UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA

CAMPOS DE BOTUCATU

INFLUÊNCIA DA FIXAÇÃO E DE MÉTODOS DE SECAGEM NA DETECÇÃO DE ALTERAÇÕES PROLIFERATIVAS ULTRAESTRUTURAIS INDUZIDAS PELO DIURON NO UROTÉLIO VESICAL DE RATOS WISTAR

RAFAELA MARONO FAVA

Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para a obtenção do título de Mestre em Patologia

Botucatu – SP
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Dedicatória

Á todos que passaram por minha vida e me ensinaram como ser uma pessoa melhor, a convivência com o outro é de fato nosso maior presente.

"Cada pessoa que passa em nossa vida, passa sozinha, é porque cada pessoa é única e nenhuma substitui a outra! Cada pessoa que passa em nossa vida passa sozinha e não nos deixa só porque deixa um pouco de si e leva um pouquinho de nós. Essa é a mais bela responsabilidade da vida e a prova de que as pessoas não se encontram por acaso"

(Charles Chaplin)

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Sumário

SUMÁRIO

Revisão de literatura

1. REVISÃO DE LITERATURA

1.1. Câncer de bexiga

O câncer de bexiga é uma doença de alta incidência na população, representando o terceiro tipo de tumor sólido mais comum em homens e o quinto em mulheres (DeGraff et al., 2012). Seu aparecimento está relacionado a fatores ambientais, ocupacionais e ao estilo de vida; o tabagismo e o crescimento industrial contribuem para o desenvolvimento dessa neoplasia (Clavel et al.,2007; Crawford, 2008).

Os modelos experimentais de carcinogênese urotelial *in vitro* ou *in vivo* podem auxiliar na identificação de fatores de risco para o câncer de bexiga (Oliveira et al., 2006; Cohen et al., 2007) e também de marcadores relacionados ao desenvolvimento da doença (De Graff et al, 2011). Por isso, podem contribuir para a redução de sua incidência e definição de seu prognóstico.

1.2. Lesões induzidas quimicamente no urotélio de roedores

A sacarina sódica (NaS) em altas concentrações na ração induz resposta proliferativa da mucosa da bexiga de ratos, levando à hiperplasia urotelial em poucas semanas de exposição e posteriormente, se administrada por longos períodos, a tumores (Cohen et al., 1990). O modo de ação (MoA) da NaS ocorre pela indução de necrose da camada celular superficial da mucosa , onde a abrasão pelos precipitados amorfos da urina, compostos principalmente por fosfato de cálcio, provocam esfoliação, claramente visualizada pela microscopia eletrônica de varredura (MEV) (Cohen et al., 1990; 1996). Por atuar como indutora de hiperplasia simples no urotélio, a NaS pode ser utilizada como controle positivo de lesões em modelos experimentais (Cohen et al., 1990; Cohen et al., 1995; de Oliveira, 1998; Nascimento et al., 2006).

O diuron é um herbicida derivado da ureia, amplamente empregado em diversos tipos de culturas como cana de açúcar, algodão, café, frutas cítricas, uva, cacau, alfafa, aspargos, entre outras (Roque

& Melo, 2000; APVMA, 2005; USEPA, 2003). É um composto pouco solúvel em água, porém altamente solúvel em solventes orgânicos sendo facilmente absorvido por vias respiratórias e pelo trato gastrintestinal (Larini, 1999). Estima-se que no ano de 2011, o Brasil tenha utilizado 9.245 toneladas deste herbicida (comunicação pessoal, Dupont Agrícola Brasil, apud da Rocha et al., 2013).

Evidências experimentais indicam que o diuron é cancerígeno para a bexiga urinária de ratos. Após dois anos de exposição contínua via oral, pela ração, à concentração de 2.500ppm, ratos Wistar de ambos os sexos apresentaram aumento da incidência de papilomas e carcinomas uroteliais na bexiga e pelve renal (USEPA, 1997; Iyer, 2002). Consequentemente, o diuron foi classificado pela U.S. Environmental Protection Agency como "provável cancerígeno para a espécie humana" (USEPA, 1997).

Estudos realizados por nosso grupo mostraram que a concentração de 2.500ppm de diuron administrada pela ração aumenta a incidência de hiperplasia urotelial simples (HS) e o índice de proliferação celular a partir de 15 semanas de exposição, indicando que esta concentração na ração pode provocar o aparecimento de lesões potencialmente neoplásicas no urotélio de ratos (Nascimento et al., 2006; da Rocha et al.,2010; Ihlaseh et al., 2011; Cardoso et al.,2013).

