 33,747-55.

Seed, J., Chapin, R.E., Clegg, E.D., Dostal, L.A., Foote, R.H., Hurtt, M.E., Klinefelter, G.R., 1996. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reprod. Toxicol. 10, 237–244.

Squire, R.A., 1998. Monographs on pathology of laboratory animals. Urinary system, second ed. Classification and differential diagnosis of neoplasms, urinary tract rats. Jones TC, Hard GC, Mohr U, eds. Springer, New York, pp. 69-74.

Tyl, R.W., Ballantyne, B., Fisher, L.C., Fait, D.L., Dood, D.E., Klonne, D.R., Pritts, I.M., Losco, P.E., 1995. Evaluation of the developmental toxicity of ethylene glycol aerosol in Cd-1 mice by nose-only exposure. Fundam. Appl. Toxicol. 27, 49-62.

Tyl, R.W., Ballantyne, B., Fisher, L.C., Fait, D.L., Dood, D.E., Klonne, D.R., Pritts, I.M., Losco, P.E. 1995. Evaluation of the developmental toxicity of ethylene glycol aerosol in the CD rat and CD-1 mouse by whole-body exposure. Fundam. Appl. Toxicol. 24, 57-75.

US Environmental Protection Agency, 1997. Carcinogenicity Peer Review of Diuron. Office of Prevention, Pesticides and Toxic Substances. Memorandum from LindaTaylor and Esther Rinde to Phillip Errico and Larry Schnaubelt.Chemicals Eval-uated for Carcinogenic Potential, Washington, DC.

U. S. Environmental Protection Agency. 2003. Registration Eligibility Decision for Diuron. List A. Office of Prevention, Pesticides and Toxic Substances. USEPA, Washington, DC.

U.S. Enviromental Protection Agency. 2004. Chemicals evaluated for carcinogenic potential. Office of Pesticide Programs, Health Effects Division. Science Information Management Branch, 106.

U.S. Environmental Protection Agency. 2005. Guidelines for Carcinogen Risk Assessment (EPA/630/P-03/001 F) March, 2005.

U.S. Environmental Protection Agency. 2005. Fact sheet: the drinking water contaminate candidate list the source of priority contaminants for the drinking water program. Washington (NY).

U.S. Dept. of Agriculture. Agricultural Research Service. 1995. ARS pesticide properties database: Diuron. http://wizard/arsusda.gov/acsl/ textfiles/DIURON

U.S. Geological Survey. 2000. National Water-Quality Assessment (NAWQA) Program. Circulars 1144, 1150, 1151, 1155-1171, 1201- 1216. http://water.usgs.gov/pubs/nawqasum/.

World Health Organization (WHO), 2010. Laboratory manual for the examination and processing of human semen, fifth edition. WHO Press, Geneva.

