UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CÂMPUS DE BOTUCATU

CARCINOGÊNESE DE MAMA EM MODELO EXPERIMENTAL DE EXPOSIÇÃO GESTACIONAL, JUVENIL E ADULTA AO HERBICIDA DIURON [3(3,4-DICLOROFENIL) 1,1, DIMETIL URÉIA] EM FÊMEAS SPRAGUE-DAWLEY

TONY FERNANDO GRASSI

Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para obtenção do título de Doutor em Patologia.

Botucatu - SP
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Doutorando: Tony Fernando Grassi
Orientador: Prof. Dr. Luís Fernando Barbisan
Co-orientador: Prof. Dr. João Lauro Viana de Camargo

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"Foste um instrumento de nosso aprendizado?".
Foste apenas um objeto de experiência?

Não!!!

Foste para nós, vítimas solicitadas pela ciência, para benefício da humanidade, porém, apesar do teu olhar mudo e de não teres a permissão da palavra, isso não nos impedirá de dizer-te sempre:

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Índice

Índice Geral

Capitulo III

Artigo Científico II

Índice de Tabelas

Capítulo II

Artigo Científico I

Capítulo III

Artigo Científico II

Índice de Figuras

Capítulo I

Artigo Científico I

Capítulo III

Artigo Científico II

Anexos

Índice de Anexos

Capítulo I

Revisão de Literatura

1. Revisão de Literatura

O câncer de mama é a neoplasia mais freqüente entre mulheres e a principal causa de morte entre mulheres de todo o mundo e sua etiologia não é totalmente conhecida.[1] Dos casos de câncer diagnosticados em mulheres, 22% dos casos novos/ano, são de mama. Na população mundial, a sobrevida média após cinco anos é de 61%. No Brasil, dados de 2008 do Instituto Nacional do Câncer (INCA) demonstram que 49.400 mulheres seriam acometidas pelo câncer de mama e com risco estimado de 51 casos a cada 100 mil mulheres. Já nos EUA, a estimativa para 2009 é de 192.370 (mulheres), 1.910 (homens) e com mortalidade de 40.170 (mulheres) e 440 (homens). (U.S. National Câncer Institute).

Como uma doença altamente heterogênea é representada por neoplasias que possuem história natural diversa, histologia complexa e resposta variável as terapias convencionais (quimioterapia, braquioterapia e radioterapia).[2] Atualmente, mesmo nos países em desenvolvimento, que tradicionalmente apresentavam baixa incidência, vêm se observando aumento nas taxas de incidência e mortalidade por câncer de mama entre as mulheres[3]. Os eventos moleculares associados ao processo de carcinogênese mamária, incluindo a iniciação, promoção e progressão, não estão bem estabelecidos e muitas alterações genéticas têm sido descritas.[4,5] Essas alterações compreendem mutações, amplificações e deleções gênicas, envolvendo oncogenes e genes supressores tumorais, entre eles, os genes receptores de

estrógenos e erb-B2 (HER2/neu). O uso de ferramentas para o estudo da expressão gênica tornou-se, também, fundamental para o entendimento dos eventos moleculares envolvidos nessa complexa enfermidade.[6,7]

Segundo a *American Cancer Society* (2002),[8] mutações gênicas específicas estão envolvidas em uma pequena porcentagem (5%) de todos os casos documentados de câncer de mama. Além disso, 50% dos casos de câncer de mama podem ser atribuídos a fatores de risco que incluem idade, estilo de vida e história familiar e reprodutiva. [9,10] Dois fatores exógenos principais têm participação importante na patogênese do câncer de mama: a exposição a agentes químicos ambientais e o consumo de fitoestrógenos na dieta.[9,11,12]

Muitas das neoplasias que ocorrem no ser humano são casualmente atribuídas à exposição a poluentes ambientais, pesticidas, drogas, luz ultravioleta, radiação e ao cigarro (tabaco).[13] Atualmente, a exposição a agentes tóxicos ambientais, entre eles, os pesticidas agrícolas tem sido correlacionada com o aumento do risco de desenvolvimento de câncer de mama.[9,11,12]

As neoplasias de mama surgem espontaneamente em algumas espécies animais, como por exemplo, cães, ratos e camundongos. Por razões práticas, a maioria dos estudos experimentais de carcinogênese mamária são conduzidos em roedores devido à baixa freqüência de tumores espontâneos observados em estudos de longa duração.[14,15] A possibilidade do desenvolvimento de neoplasias de mama em roedores tem sido utilizada para avaliar o potencial cancerígeno de compostos químicos específicos.[16,17,18] Tumores induzidos pela

administração de cancerígenos químicos constituem ferramentas úteis para o entendimento das mútiplas etapas da carcinogênese e como linha de base para testes do potencial cancerígeno de agentes químicos e para avaliação do risco.[14,19]

As neoplasias mamárias quimicamente induzidas são, em geral, carcinomas hormônio-dependentes. A incidência, multiplicidade e tipos de tumores mamários são influenciados por idade, tempo de exposição ao cancerígeno, história reprodutiva, desregulação endócrina, dieta e outros fatores que alteram o desenvolvimento e o grau de diferenciação da glândula mamária.[20-23] As substâncias químicas mais utilizadas nos modelos experimentais de indução da carcinogênese mamária em fêmeas de ratos e camundongos são a 7,12-dimetilbenz(a)antraceno (DMBA) e a N-metil-N-nitrosoureia (MNU) e as linhagens de eleição são fêmeas da linhagem de ratos Sprague-Dawley e Wistar-Furth.[24,25] Estudos experimentais indicam que substâncias ambientais como os pesticidas (DDT, policlorinadobifenis, 4-nonyphenol, 4-octylphenol e atrazina) podem promover neoplasias de mama em roedores.[26,27] O herbicida atrazina (2-cloro-4-etilamino-6-isopropilamino-*s*-triazina), por exemplo, vem sendo muito estudado pelo fato de exibir efeitos adversos sobre o sistema endócrino e gonadal, particularmente, no centro de controle hipotalámico da função hipófise/ovário e sobre a tireóide, por retardar o início da puberdade, gravidez e espermatogênese[28], além de

aumentar a da incidência de tumores mamários e pituitários em fêmeas Sprague-Dawley expostas ao herbicida atrazina.[29,30]

Nos ratos, as glândulas mamárias são formadas inicialmente pelo espessamento ectodérmico das cristas mamárias que darão origem ao broto mamário primário por volta do 12º ao 14º dia gestacional (DG). A partir do 16º DG até o nascimento, as células epiteliais do broto mamário se desenvolvem e se ramificam e o mesênquima adjacente começa a se diferenciar para formar o suporte para a ramificação ductal.[31,32,33] O crescimento da glândula mamária é isomérico (isto é, proporcional ao crescimento corpóreo), mas se torna alomérico (isto é, duas a três vezes maiores que o crescimento corpóreo) durante o período pós-natal antes do início da puberdade, e exponencial durante a puberdade.[31,32,33] Os períodos compreendidos entre o 16º-20º dia DG e a puberdade são os mais críticos para o desenvolvimento mamário e que podem ser influenciado por vários fatores, entre eles os agentes químicos atrazina e dioxinas (TCDD).[34-38]

Diversas substâncias utilizadas na agricultura são agentes teratogênicos, mutagênicos e cancerígenos.[39,40] A exposição a essas substâncias, em especial aos pesticidas, ocorre não somente por trabalhadores rurais e agricultores que manipulam diretamente estes compostos (exposição ocupacional), mas, também, pela população em geral por meio da ingestão de água e alimentos contaminados (exposição acidental). Portanto, a avaliação do potencial toxicológico (periculosidade) de pesticidas pode fornecer informações

importantes para a implementação de políticas governamentais de saúde pública que regularizem sua utilização em níveis seguros para exposição humana.[41,42]

Dentre as variedades de pesticidas encontradas no mercado de defensivos agrícolas, o Diuron [3(3,4-diclorofenil) 1,1, dimetil uréia], herbicida derivado da uréia, é utilizado para o controle seletivo de plantas daninhas nas culturas de frutas, algodão, cana-de-açúcar, alfafa e trigo, sendo prontamente absorvido pelas raízes e folhas das plantas daninhas, mostrando ação de contato e residual. Este herbicida é classificado como sendo de toxicidade nível de II a IV, dependendo do tipo de formulação[43], e se trata de uma combinação não-iônica com moderada solubilidade em água (22 a 42 mg/L a 20°C). Diuron é estável nos processos de oxidação e decomposição química, persistindo no solo por longo período.[44,45]

O Diuron é absorvido com facilidade pelo trato gastrointestinal e sistema respiratório, sofre metabolismo hepático (hidroxilação e N-dealquilação), e a maioria de seus metabólitos é encontrada na urina de animais expostos. [46] Este herbicida pertence à classe dos indutores de enzimas do sistema de oxidases de função mista (enzimas do citocromo P450), aumentando o conteúdo enzimático do sistema P450 em aproximadamente 50%, quando comparado a pesticidas similares. [47]

Em roedores, os herbicidas derivados de uréia apresentam baixa toxicidade sistêmica. O Diuron apresenta a dose letal (DL50) de 1017 mg/kg

em ratos jovens, de 437 mg/kg em ratos tratados com dieta deficiente em proteínas, ou de 2390 mg/kg em ratos tratados com dieta rica em proteínas.[48] Em estudos experimentais, o Diuron causa irritações oculares em coelhos expostos[49] e fetotoxicidade e aumento de malformações em ratos.[50] O Diuron foi identificado como agente iniciador da carcinogênese de pele em camundongos.[51]

Em dois estudos de toxicidade oral em ratos e cães (durante nove meses a dois anos) foram registrados resíduos de Diuron, especialmente no fígado e rins.[52] Os principais efeitos tóxicos da ingestão crônica de Diuron foram a perda de peso e anormalidades em sangue, fígado e baço.[46] Nesses estudos as doses médias finais testadas foram de 0, 0,625, 3,125, 6,25 e 31,25 mg/kg de peso corpóreo (p.c.) por dia durante dois anos para cães da ração Beagle (dois machos e três fêmeas) e de 0, 1,25, 6,25, 12,5 ou 125 mg/kg de p.c. por dia para ratos (machos e fêmeas). Na concentração de 125 ppm (dose de 3,125 mg/kg em cães e 6,25 mg/kg em ratos) foram observados traços de pigmentos anormais no sangue em alguns animais, embora a a incidência não tenha sido significativa. Na concentração de 250 ppm (6,25 mg/kg em cachorros e 12,5 mg/kg em ratos), foram observadas alterações hematológicas, perda de peso, hemosiderose no fígado e hiperplasia eritróide. Nesses estudos de toxicidade crônica não houve evidência de carcinogenicidade do Diuron.[46, 52]

O Diuron não mostrou atividade mutagênica na maioria dos testes *in vitro*, com ou sem ativação metabólica.[53] Entretanto, mutagenicidade positiva foi relatada em teste com *Salmonella typhimurium* com ativação metabólica e em ensaios de biosíntese de DNA testicular[54] O Diuron foi negativo em dois testes *in vitro* com células de mamíferos, o de mutação gênica em células de ovário de hamster chinês e o de síntese não programada de DNA (UDS) em hepatócitos de rato.[55] Em testes *in vivo*, a administração do Diuron nas concentrações de 170 e 340 mg/kg de peso corpóreo, induziu a formação de micronúcleo em medula óssea de camundongos após 30h e 48h[56] e mutações dominantes letais em camundongos Swiss[57], além de efeitos clastogênicos em ratos.[55]

Estudos reprodutivos de três gerações consecutivas mostraram que a reprodução de ratos não foi afetada com doses diárias de 6 mg/kg de peso corpóreo, porém esta dose foi fetotóxica e causou redução de peso corpóreo nas proles F2 e F3. O Diuron não apresentou atividade teratogênica em ratos, mas mostrou atividade fetotóxica para proles de fêmeas expostas à dose diária de 250 mg/kg de peso corpóreo. Este efeito foi caracterizado pelos pesos fetais diminuídos, costela e ossos menores e anomalias observadas. Os mesmos efeitos foram observados na dose diária de 125 mg/kg peso corpóreo, mas em incidência não significativa.[50,58]

A Agência Internacional de Pesquisa em Câncer (IARC, Lyon, França) considerou o Diuron como prioritário para ser analisado quanto ao potencial

de carcinogenicidade para seres humanos, em decorrência de seus possíveis efeitos cancerígenos e mutagênicos observados em animais experimentais.[59] Mais recentemente, em um relato publicado pela Agência de Proteção Ambiental (USEPA) americana, o Diuron foi considerado como provável cancerígeno para o ser humano em decorrência de resultados de estudos de carcinogenicidade de longa duração.[60] Estes resultados, no entanto, não estão disponíveis, pois se trata de propriedade industrial designados ao registro comercial (Bayer). Deste modo, agências de regulamentação de outros países, incluindo o Brasil, têm que se basear nas informações da EPA para definir sua regulamentação própria.

Em estudos de longa-duração foi observado que o Diuron aumenta a incidência de alguns tipos de tumores em camundongos NMR1 e em ratos Wistar.[60] O Diuron administrado na ração (maior concentração, 2500 ppm) por dois anos aumentou a incidência de papilomas e carcinomas de bexiga urinária em machos e fêmeas da linhagem Wistar; além disso, os ratos machos também apresentaram tendência a aumento do desenvolvimento de papilomas e carcinomas da pelve renal. Dois tumores renais, considerados raros para esta espécie, foram também observados em ratos Wistar machos expostos à maior concentração [61]. A deficiência neste estudo em ratos foi que vários órgãos não foram examinados, incluindo as glândulas mamárias.[60]

Camundongos NMR1 machos e fêmeas foram expostos a três concentrações de Diuron por 24 meses. As fêmeas expostas à maior

concentração de Diuron (2500 ppm) apresentaram tendência a aumento da incidência de adenocarcinomas de mama. Esta incidência foi considerada maior do que os dados de incidência histórica deste tipo de tumor em fêmeas controle em estudos com a mesma linhagem de camundongos.[60,61] Recentemente, em estudo epidemiológico realizado no estado da Califórnia, para avaliar a incidência de câncer de mama em mulheres que residiam em áreas próximas a regiões com grande uso de pesticidas, o Diuron foi incluído na lista de prováveis agentes cancerígeno para mama como um xenoestrógeno.[62]

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Objetivos

3. Objetivos

Em decorrência dos poucos dados de literatura sobre a carcinogenicidade do herbicida Diuron e das informações da USEPA que apontam possíveis efeitos cancerígenos, em especial para a mama e bexiga em roedores, o presente projeto teve como objetivo geral avaliar o potencial carcinogênico do herbicida Diuron em modelo de carcinogênese mamária induzida pela 7,12-dimetilbenz(a)antraceno (DMBA) em fêmeas Sprague-Dawley (SD).

3.1. Objetivos Específicos

3.1.1. Protocolo Experimental I

Avaliar se a exposição precoce (exposição em fase gestacional, lactacional e juvenil) ao herbicida Diuron, interfere no desenvolvimento/função do sistema reprodutivo e da glândula mamária na fase de pré-puberdade e/ou altera a susceptibilidade ao desenvolvimento da carcinogênese mamária induzida pela DMBA em fêmeas SD adulta.

3.1.2. Protocolo Experimental II

Avaliar os possíveis efeitos do herbicida Diuron nos estágios de promoção e progressão da carcinogênese mamária induzida pela DMBA em fêmeas SD e os efeitos na expressão de biomarcadores da tumorigênese mamária (proliferação celular, apoptose, célula mioepitelial e receptor de estrógeno).

Capítulo II

Artigo Científico I

Evaluation of carcinogenic potential of Diuron in a rat mammary two-stage carcinogenesis model

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Abstract

This study aimed to evaluate the carcinogenic potential of the herbicide Diuron in a two-stage rat medium-term mammary carcinogenesis model initiated by 7,12-dimethylbenz(a)anthracene (DMBA). Female seven week old Sprague-Dawley (SD) rats were allocated to six groups: Groups G1 to G4 received intragastrically (i.g.) a single 50 mg/kg dose of DMBA; Groups G5 and G6 received only a single administration of Canola oil (vehicle of DMBA). Groups G1 and G5 received a basal diet, and Groups G2, G3, G4 and G6 the basal diet added with Diuron at 250, 1250, 2500 and 2500 ppm, respectively. After 25 weeks, the animals were euthanized and mammary tumors were confirmed histologically and quantified. Tumor samples were also processed for immunohistochemical evaluation of the expressions of proliferating cell nuclear antigen (PCNA), caspase-3, estrogen-receptor-alpha (ER-alpha), p63, bcl-2 and bak. Diuron did not increase the incidence or multiplicity of mammary tumors (Groups G2, G3 and G4 *vs.* Group G1). Also, exposure to Diuron did not alter tumor growth (cell proliferation and apoptosis rates) or immunoreactivity to ER-alpha, p63 (myoephitelial marker), bcl-2 and bak (apoptosis regulatory proteins). These findings indicate that Diuron does not have a promoting potential on mammary carcinogenesis in female SD rats initiated with DMBA.

Key words: pesticides, Diuron, mammary carcinogenesis, Sprague-Dawley rats

Running title: Diuron and rat mammary carcinogenesis

1. Introduction

Breast cancer is an invasive and ultimately fatal disease whose incidence in postmenopausal women has gradually increased in most Western societies over the last few decades. The incidences have also sharply increased in younger women in more recent years, mainly in industrialized countries (Parkin *et al.* 2005; Bouchardy *et al.* 2007).

Besides genetic/familial factors including the major susceptibility genes (BRCA1 and BRCA2), other influences on breast cancer risk appear to be certain reproductive factors (i.e., older age, later age at first full-term pregnancy, no full-term pregnancies), body size/obesity, alcohol, exogenous hormones (oral contraceptives, hormone replacement therapy), menopause and possibly, some dietary habits (Parkin *et al.* 2005; Bouchardy *et al.* 2007). In the early 1990s, it was suggested that exposure to some environmental chemicals such as pesticides could play a causal role in the etiology of breast cancer through estrogen-related pathways or endocrine disrupting effects. Substantial evidence from experimental rodent studies indicate that organochlorine pesticides are potential mammary carcinogens. In contrast, direct correlations between human tissue levels of pesticides and the development of breast cancer have not been consistent, with positive and negative associations (Calle *et al.* 2002; Fenton 2006; Rudel *et al.* 2007; Salehi *et al.* 2008).