Por ser um composto não genotóxico (Nascimento et al., 2006), seu MoA cancerígeno ocorre por citotoxicidade de seus metabólitos sobre as células superficiais da bexiga levando a necrose, esfoliação e hiperplasia regenerativa (da Rocha et al., 2010). O aparecimento dessas lesões uroteliais obedece a um perfil de dose-resposta (Cardoso et al., 2013).

A ação citotóxica do diuron sobre o urotélio acontece de maneira precoce. À MEV, bexigas de ratos Wistar machos expostos ao diuron 2.500ppm via oral durante 1, 3 ou 7 dias, fixadas com Bouin e secas com hexametildizilasana (HMDS), mostrou células uroteliais com aspecto edemaciado (*swollen cells)*. À microscopia eletrônica de transmissão (MET) nestas células, no sétimo dia, mostrou alterações degenerativas caracterizadas pela distensão nuclear, citoplasmática e de organelas, às vezes associadas à ruptura e morte celular. Além disso, a reação de imunoistoquímica para a

detecção de caspase 3 realizada nestes animais não mostrou alteração na taxa de apoptose. Sugerindo-se então, que o diuron induz citotoxicidade em momentos precoces, com degeneração celular progredindo para necrose, esfoliação e consequente proliferação celular regenerativa (da Rocha et al., 2012).

No entanto, Ihlaseh (2011) verificou que a bexiga de ratos submetidos à mesma concentração de 2.500 ppm de diuron e período de exposição de sete dias, porém, fixadas com Bouin e secas com o método tradicional em aparelhos como Balzers, não apresentaram células edemaciadas, indicando que diferentes métodos de processamento utilizados para MEV poderiam influenciar o aparecimento dessas células (figuras A e B).

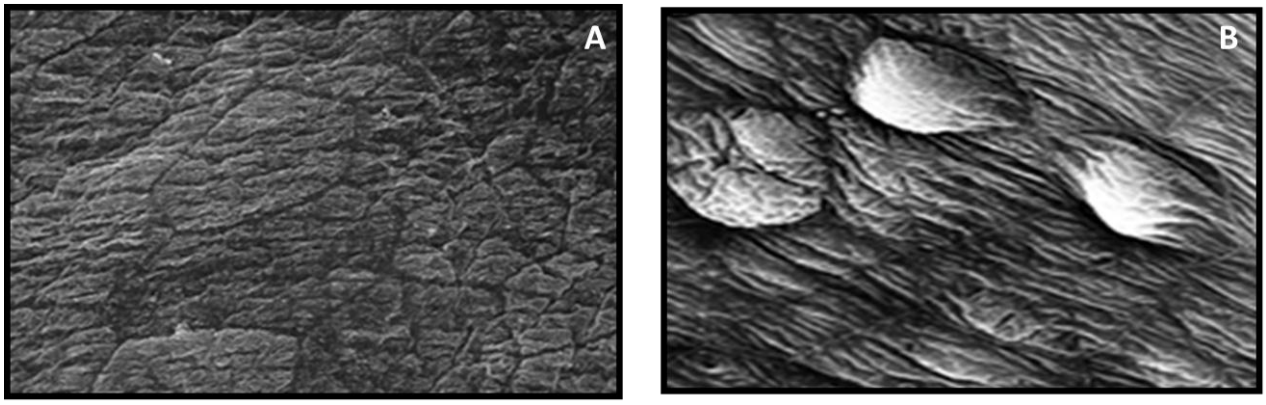

Figura A: Urotélio normal- ratos Wistar- 2.500ppm diuron /sete dias (Ihlaseh,2011)
Figura B: Urotélio com células edemaciadas – ratos Wistar – 2.500ppm diuron/ sete dias (da Rocha et al., 2012)

1.3. Microscopia Eletrônica de Varredura

A microscopia eletrônica de varredura (MEV) possibilita a ampliação da imagem dos tecidos e visão em três dimensões de estruturas superficiais (Flegler et al., 1993; Glueckert et al., 2005) e juntamente com a microscopia de luz e o índice de proliferação celular tornou-se ferramenta útil e sensível para a detecção de lesões uroteliais precoces relacionadas com a carcinogênese de bexiga (Cohen et al., 2007). No estudo de lesões uroteliais a MEV permite a detecção e identificação de cristais, precipitados, microvilos, focos de necrose, exfoliação e acúmulo de células arredondadas,

em geral interpretadas como hiperplásicas (Cano et al., 1993; Nascimento et al., 2006; Cohen et al,1990, 2007; da Rocha et al, 2010; Cardoso et al.,2013).