FIGURES AND TABLES

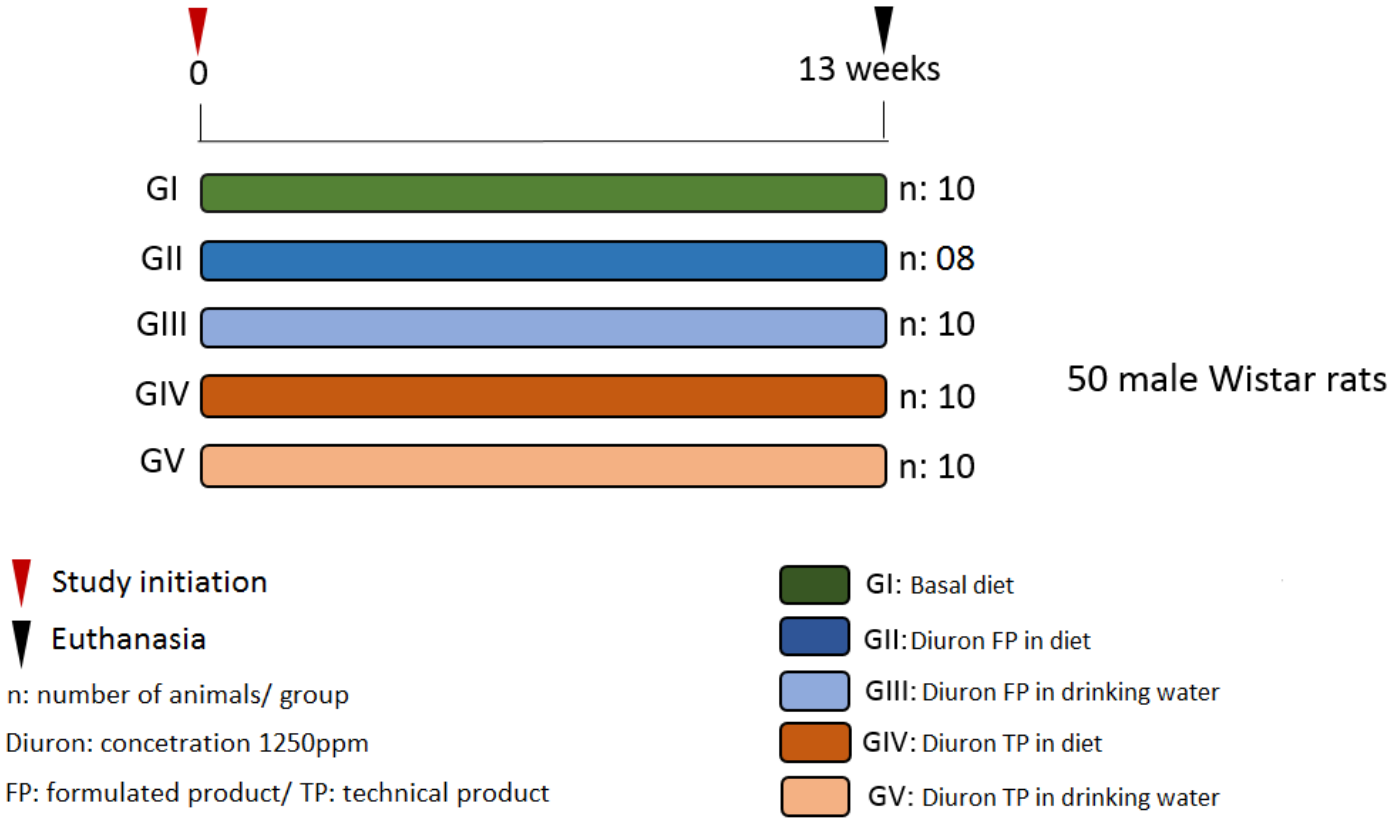

Figure 1: Experimental design

Table 1: Mean body weights and food and water consumptions of Wistar rats exposed to diuron 1250ppm FP or TP for 13 weeks

Groups	N	Body weight(g) [a]			Consumption		
		Initial	Final	Gain	Food (g/rat/day)	Water (ml/rat/day)	Diuron (mg/rat/day)
GI: Basal diet	**10**	173 ± 12	530 ± 48	355 ± 50	25.9	41.2	-
GII: Diuron FP in diet	**8**	182 ± 10	**478 ± 43[b]**	**295 ± 39[b]**	**22.6[b]**	**39.2[c]**	**27.9[e]**
GIII: Diuron FP in in drinking water	**10**	168 ± 11	**458 ± 23[b]**	**290 ± 22[b]**	23.7	**27.6[d]**	**34.9 [e]**
GIV: Diuron TP in diet	**10**	184 ± 14	**455 ± 33[b]**	**271 ± 36[b]**	**21.2[b]**	34.4	**26.5 [e]**
GV: Diuron TP in drinking water	**10**	183 ± 17	**456 ± 41[b]**	**274 ± 32[b]**	24.5	**30.4[d]**	38.0

[a] Values are expressed as mean ±SD., one-way ANOVA, Tukey's test ($p<0.05$); FP: Formulated product/ TP: Technical product
N= Effective number of animals
[b] Different from basal diet
[c] Different from GIII and GV
[d] Different from basal diet and GII
[e] Different from GV