Diuron [3-(3,4-dichlorophenyl)-1-1-dimethylurea] is a substituted phenyl urea herbicide used throughout the world to control a wide variety of annual and perennial broadleaf and grassy weeds on both crop (i.e., citrus fruit, cotton, asparagus, sugar cane, alfalfa, wheat and grapes) and non-crop sites (i.e., roads, garden paths and railways) (Iyer, 2002; Field *et al.* 2003; Giacomazzi and Cochet 2004). In Brazil, it is widely used on many agricultural crops such as sugar cane and soy (Nascimento *et al.* 2006). Diuron *per se* has low systemic toxicity to mammals and birds, and moderate toxicity to aquatic invertebrates. Its principal biodegradation product, 3-4-dichloroaniline (3-DCA), is highly toxic and relatively persistent in the environment (Valentovic *et al.* 1997; Iyer, 2002; Giacomazzi and Cochet 2004). Thus, the environmental contamination by this herbicide may represent an important public health problem (Abass *et al.* 2007; Sorensen *et al.* 2008).

In a recent epidemiological study in areas of high agricultural pesticides use, no association between Diuron exposure and breast cancer was observed in Californian women (Reynolds *et al.* 2005). However, for more than one decade Diuron has been categorized as a "known/likely" human carcinogen by the U. S. Environmental Protection Agency (USEPA) mostly based on long-term bioassays that indicated increased incidences of urothelial bladder and renal pelvis tumors after continuous dietary high concentration (2500 ppm) exposure in both genders of Wistar rats, and a trend for increased incidence of mammary adenocarcinomas in female NMRI mice (Iyer, 2002;

USEPA, 2003; 2004). In these long-term studies, the mammary glands of the Wistar female rats, a strain known to be resistant to chemically-induced breast tumors, seems to be not adequately examined, but, the NMR1female mice developed increased although relatively low incidences of adenocarcinomas: 2/50, 1/47, 1/49 and 6/50 in female mice after exposed through diet to 0, 25, 250, and 2500 ppm of Diuron, respectively (Iyer, 2002). The 12% incidence of mammary tumors led the USEPA to assume that a positive oncogenic response was seen in the highest dose group when compared to the control, after discarding the possibility of those tumors being spontaneous (USEPA, 2003). While the carcinogenic mode of action of Diuron on the rat urothelia has been more intensively studied and seems to be by a non-genotoxic pathway (Nascimento *et al.*, 2006; Rocha *et al.*, 2009), the mammary carcinogenesis process referred to Diuron exposure has not been adequately explored.

The most widely used medium-term bioassay to asses the carcinogenic potential of chemicals on mammary carcinogenesis is based on the 7-12-dimethylbenz(a)anthracene (DMBA)-induced tumors in the highly susceptible female Sprague-Dawley rats, whose tumors closely mimic human breast cancer (Russo and Russo 1996; Costa *et al.* 2002). This *in vivo* bioassay is useful for dissecting the multistep process of carcinogenesis and for detection of potential mammary carcinogens, especially those that act through endocrine disruption (Russo and Russo 1996; Costa *et al.* 2002; Fenton 2006; Rudel *et al.*

2007). Thus, the identification of potential mode of action as well as possible species-specific response of laboratory rodents may assist in the selection and further development of appropriate models for assessing the evaluation of mammary carcinogenicity of Diuron.

As breast cancer is one of the most frequent neoplasm in women and hazard detection and risk assessments of environmental chemicals for breast cancer are high public health priorities, the present study was conducted to assess the modifying effects of the herbicide Diuron on the promotion/progression stages of mammary carcinogenesis induced by DMBA in virgin female Sprague-Dawley (SD) rats. In addition, effects of Diuron on cell proliferation and apoptosis were also examined.

2. Material and Methods

2.1. Chemicals

7,12-Dimethylbenz(a)antracene (DMBA, CAS 57-97-6) and 3-(3,4-dichlorophenyl)-1-1-dimethylurea (Diuron, CAS 30-54-1, analytical standard grade) were purchased from Sigma-Aldrich Co., USA.

2.2. Experimental Design

The University Committee for Ethics in Animal Research approved the present study (Protocol number 523). Female 5-week-old Sprague-Dawley (SD) rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP, Campinas-SP, Brazil). They were kept in polypropylene cages (five animals/cage) with metallic grid covers, and maintained in a room at 22±2°C, 55±10% humidity and a 12h light/dark cycle. They were fed commercial Purina chow (Paulínia, SP, Brazil) and water *ad libitum* during a 2-week acclimatization period.

The animals were randomly allocated to six groups, consisting of 18 rats in groups G1 to G4, 10 rats in group G5 and 15 rats in group G6. After two weeks of acclimatization, with seven weeks of age, groups G1 to G4 were initiated for mammary carcinogenesis by a single intragastric (i.g.) dose of 50 mg/kg body weight of DMBA; groups G5 and G6 received a single i.g. dose of Canola oil (DMBA vehicle, 1ml/kg). After one week, Groups G1 and G5 were kept in the commercial basal diet and Groups G2, G3, G4 and G6

received the same diet added with 250, 1250, 2500 or 2500 ppm of Diuron, respectively, for 25 weeks. During the experimental period, the animals were carefully checked once a week for the presence of gross mammary tumors; data on the number and localization of each palpable mass in the six mammary complexes were recorded. Sacrificed moribund rats and deceased animals were autopsied; those surviving for 23 or more weeks of Diuron treatment were included in the effective number of rats for histopathology and immunohistochemistry analysis. All animals were euthanatized by exsangüination under sodium pentobarbital anesthesia (45 mg/kg b.w.). Individual body weights and food consumption were recorded weekly during the experimental period.

2.3. Hormone analysis and tissue processing for histology

Immediately before sacrifice, samples of peripheral blood were collected for estrogen and progesterone plasma determinations. Estrogen and progesterone levels were determined automatically (VITROS ECi-Johnson and Johnson Ultra-Sensitive Chemiluminescence analysis, USA) using specific reagents supplied by Johnson and Johnson Orthoclinical (São Paulo-SP, Brazil). At necropsy, the whole skin with mammary glands and tumors, liver, kidneys, spleen, ovaries, uterus and vagina were removed and fixed for 24h in 10% phosphate-buffered formalin. Before fixation, mammary tumors, liver, kidneys, spleen, ovaries and uterus were weighed. Samples of organs/tissues

were processed in order to provide 5 μm thick paraffin sections for histological (hematoxylin-eosin – H&E) and immunohistochemical analysis.

Proliferative or neoplastic lesions in mammary glands and in reproductive system (i.e., oviduct, ovary, uterus, cervix and vagina) were classified according to published criteria by The Society of Toxicologic Pathology (SSNDC Guides, 2006).

2.4. PCNA, caspase-3, p63, RE, bcl-2 and bak immunostaining

Histological sections were put on poly-l-lysine coated slides, deparaffinized and rehydrated with graded alcohol. Sections were subjected to microwave antigen retrieval in citric acid buffer at pH 6.0 for 3 x 5 min (PCNA, caspase-3, p63, bcl-2 and bak primary antibodies) or Pascal pressure chamber retrieval in citrate acid buffer at pH 6.0 at 120ºC for 3 min (estrogen receptor-alpha antibody). Endogenous peroxidase was blocked with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min in dark. After washing with PBS, slides were incubated with non-fat milk in PBS for 60 min. Sections were then incubated with primary antibodies mouse monoclonal anti-PCNA/PC10 (1:200 dilution) (DakoCytomation Denmark A/S, Glostrup, Denmark), rabbit polyclonal anti-caspase-3 cleaved /Asp 175 rabbit (1:100 dilution) (Cell Signalizing Technology Inc., Danvers, MA - USA), mouse monoclonal anti-ER-alpha/6F11 (1:50 dilution) (BioCare Medical – Concord, CA – USA), monoclonal mouse anti-p63/4A4 (1:100 dilution) (Dako Cytomation

Denmark A/S) and anti-polyclonal rabbit anti-bcl2/N-19 (1:200 dilution) and polyclonal rabbit anti-bak/G-23 (1:200 dilution) (Santa Cruz Biotechnology Inc., CA - USA) for overnight. This was followed by biotinylated secondary antibodies horse anti-mouse or goat anti-rabbit (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min and incubated with performed streptoavidin-biotin complex/horseradish peroxidase (Vector Laboratories Inc.), both at room temperature. Chromogen color development was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Co.) as the substrate to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris's hematoxylin. A negative control was performed in all cases by omitting incubation with the primary antibodies for PCNA, caspase-3, ER-alpha, p63, bcl-2 and bak, which in all instances resulted in negative immunoreactivity.

The rates of PCNA S-phase, and of caspase-3 cleaved labeling and of apoptosis (HE staining) in mammary tumor sections were calculated as the number of positively marked cells divided by the total number of cells scored x 100 (10 representative microscopic fields without necrosis with ~ 10,000 tumor cells in each tumor section). The immunoreactivities for p63, ER-alpha, bcl-2 and bak were evaluated using a score system. The score is based on estimating both the percentage of positively stained cells on a slide (proportion score) and the general strength of the staining (intensity score). The proportion score is ranked as 0 for negative; 1 for < 10%, 2 for >10%

and < 50% or 3 for > 50% of cells stained. The intensity score is ranked as 1 for weak, 2 for moderate or 3 for strong expression. The combined score was represented by multiplication product of the proportion score and the intensity score (Ip *et al.* 2000).

2.5. Statistical Analysis

Data for body weight and body-weight gain, food consumption, relative liver kidney, spleen weights, tumor weights, estrogen and progesterone plasma levels were analyzed by ANOVA when the results presenting normal distribution or Kruskal-Wallis test when this did not occur. Analyses of the semi-quantitative combined scores were performed using the Kruskal-Wallis test. Contrast among groups was analyzed by the Tukey or Student-Newman-Keuls methods. Incidences of mammary tumor were examined using the chi-squared or the Fischer test. Significant differences were assumed when $P < 0.05$. The statistical analyses were performed using the Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA, USA).

3. Results

3.1. Mortality, body and organ weights, food consumption and hormonal analysis

At the end of the experimental period, survival rates were 72.2% in DMBA-initiated group (G1, n= 13), 77.8%, 88.9% and 83.3% in DMBA-initiated and Diuron-treated groups to 250 (G2, n= 14), 1250 (G3, n= 16) and 2500 ppm (G4, n= 15) respectively, and survival rates were 100% in both non-initiated groups (G5, n= 10 and G6, n = 15, respectively) (Table 1).

During Diuron treatment, food consumption and body-weight gain (Figure 1) were significantly reduced ($P < 0.001$) in DMBA-initiated/Diuron-treated 1250 and 2500 ppm and non-initiated/Diuron-treated 2500 ppm groups (G3, G4 and G6, respectively) when compared to the respective control groups (Table 1). At week 25, DMBA-initiated/Diuron-treated 2500 ppm and non-initiated/Diuron-treated 2500 ppm groups (G4 and G6, respectively) had lower final body weights ($P < 0.001$) than their respective control groups (Table 1). Increased relative liver and spleen weights ($P < 0.001$) were observed in DMBA-initiated/Diuron-treated 1250 and 2500 ppm groups (G3 and G4) and in non-initiated and Diuron-treated 2500 ppm (G6) (Table 2). Increased relative right and left kidney weights (P= 0.026; P = 0.006, respectively) and decreased relative left ovary weight (P = 0.004) were observed in non-initiated and Diuron-treated 2500 ppm group (G6) when compared to the non-initiated group (G5) (Table 2).

Diuron treatment did not cause any significant alterations in estrogen (pg/ml) or progesterone (ng/ml) plasma levels when compared to the respective controls (Table 3).

3.2. Histopathologic and immunohistochemical analysis

Sixty mammary tumors were histologically confirmed in DMBA-initiated groups. The majority of them was classified as adenocarcinomas with either tubular, papillary or comedo-cribriform patterns or mixed structures (57/60); the others were adenomas (2/60) and fibroma (1/60) (Table 3). The prevailing histological tumor pattern was considered for classification. In general, adenocarcinomas induced by DMBA presented an expansive pattern with local invasive areas. In non-initiated/Diuron treated 2500 ppm animals (G6) no hyperplastic lesion (lobular or atypical) or benign or malignant neoplasms were observed in the mammary glands.

Treatment with Diuron did not alter the latency period (i.e., time to first palpable mammary tumor), incidence, multiplicity, tumor weights and histological patterns in female SD rats initiated with DMBA (Table 3). Also, malignant mammary neoplasms were further characterized by a number of immunohistochemical markers such as PCNA, caspase-3 cleaved, p63 (myoepithelial marker), bcl-2 and bak (apoptosis regulatory proteins) and ER-alpha (Figure 2A-F). As the rates of caspase-3 cleaved positive apoptotic cells correlated well with the corresponding rates of H&E stained apoptotic cells

(Figure 2B1), the apoptosis indexes (AI%) were estimated in both caspase-3 cleaved expression and morphological aspects (H&E stained sections) (Eckle *et al.* 2004).

The different Diuron dietary levels did not change the indexes of cell proliferation (PCNA LI%) and apoptosis (AI%) in mammary tumors. Immunoreactivity for p63 nuclear protein and for apoptosis regulatory proteins bcl-2 and bak and ER-alpha were variably expressed in different regions of the tumor (central, periphery, or in proliferating or necrotic areas), irrespective of their histological patterns. The semi-quantitative combined score system herein adopted indicated that the treatment with Diuron also did not change the patterns of immunoexpression of p63, bcl-2, bak or ER-alpha in the DMBA-induced neoplasms (Table 4).

Some female SD rats exposed to Diuron 2500 ppm (groups G4 and G6) developed liver centrilobular hypertrophy, splenomegaly and urothelial hyperplasia in the urinary bladder and renal pelvis. These changes were related to the influence of high concentrations of Diuron since they have been already reported in previous studies (Nascimento *et al.* 2006; Grassi *et al.* 2007; Fernandes *et al.* 2007).

4. Discussion

The results of the present study indicate that a 25-week long exposure to high dietary concentrations of Diuron does not modify the mammary carcinogenesis process initiated by DMBA in the highly susceptible female SD rats. Also, different Diuron exposure levels did not alter the histological patterns, the rates of cell proliferation and apoptosis, and the immunoreactivity of ER-alpha, p63, bcl-2 and bak biomarkers in developing tumors. These findings were obtained using a dietary concentration of Diuron reported to be carcinogenic to the urinary bladder male and female Wistar rats and to the mammary gland of female NMRI mice in long-term feeding studies (Iyer, 2002); (USEPA, 2003).

The oral exposure to Diuron at 2500 ppm for 25-week also did not initiate the mammary carcinogenesis process since no preneoplastic or neoplastic lesion were histologically detected in the mammary glands of non-initiated female SD rats. It is not surprising that Diuron alone did not initiate the carcinogenesis process in this organ. Although some reports in the literature suggest that Diuron is genotoxic (Agrawal *et al.* 1996; Agrawal and Mehrota 1997; Bouilly *et al.* 2007), studies developed in our laboratory indicated that the herbicide does not damage DNA *in vivo* (Nascimento *et al.* 2006) and does not induce *crosslinks in vitro* (Rocha *et al.*, 2009) when tested by the comet (single cell gel eletrophoresis) assay. Besides, Diuron did not exert initiating or promoting potentials in the skin of Swiss albino mice (Ferrucio *et*

al., 2009). These observations are in line with others reported in the literature stating that diuron is a non-genotoxic agent (Liu, 2001; Iyer, 2002; USEPA, 2003).

In the present study, oral exposure to Diuron did not exert any promoting effect on the mammary carcinogenesis process initiated by DMBA when latency, incidence, multiplicity and histological patterns were used as parameters. In fact, although not significantly, fewer tumors (tumor burden) were observed in DMBA-initiated groups fed higher dietary levels of Diuron (1250 and 2500 ppm). This finding could be directly associated to the reduction of body weight gain (-15 % and -33%, respectively) and food consumption (-4% and -13%, respectively) observed in these two groups at the end of the experiment (Table 1). Chemical treatments that decrease body weight gain associated with toxicity, to non-palatability, or to indirect food restriction are likely to inhibit the development of rodent mammary tumors in medium- and in long-term bioassays (Rudel *et al.* 2007).

Cell proliferation and apoptosis play an important role during the progression of the rat mammary gland tumorigenesis (Strange *et al.* 2001; Al-Dhaheri *et al.* 2008). PCNA, a co-factor for delta-DNA-polymerase leading to DNA replication and DNA-damage repair, has been considered a feasible marker for cell proliferation in mammary carcinogenesis (Al-Dhaheri *et al.* 2008). Bcl-2 family members are pivotal in the regulatory processes that either repress (e.g., bcl-2 and bcl-x1) or induce (e.g., bak, bax, and bad) apoptosis

(Kuwana and Newmeyer, 2003; Brunelle and Letai, 2009). In premalignant lesions and during mammary tumor progression in the female rat, increases in cell proliferation and imbalances of the anti-apoptotic bcl-2 and bcl-x and pro-apoptotic bax or bak proteins have been described (Xie *et al.* 1999; Shilkaitis *et al.* 2000; Strange *et al.* 2001; Al-Dhaheri *et al.* 2008). However, in the present study Diuron did not alter significantly cell proliferation or apoptosis indexes or the expressions of bcl-2 and bak proteins in the DMBA-initiated mammary tumors.

p63 is a p53-related DNA-binding protein that has been described as a nuclear transcriptional factor involved with differentiation, maintenance and proliferation of epithelial progenitor cells (stem cells) (Barbieri and Pietenpol, 2006). This myoepithelial cell marker has been observed in normal and neoplastic tumors of both human and rat mammary tissues (Ribeiro-Silva *et al.*, 2005; Chan *et al.*, 2005; Rakha *et al.*, 2006). In human breast cancer, p63 has been shown to be expressed in *in situ* ductal carcinoma and in benign neoplasms, but is frequently absent in invasive lesions (Ribeiro-Silva *et al.*, 2005; Rakha *et al.*, 2006). In this study, p63 immunoreactivity was observed in basal cells of most neoplastic ducts-like structures or in focal areas not directly lining epithelial mammary neoplastic cells. Thus, mammary tumors induced by DMBA in SD female rats can be constituted by a mixed cellular lineage (i.e., epithelial and myoepithelial cells) as previously described for mammary

neoplasms induced by MNU (Chan *et al.*, 2005) and this structural pattern was not altered by exposure to Diuron.