A identificação de lesões uroteliais relacionadas ao processo cancerígeno pode ser utilizada em estudos toxicológicos para caracterização de doses críticas, como nível de efeito adverso não observado (NOAEL) e menor nível com efeitos adverso (LOAEL). Por isso, é importante discernir lesões "reais" daquelas que são artefatos de procedimentos (Cohen et al., 2007), para que esses níveis críticos e os eventos chaves do modo de ação das substâncias testadas sejam consistentes e possam elucidar o real impacto dessas substâncias sobre o órgão alvo em estudo. Para que os resultados desses estudos não sejam comprometidos por falhas metodológicas é indispensável a escolha de um processamento adequado que preserve a integridade das amostras e possibilite a realização da técnica escolhida (Cohen et al.,2007) .

O processamento de amostras para MEV inclui fixação, desidratação, secagem e metalização (Buravkov, et al., 2011). Estes procedimentos, muitas vezes, podem induzir artefatos e perda da integridade morfológica dos tecidos (Postek et al., 1980).

1.4.Fixação

O objetivo da fixação é manter a estrutura morfológica e química do tecido (Falconi et al., 2007) evitando alterações e distorções durante os procedimentos subsequentes (Postek et al., 1980) e o ataque microbiano (EMBRAPA, 1998). A interpretação da estrutura e/ou ultraestrutura de tecidos normais ou patológicos depende da preservação das amostras (Falconi et al., 2007), por isso, quanto mais próximo do estado natural e funcional as células permanecerem, melhor será a análise e os resultados obtidos (EMBRAPA, 1998).

A fixação química pode ser realizada com fixadores coagulativos como formol-acético, ácido fórmico e Bouin ou não coagulativos como glutaraldeído, paraformoldeído e tetróxido de ósmio (Postek et al., 1980).

Apesar de os fixadores coagulativos serem amplamente utilizados no processamento para microscopia de luz, suas propriedades podem afetar a morfologia do tecido pela coagulação dos componentes celulares, especialmente das proteínas, sendo muitas vezes inadequados para microscopia eletrônica (Postek et al., 1980; Bignold, 2002).

Entre os fixadores empregados na microscopia eletrônica, o glutaraldeído é o mais recomendado (EMBRAPA, 2002; Cohen et al.,2007; Falconi et al., 2007). Seu uso seguido por pós-fixação em tetróxido de ósmio proporciona boa preservação da organização celular no tecido (Falconi et al., 2007). Entretanto, por alterar a estrutura terciária dos antígenos e dificultar a ligação antígeno-anticorpo (Bozzola & Russell, 1992), sua utilização não é indicada em estudos imunoistoquímicos (Cohen et al., 2007)

Em nossos estudos, a bexiga é fixada pela injeção intraluminal de Bouin, cortada sagitalmente ao meio e processada para microscopia de luz e MEV. O uso do fixador de Bouin permite a realização concomitante de histologia, imunoistoquímica e MEV (Bozzola & Russell, 1992; Cohen et al., 2007). Em amostras fixadas com Bouin e secas com HMDS, da Rocha et al. (2012), detectaram células edemaciadas na superfície urotelial nunca antes visualizadas em nossos experimentos, sugerindo que o uso do Bouin em combinação com método de secagem em aparelho Balzers poderia mascarar a detecção de alterações uroteliais precoces, causar artefatos e diminuir a nitidez da visualização do epitélio.

1.5.Métodos de secagem

Os materiais biológicos apresentam uma condição natural hidratada, o que implica em certa complexidade em seu processamento para MEV. Diferentemente de objetos rígidos, como sementes vegetais, que podem ser observados à MEV com um mínimo de preparação, os tecidos não rígidos necessitam em sua maioria passar por diversas etapas em seu processamento (Silveira, 1989).

Na MEV as amostras são analisadas a vácuo, por isso, é necessário que estejam totalmente "secas". Embora existam muitos métodos de secagem do material, o Ponto Crítico de Secagem (PCS) realizado em aparelhos como o Balzers CPD-020 é o mais utilizado (Postek et al.,1980). Neste método, após a fixação, o material é colocado em câmara selada a temperatura de aproximadamente 42°C e elevada pressão que resulta em sua total desidratação (EMBRAPA, 2002). Apesar do uso do PCS realizado em aparelho Balzers não induzir artefatos e ser considerado um método seguro para a secagem de materiais para MEV, sua utilização tem como desvantagens maior complexidade e alto custo.