Table 2: Organ weights of Wistar rats exposed to diuron 1250ppm FP or TP for 13 weeks

Groups	N	Absolute organ weight [a] (g)			Relative organ weight [a] (g/kg b.w.)		
		Liver	Spleen	Kidneys	Liver	Spleen	Kidneys
GI: Basal diet	**10**	22.77± 9.33	1.30 ± 0.47	3.79 ± 1.45	4.27 ± 1.57	0.24 ± 0.08	0.71 ± 0.24
GII: Diuron FP in diet	**8**	20.05± 7.12	1.82 ± 0.58	3.17 ± 1.05	4.25 ± 1.61	0.38 ± 0.13	0.67 ± 0.24
GIII: Diuron FP in in drinking water	**10**	18.25 ± 5.43	1.67 ± 0.79	3.77 ± 1.35	4.00 ± 1.16	0.36 ± 0.17	0.82 ± 0.29
GIV: Diuron TP in diet	**10**	21.22 ± 8.00	1.82 ± 0.66	3.71 ± 1.39	4.63 ± 1.60	0.39 ± 0.13	0.80 ± 0.26
GV: Diuron TP in drinking water	**10**	19.75 ± 8.25	1.43 ± 0.67	3.66 ± 1.34	4.32 ± 1.73	0.31 ± 0.13	0.79 ± 0.27

[a] Values are expressed as mean ±SD.one-way ANOVA, Tukey's test (p<0.05); FP: Formulated product/ TP: Technical product;
N= Effective number of animals;

Table 3: Reproductive organ weights of Wistar rats exposed to diuron 1250ppm FP or TP for 13 weeks

Groups	N	Absolute organ weight [a](g)			Relative organ weight [a] (g/kg b.w.)		
		Right Epididymis	Right Seminal vesicle	Right Testis	Right Epididymis	Right Seminal vesicle	Right Testis
GI: Basal diet	**10**	0.84 ± 0.34	0.86 ± 0.35	2.43 ± 1.00	0.16 ± 0.06	0.16 ± 0.06	0.45± 0.17
GII: Diuron FP in diet	**8**	0.81 ± 0.32	0.77 ± 0.27	2.43 ± 1.03	0.17 ± 0.06	0.16 ± 0.05	0.51 ±0.21
GIII: Diuron FP in in drinking water	**10**	0.77 ± 0.25	0.73 ± 0.27	2.38 ± 0.81	0.16 ± 0.05	0.16 ± 0.06	0.51 ± 0.17
GIV: Diuron TP in diet	**10**	0.84 ± 0.32	0.85 ± 0.38	2.49 ± 0.90	0.18 ± 0.06	0.18 ± 0.07	0.54 ± 0.17
GV: Diuron TP in drinking water	**10**	0.79 ± 0.27	0.87 ± 0.37	2.41 ± 0.80	0.17 ± 0.05	0.19 ± 0.07	0.52 ± 0.16

[a] Values expressed as mean ±SD.one-way ANOVA, Tukey's test (p<0.05); FP: Formulated product/ TP: Technical product;
N= Effective number of animals

Table 4: Incidence of proliferative changes in the urinary bladder and kidney pelvis of Wistar rats exposed to diuron 1250ppm FP or TP for 13 weeks.

Groups	Incidence of urinary bladder hyperplasia by LM [a]			Classes of increased severity of urinary bladder alterations (SEM) [b]					Incidence of kidneys pelvis hyperplasia by LM [a]		
	N	Normal	SH [c]	N	Normal (Class 1- 3)	Altered (Class 4)	Altered (Class 5)	Total of altered Classes 4-5	N	Normal	SH [c]
GI: Basal diet	**10**	10	0	**6**	6	0	0	0	**10**	**10**	0
GII: Diuron FP in diet	**8**	7	1	**6**	0	**3**	**3**	**6** [d]	**8**	3	**5[d]**
GIII: Diuron FP in in drinking water	**10**	6	4	**6**	0	**2**	**4**	**6** [d]	**10**	3	**7[d]**
GIV: Diuron TP in diet	**10**	9	1	**6**	1	**3**	**2**	**5** [d]	**10**	2	**8[d]**
GV: Diuron TP in drinking water	**10**	8	2	**6**	1	**4**	**1**	**5** [d]	**10**	1	**9[d]**