Some pesticides with potential endocrine disrupting properties have been categorized as mammary carcinogens in rodent studies (Rudel *et al.* 2007; Ueda *et al.* 2005). These pesticides, including some substituted urea herbicides, are able to block or inactivate the steroid hormone receptors and/or affect the levels of pituitary hormones with potential to alter the development and function of the male and female reproductive systems (Bauer *et al.* 1998; Cook *et al.* 1993; Vinggaard *et al.* 1999; 2000; Kojima *et al.* 2004; Noguerol *et al.* 2006). *In vitro* assays indicate that Diuron did not inhibit 5alpha-reductase activity but has the capability to connect to the androgen receptor, thus acting as a male endocrine disruptor (Bauer *et al.* 1998). Also, Diuron did show estrogen receptor (ER)-mediated response in human MCF-7 breast cancer cells, Chinese hamster ovary cells or recombinant yeast strains (Vinggaard *et al.* 1999; Kojima *et al.* 2004; Noguerol *et al.* 2006). However, Diuron did not affect CYP19 aromatase activity in the human placental microsomes assay, indicating that it does not interfere with the conversion of androgens to estrogens, which could alter the balance between male and female sex hormones (Vinggaard *et al.* 2000). The present findings showing absence of promoting effects of Diuron on mammary carcinogenesis are in agreement with these *in vitro* assay negative results.

Estrogens have important physiological effects on the growth and function of hormone-dependent tissues, including the mammary gland but its are also associated with the development and progression of breast cancer (Matthews and Gustafsson, 2003; Petterson and Gustafsson, 2001; Couse and Korach, 1999). Estrogens exert their carcinogenic effects by both estrogen receptors (ER)-dependent and ER-independent pathways. In special, the ER-dependent pathway involves the activation of the ER by estrogens, leading to the expression of estrogen responsive genes, as well as stimulation of cell growth and proliferation (Khan *et al.*, 1998). The ER-alpha is a ligand dependent transcription factor that regulates a large number of genes in many different target tissues and is important in the development and progression of estrogen-responsive neoplastic cells (DeSombre, 2000). In the present study, the positive expression rate of ER-alpha was approximately 80 % in a total of 60 tumors. In fact, as most mammary tumors induced by DMBA are estrogen-(ER) positive and dependent of hormonal status (Russo and Russo, 1996), our results also indicate that the rat mammary gland is not a potential target for the carcinogenic potential of Diuron since the serum estradiol levels, tumor burden and the immunoexpression of ER-alpha in the tumors were not altered.

Finally, no histological evidences of Diuron toxicity on reproductive organs like ovary, uterus and vagina were observed in this study. This is at difference with the herbicide atrazine which has been categorized as endocrine

disruptor and a potential mammary carcinogen for female SD in chronic feeding studies. In high-doses, atrazine has been related to an acceleration of age-related endocrine changes leading to an earlier onset and/or increased incidence of chemically-induced mammary tumors in females SD rats. This sex/strain endocrine-mediated response, which appears to be unique to the SD female rat, occurs only at high doses that interfere with normal estrous cycling, promoting prolonged exposure to endogenous estrogen and prolactin (Wetzel *et al.*, 1994; Stevens *et al.*, 1999). Therefore, the present results suggest that Diuron also does not have a female endocrine disruptor potential like the herbicide atrazine.

Since in the present study no initiating or promoting influences of Diuron on the chemically-induced mammary gland carcinogenesis process were registered in female SD rats, it can be assumed that the rat mammary glands are not potential targets for the toxicity of this herbicide. Studies designed to understand the mode of action of high dose of Diuron on the mammary gland of female NRMI mice, as reported by others (Iyer, 2002), may lead to better understanding of the possible species-specific mammary carcinogenic activity of this herbicide and its potential relevance to humans.

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Legend for Figures

Figure 1 – Evolution of body weight in different groups during the experimental period.

Figure 2 - Immunohistochemically or HE-stained sections of mammary tumors from DMBA-initiated rats: A) PCNA-positive neoplastic cells (brown nuclei, 40x objective); B1and 2) Apoptosis cells identified in HE-stained section (black arrows, 100x objective) or by expression of caspase-3-cleaved (black arrows, 60x objective), respectively; C) p63-positive myoepithelial cells (nucleus brown; 40x objective); D-E) Immunostaining for bak and bcl-2 mitochondrial proteins (brown-stained cytoplasm, 40x objective; F) Immunostaining for ER-alpha (brown nuclei; 40x objective).

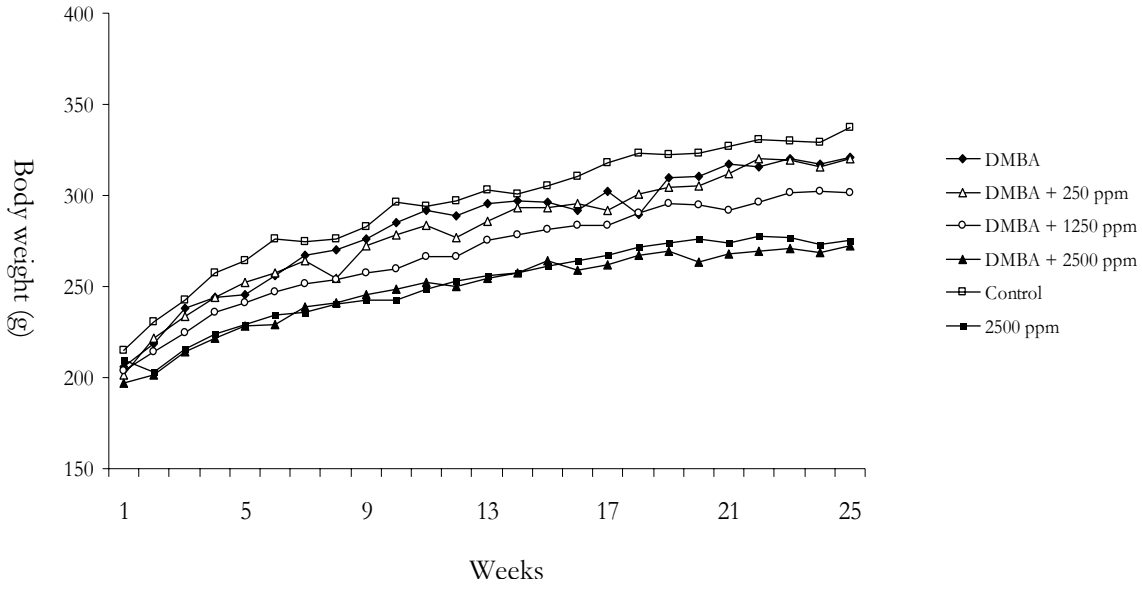
400
350
300
250
200
150
Body weight (g)
1
5
9
13
17
21
25
Weeks
DMBA
DMBA + 250 ppm
DMBA + 1250 ppm
DMBA + 2500 ppm
Control
2500 ppm

Figure 1

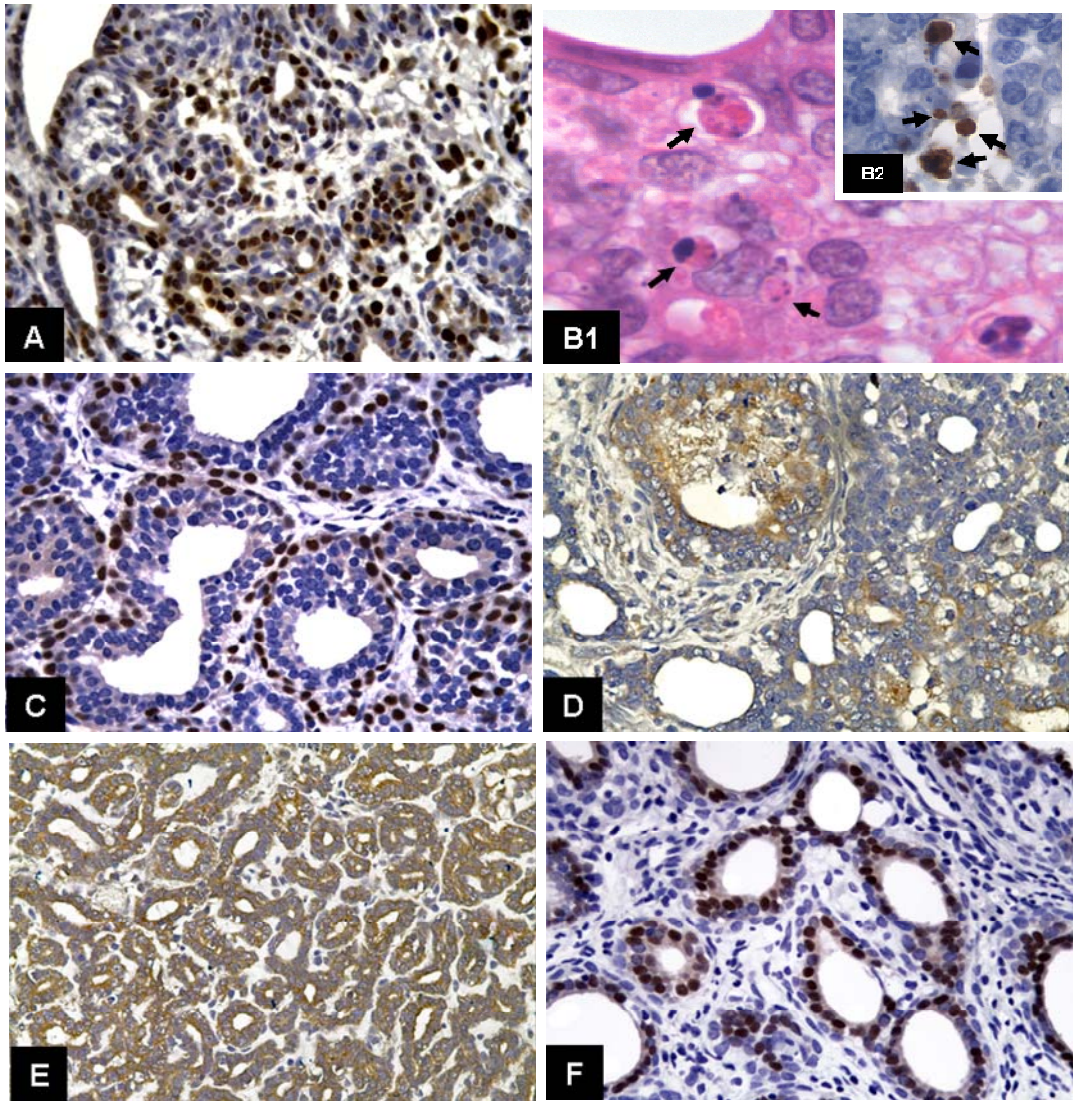

Figure 2

Table 1 – Final body weights, body-weight gain, food and Diuron consumption and feed efficiency of different experimental groups at the end of the 25-week experiment.[1]

	Group/Treatment[2]	Effective number of rats (survivors)	Final body weights (g)	Body-weight gain[3] (g)	Food consumption (g/rat/day)	Feed efficiency[4]	Diuron consumption (mg/kg/day)
	Initiated						
G1	DMBA	13	321.23 ± 36.13[a]	114.85 ± 24.19[a]	18.07 ± 2.08[a]	0.17 ± 0.04[a]	-
G2	DMBA+250 ppm	14	320.50 ± 30.79[a]	119.79 ± 27.47[a]	17.78 ± 2.54[b]	0.15 ± 0.04[b]	16.21 ± 2.43
G3	DMBA+1250 ppm	16	301.25 ± 28.44[a]	97.81 ± 27.43[a]	17.23 ± 1.72[c]	0.19 ± 0.07[c]	81.42 ± 9.12
G4	DMBA+2500 ppm	15	272.20 ± 31.69[b]	77.20 ± 29.17[b]	15.68 ± 1.66[d]	0.22 ± 0.07[d]	159.57 ± 17.35
	P value		*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	-
	Non-initiated						
G5	Control	10	337.30 ± 24.10[a]	122.30 ± 23.95[a]	18.61 ± 1.75[a]	0.16 ± 0.03[a]	-
G6	2500 ppm	15	275.67 ± 14.41[b]	66.20 ± 17.91[b]	15.94 ± 1.76[b]	0.23 ± 0.04[b]	160.59 ± 18.61
	P value		*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	-

[1]Values are mean ± SD; [2]DMBA = (7,12-dimethylbenz[a]anthracene, 50 mg/kg i.g.) plus 250, 1250 or 2500 ppm of Diuron in diet. [3]Diuron exposure period (1st to the 25th week of the experiment);[4] Weight gain/food consumed. Groups G2, G3 and G4 and Group G6 were compared only to the respective control groups (groups G1 and G5, respectively).

Table 2 – Organ relative weights of the different experimental groups at the end of experiment.[1]

	Group/Treatment[2]	Effective number of rats (survivors)	Organs relative weight (%)						
			Liver	Spleen	R Kidney [3]	L Kidney[3]	R Ovary	L Ovary	Uterus
	Initiated								
G1	DMBA	13	3.33 ± 0.43[a]	0.23 ± 0.06[a]	0.30 ± 0.03	0.30 ± 0.04	0.08 ± 0.02	0.08 ± 0.03	0.92 ± 0.35
G2	DMBA+250 ppm	14	3.42 ± 0.40[a]	0.34 ± 0.07[a]	0.31 ± 0.02	0.31 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.97 ± 0.20
G3	DMBA+1250 ppm	16	3.90 ± 0.49 [b]	0.46 ± 0.07[b]	0.32 ± 0.03	0.32 ± 0.03	0.07 ± 0.01	0.08 ± 0.02	1.21 ± 0.58
G4	DMBA+2500 ppm	15	3.84 ± 0.39[b]	0.50 ± 0.12[b]	0.33 ± 0.06	0.31 ± 0.04	0.06 ± 0.02	0.06 ± 0.02	1.23 ± 0.60
	P value		*P = < 0.001*	*P < 0.001*	*P = 0.413*	*P = 0.136*	*P = 0.060*	*P = 0.074*	*P = 0.114*
	Non-initiated								
G5	Control	10	3.33 ± 0.45[a]	0.20 ± 0.03[a]	0.31 ± 0.02[a]	0.31 ± 0.02[a]	0.07 ± 0.03	0.09 ± 0.02[a]	1.29 ± 0.98[a]
G6	2500 ppm	15	4.18 ± 0.46[b]	0.51 ± 0.07[b]	0.36 ± 0.05[b]	0.35 ± 0.08[b]	0.06 ± 0.01	0.06 ± 0.02[b]	1.02 ± 0.39[b]
	P value		*P = < 0.001*	*P < 0.001*	*P = 0.026*	*P = 0.006*	*P = 0.120*	*P = 0.004*	*P = 0.803*

[1]Values are mean ± SD; [2]DMBA= (7, 12-dimethylbenz(a)anthracene, 50 mg/kg i.g.) plus 250, 1250 or 2500 ppm of Diuron in diet; [3]R= Right, L= Left. Groups G2, G3 and G4 and Group G6 were compared only to the respective control groups (groups G1 and G6, respectively).

Table 3 – Hormonal plasma levels and mammary tumor data of DMBA-initiated groups.

	Group/Treatment[1]			
	G1 DMBA	G2 DMBA+250 ppm	G3 DMBA+1250 ppm	G4 DMBA+2500 ppm
Effective number of rats	13	14	16	15
Hormone levels				
Estradiol (μg/ml)	29.67 ± 13.89	22.33 ± 8.65	26.66 ± 13.78	21.06 ± 9.99
Progesterone (ng/mL)	26.26 ± 15.53	23.39 ± 18.35	26.02 ± 18.75	11.31 ± 6.16
Period of tumor appearance (days)	83.13 ± 36.17	86.80 ± 37.07	84.00 ± 28.69	85.75 ± 42.53
Day of 1st tumor	42º	35º	49º	49º
Incidence of tumor bearing rats (%)[2]	7/13 (53.8)	10/14 (71.4)	6/16 (37.5)	5/15 (33.3)
Multiplicity[3] (No./rat)	2.71 ± 1.70	1.90 ± 0.99	2.17 ± 0.75	1.67 ± 1.21
Tumor weight (g)	10.87 ± 11.85	9.64 ± 9.77	13.02 ± 8.38	14.86 ± 12.84
Total number of mammary tumors	21	20	11	8
Histological Types				
Fibroma	1 (4.7%)	0	0	0
Adenoma	1 (4.7%)	0	0	1 (12.5)
Adenocarcinoma	19 (91.5%)	20 (100%)	11 (100%)	7 (87.5%)

[1]DMBA = (7,12-dimethylbenz(a)anthracene, 50 mg/kg i.g.) plus 250, 1250 or 2500 ppm of Diuron in diet.; [2]Incidence: percentage of tumor-positive rats surviving for 23 or more weeks of Diuron treatment; [3]Multiplicity: average number of tumors/tumor-bearing rat. No significant different was observed among groups.