Uma alternativa frequentemente utilizada no processamento de materiais para MEV é a secagem química com hexametildisilazana (HMDS) (Bray, 2000; Araújo et al., 2003), além de não ocasionar tensão superficial das amostras (Buravkov et al.,2011) o uso dessa substância é considerado um método confiável para a secagem de materiais no processamento para MEV (Bray et al., 1993; Araújo et al., 2003; Buravkov et al.,2011), tendo como vantagens o baixo custo, a rapidez e praticidade (Bray et al., 1993).

Devido a tensão superficial que exercem no tecido durante a evaporação, a utilização de alguns métodos químicos para secagem de materiais no processamento para MEV pode ocasionar deformação irreversível das estruturas teciduais (Bozzola & Russell, 1992; Buravkov et al.,2011) Mesmo sendo uma alternativa frequentemente utilizada pelos pesquisadores, a presença de artefatos pelo uso do HMDS já foi observada em alguns estudos (Slízová et al.,2003)

Em estudos prévios, a fixação com Bouin seguida por processamento com PCS possibilitou a detecção de necrose, exfoliação e hiperplasia no urotélio de animais expostos ao diuron por mais de 15 semanas (Nascimento et al., 2006; da Rocha et al, 2010; Cardoso et al.,2013) Entretanto, em estudo de sete dias, a detecção de determinadas alterações uroteliais precoces (swollen cells) só foram descritas em amostras fixadas com Bouin e processadas com HMDS (da Rocha et al.,2012).

Assim, o presente estudo verificou qual o melhor método de processamento das bexigas para detecção à MEV de alterações precoces (swollen cells) induzidas por substâncias químicas na superfície urotelial

2. REFERÊNCIAS

Araújo, J.C., Téran, F.C., Oliveira, R.A., Nour, E.A., Montenegro, M.A., Campos, J.R., Vazoller, R.F. (2003) Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. *J Electron Microsc* **52**, 429–433

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

Bignold, L.P. (2002).Hypothesis for the influence of fixatives on the chromatin patterns of interphase nuclei, based on shrinkage and retraction of nuclear and perinuclear structures. *Br J Biomed Sci* **59**, 105-113

Bozzola, J. J., Russell, D.L. Electron microscopy: Principles and techniques for biologists. 1ed. Local: Jones and Bartlett Publihers, 1992, p.41 -63 , 234-251.

Bray, D.F., Bagu, J., Koegler, P. (1993). Comparison of hexamethyldisilazane (HMDS), Peldri ll, and critical-point drying methods for scanning electron microscopy of biological specimens.*Microsc Res Tech* **26**, 489-495

Bray, D.Critical Point Drying of Biological Specimens for Scanning Electron Microscopy. Supercritical Fluid Methods and Protocols Methods In Biotechnology ™ V. 13, 2000, 00. 235, 243

Buravkov, S.V., Chernikov, V.P., Buravkova, L.B. (2011) Simple Method of Specimen Preparation for Scanning Electron Microscopy. *Bull Exp Biol Med* **151**,378-382

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

Cano, M., Suzuki, T., Cohen, S.M. (1993).Application of scanning electron microscopy and X-ray analysis to urinary tract cancer in animals and humans.*Scanning Microsc* **7**, 363-370

Cohen, S.M., Fisher, M.J., Sakata, T. (1990). Comparative analysis of the proliferative response of the rat urinary bladder to sodium saccharin by light and scanning electron microscopy and autoradiography.*Scanning Microsc* **4**, 135-142

Cohen, S.M., Garland, E.M., Cano, M.,St John, M.K., Khachab, M., Wehner, J.M., Arnold, L.L. (1995). Effects of sodium ascorbate, sodium saccharin and ammonium chloride on male rat urinary bladder. *Carcinogenesis* **16**, 2743-2750.

Cohen, S.M., Cano, M., Anderson, T., Garland, E.M. (1996). Extensive handing of rats leads to mild urinary bladder hyperplasia. *Toxicol Pathol* **24**, 251-257

Cohen, S.M., Ohnishi, T., Clark, N.M., He, J., Arnold, L.L. (2007). Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. *Toxicol Pathol* **35**, 337-347

Clavel, J. (2007) Progress in the epidemiological understanding of gene-environment interactions in major diseases: cancer. *C R Biol* **330**, 306-317

Crawford, J.M. (2008) The origins of bladder cancer. *Lab Invest* **88**, 686-693

Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). (1998). Técnicas microscópicas aplicadas na identificação e localização de bactérias fixadoras de nitrogênio e biomacromoléculas em tecidos vegetais

Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). (2002). Processamento de Amostras para Microscopia Eletrônica de Varredura

Falconi, M., Teti, G., Zago, M., Pelotti, S., Gobbi, P., Breschi, L.,Mazzotti, G. (2007) Effect of fixative on chromatin structure and DNA detection. Microsc Res and Tech **70**, 599-606