[a] Values expressed per animal: Fisher's Exact test (2-tail); FP: Formulated product/ TP: Technical product;
N= Effective number of animals; [a] LM: Light microscopy, [b] SEM: Scanning electron microscopy, [c] SH: Simple hyperplasia
SEM: Classes 1-5: Increased severity of urothelial alterations (Cohen et al., 2007)
[d] Different from basal diet (p< 0.05)

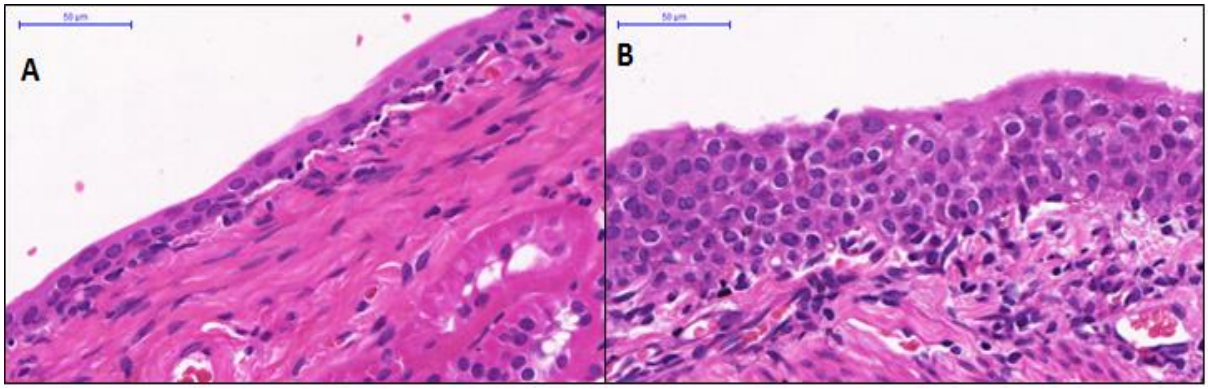

Figure 2. LM - Figure 2. Histology of kidney pelvis (Hematoxylin & Eosin): A) Normal urothelium (GI, 40x); B) Simple Hyperplasia (GIV, 40x).