Table 4 – Growth kinetic and expression immunohistochemical of biomarkers in mammary neoplasms from DMBA-initiated groups.[1]

Group/Treatment[2]		Growth kinetic[3]		Immunohistochemical score[4]			
		PCNA LI%	AI%	ER-alpha	p63	bak	bcl-2
G1	DMBA	12.65 ± 7.14	0.57 ± 0.24	2.94 ± 1.78	1.89 ± 1.59	1.00 ± 0.63	2.33 ± 1.86
G2	DMBA+250 ppm	10.72 ± 4.99	0.57 ± 0.20	3.94 ± 1.69	3.42 ± 2.83	1.86 ± 2.27	2.00 ± 1.55
G3	DMBA+1250 ppm	12.84 ± 5.32	0.45 ± 0.14	2.14 ± 1.07	3.82 ± 2.99	1.63 ± 0.74	2.33 ± 2.16
G4	DMBA+2500 ppm	14.90 ± 4.86	0.58 ± 0.18	5.14 ± 2.48	2.13 ± 2.03	1.86 ± 1.68	2.29 ± 2.06

[1]Values are mean ± SD; [2]DMBA= (7, 12-dimethylbenz(a)anthracene, 50 mg/kg i.g.) plus 250, 1250 or 2500 ppm of Diuron in diet.; [3]PCNA labeling index and apoptosis index; [4]Score = Multiplication product of the proportion score and the intensity score. Groups G2, G3 and G4 were compared only to the respective control group (group G1). No significant different was observed among groups.

Capítulo III

Artigo Científico II

Early life stage exposure to the herbicide Diuron: effects on reproductive development and function and susceptibility to the mammary carcinogenesis in female Sprague Dawley rats.

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Abstract

Diuron is widely used in agriculture and its deleterious effects on the reproductive system and mammary gland are still poorly known. Thus, this study evaluated if early life stage exposure to Diuron alter puberty onset or susceptibility to mammary carcinogenesis in female Sprague-Dawley (SD) rats. Pregnant rats received basal diet or diet containing Diuron at 500, 750 and 1250 ppm, from gestational day 12 (GD12) to the end of lactation period. After weaning, female offspring rats continued receiving basal diet or diet containing Diuron until PND 51. The animals were sacrificed on PND 51, 75 and 25-weeks after 7,12-dimethly(a)antracene (DMBA) administration. There was no significant difference among groups on vaginal opening, estrous cycle or mammary gland morphology or carcinogenesis. However, a reduction on ovary weight and corpora lutea was observed PND 75 in Diuron-treated 1250 ppm rats suggesting that Diuron exposure may have been toxic to the ovaries.

Key words: pesticides, Diuron, mammary carcinogenesis, Sprague-Dawley rats, maternal exporure, female offspring, reproduction

1. Introduction

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a urea-derived herbicide acting by inhibiting photosynthesis in microorganisms and plants [1,2]. This pesticide is indicated for the control of weeds, as well as mosses, in non-crop areas and in many agricultural crops such as fruit, cotton, alfalfa, wheat and soy or as antifouling agent in aquatic environmental [1,2]. Due to it's moderately to highly persistence, Diuron can be found in many environments such as soil, sediments and water [2,3].

Diuron *per si* has low systemic toxicity to mammals and birds, and moderate toxicity to aquatic invertebrates [2]. In mammals, it is biotransformated by dealkylation of methylureic groups of carbons two and/or six from the benzene ring via cytochrome (CYP) P450s enzymes, generating 3,4-dichlorophenyl and 3,4-dichloroaniline (3,4-DCA) [2-6]. Thus, a possible adverse effect could be operative for humans exposed to larger amounts of Diuron and that present a high CYP1A 1 and 2 contents, which may be able to metabolize Diuron more efficiently [7].

Several chemicals have been identified as endocrine disruptors, including natural and synthetic hormones, pesticides, plasticizers and industrial by-products which can interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction [8]. Some substituted urea herbicides are able to block or inactivate the steroid hormone receptors and/or affect the levels of sex or

pituitary hormones with potential to affecting the development and function of the male and female reproductive system [9-15]. Findings from *in vitro* assays indicate that Diuron did not inhibit 5alpha-reductase activity but has the capacity to connect to the androgen receptor, thus allowing that this herbicide to act as a male endocrine disruptor [11,16]. Besides, Diuron did not affect CYP19 aromatase activity in the human placental microsomes assay, indicating that this herbicide did not interfere in the conversion of androgens to estrogens, which could alter the balance between the male and female sex hormones [13,17]. *In vitro* assays indicated that this herbicide did not appear to show estrogenic activity in human MCF-7 breast cancer cells, Chinese hamster ovary cells or recombinant yeast strains [13-15].

Diuron have been considered an aryl hydrocarbon receptor (AhR) partial agonist with major efficacy in rat cells than in the mouse, guinea pig or human cell lines [18]. The AhR, known for mediating the toxicity of dioxins and related compounds, is a helix-loop-helix PAS-containing transcription factor which actives targets gene transcription in a ligand-dependent manner [19]. Some AhR ligand has been reported to induce formation of an AhR-estrogen receptor complex (Ahr/ER complex), which stimulates transcription of ER target genes [20,21,22]. There is an extensive evidence showing that crosstalk between the ER and AhR/aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimer could be leads to inhibition/activation of estrogenic signaling both *in vitro* and *in vivo* [20,21,22]. Thus, Diuron could be

acting as an endocrine disruptor to the reproductive organs and mammary gland via activation of Ahr/ER complex.

In order to knowledge of possible adverse effects on reproductive toxicity and development from Diuron, the present study was delineated to investigate if early life stage (gestational, lactational and juvenile) exposure to the herbicide Diuron affect reproductive development and function and/or alter the susceptibility to the mammary carcinogenesis in adult female Sprague Dawley rats.

2. Material and Methods

2.1. Animals and Treatments

The animals were handled in accordance with Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee for Ethics in Animal Experimentation of the Faculty of Medicine, UNESP, Botucatu-SP, Brazil (Protocol nº 523).

Sprague-Dawely (SD) outbred male and female rats were obtained from colonies under SPF-conditions from Multidisciplinary Center for Biological Investigation (CEMIB-UNICAMP, Campinas-SP, Brazil). The animals were housed in polypropelyne cages with white pine shavings autoclaved and maintained in rooms under controlled environmental conditions (temperature 22 ± 2°C, relative humidity 55 ± 20%, a 12/12h light-dark cycle and 4 daily exhaust periods). All animals received Nuvilab CR-1 commercial chow (Nuvital, PR, Brazil), filtered drinking water *ad libitum*.

After a 2-week acclimation period, 8-week–old female SD rats (n=26) were mated to 12-week-old male SD rats (n=12) by placing two females in a cage with one male. Mating was realized during the dark period of the cycle and the gestational day (GD) 0 was determined by the presence of sperm in vaginal smears of females in estrus (sexually receptive). The pregnant or lactating dams were weighed on alternating days to permit calculation of food and Diuron intake and investigation of clinical signs of maternal toxicity. Data

for food and water consumption of dams and litters were registered during the experimental period.

Pregnant SD rats were non-treated or orally treated with 250, 500 e 1250 ppm of Diuron in the basal diet from day GD 12 up to GD 21 to the end of lactation period (postnatal day 21- PND21). After weaning, female offspring were fed basal diet or basal diet containing Diuron at 250, 500 e 1250 ppm until PND 51. Some female were sacrificed on PND 51 (mammary gland and hormonal analysis), PND 75 (reproductive organs and hormonal analysis) or 25-weeks after 7,12-dimethly(a)antracene (DMBA) administration for initiation of mammary carcinogenesis.

2.2. Reproductive organs development and function

2.2.1. External signs of puberty onset and estrous cycle

The time of vaginal opening (VO, assessed daily starting at PND 30) was registered as an indicator of puberty. All female offspring rats from Diuron-treated 500, 750 and 1250 ppm and non-treated groups were evaluated daily and the day of complete VO was adequately recorded. On PND 60, the estrous cyclicity of female offspring rats was assessed on cells from daily vaginal smears, collected over a period of 15 days. Every morning 10 μL of 0.9% saline solution was instilled into the vagina and subsequently aspirated. The material was observed under light microscopy and the estrous cycle phase was determined by cytology [23]. The total frequency of each

phase for every rat observed in this period was used to calculate the total length of the proestrus, estrus, metaestrus and diestrus (in days) and the estrous cycle length.

2.2.2. Analysis of reproductive organs

At PND 75, ovaries and uteri from Diuron-treated 750 and 1250 ppm and non-treated groups were collected of female offspring rats in estrus phase, weighed on precision balance, fixed in Alfac's solution, dehydrated in ethanol and embedded in paraplast. Three sections (5 μm) per animal, with 50 μm of distance among them, were obtained, mounted on glass slides and stained with hematoxylin and eosin. In each ovary, ovarian follicles and corpora lutea were counted in 3 sections per animal and expressed as number per unit area (mm^2). Follicles were classified according to Guerra et al. (2009). [23] Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as pre-antral when containing 2–4 layers of granulosa cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane. In the uterus, the endometrial height was measured, in 3 sections per animal using a light microscope. In each section, five

different regions were analyzed, resulting in a total of 15 measurements per animal.

2.2.3. Hormonal analysis

Female offspring rats were sacrificed on PND 51 and 75, during the estrus phase, between 8:00 and 10:00 a.m. After decapitation, trunk blood was collected and allowed to clot on a refrigerator (4 C) for 30 min. Serum was collected after centrifugation and stored at −20 C until analysis. At PND 75, serum FSH and LH concentrations were measured using a double-antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases–NIADDK, USA).At PND 51, estrogen and progesterone levels were determined by means of automatic equipment (VITROS ECi-Johnson and Johnson Ultra-Sensitive Chemiluminescence analysis, USA) using specific reagents supplied by Johnson and Johnson Orthoclinical (São Paulo-SP, Brazil). All the samples were analyzed at the same assay to avoid inter-assay variability.

3. Effects of Diuron exposure in early life stage on mammary gland development and tumorigenesis

3.1 Analysis of the mammary gland morphology and function

At PND 51, female offspring rats were sacrificed and whole skin with mammary glands were removed and fixed for 24h in 10% phosphate-buffered

formalin. Samples of organs/tissues were processed in order to provide 5 μm thick paraffin sections for histological (hematoxylin-eosin – H&E) and immunohistochemical analysis for proliferating cell nuclear antigen (PCNA), cleaved caspase -3 and estrogen-receptor-alpha (ER-α).

Mammary tissue sections were put on poly-l-lysine coated slides, desparaffinized and rehydrated with graded alcohol. Sections were subjected to microware antigen retrieval in citric acid buffer at pH 6.0 for 3 x 5 min (PCNA and caspase-3 markers) or Pascal pressure chamber retrieval in citrate acid buffer at pH 6.0 at 120ºC for 3 min (estrogen receptor-alpha maker). Endogenous peroxidase was blocked with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min in dark. After washing with PBS, slides were incubated with non-fat milk in PBS for 60 min. Sections were then incubated with primary antibodies mouse monoclonal anti-PCNA/PC10 (1:200 dilution) (DakoCytomation Denmark A/S, Glostrup, Denmark), rabbit polyclonal anti-cleaved caspase-3 /Asp 175 rabbit (1:100 dilution) (Cell Signalizing Technology Inc., Danvers, MA - USA) and mouse monoclonal anti-ER-α/6F11 (1:50 dilution) (BioCare Medical – Concord, CA – USA) for overnight. This was followed by secondary antibodies anti-mouse and anti-rabbit conjugated with polymer/peroxidase (Max Polymer, Novolink TM - Novocastra TM- Leica Microsystems, UK) for 30 min at room temperature. Chromogen color development was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Co.) as the substrate to demonstrate

the sites of peroxidase binding. The slides were counterstained with Harris's hematoxylin. A negative control was performed in all cases by omitting incubation with the primary antibodies, which in all instances resulted in negative immunoreactivity.

The PCNA S-phase, caspase-3 cleaved and ER-α labeling indexes in mammary glands were calculated as the number of positively marked cells divided by the total number of cells scored x 100 (~ 300 to 500 epithelial cells in each section).

3.2 Mammary carcinogenesis assay

On PND 51, female offspring rats from Diuron-treated at 500, 750 and 1250 ppm and non-treated groups (12 rats/group, 2 female/litter) received a single intragastric (i.g.) dose of 50 mg/kg body weight (b.w.) of DMBA. After DMBA administration, all groups received basal diet and drinking water ad libitum for 25 weeks. During the experimental period, the animals were carefully checked once a week for the presence of gross mammary tumors; data on the number and localization of each palpable mass in the six mammary complexes were recorded. Sacrificed moribund rats and deceased animals were autopsied. Moribund and surviving rats were included in the effective number of rats for histopathology analysis. All animals were euthanatized in estrus phase by exsangüination between 9:00 to 11:00 hr, under sodium pentobarbital anesthesia (45 mg/kg b.w.).

At necropsy, mammary tumors, ovaries, uterus and vagina were removed and fixed for 24h in 10% phosphate-buffered formalin. Before fixation, mammary tumors, ovaries and uterus were weighed. Samples of organs/tissues/tumors were processed in order to provide 5 μm thick sections for histological analysis. Proliferative or neoplastic lesions in mammary glands and in reproductive system (i.e., ovary, uterus, cervix and vagina) were classified according to published criteria by The Society of Toxicologic Pathology (SSNDC Guides, 2006). [24]

4. Statistical Analysis

Data for body weight and body-weight gain, food consumption, tumor weights, ovary and uterus weights, reproductive parameters and FSH, LH, estrogen and progesterone serum levels were analyzed by ANOVA when the results presenting normal distribution or Kruskal-Wallis test when this did not occur. Analyses of the semi-quantitative combined scores were performed using the Kruskal-Wallis test. Contrast among groups was analyzed by the Tukey or Student-Newman-Keuls methods. Incidences of mammary tumor were examined using the chi-squared or the Fischer test. Significant differences were assumed when $P < 0.05$. The statistical analyses were performed using the Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA, USA).

3. Results

3.1. Effects of early life Diuron exposure on dams (F0) and females offspring

Dam weight was recorded during GD12-20 Diuron exposure period and weight gain was compared among groups (Table 1). Dams treated with Diuron 1250 ppm gained less weight and presented a significantly lower body weight at DG20 than control dams (G1 group vs. G4 group, $P = 0.042$). Female offspring rats) exposed to Diuron during gestation, lactation and pre-puberty presented a significantly lower body weight at PND 10 (750 and 1250 ppm), PND 21 (500, 750 and 1250 ppm) and PND 51 (1250 ppm) when compared to female pups non-treated ($0.042 < P < 0.001$). During PND 21-51, no significant difference in food consumption was observed among female offspring treated or non-treated (Table 1).

3.2. Effects of early life Diuron exposure on reproductive and mammary parameters in females offspring

Vaginal opening (all groups) and estrous cyclicity (Diuron-treated 750 and 1250 ppm and non-treated groups) were evaluated as physical signs of female reproductive development. Following VO, estrous cyclicity patterns of these animals were observed until PND 60. The age at vaginal opening was not significantly altered, occurring on days 39.08 ± 0.67 (n=6 litters), 39.42 ± 1.31(n=6 litters), 38.70 ± 0.63 (n=6 litters), 39.25 ± 1.10 (n=6 litters) for non-

treated and early in life exposure to Diuron at 500, 750 and 1250 ppm groups, respectively. Also, no significant difference due to early in life exposure to Diuron was observed from analysis of estrous cyclicity in female offspring rats (Table 2). Besides, ovary, uterus morphology analysis and FSH and LH serum concentrations were also evaluated at PND75. Absolute ovary weight and corpora lutea counting was lower in Diuron-treated 1250 ppm and 750 and 1250 ppm groups, respectively, when compared to the female offspring rats from non-treated group (Table 2). For others reproductive parameters including FSH and LH serum levels found no significant difference among groups (Table 2 and Figure 1).

In addition to the traditional indicators of rat puberty, mammary gland development was also evaluated in female offspring at PND51. To evaluate the effects of Diuron following gestational, lactational and pre-puberty exposure were evaluated progesterone, estrogen levels and morphology (all groups) and growth (cell proliferation and apoptosis analysis) and ER-α expression in histological sections from Diuron-treated 1250 ppm and non-treated groups. At PND 51, no significant alterations in estradiol and progesterone serum levels was observed among Diuron-treated 750 and 1250 ppm and non-treated groups (Figure 1). Female offspring Diuron-treated 1250 ppm had mammary gland morphology with a normal appearance of lobular/acinar units surrounded by abundant adipose tissue similar to the non-treated animals (data not shown). Moreover, Diuron-treatment at 1250 ppm

did not alter the PCNA, cleaved caspase-3 labeling indexes or the immunoreactivity for ER-α in the mammary gland sections (Figure 2).

3.3. Effects of early life Diuron exposure on mammary carcinogenesis susceptibility

To evaluate if exposure to Diuron early in life would elicit developmental changes in the mammary tissue and cause a predisposition for mammary cancer, female offspring were initiated with DMBA at PND 51. At the end of experiment at week 25, survival rates were 66.67% in DMBA-initiated group (G1), 100%, 75% and 66.67% in early life Diuron-treated 500 (G2), 750 (G3) and 1250 ppm (G4) and DMBA-initiated groups, respectively (Table 3). Final body weight were significantly reduced (*P = 0.010)* in early life Diuron-treated 1250 ppm and DMBA-initiated group (G4) when compared to solely DMBA-initiated group (G1). Diuron early in life exposure and DMBA initiation did not alter the body-weight gain or ovary and uterus weights when compared to respective control group (G1) (Table 3).

Data from mammary tumor analysis in DMBA-initiated groups collected during experimental period is presented in Table 4. More than 50% of the animals developed mammary tumors histologically classified as fibroma (15.2%) and adenocarcinoma (84.9%). In general, mammary adenocarcinomas induced by DMBA presented an expansive pattern with local invasive areas. Exposure to Diuron early in life did not alter the latency period (i.e., time to

first palpable mammary tumor), incidence, multiplicity, tumor weights or histopathologic patterns in adult female SD rats initiated with DMBA for any treatment group (Table 4).

4. Discussion and Conclusions

Diuron and others pesticides with potential endocrine disrupting properties have been categorized as potential mammary carcinogens in rodent carcinogenesis assays [25,26]. These pesticides, including some substituted urea herbicides, are able to block or inactivate the steroid hormone receptors and/or affect the levels of pituitary hormones with potential to alter the development and function of the male and female reproductive systems [10,13,14,15,16,17]. Thus, the current study was performed to assess the potential adverse of early life stage exposure to the herbicide Diuron on reproductive system and mammary gland development since during these critical phases of development (i.e., *in utero*, lactation and pre-puberty) the fetuses and newborns appear to be more sensitive than adults to endocrine disruption [27,28].