Flegler, S.L., Heckman, J.W. Jr., Klomparens, K.L.: Scanning and transmission electron microscopy- an introduction. W.H Freeman & Company, New York (1993) 1-225

Glueckert, R., Pfaller, K., Kinnefors, A., Schrott-Fischer, A., Rask-Andersen, H. (2005) High resolution scanning electron microscopy of the human organ of Corti. A study using freshly fixed surgical specimens. *Hear Res* **199**, 40-56

DeGraff, D.J., Cates, J.M., Mauney, J.R., Clark, P.E., Matusik, R.J., Adam, R.M. (2011) when urothelial differentiation pathways go wrong: Implications for bladder cancer development and progression. *Urol Oncol* **31** 802-811

DeGraff, D.J., Robinson, V.L., Shaah, J.B., Brandt, W.D., Sonpavde, G., Kang, Y., Liebert, M., Wu, X.R., Taylor, J.A. (2012)Current preclinical models for the advancement of translational bladder cancer research. Mol Cancer Ther **12**, 121-130

Ihlaseh, S.M. Estudo dose-resposta e perfil de expressão gênica do herbicida diuron [3,(3,4-diclofenil)-1,1-dimetiluréia] em bexiga urinária de ratos Wistar machos. 2011. 110 folhas. Tese (Doutorado em Patologia) – Universidade Estadual Paulista Júlio de Mesquita Filho. Botucatu, 2011

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

Iyer, P. (2002). Evidence on the development and Reproductive Toxicity of Diuron. Draft. Reproductive and Cancer Hazard Assessment Section. Office of Environmental Health Hazard Assessment, California Environmental Protection Agency,pp. 43.(ok ana)

Larini, L. (Ed.). *Toxicologia dos praguicidas*. Local: São Paulo: Manole, 1999. 230p.

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

de Oliveira, M. L. C. S. (1998). Efeitos de sais de sódio, potássio e cálcio sobre a composição da urina sobre o epitélio da bexiga de ratos machos F344. Faculdade de Medicina, Universidade Estadual Paulista, Botucatu, tese (Doutorado)

Oliveira, P.A, Colaço, A., De la Cruz P, L.F., Lopes, C. (2006) Experimental bladder carcinogenesis rodent models. *Exp Oncol* **28**, 2-11

Postek, T.M., Howard, K.S., Johnson, A.H., M^{c}Michael, K.L. Scanning electron microscopy: A student´s handbook. 1ed. Local: Ladd Research Industries, 1980, p.115-238.

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

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

da Rocha, M.S., Arnold, L.L., Dodmane, P.R., Pennington, K.L., Qiu, F., de Camargo, J.L., Cohen, S.M. (2013). Diuron matabolites and urothelial cytotoxicity: In vivo, in vitro and molecular approaches. *Toxicology* **314**, 238-246

Roque M. R. A., Melo, I.S. (2000). Isolamento e caracterização de bactérias degradadoras do herbicida diuron. *Scientia Agricola* **57**, 723-72

Silveira, M. (1989). Preparação de amostras biológicas para microscopia eletrônica de varredura. *Manual sobre técnicas básicas em microscopia eletrônica.* São Paulo: USP.**1**, 71-79

Slízová, D., Krs,O., Pospísilová, B. (2003) Alternative method of rapid drying vascular specimens for Scanning Electron Microscopy. *J EndovascTher* **10**, 285-287

USEPA (1997). Carcinogenicity peer review of diuron: Office of Prevention, Pesticides and Toxic substances. [Memorandum]. Cincinnati, 22p

USEPA (2003) U.S. Environmental Protection Agency, 2003.Registration Eligibility Decision for Diuron. List A. Case 0046. Office of Prevention, Pesticides and Toxic Substances.USEPA, Washington, DC

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Manuscrito

INFLUENCE OF FIXATION AND DRYING PROCESSING METHODS TO SCANNING ELECTRON MICROSCOPY IN THE DETECTION OF ULTRASTRUCTURAL ALTERATIONS CHEMICALLY INDUCED BY DIURON ON THE URINARY BLADDER OF MALE WISTAR RAT

