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Evaluation of early changes induced by diuron in the rat urinary bladder using different processing methods for scanning electron microscopy

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ABSTRACT

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a substituted urea herbicide carcinogenic to the rat urinary bladder at high dietary levels. The suggested non-genotoxic mode of action (MOA) of diuron encompasses cytotoxicity and necrosis followed by regenerative hyperplasia. Prenecrotic swollen cells as observed under scanning electron microscopy (SEM) have been reported as early morphological alterations, putatively related to diuron cytotoxicity. However, these changes were not observed in a previous SEM study conducted in this laboratory. This study evaluated whether these early alterations are actually due to diuron cytotoxicity or artifacts related to different processing methods used for SEM analysis. Male Wistar rats were fed *ad libitum* with basal diet, 7.1% sodium saccharin (NaS) or 2.500 ppm diuron for seven days or 15 weeks. The urinary bladders were processed for histological and labeling indices examinations and for SEM using two different processing methods. The incidence of simple hyperplasia after 15 weeks of exposure to diuron or to NaS was significantly increased. By SEM, the incidences and severity of lesions were significantly increased in the diuron group independently of exposure time. The different SEM processing methods used allowed for visualization of swollen superficial cells after seven days of diuron exposure. Probably the absence these cells in a previous study was due to the use very few animals. Our results support the hypothesis that the swollen cell is an early key event due to diuron-induced cytotoxicity and is the result of a degenerative process involved in the non-genotoxic carcinogenic mode of action of high doses of diuron.

1. Introduction

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a substituted urea herbicide widely used in several types of crop and non-crop areas (Oturan et al., 2008). In a 2-year bioassay, diuron was carcinogenic to the urinary bladder of Wistar rats at high dietary exposure levels (2500 ppm) (APVMA, 2005, 2011), and was classified by the U.S Environmental Protection Agency (USEPA) as a "known/likely human carcinogen" (USEPA, 1997).

In the rat urinary bladder, changes in urinary solids or pH have been shown not to be key events in diuron-induced carcinogenesis. The suggested non-genotoxic carcinogenic mode of action (MOA) of diuron at high doses involves metabolic activation to toxic metabolites, mainly *N*-(3,4-dichlorophenyl) urea (DCPU) and 4,5-dichloro-2-hydroxyphenyl urea (2-OH-DCPU) leading to cytotoxicity as evidenced by necrosis and exfoliation, followed successively by regenerative cell proliferation, urothelial hyperplasia, and eventually urinary bladder tumors (da Rocha et al., 2010, 2012, 2013, 2014).

Abbreviations: BrdU, bromodeoxyuridine; CPD, critical point drying; Diuron, [3-(3, 4-dichlorophenyl)-1,1-dimethylurea]; HMDS, hexamethyldisilazane; LI, labeling index; LM, light microscopy; MOA, mode of action; NaS, sodium saccharin; PNH, papillary and nodular hyperplasia; SEM, scanning electron microscopy; SH, simple hyperplasia.

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Scanning electron microscopy (SEM) is considered the most sensitive technique for the detection of early urothelial changes associated with the carcinogenic process in the rat urinary bladder (Cohen et al., 1990), and it has been used by our group to identify urothelial changes such as necrosis, exfoliation and regenerative hyperplasia associated with diuron exposure (Nascimento et al., 2006; da Rocha et al., 2010, 2014; Cardoso et al., 2013).

The correct interpretation of the structure and/or ultrastructure of normal and pathological tissues depend on good tissue morphologic preservation. Specimen preparation for SEM includes fixation, dehydration, drying, and sputter coating (Buravkov et al., 2011). Fixation is probably the most critical step in the preparation of biological specimens for SEM (Postek et al., 1980). Although glutaraldehyde is the most recommended fixative for SEM (Cohen et al., 2007; Falconi et al., 2007), in our studies Bouin's fixative has been used to allow concurrent evaluation using SEM, and histological and immunohistochemical analyses.

Following fixation, the biological samples generally are dehydrated with a graded ethanol series before drying. The most common procedure for drying biological specimens for SEM is critical point drying (CPD) using proper dryer equipment (Postek et al., 1980). Some authors have described an alternative chemical drying method using hexamethyldisilazane (HMDS) (Bray et al., 1993; Araújo et al., 2003), however, there are reports indicating that this substance can induce retraction artefacts in samples (Slízová et al., 2003). To complete processing for SEM examination, the biological materials are placed in a sputter coater and coated with a thin layer of gold.