Analysis of body weight through the course of the experiment supplies data on the general health of the animal and can be important in interpreting the effects of a toxic substance on the reproductive system. A sharp decline in animal body weight may be a consequence of systemic toxicity [29]. The decrease in body weight of the dams treated with Diuron at 1250 ppm observed in our study may be related to fewer pups, although this parameter is not statistically significant, or even then represent a sign of maternal toxicity. Our results corroborates with Khera (1979) [30], which administered Diuron in doses of 250 e 500 mg/b.wt. at DG 15 and 22, found a decrease in body

weight of dams, suggesting that Diuron increased incidence of maternal toxicity in these experimental conditions. In a previous study, a decrease in number of fetuses in female rats inseminated by males treated with the dose of 125 mg/kg was observed, indicating a possible increase in number of resorptions and in frequency of post-implantation losses [31]. Besides, the reduction in body weight of female offspring during treatment indicates that Diuron may have interfered with growth and development theses animals in this critical development period. Thus, the treatment with Diuron caused a significant reduction in weight of mothers and of uteruses with fetuses.

In the present study, the herbicide Diuron was incorporated into chow, which could made it less palatable, leading to diminished intake by rats or even rejection of the diet containing chemicals [32]. However, the consumption of basal diet containing Diuron was similar among the experimental groups showing that the observed body weight reduction in dams and female pups was not related to food consumption.

Puberty, an event indicated by the age of vaginal opening and a gradual increase in the secretion of gonadotropic hormones by the pituitary, which leads, in turn, to an increase in blood estradiol levels [29,33]. In the present study, no significant changes was observed in the day of vaginal opening among the groups, indicating absence of a adverse effect of Diuron on puberty onset. Sex steroids influence the growth, function and differentiation on female reproduction organs and make them susceptibly to endocrine

disruption. During the estrous cycle, fluctuating levels of estrogens and progesterone elicit profound effects on epithelial proliferation and cytodifferentiation [34]. In this study, no alterations in uterine endometrial height and estrous cycle was observed in the female offspring rats exposed to Diuron in early in life, indicating normal hormonal responsiveness of the female reproductive tract, a finding supported by hormonal results, whereas no difference in LH and FSH serum levels among groups was observed.

Significant increases or decreases in ovarian weight compared with controls should be considered an indication of female reproductive toxicity. Although ovarian function shifts throughout the estrous cycle, ovarian weight in the normal rat does not show significant fluctuations. Still, changes as inhibition of corpus luteum formation may be associated with changes in ovarian weight [29]. The reduction of ovarian weight observed in female pups treated with Diuron at 750 and 1250 ppm was correlated with a decrease in number of corpora lutea, indicating a possible toxic action of Diuron on this organ. In the chronic toxicity study in Wistar rats, no significant effects on the female reproductive system were observed in the animals examined at 12 months [35]. Moreover, the possible impact of treatment with the herbicide Diuron on ovary morphology and function requires further examination.

The exposure to Diuron during critical period of development of the mammary gland did not induced delayed development (atrophy) or tubuloalveolar hyperplasia or serum progesterone and estradiol levels,

which biomarkers indicative of endocrine-disrupting effects [36]. Also, Diuron-treatment did no alter the levels of cell proliferation (PCNA marker) and apoptosis (cleaved caspase-3 marker) neither the immunoreactivity for estrogen receptor alpha in alveolar and ductal ephitelial mammary cells. Thus, the herbicide Diuron did no induce systemic hormonal perturbation or mammary changes in conditions of present experiment.

7,12-Dimethylbenz[a]anthracene (DMBA) is a prototype carcinogen that induces mammary carcinogenesis in rodents DMBA. It is a procarcinogen that requires metabolic conversion to its ultimate carcinogenic metabolite by oxidation, which is conducted by CYP1A1 and 1B1 (CYP1) the CYP1A1 and CYP1B1 (CYP1) [37]. Both Diuron and DMBA bind and activate the aryl hydrocarbon receptor (AhR) [38], resulting in activation of AhR and subsequent nuclear translocation, where it heterodimerizes with another bHLH partner, the AhR nuclear translocator protein (ARNT) [39]. The AhR–ARNT dimer binds to specific regulatory elements, xenobiotic responsive elements (XREs), upstream of the responsive genes and enhances their transcripts, the CYP1 enzyme family which is a basic helix–loop–helix (b-HLH) protein. As liver is a primary role in the biotransformation of both DMBA and Diuron involving CYP1 enzyme family and centrilobular hypertrophy induced by Diuron has been described [31, 40], we hypothesized that an exposure to Diuron early in life could produce the biological character to potentially increase the risk of DMBA-induced mammary carcinoma.

However, our findings indicate that Diuron did not interfere in development of mammary neoplasms when latency period, incidence, multiplicity, tumor weights or histopathologic patterns were investigated.

No studies of human exposure and developmental and reproductive effects were located and a few reports in the literature on reproductive toxicity of Diuron in female rat have been described [41]. This present study provide the first evidence that early life stage exposure to the herbicide Diuron did not modify mammary gland development and carcinogenesis in adult female Sprague-Dawley rats but the impact on ovary morphology and function requires further examination.

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Legend for Figures

Figure 1 - Hormonal serum levels: PND 51- Estradiol (pg/ml) and Progesterone (ng/ml); PND 75- LH (ng/ml) and FSH (ng/ml).

Figure 2 – Mammary gland sections from female offspring rats at PND 51: A) PCNA-positive epithelial cells (brown nuclei, 60x objective); B) Apoptotic bodies identified by expression of cleaved caspase-3 (black arrows, 100x objective) and C) ER-α-positive epithelial cells (brown nuclei; 60x objective). A1-C1) PCNA, cleaved caspase-3, and ER-α-positive labeling indexes (LI%) in the mammary gland from non-treated and Diuron 1250 ppm treated groups, respectively. No significant different was observed between groups.

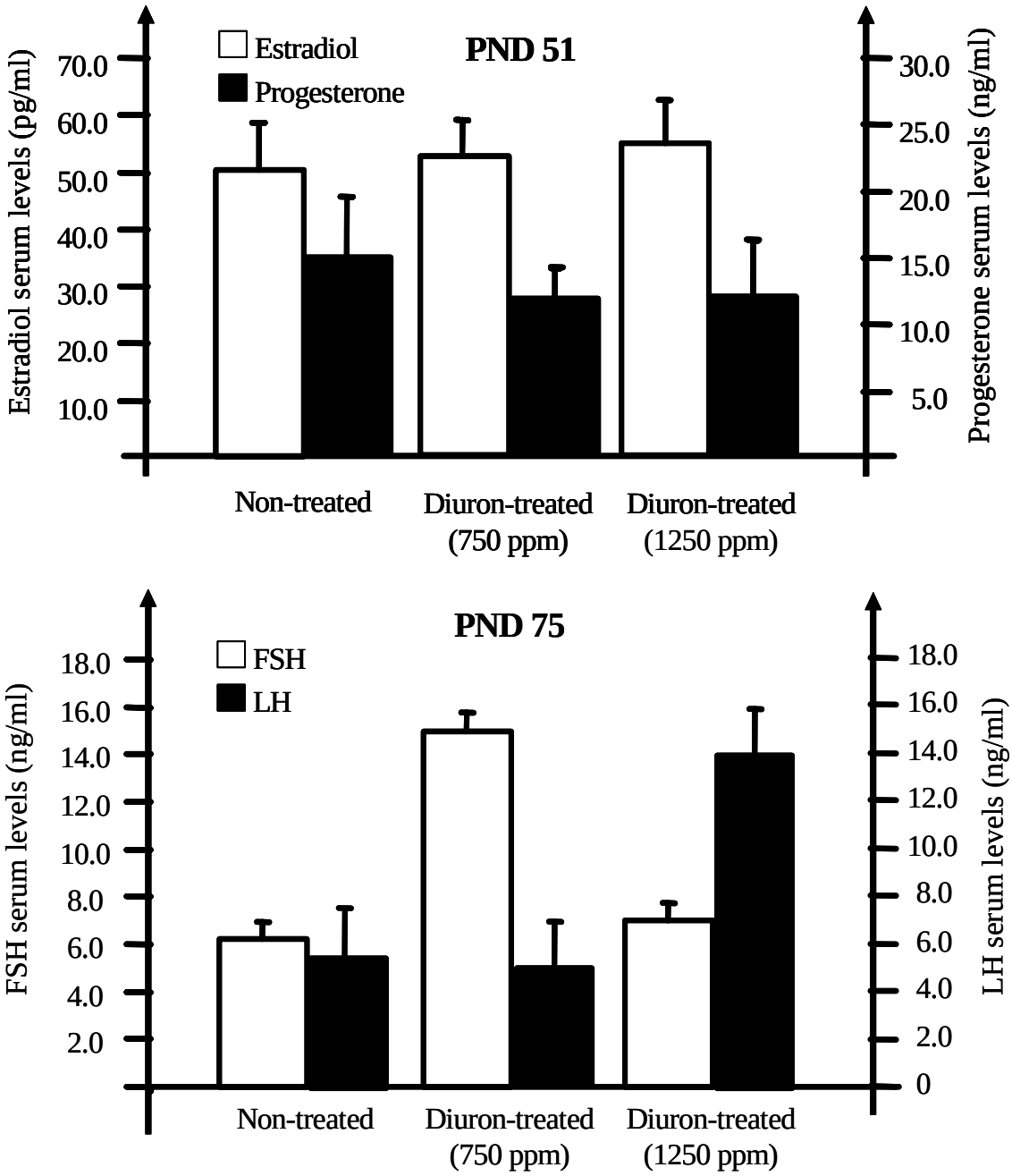
Estradiol
Progesterone
PND 51
Estradiol serum levels (pg/ml)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
30.0
25.0
20.0
15.0
10.0
5.0
Progesterone serum levels (ng/ml)
Non-treated
Diuron-treated (750 ppm)
Diuron-treated (1250 ppm)
PND 75
FSH
LH
FSH serum levels (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
LH serum levels (ng/ml)
Non-treated
Diuron-treated (750 ppm)
Diuron-treated (1250 ppm)

Figure 1

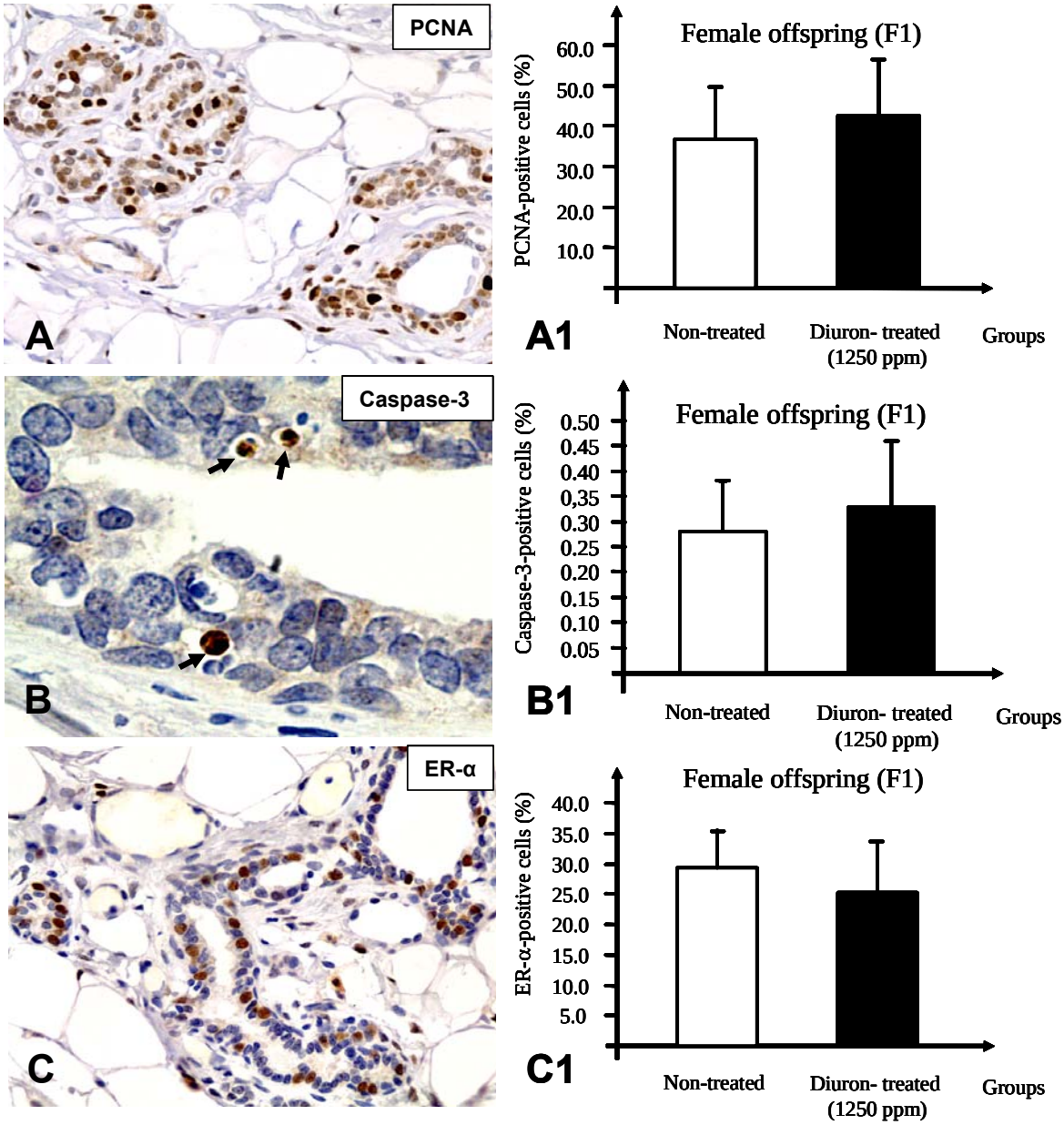
PCNA
A
Female offspring (F1)
PCNA-positive cells (%)
60.0
50.0
40.0
30.0
20.0
10.0
A1
Non-treated
Diuron- treated (1250 ppm)
Groups
Caspase-3
B
Female offspring (F1)
Caspase-3-positive cells (%)
0.50
0.45
0.40
0,35
0.30
0.25
0.20
0.15
0.10
0.05
B1
Non-treated
Diuron- treated (1250 ppm)
Groups
ER-α
C
Female offspring (F1)
ER-α-positive cells (%)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
C1
Non-treated
Diuron- treated (1250 ppm)
Groups

Figure 2

Table 1- Reproductive outcomes from dams and female offspring rats in Diuron-treated and non-treated groups.[1]

Parameters	Group/Treatment[2]			
	G1	G2	G3	G4
	Non-treated	Diuron 500 ppm	Diuron 750 ppm	Diuron 1250 ppm
Dams				
Gestation length (days)	21.40 ± 9.57	21.43 ± 8.10	21.50 ± 8.78	21.50 ± 8.78
Litter size	11.40 ± 5.10	12.57 ± 4.75	13.67 ± 5.58	9.67 ± 3.95
Sex ratio (F:M)	1.03 ± 0.46	1.26 ± 0.48	1.33 ± 0.54	2.45 ± 1.00
Body weight (g) at GD20º	364.0 ± 148.60	352.0 ± 133.04	391.3 ± 159.76	316.5 ± 129.21*
Body weight gain (g) (GD12 -GD 20)	80.17 ± 32.73	73.86 ± 27.92	87.67 ± 35.79	52.00 ± 21.23
Female pups				
Body weights (g)				
PND 10	18.62 ± 3.65	17.80 ± 2.68	16.18 ± 2.70**	16.39 ± 2.85**
PND 21	41.53 ± 8.31	34.67 ± 5.23**	37.11 ± 6.19**	37.45 ± 6.62**
PND 51	180.67 ± 52.15	180.58 ± 52.13	173.75 ± 50.16	160.50 ± 46.33***
Food consumption	(g/rat/day)	(g/rat/day)	(g/rat/day)	(g/rat/day)
Diuron intake	13.68 ± 2.14	13.13 ± 2.13	14.25 ± 2.23	12.61 ± 1.97
(PND 21-PND51)	(mg/rat/day)	(mg/rat/day)	(mg/rat/day)	(mg/rat/day)
	0	6.67 ± 1.07	10.69 ± 1.67	15.76 ± 2.46

[1]Values are mean ± S.E.M.; [2]500, 750 or 1250 ppm of Diuron in basal diet. Different from G1 group, **P = 0.042, ** P <0.001* and ****P= 0.003.*

Table 2 - Assessment of estrous cycle length and frequency of each phase over a 15-day period of evaluation in the female offspring on PND 60[1].

Estrous cycling	G1 Non-treated	G3[2] Diuron 750 ppm	G4 Diuron 1250 ppm
Number of females evaluated / litters	14 / 4	9 / 3	10 / 3
Estrous cycle length (days)	2.75 (2.38 - 3.25)	3.00 (2.67 - 3.25)	3.00 (2.50 - 3.00)
Frequency of proestrus (days)	3.25 (3.00 - 4.38)	3.33 (3.29 - 3.42)	3.30 (3.15 - 4.03)
Frequency of estrus (days)	3.38 (2.69 - 3.63)	3.00 (2.88 - 3.00)	2.75 (1.71 - 2.88)
Frequency of metaestrus (days)	6.25 (5.94 - 6.56)	5.75 (5.63 - 6.71)	7.00 (6.75 - 7.50)
Frequency of diestrus (days)	1.88 (1.44 - 2.13)	3.00 (2.00 - 3.13)	2.00 (1.38 - 2.50)

[1]Values are expressed median (Q1 quartile–Q3 quartile). [2]750 or 1250 ppm of Diuron in basal diet. No significant different was observed among groups.