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3.1. ABSTRACT

Diuron is a substituted urea herbicide, carcinogenic to the rat urinary bladder at high dietary levels (2500 ppm). Different scanning electron microscopy (SEM) processing methods resulted in differing observations of early urothelial changes induced by exposure to this herbicide. This study evaluated the influence of SEM processing methods on the detection of urothelial alterations induced by diuron. Male Wistar rats were divided into 3 groups: control (basal diet), 7.1% Sodium Saccharin (NaS) (positive control) and 2500 ppm diuron, and fed for 7 days or 15 weeks. Urinary bladders were fixed with Bouin's or glutaraldehyde fixative and processed by critical point drying (CPD) or by hexamethyldisilazane (HMDS) for comparison of the cytotoxic and proliferative changes induced by diuron in the urothelium. Histological and cell proliferation evaluations were also performed. After seven days, no differences in the incidence of histological urothelial lesions or labeling indices were detected among the groups; however, the incidence of urothelial lesions after 15 weeks was significantly increased in the animals fed diuron or NaS. After seven days or 15 weeks, the severity of urothelial alterations was significantly higher in animals fed with diuron. Both fixative and drying methods allowed for the identification of prenecrotic swollen superficial cells in the urothelium after seven days exposure to 2500 ppm diuron. Our results confirmed that the presence of prenecrotic swollen cells in the urothelium is an early key event due to diuron cytotoxicity and is not an artifact related to the processing methods used.

Key word: diuron, scanning electron microscopy, swollen cells, urinary bladder, SEM processing

3.2. INTRODUCTION

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). This method provides a wide 3-D view of tissue structures, high resolution and amplification of the urothelial surface (Cano et al., 1993). Together with light microscopy and labeling indices, SEM plays an important role in the identification, localization and classification of cytotoxic and proliferative changes in the urinary bladder (Cohen et al., 2007)

Specimen preparation for SEM includes fixation, dehydration, drying, and sputter coating (Buravkov, et al., 2011). The fixative used is important for preserving the characteristics and morphological changes of the samples and for correct interpretation of the structure and/or ultrastructure of normal and pathological tissues (Falconi et al., 2007).

There are several methods for drying biological materials for SEM examination; the most common procedure is critical point drying (CPD) using the instrument such as Balzers (Postek et al., 1980). Some authors have also described the use of hexamethyldisilazane (HMDS) as an alternative chemical drying method (Bray et al., 1993; Araújo et al., 2003). However, there are reports that HMDS can induce artefacts of retraction in the samples (Slízová et al.,2003).

SEM has been used by our group to identify urothelial proliferative changes such as necrosis, exfoliation and regenerative hyperplasia induced by oral exposure of Wistar rats to 2500 ppm diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] for 15 weeks or longer (Nascimento et al, 2006, da Rocha et al 2010, Cardoso et al., 2013). Diuron is an herbicide widely used in several types of crop and non-crop areas (Outran et al., 2008). In a two-year bioassay, diuron was carcinogenic to the urinary bladder of rats at high dietary exposure levels (2500 ppm) (APVMA, 2005, 2011), and for this reason, diuron was classified by the U.S Environmental Protection Agency (USEPA) as a “known/likely human carcinogen” (USEPA, 1997).

Diuron is a non-genotoxic compound (Nascimento et al., 2006). Its suggested mode of action (MoA) for rat urothelial carcinogenesis encompasses cytotoxicity, as evidenced by prenecrotic swollen superficial cells, necrosis and exfoliation, followed successively by regenerative cell proliferation, urothelial hyperplasia and ultimately urinary bladder tumors (da Rocha et al., 2010; 2012; 2013).

In a short-term study (1, 3 or 7 days), male Wistar rats fed 2500 ppm diuron showed by SEM, swollen superficial cells as the first key event for the carcinogenic mode of action (MoA) of diuron in urinary bladders fixed with Bouin's fixative and dried with HMDS. Transmission electron microscopy (TEM) revealed that these swollen cells had degenerative alterations such as cytoplasmic and nuclear swelling that could lead to rupture, necrosis and exfoliation (da Rocha et al., 2012). However, after a seven day exposure period in another study using the same dose of diuron and, in which Bouin's fixative and CPD processing were used prior to SEM examination, these alterations were not detected (Ihlaseh, 2011). Initially, it was thought that the presence of swollen superficial cells in the urinary bladder could be related to the different processing methods for SEM.

The present study was undertaken to identify the more sensitive processing method for SEM in the detection of early changes in the urothelium of rats exposed to diuron; for that, we compared Bouin's and glutaraldehyde fixatives, and CPD using the Balzers instrument and HMDS drying procedures in the processing of urinary

3.3. MATERIAL AND METHODS

3.3.1. 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 shaving wood bedding in a room with a targeted temperature of 21 ± 3◦C and relative humidity of 50 ± 20% and on a 12h light/dark schedule, with the light changing at 0700 and 1900h

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). The chemicals were mixed into a powdered commercial diet (Nuvilab CR1; Nuvital, Colombo, PR, Brazil) at final concentrations of 7.1% NaS and 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; de Oliveira, 1998, Nascimento et al., 2006). After two weeks acclimation, the rats were randomized and allocated to two experiments of seven days and 15 weeks with three groups of 14 animals each: Control (Basal diet), 7.1% NaS (positive control) and 2500 ppm diuron. Water and food were provided *ad libitum*.