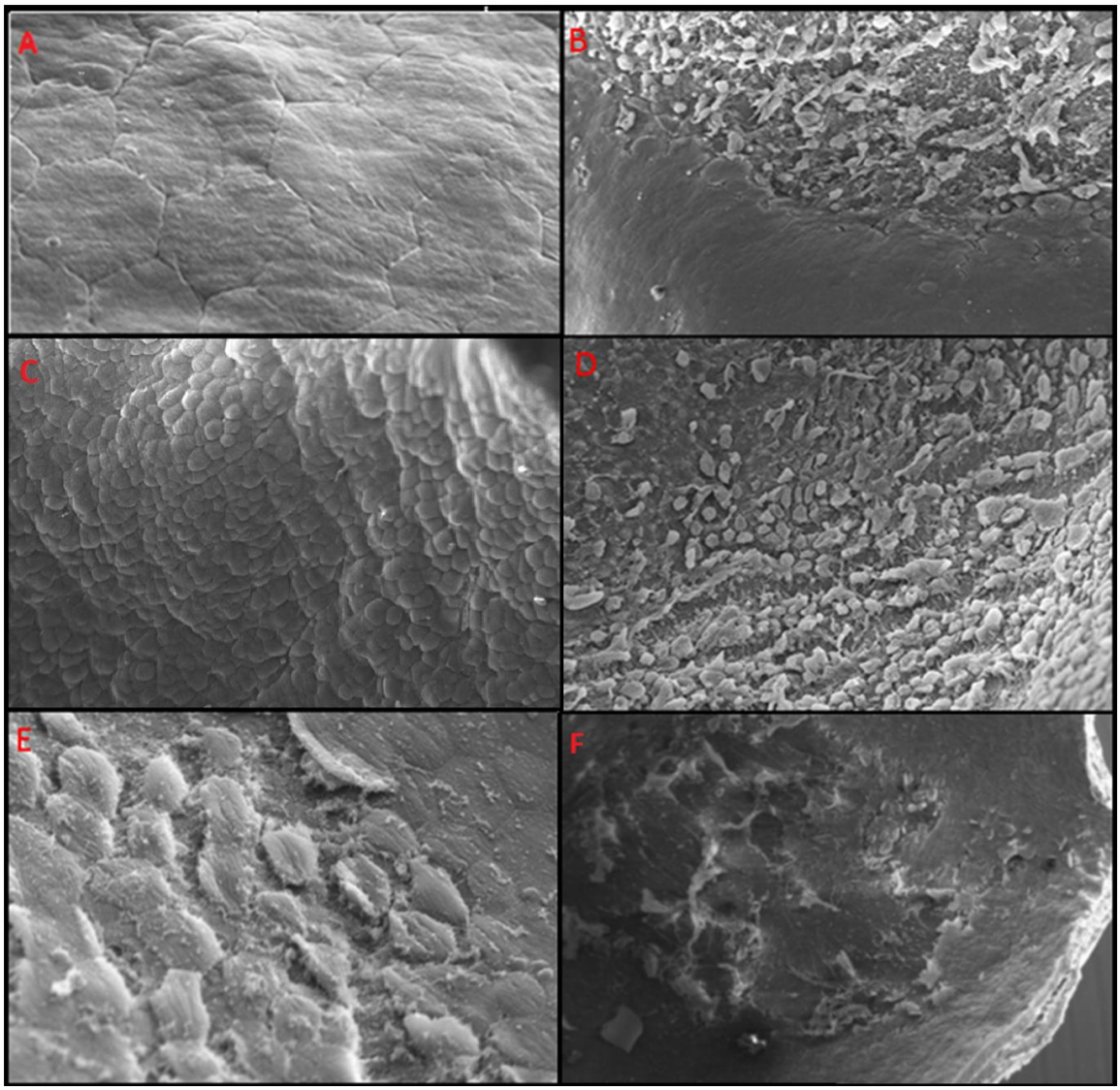

Figure 3. Urinary bladder (Scanning Electron Microscopy): A) Normal urothelium (GI, 800x); B) Necrosis and exfoliation (GII, 300x); C) Hyperplasia (GIII, 310x); D) Necrosis, exfoliation and hyperplasia (GIV, 300x); E) and F) Hyperplasia and necrosis, exfoliation and (GV,120x and 800x, respectively).

Table 5: Sperm analysis of Wistar rats exposed to diuron 1250ppm FP and TP for 13 weeks

Groups	N	Sperm Concentration ($x10^6$/ml) [a]	Normal sperm morphology (%) [b]	Vitality	Motility
				Live Sperm (%) [b]	Mobile sperm (%) [b]
GI: Basal diet	**10**	43.04 ± 0.86	95.00 (93.00 – 96.00)	94.00 (91.00 – 97.00)	65.00 (49.00 – 95.00)
GII: Diuron FP in diet	**8**	**29.54 ± 1.60[c]**	**89.50 (87.00 – 96.00)[c]**	**86.50 (82.00– 93.00) [c]**	**41.50 (34.00– 60.00) [c]**
GIII: Diuron FP in in drinking water	**10**	**28.45 ±1.82[c]**	**89.50 (87.00 – 91.00)[c]**	**87.00 (81.00 –93.00) [c]**	**38.50 (31.00 – 59.00) [c]**
GIV: Diuron TP in diet	**10**	**29.76 ± 2.01[c]**	**90.00 (87.00 – 95.00)[c]**	**89.00 (82.00 -95.00) [c]**	**43.50 (33.00 – 69.00) [c]**
GV: Diuron TP in drinking water	**10**	**28.77 ± 2.04[c]**	**89.00 (87.00 – 92.00)[c]**	**86.05 (80.00– 93.00) [c]**	**44.00 (35.00 – 70.00) [c]**