Table 3- Body weight, absolute and relative organ weights and ovarian and uterus analysis in the female offspring (PND75) in estrus phase.[1]

Parameters	Group/Treatment[2]		
	G1	G3	G4
	Non-treated	Diuron 750 ppm	Diuron 1250 ppm
General	(9 litters)	(9 litters)	(9 litters)
Body weight (g)	254.66 ± 7.21	239.31 ± 4.18	235.34 ± 6.93
Ovaries (mg)	84.44 ± 5.23	71.89 ± 1.70	67.33 ± 3.65*
Ovaries/body weight (mg/g)	0.33 ± 0.01	0.30 ± 0.01	0.29 ± 0.02
Uterus with fluid (mg)	598.56 ± 52.42	635.78 ± 68.61	728.44 ± 73.58
Uterus with fluid/body weight (mg/g)	2.36 ± 0.20	2.67 ± 0.30	3.09 ± 0.29
Structures	(6 litters)	(6 litters)	(6 litters)
Primordial and primary follicles	16.33 ± 2.59	12.40 ± 2.77	15.4 ± 5.37
Pre antral follicles	12.17 ± 1.58	8.40 ± 1.50	10.6 ± 1.70
Antral follicles	9.33 ± 1.80	6.20 ± 1.85	6.80 ± 1.16
Atretic follicles	12.67 ± 1.76	11.40 ± 1.86	13.00 ± 3.02
Corpora lutea	32.67 ± 2.42	18.80 ± 2.85*	19.60 ± 2.69*
Thickness of uterine endometrium	632.40 ± 39.22	596.54 ± 39.79	515.54 ± 86.64

[1]Values are mean ± S.E.M.; [2]750 or 1250 ppm of Diuron in basal diet. Different from G1 group, *P<0.0 1.

Table 4 – Data from body weights, body-weight gain and ovary and uterus relative weights after week 25 of DMBA administration.[1]

Group/Treatment[2]		Effective number of rats[3]	Final body-weight (g)	Body-weight gain (g)	Organs relative weight[4] (%)		
					R. Ovary	L. Ovary	Uterus
G1	DMBA	12/8	318.33 ± 91.89[a]	137.67 ±39.74	0.06 ± 0.02	0.06 ± 0.02	0.99 ± 0.29
G2	500 ppm +DMBA	12/12	327.92 ± 94.66[a]	147.33 ± 2.53	0.06 ± 0.02	0.06 ± 0.02	0.70 ± 0.21
G3	750 ppm +DMBA	12/9	313.00 ± 90.36[a]	139.25 ±40.20	0.06 ± 0.02	0.06 ± 0.02	0.96 ± 0.28
G4	1250 ppm +DMBA	12/8	294.67 ± 85.06[b]	134.17 ±38.73	0.06 ± 0.02	0.07 ± 0.02	0.72 ± 0.21

[1]Values are mean ± S.E.M.; [2]DMBA = (7,12-dimethylbenz(a)anthracene, 50 mg/kg i.g.) plus 500, 750 or 1250 ppm of Diuron in basal diet. [3] (51 DPN to the 25th week of the experiment).[4]R= Right. L= Left.

Table 5 – Data from mammary tumor analysis in DMBA-initiated groups obtained during experimental period.

	Group/Treatment[1]			
	G1 DMBA	G2 500 ppm +DMBA	G3 750 ppm +DMBA	G4 1250 ppm +DMBA
Effective number of rats[2]	12/08	12/12	12/09	12/08
Period of first tumor appearance (days)	86.80 ± 38.82	119.00 ± 84.15	99.40 ± 44.45	96.60 ± 43.20
Day of 1st tumor	35º	105º	56º	63º
Incidence of tumor bearing rats (%)	08 (67)	04 (33)	07 (58)	07 (58)
Multiplicity[3] (No./rat)	1.63 ± 0.57	2.00 ± 1.15	1.43 ± 0.54	1.14 ± 0.43
Tumor weight (g)	16.25 ± 6.64	8.51 ± 6.02	11.03 ± 4.50	10.61 ± 4.75
Histological Types	10[4]	6	9	8
Fibroma (%)	2 (20)	1 (17)	0	2 (25)
Adenocarcinoma (%)	8 (80)	5 (83)	9 (100)	6 (75)

[1]DMBA = (7,12-dimethylbenz(a)anthracene, 50 mg/kg i.g.) plus 500, 750 or 1200 ppm of Diuron in basal diet.; [2]Number of animals in beginning and end of experiment; [3]Multiplicity: average number of tumors/tumor-bearing rat. [4]Total number of mammary tumors. No significant different was observed among groups.

Conclusões Gerais

Conclusões Gerais

A partir dos resultados obtidos e sob as condições experimentais empregadas no presente trabalho, podemos concluir que:

1. O herbicida Diuron não apresenta atividade promotora da carcinogênese mamária em fêmeas SD adultas. A incidência, tipos e multiplicidade das neoplasias mamárias induzidas pela DMBA não foram alteradas pela exposição ao Diuron durante a fase de pós-iniciação da carcinogênese mamária.

2. Os níveis de expressão imunoistoquímica do RE alpha, p63, anti-blc-2, anti-bak e PCNA não foram modificados em decorrência do tratamento crônico com o herbicida Diuron.

3. O tratamento com o herbicida Diuron nas fases gestacional, lactacional e juvenil não alterou o dia da abertura vaginal, indicando ausência de efeitos adversos sobre o início da puberdade.

4. O tratamento com o herbicida Diuron nas fases gestacional, lactacional e juvenil não alterou o ciclo estral e a espessura da camada epitelial do

endométrio, indicando a capacidade de resposta hormonal normal do aparelho reprodutivo feminino de fêmeas SD previamente expostas ao Diuron.

5. As alterações observadas nos ovários poderiam ser consideradas um indicativo de toxicidade no sistema reprodutor feminino, mas o impacto da exposição sobre a morfologia do ovário e função requer estudos complementares.

6. A exposição ao herbicida Diuron durante o período crítico de desenvolvimento da glândula mamária, não alterou o desenvolvimento (atrofia) ou induziu hiperplasia túbulo-alveolar nem alterou os níveis séricos de progesterona e estradiol, considerados biomarcadores de efeitos de desregulação endócrina.

7. O tratamento com o herbicida Diuron não alterou a cinética de proliferação celular, apoptose e imunoreatividade para receptor de estrógeno no epitélio ductal e alveolar das glândulas mamárias, indicando que a exposição precoce ao herbicida Diuron não induz perturbação no sistema hormonal ou danos mamários.

8. A exposição precoce ao herbicida Diuron não modificou o desenvolvimento estrutural ou neoplásico da glândula mamária na prole de fêmeas adulta Sprague-Dawley.

9. Estudos complementares devem ser conduzidos utilizando-se a linhagem de camundongos NMR1 em exposição a altas concentrações do herbicida Diuron para o melhor entendimento do modo de ação na glândula mamária e das possíveis atividades carcinogênicas espécie-específica visto que Moura *et al.* 2009 não observaram efeitos carcinogênicos na glândula mamária de camundongos da linhagem Swiss quando expostos a altas concentrações de Diuron. (*ver anexo*)

Anexos

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GENOTOXICITY AND CARCINOGENICITY

Potential effects of the herbicide Diuron on mammary and urinary bladder two-stage carcinogenesis in a female Swiss mouse model

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Abstract The potential promoting effect of Diuron was investigated in a mouse model of mammary and urinary bladder carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). Four-week old female Swiss mice were allocated to five groups: Groups G1-G3 received DMBA (5 × 1.5 mg/mouse) and BBN (8 × 7.5 mg/mouse) and G4 and G5 groups received only vehicles during the first 6 weeks. At week 7, G1 and G5 groups received basal diet and G2, G3 and G4 groups were fed a diet containing Diuron at 1,250, 2,500 and 2,500 ppm, respectively, during 13 weeks. At week 20, the animals were euthanized and the gross tumors were registered. Mammary glands and urinary bladder were processed for histopathological analysis. Samples from non-tumor areas were evaluated for cell proliferation by 5-bromodeoxyuridine labeling index (BrdU-LI%) and apoptosis. Dietary treatment with Diuron at 1,250 and 2,500 ppm significantly increased BrdU-LI% ($P < 0.05$) and the incidence of simple/nodular urothelial hyperplasia in the urinary bladder from DMBA/BBN-initiated groups (G2 and G3 vs. G1, $P < 0.02$) and in the non-initiated group (G4 vs. G5, $P = 0.042$). Two transitional cell carcinomas were observed in the group initiated and fed Diuron 2,500 ppm (G3). In contrast, in the mammary gland, Diuron feeding for 13 weeks did not significantly alter cell proliferation and apoptosis indexes or the incidence of hyperplastic lesions or neoplasms in the DMBA/BBN-initiated groups. These findings suggest that Diuron is a promoting agent to the urinary bladder but not to the mammary gland in female Swiss mice submitted to a medium-term two-stage carcinogenesis bioassay.

Keywords Pesticides · Diuron · Mammary and urinary bladder carcinogenesis · Female Swiss mouse

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Introduction

Diuron [3-(3,4-dichlorophenyl)-1-1-dimethylurea], a substituted phenyl urea herbicide has been used throughout the word to control a wide variety of annual and perennial broadleaf and grassy weeds on both crop (i.e., citrus fruit, cotton, asparagus, sugar cane, alfalfa, wheat and grapes) and non-crop sites (i.e., roads, garden paths and railways) (Iyer 2002; Field et al. 2003; Giacomazzi and Cochet 2004). In Brazil, it has been widely employed in the cultivation of sugar cane (Nascimento et al. 2006). Diuron *per se* has low systemic toxicity to mammals and birds, and moderate toxicity to aquatic invertebrates. This herbicide is biotransformed in the liver microsomes from human, rat and mouse and the urine is the primary route of excretion for Diuron and its metabolites (Verheij et al. 1989; Van Boven et al. 1990; Giacomazzi and Cochet 2004; Abass et al. 2007). Its principal biodegradation product, 3-4-dichloroaniline (3-DCA), is highly toxic and relatively persistent in the environment (Iyer 2002; Giacomazzi and Cochet 2004). Due to the high persistence in the soil, water and groundwater, contamination by this herbicide may represent an important public health problem.

In the absence of convincing epidemiological evidences, but taking into account that Diuron has been shown to be

carcinogenic to rodents, the United States Environmental Protection Agency (US EPA) considered Diuron as a "known/likely" carcinogen to humans (U.S. EPA 2003, 2004). In a 2-year feeding study, male and female Wistar rats exposed to Diuron at 2,500 ppm presented increased incidences of urinary bladder and renal pelvis tumors (Iyer 2002). Besides, female NMRI mice chronically exposed to Diuron at 2,500 ppm developed an increased incidence of mammary adenocarcinomas (Iyer 2002). Also, we have recently demonstrated that 20-week dietary treatment with Diuron at 2,500 ppm is associated with urothelial necrosis and continuous regenerative cell proliferation in male Wistar rats, resulting in the development of urothelial hyperplasia (Nascimento et al. 2006).

The most widely used carcinogens in medium-term bioassay for mammary and urinary bladder carcinogenesis are 7-12-dimethylbenz(a)anthracene (DMBA) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), respectively (Fischer et al. 1992; Qing et al. 1997; Cohen 1998, 2002). Treatment of female mice with DMBA induces the development of hyperplastic lesions like alveolar/ductal hyperplasia, benign tumors such as adenoma, fibroma, fibroadenoma and malignant neoplasms like adenocarcinomas similar to those seen in humans (Fischer et al. 1992; Qing et al. 1997; Cardiff and Wellings 1999). Moreover, treatment of male and female mice with BBN produces urinary bladder tumors that strongly resemble human high-grade invasive urothelial neoplasia. In BBN-treated mice, urothelial carcinogenesis proceeds through a sequence of morphologic changes: simple hyperplasia that becomes nodular and/or papillary hyperplasia (the first is more common), and then progression to invasive urothelial carcinomas (Cohen 1998, 2002).

Since Diuron did not present a convincing carcinogenic activity to the urinary bladder and mammary gland of non-initiated female NMRI mice, the present study was conducted to assess the potential promoting effects of the herbicide Diuron on mammary and urinary bladder carcinogenesis initiated by DMBA/BBN in a medium-term two-stage bioassay in female Swiss mice. This mouse multi-organ model was established to detect the possible enhancing influence of Diuron in a single experiment of carcinogenesis. In addition, the effects of Diuron on cell proliferation, as estimated by nuclear incorporation of 5-bromodeoxyuridine (BrdU), and apoptosis were also examined.

Materials and methods

Chemicals

7,12-Dimethylbenz(a)antracene (DMBA, CAS 57-97-6) and 3-(3,4-dichlorophenyl)-1-1-dimethylurea (Diuron, CAS 30-54-1, analytical standard grade) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) was purchased from Tokyo Kasei Industries Co. (Tokyo, Japan).

Experimental design

The University Committee for Ethics in Animal Research approved the protocol used in this study (Protocol number 78/07). Female 3-week-old Swiss mice were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP Campinas-SP, Brazil). The animals were housed in polypropylene cages covered with metallic grill in a room maintained at 22 ± 2°C, 55 ± 10% humidity and a 12 h light/dark cycle. They were fed commercial Purina chow (Paulínia, SP, Brazil) and water ad libitum for a 1-week acclimatization period.

Mice were randomly allocated into five groups, consisting of 15 animals (Fig. 1). Groups G1 to G3 were given intragastric administration (i.g.) of DMBA (5 × 1.5 mg/mouse) and BBN (8 × 7.5 mg/mouse) and G4 and G5 groups received only the vehicle Canola oil and ethanol:water (20:80) (i.e., DMBA and BBN vehicles, respectively) during the first six weeks. This experimental design using DMBA to initiate mammary carcinogenesis and BBN to initiate urinary bladder carcinogenesis was adapted from previously described protocols (Rao et al. 1996; Quin et al. 1997). At week 7, G1 and G5 groups were fed basal diet and G2, G3, G4 groups received basal diet containing Diuron at 1,250, 2,500 or 2,500 ppm, respectively, for 13 weeks. The higher dietary concentration of Diuron was chosen due to the carcinogenic potential detected in chronic feeding studies in Wistar rats and NMRI mice (Iyer 2002).

During the experimental period, the mice were carefully checked for the presence of gross mammary tumors once a week and data on the number and localization of each palpable mass in the different mammary gland complexes were recorded. All animals were euthanatized by exsanguination under sodium pentobarbital anesthesia (45 mg/kg b.w.). Individual body weights and food consumption were recorded weekly during the experimental period. Two hours before sacrifice, mice were administered a single i.p. injection of 100 mg/kg b.w. of bromodeoxyuridine (BrdU) (Sigma–Aldrich Co.) between 8:00 and 11:00 AM.

Tissue processing, histology and immunohistochemical procedures

At necropsy, the whole skin with mammary glands and tumors, liver, kidneys, spleen, and urinary bladder were removed and fixed in 10% phosphate-buffered formalin for 24 h. Before fixation, liver, kidneys and spleen were weighed. Each urinary bladder was cut into 4–6 strips. Samples of selected organs were processed for paraffin

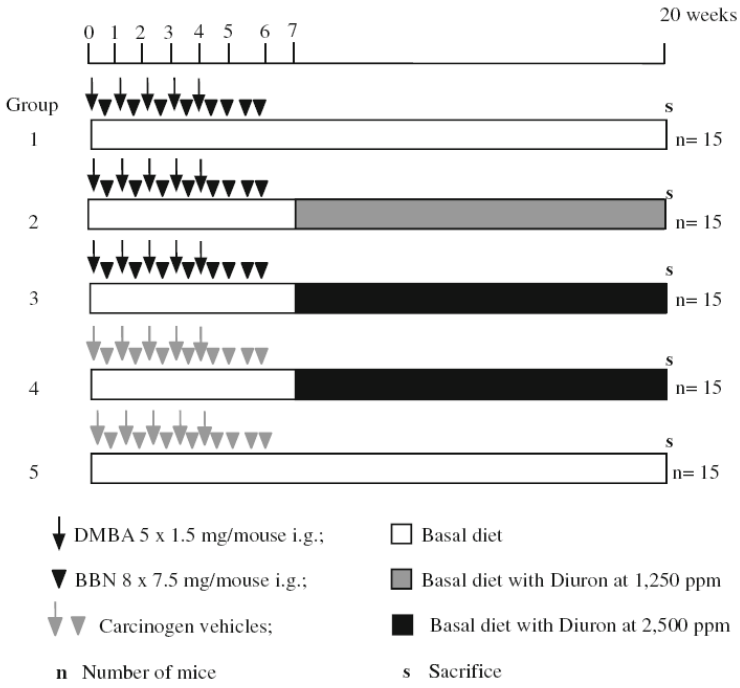

Fig. 1 Experimental design. DMBA = 7,12-dimethly-benz(a)anthracene and BBN = *N*-butyl-*N*-(4-hydroxybutyl). See details in "Material and methods"

embedding and stained with hematoxylin-eosin (HE) for histophatological and immunohistochemical analysis. Mammary lesions were classified as alveolar/ductal hyperplasia, in situ carcinoma, adenocarcinoma and acanthoma and urothelial lesions were classified as simple or nodular hyperplasia and transitional cell carcinoma (TCC) according to previously published criteria (Cardiff and Wellings 1999; Cohen 1998, 2002).

After deparaffinization, 5-μm-thick sections on poly-l-lysine coated slides were sequentially incubated with 2N HCl for 30 min at 37°C and 0.025% trypisin (Sigma–Aldrich, USA) for 10 min, 3% H_2O_2 in phosphate-buffered saline (PBS) for 15 min in dark and 1% non-fat milk in PBS for 60 min at room temperature. Sections were overnight incubated at 4°C with a mixture containing primary antibody mouse monoclonal anti-BrdU/Clone BU33 (Sigma Co. St. Louis, MO, USA), biotinylated secondary antibody horse anti-mouse (Vector Laboratories, Inc., Burlingame, CA, USA) and normal serum mouse at final concentration 1:100/1:200/1:2,400, respectively (Fung et al. 1992). Then, the slides were incubated with streptoavidin–biotin-peroxidase solution (TissuGnost Kit Merck, Darmstadt, Germany, 1:1:50 dilution) for 45 min at room temperature. Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich Co.) as the substrate to demonstrate the sites of peroxidase binding (brown nuclear staining). The slides were counterstained with Harris's hematoxylin. A segment of the small intestine from each animal was included to confirm nuclear incorporation of BrdU and a negative control was performed in all cases by omitting incubation with the primary antibody for BrdU, which in all instances resulted in negative immunoreactivity.