3.3.2. Urinary bladder, duodenum, kidney, liver and spleen removal

After treatment for seven days or 15 weeks, animals were anesthetized with a mixture of ketamine (80ml, 90 mg/Kg) and xylazine (2%, 10 to 13 mg/kg) i.p. One hour prior to anesthesia, bromodeoxyuridine (120 mg/kg body weight) (BrdU, Sigma Chemical Co., St Louis, MO, USA) was injected intraperitoneally (i.p.). Before euthanasia, the urinary bladder was exposed, injected with Bouin's 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. For histology, the bladder samples remained in Bouin's fixative for 4h or glutaraldehyde for 3h; for SEM examination, the bladders fixed in Bouin's fixative were washed in 70% ethanol; those fixed in glutaraldehyde remained in this fixative until processing. After fixation the urinary bladder was cut mid-sagittally, one half was processed for SEM and the other half for light microscopy. The kidneys, liver and spleen were also removed, weighed and immersed in 10% buffered formalin. A fragment of duodenum was collected and placed together with the urinary bladder in Bouin's or glutaraldehyde fixative to be used as a positive control for BrdU immunohistochemistry

3.3.3. Scanning Electron Microscopy processing

One half of the urinary bladder was sectioned again longitudinally into two fragments and processed by two different drying methods as described below

3.3.3.1. Critical Point Drying (CPD):

After fixation in 2.5% glutaraldehyde, the urinary bladder samples were rinsed in 0,1 phosphate buffer and in distilled water and then post fixed in osmium tetroxide OsO_4. After 4h in the Bouin's fixative the urinary bladders were rinsed three times in 70% ethanol and stored in the same until SEM processing. The samples fixed in 2.5% glutaraldehyde were dehydrated in a graded ethanol series of 15%, 30%, 50%, 70%, 90% and 100%, and the samples fixed in Bouin's fixative were dehydrated in 90% and 100% ethanol, immersed in liquid carbon dioxide and placed in the critical point dryer (Balzers CPD-020, Bal-tec, Wiesbaden, Germany) for drying.

3.3.3.2. Hexamethyldisilazane

After fixation in 2.5% glutaraldehyde, the urinary bladder was rinsed a minimum of three times in 0,1 phosphate buffer and dehydrated in a graded ethanol series of 50% and 70%. The samples fixed in Bouin's fixative and glutaraldehyde were rinsed in 70% ethanol and dehydrated in a graded ethanol series of 80%, 90%, 95% and 100%. The specimens were rinsed in a 1:1 mixture of 100% ethanol and HMDS (CAS: 999-97-3; Sigma Aldrich; Co., St Louis; MO; USA) for 15 minutes and then immersed in 100% HMDS for 15 minutes.

After drying by CPD or in HMDS, the urinary bladders were mounted on aluminum SEM stubs fixed with nail polish mixed with a silver conductor. Samples were placed in a sputter coater (BAL-TEC- SCD 050) and coated with a thin layer of gold prior to examination by SEM.

3.3.3.3. SEM analysis

SEM analysis was performed using a Quanta 200 Scanning Electron Microscope (Fei Company, Oregon, USA) in the Electron Microscopy Center at the Institute of Biosciences, UNESP, Botucatu, São Paulo, Brazil. The urothelial alterations were classified according to the following five categories (Cohen et al., 2002): Class 1, flat urothelium composed of large polygonal superficial cells, with a few necrotic or exfoliated cells due to normal cell death, (figure 1) ; Class 2, occasional small foci of superficial urothelial necrosis and exfoliated cells; Class 3, pleomorphic polygonal superficial cells with numerous small foci of superficial urothelial necrosis and exfoliated cells; Class 4, extensive superficial urothelial necrosis; Class 5, extensive necrosis and exfoliation with piling up of small rounded cells (urothelial hyperplasia). Usually, normal urothelium and slightly altered mucosa are included in Classes 1 to 3, and progressively more altered mucosa are classified in Classes 4 and 5 (Cohen et al., 2002). The presence of enlarged urothelial cells protruding into the lumen of the bladder, namely, the "swollen cells", were evaluated as part of the classification for cytotoxicity as described by da Rocha et al. (2012).