Although the alterations induced by 2500 ppm diuron after 15 weeks of exposure or longer are well established (Nascimento et al., 2006; da Rocha et al., 2010, 2014; Cardoso et al., 2013), there was no conclusive evidence documenting the early morphological urothelial changes induced by short-term exposure to these dose of this herbicide. In a short-term study (1, 3 or 7 days), the urinary bladders of male Wistar rats fed 2500 ppm diuron showed by SEM swollen urothelial superficial cells after fixation with Bouin's fixative and drying with HMDS. Under transmission electron microscopy (TEM), these swollen cells presented degenerative alterations such as cytoplasmic and nuclear swelling that could lead to rupture, necrosis and exfoliation (da Rocha et al., 2012). This alteration was assumed to be an early key event of the carcinogenic mode of action by diuron in the urothelium (da Rocha et al., 2012, 2014). However, in a study conducted by our laboratory using the same dose of diuron for seven days, and in which a dose-response relationship in the number of transcriptionally-altered genes was observed, these swollen cells were not detected by SEM after Bouin's fixation and CPD processing (Ihlaseh-Catalano et al., 2014).

The present study was undertaken to elucidate if the swollen cells in the urothelium actually represent an early event of the MOA proposed for diuron, or if they are artifacts of the processing methods used for SEM. For that, we compared the effects of Bouin's and glutaraldehyde fixatives and CPD and HMDS drying procedures in the processing of urinary bladders of rats exposed to a carcinogenic dose of diuron.

2. Material and methods

2.1. Experimental outline

This study was approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, SP, Brazil, Protocol no. 928/2012. Eighty-four four-week old male Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, São Paulo, Brazil) and housed four per plastic cage with dry pine wood shaving bedding in a room with a targeted temperature of 21 ± 3 °C and relative humidity of 50 ± 20% and on a 12 h light/dark cycle.

The chemicals used were diuron (CAS no. 30-54-1; Sigma Chemical Co., St Louis, MO, 97% purity) and sodium saccharin (NaS) (CAS no. 82385-42-0; Sigma Chemical Co., St Louis, MO, ≥98% purity). Each of these chemicals was mixed into a powdered commercial diet (Nuvilab CR1; Nuvital, Colombo, PR, Brazil) at final concentrations of 7.1% NaS or 2500 ppm diuron by weight and pelleted. The concentration of 7.1% NaS was chosen since it was equimolar to the concentration used in previous experimental urinary bladder carcinogenesis studies (Cohen et al., 1990, 1995; Garland et al., 1993; Nascimento et al., 2006). After two weeks acclimation, the rats were randomized into three groups of 14 animals each: control (basal diet), 7.1% NaS (positive control) and 2500 ppm diuron and treated for seven days or 15 weeks. Water and food were provided *ad libitum*.

After seven days or 15 weeks of treatment, the animals were anesthetized with a mixture of ketamine (80 ml, 90 mg/kg) and xylazine (2%, 10–13 mg/kg) intraperitoneally (i.p.). One hour prior to anesthesia, bromodeoxyuridine (BrdU, Sigma Chemical Co., St Louis, MO, USA) was injected 120 mg/kg body weight intraperitoneally. Before euthanasia, the urinary bladder was exposed, injected with Bouin's fixative or 2.5% glutaraldehyde fixative *in situ*, rapidly removed, and immersed in the same fixative. Immediately after, the animals were euthanized under anesthesia by opening the abdominal cavity and sectioning the inferior vena cava. Urinary bladder samples remained in Bouin's fixative for 4 h or glutaraldehyde for 3 h. After fixation the urinary bladder was cut mid-sagittally, one half was processed for SEM and the other half for light microscopy (LM). Bladders fixed in Bouin's fixative were rinsed in 70% ethanol before further processing; those fixed in glutaraldehyde remained in this fixative until processing.

The kidneys, liver and spleen were also removed, weighed and immersed in 10% buffered formalin. A section of duodenum was collected and placed together with the urinary bladder in Bouin's fixative or glutaraldehyde fixative to be used as a positive control for BrdU immunohistochemistry.

2.2. Histological analysis

Following fixation with either fixative, one half of the urinary bladder from each animal was cut longitudinally into four strips. A section of duodenum was processed for paraffin embedding in the same cassette as the urinary bladder tissue. Hematoxylin and eosin (H&E) stained sections were used for histopathological examination and unstained sections were used for immunohistochemical staining of BrdU.

The urinary bladder sections were analyzed using a conventional optical microscope (Olympus Optical Co., Ltd., Japan) for the occurrence of proliferative lesions, particularly simple hyperplasia (SH) and papillary and nodular hyperplasia (PNH) according to Cohen's criteria (1983).

2.3. Evaluation of cell proliferation

For evaluation of cell proliferation, unstained slides of paraffin-embedded sections were deparaffinized and rehydrated. Pretreatment of tissue sections was performed using 2 N HCl followed by 0.005% trypsin, both at 37 °C, and incubation in BLOXALL™ (Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution – Vector Laboratories, Inc., Burlingame, CA, USA). The sections were incubated for two hours at room temperature with the primary antibody (mouse monoclonal anti-BrdU, Becton Dickinson, San Jose, CA, USA) at a dilution of 1:200 followed by incubation with the LSAB + System-HRP kit (Dako North America, Inc., Carpinteria, CA, USA) was used as specified by the