BrdU-labeling index and apoptosis analysis

BrdU labeling (BrdU-LI%) and apoptotic (AI%) indexes in mammary glands and urinary bladder epithelium were determined by dividing the number of cells positive for BrdU or in apoptosis (Fig. 2), respectively, by the total number of cells scored and multiplying by 100. The morphological criteria for the apoptotic cells identification was used as previously described (Levin et al. 1999). Approximately, 350–500 mammary epithelial cells and 1,000 urothelial cells in microscopic fields were scored under a 40× objective. Non-altered (non-initiated groups, G4 and G5) and non-altered and hyperplastic areas (initiated groups, G1 to G3), but not tumors, were randomly analyzed in mammary glands and urinary bladder urothelium.

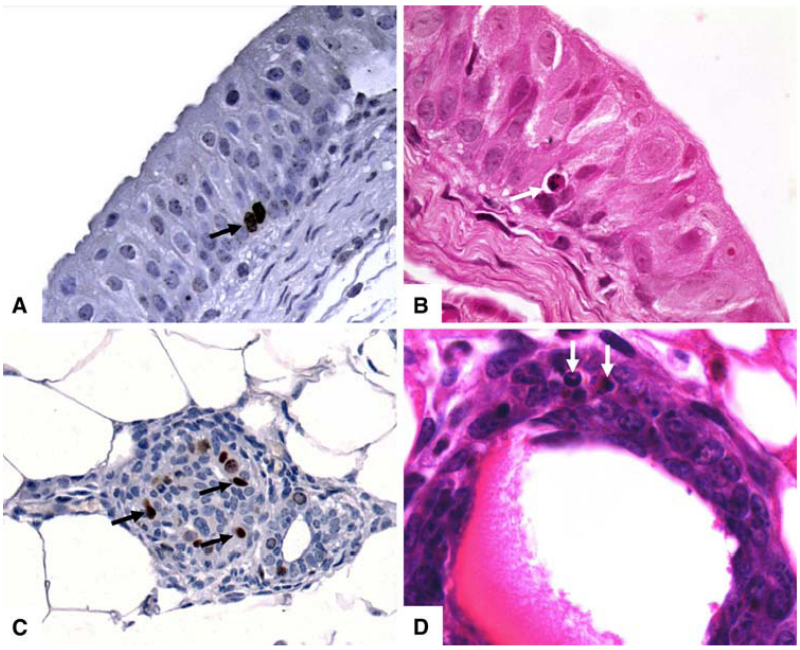

Fig. 2 Bromodeoxyuridine-labeling cells (dark nuclei in immunohistochemically stained sections, *black arrow*.) and apoptotic cells (*white arrows*. in HE stained section) in bladder urothelium (**a** and **b**) and mammary glands (**c** and **d**) from DMBA/BBN-initiated or non-initiated mice groups. **a** BrdU-positive urothelial cells, non-initiated/Diuron-treated 2,500 ppm mouse (G4 group); **b** Apoptosis in urothelial cell, non-initiated/Diuron-treated 2,500 ppm mouse (G4 group); **c** BrdU-positive mammary alveolar cells, DMBA/BBN-initiated mouse (G1 group); **d** Apoptosis in mammary alveolar cells, DMBA/BBN-initiated/Diuron-treated 1,250 ppm mouse (G2 group)

Statistical analysis

Data for body weight and body-weight gain, food consumption, relative liver, kidney and spleen weights were analyzed by the ANOVA or Student T tests when the results presenting normal distribution or Kruskal–Wallis or Mann-Whitney tests when this did not occur. The contrast among groups for ANOVA or Kruskal–Wallis tests was analyzed by the Tukey or Student–Newman–Keuls methods. Incidences of preneoplastic lesions or neoplasms were examined using the chi-squared or the Fischer tests. Significant differences were assumed when $P < 0.05$. The statistical analyses were performed using the Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA, USA).

Results

Mortality, body and organ weights and food consumption

At the end of the experimental period, survival rates were 93.3% in DMBA/BBN-initiated group (G1), 73.3% in both DMBA/BBN-initiated/Diuron-treated 1,250 and 2,500 ppm groups (G2 and G3, respectively) and 100% in both non-initiated/Diuron-treated 2,500 ppm and control groups (G4 and G5, respectively). A complete necropsy was not carried out on dead mice due to the advanced postmortem changes. The presumed cause of death was probably related to the induction of lymphomas by multiple doses of DMBA (Qing et al. 1997; Buters et al. 1999).

Food consumption, body-weight gain and final body weight were not significantly altered in DMBA/BBN-initiated/Diuron-treated 1,250 and 2,500 ppm groups (G2 and G3) or in non-initiated/Diuron-treated 2,500 ppm group (G4) when compared to the respective control groups (G1 and G5, respectively) (Table 1). At week 20, an increase in relative liver ($P < 0.05$) and spleen ($P < 0.005$) weights were simultaneously observed in DMBA/BBN-initiated/Diuron-treated 2,500 ppm group (G3) and in non-initiated-Diuron-treated 2,500 ppm group (G4) (Table 2). Diuron feeding did not cause any significant alteration in relative weights of right or left kidney compared to the respective controls (Table 2).

Histophatologic and immunohistochemical analysis

Some female Swiss mice exposed to Diuron at 2,500 ppm (G3 and G4 groups) developed hepatic centrilobular hypertrophy and splenomegaly characterized by congestion, extramedullary hemocytopoiesis and hemosiderosis. These changes in the liver and spleen were related to toxicity of high doses of Diuron as previously described by our group in male Wistar rats (Nascimento et al. 2006; Grassi et al. 2007; Fernandes et al. 2007). Also, female Swiss mice exposed to Diuron at 2,500 ppm developed simple and nodular hyperplasia in renal pelvis but no tumor was detected in non-initiated or initiated groups.

Table 1 Body weight data, water and food and Diuron intake values in the different experimental groups

Group/treatment	Number of mice	Body weight (g)			Consumption		
		Initial	Final	Gain	Water (ml/mouse day)	Food (g/mouse day)	Diuron (mg/mouse day)
Initiated							
(G1) DMBA/BBN	14	21.33 ± 2.50	46.71 ± 5.95	25.14 ±7.26	6.58 ± 0.82	6.17 ± 1.43	–
(G2) DMBA/ BBN + 1,250 ppm	11	21.66 ± 3.33	42.18 ± 7.4	21.09 ± 5.6	6.20 ± 0.86	6.19 ± 1.44	85.61 ± 9.36
(G3) DMBA/ BBN + 2,500 ppm	11	22.20 ± 3.34	40.90 ± 4.72	19.45 ± 4.84	6.50 ± 0.73	6.09 ± 1.10	142.29 ± 18.59
Non-initiated							
(G4) 2,500 ppm	15	21.61 ± 1.90	46.68 ± 5.0	24.33 ± 5.1	6.74 ± 0.75	6.82 ± 1.44	159.66 ± 11.36
(G5) Control	15	22.46 ± 2.70	50.0 ± 4.67	27.54 ± 5.5	6.85 ± 0.46	6.86 ± 1.07	–

Values are mean ± SD

DMBA 7,12-dimethlybenz(a)anthracene (4 × 1.5 mg/mouse, i.g.); *BBN* *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (8 × 7.5 mg/mouse, i.g.); 1,250 or 2,500 ppm of Diuron in the basal diet for 13 weeks

Table 2 Relative weights values of liver, spleen and kidney in the different experimental groups

Group/treatment	Number of mice	Relative weight organs (%)			
		Liver	Spleen	Right kidney	Left kidney
Initiated					
(G1) DMBA/BBN	14	4.20 ± 0.55	0.24 ± 0.68	0.48 ± 0.06	0.47 ± 0.10
(G2) DMBA/BBN + 1,250 ppm	11	4.16 ± 0.45	0.32 ± 0.96	0.50 ± 0.09	0.49 ± 0.09
(G3) DMBA/BBN + 2,500 ppm	10	4.93 ± 0.67[a]*	0.42 ± 0.12[b]*	0.50 ± 0.08	0.52 ± 0.13
Non-initiated					
(G4) 2,500 ppm	14	3.87 ± 0.32[a]**	0.29 ± 0.07[b]**	0.47 ± 0.05	0.48 ± 0.06
(G5) Control	15	3.52 ± 0.49	0.22 ± 0.05	0.45 ± 0.08	0.45 ± 0.06

Values are mean ± SD

DMBA 7,12-dimethlybenz(a)anthracene (4 × 1.5 mg/mouse, i.g.); *BBN* *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (8 × 7.5 mg/mouse, i.g.); 1,250 or 2,500 ppm of Diuron in the basal diet for 13 weeks

[a,b] Different from G1 or G5 groups, respectively, * $P < 0.05$, ** $P < 0.005$

The modifying effects of Diuron on the incidence of preneoplastic and neoplastic lesions in the mammary gland and urinary bladder are shown in Table 3. The DMBA/BBN-initiated mice developed preneoplastic (i.e., ductal or alveolar hyperplasia) and neoplastic (i.e., adenocarcinomas/acanthoma) lesions in the mammary gland at the end of week 20 (Fig. 3). Mammary tumors were histologically classified as adenocarcinomas (Fig. 3e) or with squamous areas (acanthomas). In general, DMBA-induced tumors were non-metastatic but frequently presented an invasive pattern. Diuron feeding for 13-week did not significantly alter the incidence or burden of hyperplastic lesions or neoplasms in the mammary gland from DMBA/BBN-initiated mice. Also, no preneoplastic or neoplastic lesions were observed in the mammary glands from non-initiated/Diuron treated 2,500 ppm mice (G5 group).

In the urinary bladder, Diuron feeding at 1,250 and 2,500 ppm resulted in a significant increase in the incidence of simple/nodular urothelial hyperplasia (Fig. 2) in both DMBA/BBN-initiated (G2 and G3 vs. G1, $P < 0.02$) and non-initiated groups (G5 vs. G4, $P = 0.042$) (Table 3). Two invasive transitional cell carcinomas were observed in DMBA/BBN-initiated/Diuron treated 2,500 ppm group (G3). Non-initiated/Diuron-treated 2,500 ppm mice developed only simple hyperplasia.

The rates of BrdU-labeling (BrdU-LI%) and apoptosis (AI%) in the mammary gland and bladder urothelium are shown in Fig. 4. In the mammary gland, the rates of cell proliferation and apoptosis did not differ among initiated or non-initiated groups after 13-weeks Diuron feeding. In contrast in the urinary bladder, Diuron feeding induced a significant increase in urothelial BrdU-LI%, but not in AI%, in both initiated (G2 and G3) and non-initiated groups (G4) when compared to the respective control groups (G1 and G5) ($P < 0.042$ and $P < 0.020$, respectively).

Table 3 Incidence (%) of mammary and urothelial preneoplastic and neoplastic lesions in the different experimental groups

Group/treatment	Number of mice	Mammary lesions		Urothelial lesions	
		DH/AH	ADENOCAR	SH/NH (%)	TCC
Initiated					
(G1) DMBA/BBN	14	6 (43%)	5 (36%)	8 (57)	0
(G2) DMBA/BBN + 1,250 ppm	11	5 (45%)	7 (64%)	11 (100)[a]*	0
(G3) DMBA/BBN + 2,500 ppm	11	3 (27%)	7 (64%)	11 (100)[a]*	2 (18%)
Non-initiated					
(G4) 2,500 ppm	15	0	0	5 (33)[b]**	0
(G5) Control	15	0	0	0 (0)	0

DMBA 7,12-dimethlybenz(a)anthracene (5 × 1.5 mg/mouse, i.g.); *BBN N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (8 × 7.5 mg/mouse, i.g.), 1,250 or 2,500 ppm of Diuron in the basal diet for 12 weeks; *DH/AH* ductal hyperplasia or alveolar hyperplasia; *ADENOCAR* adenocarcinoma; *SH/NH* simple hyperplasia or nodular hyperplasia; *TCC* transitional cell carcinoma

[a,b] Different from G1 or G5 groups, respectively, * $P < 0.02$, ** $P < 0.042$

Discussion

In the present study sub-chronic treatment with Diuron at high concentrations did not modify mammary carcinogenesis but enhanced tumorigenesis in the urinary bladder from DMBA/BBN-initiated female Swiss mice. These findings were obtained using dietary concentrations of Diuron known to have carcinogenic potential to urinary bladder in both genders of Wistar rats and NMRI mice and mammary gland of female NMRI mice in a long-term feeding study (Iyer 2002, U.S. EPA 2003).

Female NRMI mice exposed for 12 months to Diuron at 2,500 ppm developed a low incidence of mammary adenocarcinomas. The incidence of these neoplasms were 2/50, 1/47, 1/49 and 6/50 in the groups fed Diuron at 0, 25, 250, and 2,500 ppm, respectively (Iyer 2002). Therefore, the occurrence of mammary tumors in this murine model of exposure to Diuron may be considered "spontaneous" and no significant effects on the reproductive system have been described (U.S. EPA 2003). In the present study, treatment with Diuron at 2,500 ppm for 13-weeks did not alter the rates BrdU-labeling or apoptosis in the mammary gland neither had a promoting effect on mammary gland carcinogenesis in female Swiss mice initiated with DMBA. These findings are not conflicting with previous results in female NMR1 mice carcinogenic study since this later study was not conclusive and were based on a statistical trend of mammary tumors of spontaneous occurrence.

Some pesticides with potential endocrine disrupting properties have been categorized as mammary carcinogens in rodent studies (Ueda et al. 2005; Rudel et al. 2007). These pesticides, including some substituted urea herbicides, are able to block or inactivate the steroid hormone receptors and/or affect the levels of sex hormones with potential to affecting the development and function of the male and female reproductive system (Cook et al. 1993; Bauer et al. 1998; Vinggaard et al. 1999, 2000; Kojima et al. 2004; Noguerol et al. 2006). Findings from in vitro assays indicate that Diuron has the capacity to connect to the androgen receptor, thus allowing this herbicide to act as an endocrine disruptor, as the herbicide linuron, a related substituted urea compound (Cook et al. 1993; Bauer et al. 1998). Diuron did not affect CYP19 aromatase activity in the human placental microsomes assay, indicating that this herbicide did not interfere in the conversion of androgens to estrogens, which could alter the balance between male and female sex hormones (Vinggaard et al. 2000). In a recent study in California with breast cancer bearing women living in areas of recent and high agricultural pesticides use, Diuron was selected for individual analysis as a potential xenoestrogenic carcinogen (Reynolds et al. 2005). However, in vitro assays indicated that this herbicide did not appear to show estrogen receptor (ER)-mediated response using human MCF-7 breast cancer cells, Chinese hamster ovary cells or recombinant yeast strains (Vinggaard et al. 1999; Kojima et al. 2004; Noguerol et al. 2006). These in vitro results are in keeping with our findings on the absence of promoting effects of Diuron on mammary carcinogenesis, thus indicating that mammary gland is not a potential target organ for the carcinogenic effect of this herbicide.

In the urinary bladder, long-term treatment with Diuron at 2,500 ppm induced the development of papillomas and carcinomas in both male and female Wistar rats (Iyer 2002). The incidence of these tumors in the urinary bladder were 1/49, 0/50, 1/49 and 35/48 in male and 1/47, 0/49, 1/50 and 13/49 in female rats exposed to Diuron at concentrations of 0, 25, 250, and 2,500 ppm, respectively. Moreover, an increase in hyperplasia in the urinary bladder was observed in female NMRI mice exposed to Diuron at 2,500 ppm for 24 months (Iyer 2002). In the present study, treatment with Diuron at 2,500 ppm for 13-week resulted in a significant increase in the incidence of simple/nodular

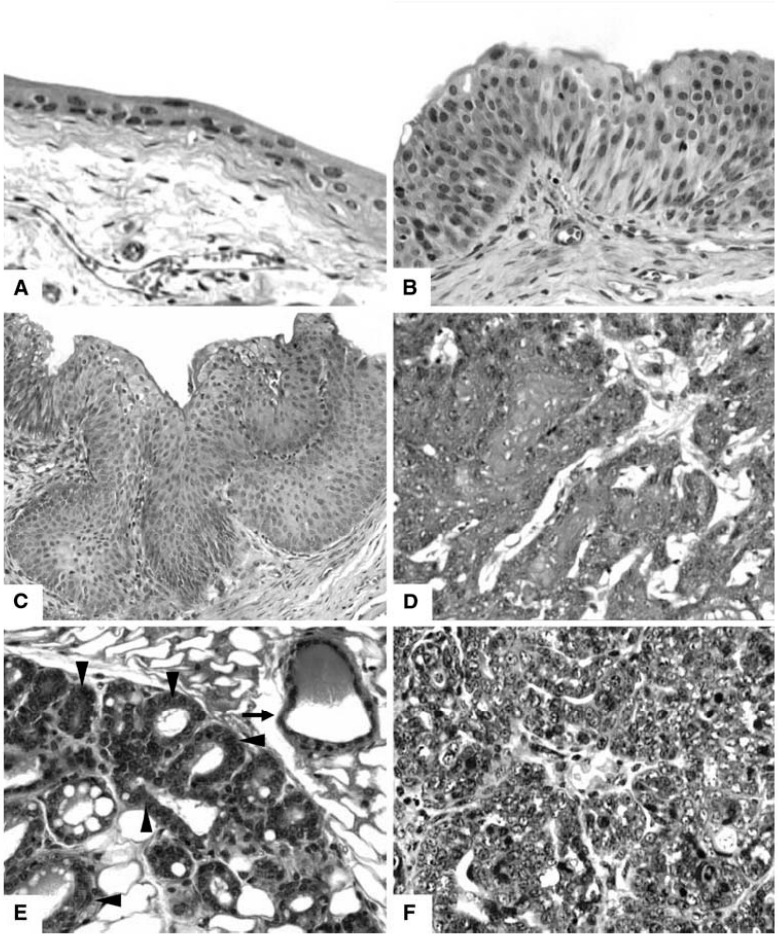

Fig. 3 Representative microscopic lesions in urinary bladder (**a-d**) and mammary gland (**e** and **f**) of DMBA/BBN-initiated or non-initiated mice groups. **a** Normal urothelium, non-initiated mouse (G5 group); **b** Simple hyperplasia, DMBA/BBN-initiated mouse (G1 group); **c** Nodular hyperplasia, DMBA/BBN-initiated/Diuron treated 1,250 ppm mouse (G2 group); **d** Transitional cell carcinoma, DMBA/BBN-initiated/Diuron-treated 2,500 ppm mouse; **e** Alveolar mammary hyperplasia, DMBA/BBN-initiated mouse (G1 group). Hyperplasic alveoli (*arrowheads*) and non-altered alveolus (*arrow*); **f** Mammary adenocarcinoma, DMBA/BBN-initiated/Diuron-treated 2,500 ppm mouse (G3 group)

hyperplasia in the urinary bladder from both DMBA/BBN-initiated or non-initiated groups. These lesions are a predisposing condition to urinary bladder carcinogenesis in rodents and considered as early precursors of papilloma and transitional invasive urothelial carcinomas (Cohen 1998, 2002).