[a] Values expressed as mean ± SEM., One-way ANOVA, Tukey's test ($p<0.05$); FP: Formulated product/ TP: Technical product; N= Effective number of animals; [b] Values expresses as median (Q_1-Q_3)., Kruskal -Wallis test, followed by Dunn test ($p<0.05$)
[c] Different from control

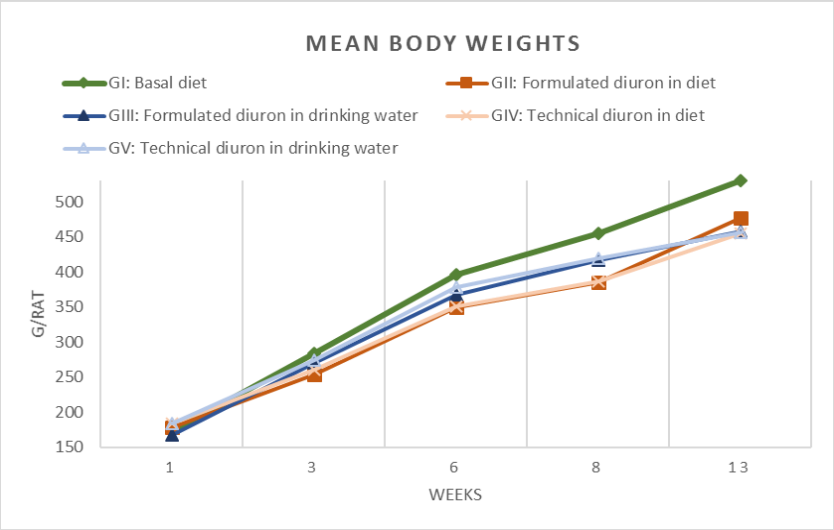

Figure 4. Body weight of Wistar rats exposed to diuron FP or TP 1250ppm for 13 weeks

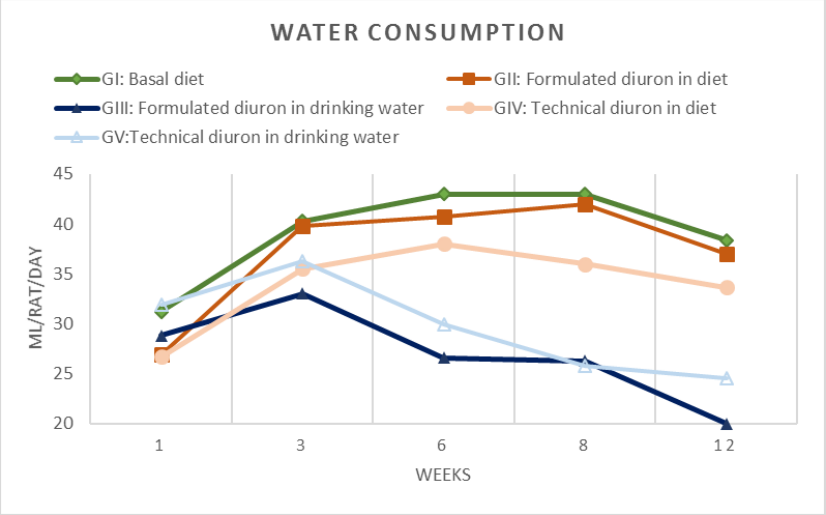

Figure 5. Water consumption of Wistar rats exposed to diuron FP or TP 1250ppm for 13 weeks