Increased urothelial cell proliferation can occur by cytotoxicity and regeneration (Cohen 1998). Diuron feeding at high concentrations to male Wistar rats does not induce urothelial DNA damage, but increases cell necrosis and regenerative cell proliferation that leads to urothelial hyperplasia (Nascimento et al. 2006). Although some evidence of mutagenic potential have been observed in Swiss mice submitted to the bone marrow micronucleus assay and in the dominant lethal test, Diuron was negative in most in vitro and in vivo genetic toxicology assays (Liu 2002; Iyer 2002; U.S. EPA 2003). Thus, the most probable mode of carcinogenic action (MOA) of Diuron on the urinary bladder urothelium in male Wistar rats may be as a non-genotoxic pathway associated to persistent cytotoxicity and regenerative proliferation (Nascimento et al. 2006).

In rodent studies with genotoxic agents, such as 4-aminobiphenyl, 2-acethylaminofluorene and cyclophosphamide, an increased incidence of bladder tumors was detected only at doses at which there was a relationship between DNA damage and increased urothelial cell proliferation (Cohen and Ellwein 1990, Cohen et al. 2006; McCarroll et al. 2008). On the other hand, non-genotoxic agents can induce the development of hyperplasic lesions and bladder tumors through continuous stimulation of urothelial cell proliferation (Cohen 1998). In the present study, a significant increase in BrdU-labeling index was observed

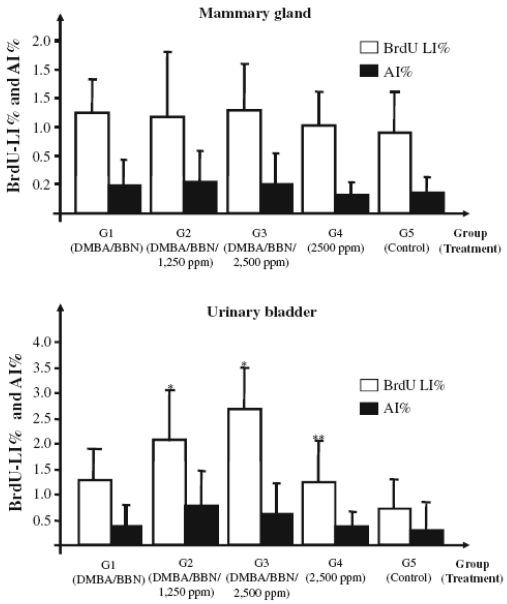

Fig. 4 Effects of Diuron on cell proliferation and apoptosis in the mammary gland and urinary bladder from initiated and non-initiated groups. BrdU LI% = bromodeoxyuridine-labeling indexes; AI (%) = Apoptosis indexes. *,** Significantly different from G1 or G5 groups, $P < 0.01$ and $P < 0.05$, respectively

in either non-initiated or initiated female Swiss mice fed Diuron at 2,500 ppm. Regenerative urothelial cell proliferation secondary to cytotoxicity can be produced by the presence of urinary solids such as precipitates, microcrystals, or calculi (Cohen 1998). In a previous study, magnesium ammonium phosphate crystals and urothelial cytotoxicity was detected by SEM in male Wistar rats exposed to Diuron 2,500 ppm, but the cause of urothelial necrosis was not completely determined (Nascimento et al. 2006). In this way, diffuse necrosis with consequent regenerative hyperplasia in the absence of a clear genotoxic potential could be considered as the MOA of Diuron at high doses on rodent urinary bladder tumorigenesis, as detected in standard feeding long-term bioassays (Iyer 2002).

Using a cytotoxic MOA as a paradigm for urinary bladder carcinogenesis, the sustained cytotoxicity and regenerative cell proliferation could be considered relevant for evaluating human cancer risk, if metabolic and oncogenic pathways are common to animal models and in humans (Cohen et al. 2004, 2006). This persistent urothelial cytotoxicity could be related to reactive metabolites in urine, generated during mouse hepatic Diuron *N*-demethylation (Abass et al. 2007), leading to development of hyperplasia and ultimately in tumors. As Diuron has been reported to be biotransformated by in vitro and in vivo human cytochrome (CYP) P450s (Abass et al. 2007), a possible cytotoxic MOA could be operative for humans exposed to larger amounts of Diuron and that present a high CYP1A2 content, which may be able to metabolize Diuron more efficiently (Abass et al. 2007).

3-4-dicloroaniline (3,4-DCA) is an intermediate in the industrial production of various substituted phenyl urea herbicides, including Diuron (Giacomazi and Cochet 2004). The 3,4-DCA is a major metabolite of Diuron frequently detected in urine from human exposed to substituted phenyl urea herbicides (Verheij et al. 1989; Van Boven et al. 1990; Nguyen et al. 2007). Valentovic et al. (1997) have described early toxic effects of 3,4-DCA on liver, kidney and urinary bladder of male F344 rats. Thus, urothelial toxicity by Diuron metabolites present in urine could contribute to urinary bladder carcinogenesis in non-initiated and initiated female Swiss mice.

In conclusion, the results of present study indicate that Diuron at high concentrations enhanced tumor development in the urinary bladder, but not in the mammary gland in a murine model of carcinogenesis. As there is no report of human tumors related to exposure to Diuron, the characterization of a carcinogenic mode of action of Diuron and its biological plausibility to exposed humans should continue to be investigated.

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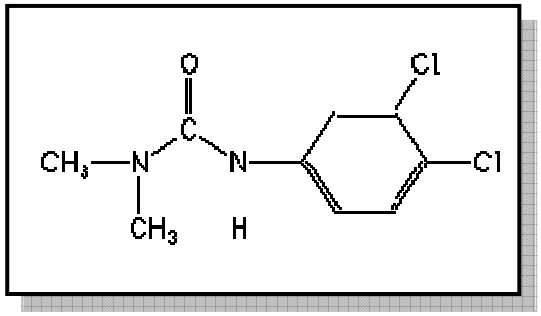

Figura 1

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CEEA Ética



Comissão de Ética em Experimentação Animal

CERTIFICADO

CERTIFICAMOS *que o Protocolo n.º* ***523*** *sobre o Projeto de Pesquisa* ***"Avaliação da carcinogenicidade do pesticida Diuron [3,(3,4-diclorofenil) 1,1 dimetil uréia] em modelo experimental de carcinogênese mamário"****, sob a responsabilidade do* ***Prof. Dr. Luis Fernando Barbisan****, com a colaboração de Tony Fernando Grassi, e do* ***Prof. Dr. João Lauro Viana de Camargo****, esta de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os* ***ratos*** *são provenientes de biotério convencional sem condições de emitir Atestado de Sanidade.*

Projeto de Pesquisa Aprovado em 22 de março de 2.006

Profª Drª Norma Sueli P. Modolo
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

ANEXO 4

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA
DEPARTAMENTO DE PATOLOGIA
FACULDADE DE MEDICINA

BOTUCATU, SP - RUBIÃO JÚNIOR - CEP 18618-970 PBX (014) 6802- 6238

Botucatu, 13 de janeiro de 2010

Ilma Srª
Regina Helena Garcia Martins
Presidente da Comissão de Ética em Experimentação Animal

Prezada Sra.:

Vimos por meio deste, solicitar alteração do título do Projeto de Pesquisa **"Avaliação da carcinogenicidade do pesticida Diuron [3, (3,4-diclorofenil) 1,1 dimetil uréia] em modelo experimental de carcinogênese mamária"** sob a responsabilidade do Prof. Dr. Luís Fernando Barbisan com a colaboração de Tony Fernando Grassi e do Prof. Dr. João Lauro Viana de Camargo correspondente ao **Certificado nº 523** aprovado em 22 de março de 2006 pela Comissão de Ética em Experimentação Animal – Universidade Estadual Paulista – Faculdade de Medicina de Botucatu – UNESP para o título **"Carcinogênese de mama em modelo experimental de exposição gestacional, juvenil e adulta ao herbicida Diuron [3(3,4-Diclorofenil) 1,1, dimetil uréia] em fêmeas Sprague-Dawley"**

Atenciosamente

Prof. Dr. Luís Fernando Barbisan
Orientador

Tony Fernando Grassi
Doutorando

Universidade Estadual de Campinas - UNICAMP
Centro Multidisciplinar para Investigação Biológica na
Área da Ciência em Animais de Laboratório – CEMIB
International Council For Laboratory Animal Science
ICLAS Network Member for Promotion of Animal Quality in Research
www.cemib.unicamp.br

Prof. Dr. Tony Fernando Grassi
Faculdade de Medicina de Botucatu – UNESP
Departamento de Patologia – TOXICAM
Botucatu - SP

Atestado de Saúde Animal

Atestamos que os ratos heterogenéticos da linhagem **NTacUnib:SD,** provenientes da Expansão de Matrizes 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. No atestado consta a 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: Março/2009.

Campinas, 11 de maio de 2009.

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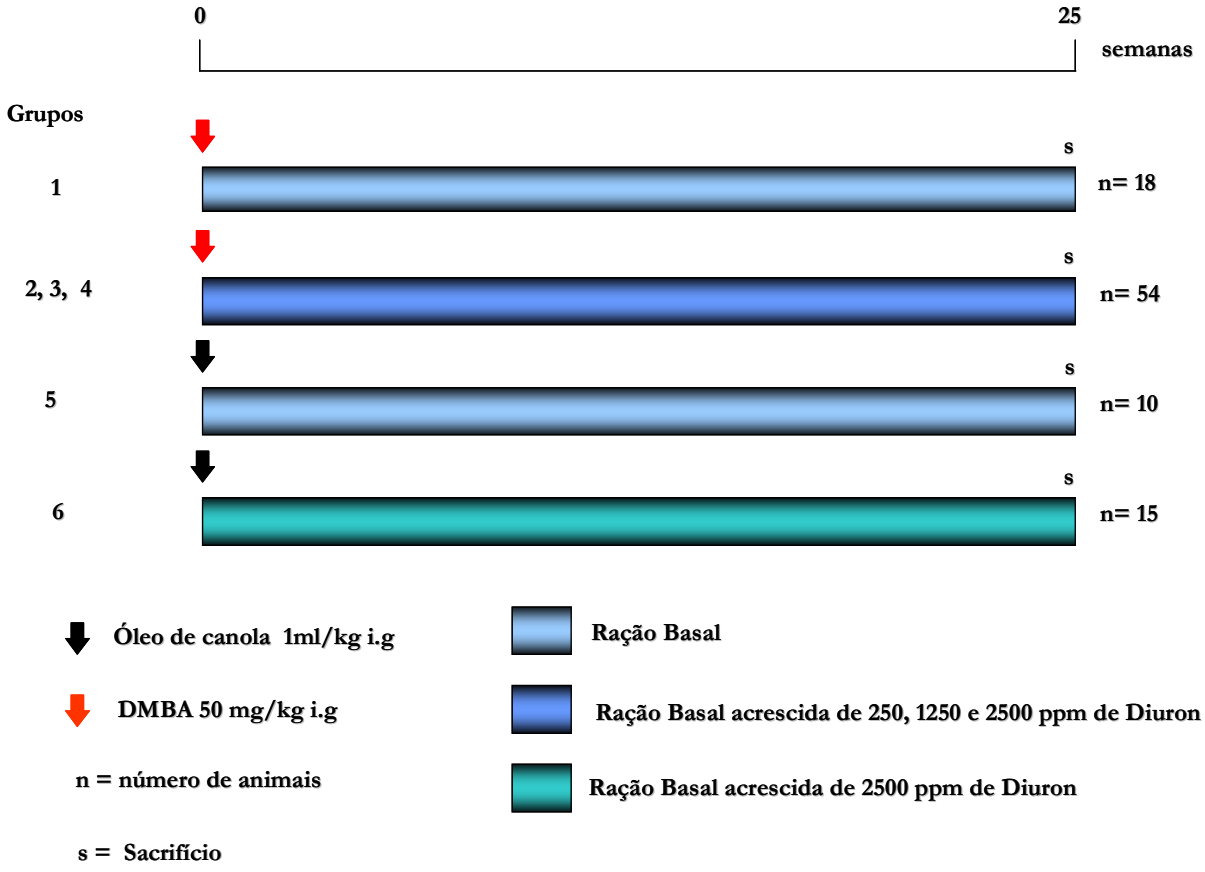
0
25
semanas
Grupos
1
s
n= 18
2, 3, 4
s
n= 54
5
s
n= 10
6
s
n= 15
Óleo de canola 1ml/kg i.g
DMBA 50 mg/kg i.g
n = número de animais
s = Sacrifício
Ração Basal
Ração Basal acrescida de 250, 1250 e 2500 ppm de Diuron
Ração Basal acrescida de 2500 ppm de Diuron

Figura 2

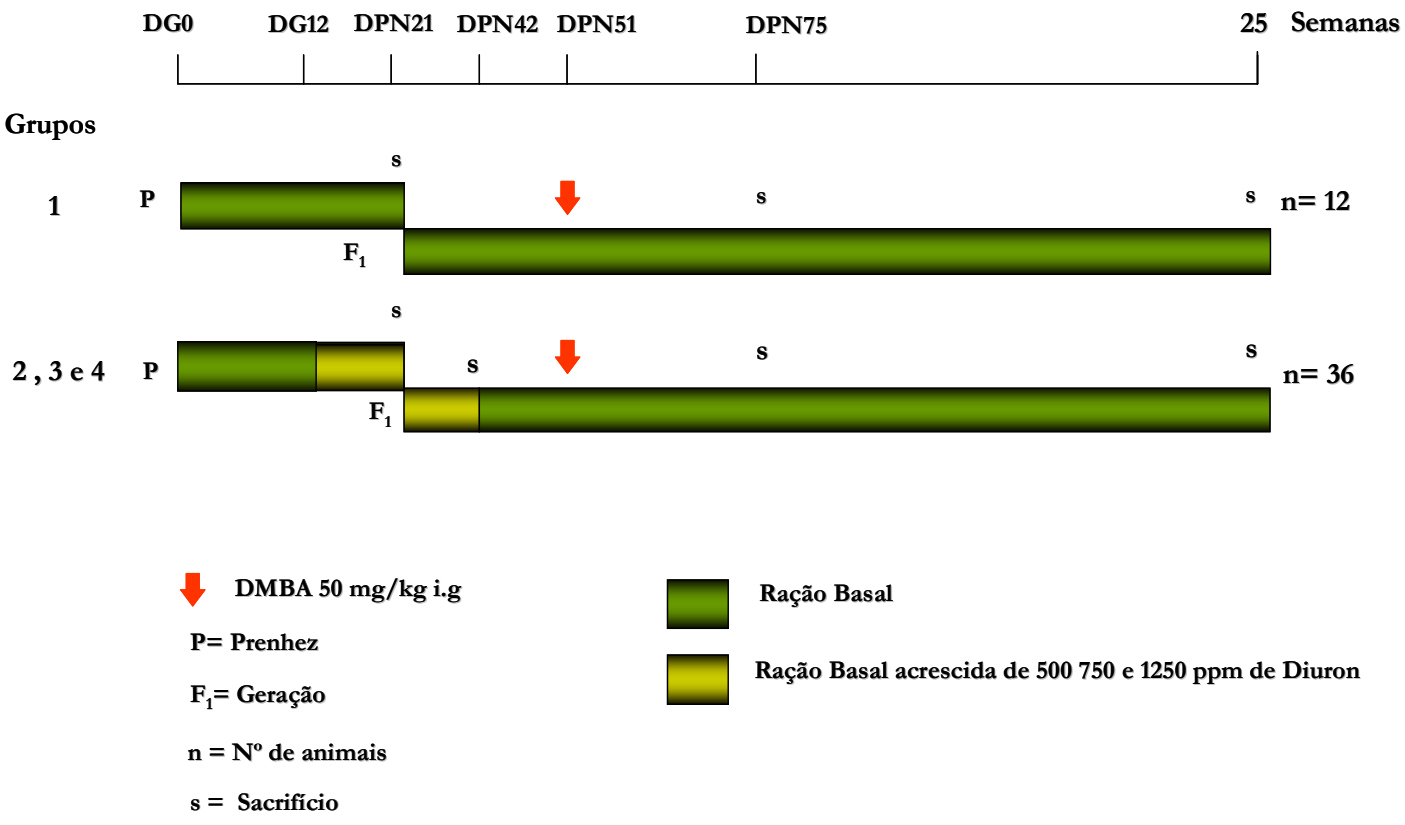
DG0
DG12
DPN21
DPN42
DPN51
DPN75
25 Semanas
Grupos
1
P
s
s
s
n= 12
F1
2 , 3 e 4
P
s
s
s
s
n= 36
F1
DMBA 50 mg/kg i.g
P= Prenhez
F1= Geração
n = Nº de animais
s = Sacrifício
Ração Basal
Ração Basal acrescida de 500 750 e 1250 ppm de Diuron

Figura 3

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