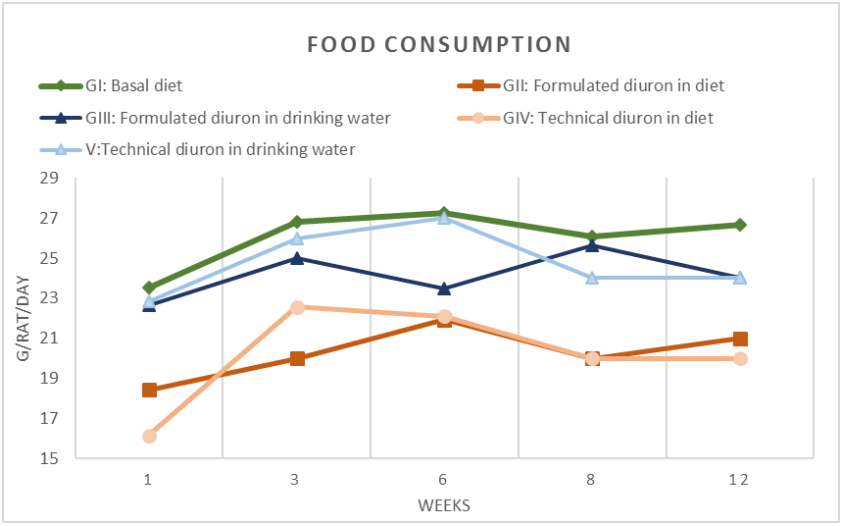

Figure 6. Food consumption of Wistar rats exposed to diuron FP or TP 1250ppm for 13 weeks

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Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO-RETIFICADO

Certificamos que a alteração do título com objetivo acadêmico: "*Comparação dos efeitos do diuron formulado e técnico no urotélio e nos parâmetros reprodutivos de ratos Wistar machos*", conduzida pela Pesquisadora: **Bianca Camargo Penteado Sales**, Orientada pela Profa. Dra. Maria Luiza Cotrin Sartor de Oliveira, Coorientada pelo Prof. Dr. João Lauro Viana de Camargo, com a participação das Colaboradoras: Merielen Garcia Nascimento e Patricia Carvalho Garcia, registrado e com o nº 1181/2016, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Alteração de Título APROVADA em 17 de janeiro de 2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Espécie/Linhagem/Raça	Ratos Wistar
Nº de animais	50
Idade/Peso	4 semanas – 150 gramas
Sexo	Macho
Origem	Biotério CEMIB-UNICAMP

Graziela Nogueira Bertani
Secretária Responsável
Comissão de Ética no Uso de Animais – CEUA

Prof. Dr. Guilherme Antônio Moreira de Barros
Presidente
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Profª. Maria Luiza Cotrin
CPF 039.004.168-85
CNPJ- 48.031.918/0019-53
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Botucatu - SP

Atestado de Saúde Animal Nº406/2016

Atestamos que os Ratos (50 machos) da linhagem **HanUnib:WH,** provenientes da Divisão de Produção de Animais S.P.F. (Specific Pathogen Free) deste Centro, pertencem à categoria sanitária S.P.F. e apresentam-se isentos dos agentes patogênicos pesquisados pelo laboratório de controle de qualidade sanitária. Informamos que os mesmos encontram-se livres de outros agentes infecciosos capazes de causarem riscos à saúde humana. A validade deste Atestado consta da data dos últimos testes do programa de monitorização sanitária, rotineiramente realizados pelo Laboratório de Controle de Qualidade Animal - C.Q.S.**(*)**.

Observação - O estado sanitário dos animais retirados do CEMIB nesta data será mantido se os mesmos forem acondicionados em equipamento adequado e o mesmo não for violado durante o transporte. A Instituição receptora deverá oferecer infra-estrutura e condições adequadas para a manutenção de animais da Categoria Sanitária livres de agentes patogênicos especificados (S.P.F.), alojando os animais em equipamentos e/ou salas dotadas de sistema de barreiras de proteção sanitária. Torna-se necessário manejo correto e a esterilização de todo material utilizado na rotina como: ração, maravalha/cama, bebedouros, água, gaiolas, tampas, e outros.

(*) Data dos últimos testes de monitorização sanitária realizados em Outubro de 2016.

Campinas, 21 de novembro de 2016.

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