3.3.4. Light Microscopy

3.3.4.1. Light Microscopy processing:,

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 to act as the positive control for BrdU immunohistochemical staining. Hematoxylin and eosin (H&E) stained sections were used for for histopathological examination and unstained sections were used for immunohistochemical staining of BrdU

3.3.4.2. Light Microscopy Analysis

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) which were diagnosed according to Ito & Fukushima (1989) and Cohen (1983) criteria´s

3.3.4.3. Immunohistochemistry processing:

For evaluation of cell proliferation, unstained slides of paraffin-embedded sections were deparaffinized and rehydrated. Pretreatment of tissue sections was performed using 2N HCl followed by 0.005% trypsin, both at 37ºC, and incubation in BLOXALL (^{TM} Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution (Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010-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 manufacturer. The presence of peroxidase was detected using the DAB-Peroxidade substrate Kit (Vector laboratories, Inc. Burlingame, CA, USA) at room temperature for 2 minutes and counterstained with Harris hematoxylin. This procedure was performed only on bladder tissue collected after treatment with diuron for seven days and fixed with Bouin's

3.3.4.4. Cell proliferation analysis:

The urothelial nuclei incorporating BrdU were counted using a conventional optical microscope at 400× magnification. All cells in the epithelial layer of the urinary bladder from each animal were counted. Labeling Index (LI) in BrdU immunostained material was determined by using: dividing the total number of labeled nuclei by the total number of nuclei (labeled and unlabeled nuclei) and multiplying by 100.

3.3.5. Statistical Analysis

Body weights, water and food consumptions and organ weights of the experimental groups were initially compared by ANOVA; when a significant ($p < 0.05$) difference was detected, the Tukey´s test followed. The Fisher's Exact test (two-tail) was used to analyze the incidences of urinary bladder lesions evaluated by histology and SEM and the incidence of kidneys pelvis lesions evaluated by histology. Labeling index was analyzed using Duncan's multiple range tests. The statistical analyses were performed using SAS for Windows (version 9.1) and GraphPad InStat-[DATASET1.ISD].

3.4. RESULTS

3.4.1. Seven days study:

3.4.1.1. Body weights, food and water consumption and organ weights

Mean body weight and weight gain in the NaS and 2500 ppm diuron groups were significantly decreased compared to control ($p<0.05$). Although not significant, the food consumption in the NaS group was lower compared to the diuron and control groups. Water consumption was significantly increased in the NaS group ($p<0.05$) compared to the diuron and control groups and significantly decreased ($p<0.05$) in the diuron group compared to control (Table1).

The mean absolute liver weights were higher ($p<0,05$*)* in the NaS group compared to the diuron and control groups, however the relative liver weights were significantly higher *(*$p<0,05$*)* in the diuron group compared to NaS group. No differences were found among the groups in the absolute weights kidneys, but the relative kidney weights were significantly higher in the NaS and diuron groups compared to control. The group fed diuron showed significantly increased mean absolute and relative spleen weighs when compared to the NaS and control groups (Table 2)

3.4.1.2. Histological analyses

By light microscopy, there were no significant differences among the groups in the incidence of histopathological lesions of the urinary bladders or kidneys regardless of the fixative used (Table 3)

3.4.1.3. BrdU labeling index

Although not significant, the BrdU labeling index in the Bouin's fixed bladder urothelium of animals in the NaS and the diuron groups was higher compared to the control group (Table 3, Figure 2). We were unable to evaluate the BrdU labeling index in the glutaraldehyde fixed tissue since it induced numerous artifacts in the cellular structure and tissue morphology, and intense background staining (Figure 3-4).

3.4.1.4. Scanning electron microscopy

By SEM, the diuron treated group had a significantly increased ($p<0.03$) incidence (5/13) of severe urothelial alterations compared to the control (Classes 4-5), characterized by areas of exfoliation, necrosis and urothelial cell hyperplasia. In addition, a few animals (3/13) in the diuron treated group had prenecrotic swollen cells present on the luminal surface of the urothelium These cells appeared only in the diuron group in classes 1-3 which are considered normal bladder classes (Table 4 and Figure 5-6).

The identification and localization of prenecrotic swollen cells, exfoliation, necrosis, and hyperplasia on the surface of the urothelium was possible with either fixative or drying method used to process samples for SEM examination. The glutaraldehyde fixative did induced more shrinkage artifacts on the urinary bladder surface processed with HMDS compared to those processed by CPD, but these did not influenced the analysis. Both drying methods produced well preserved samples in the Bouin's fixed tissue (figure 7).

3.4.2. Fifteen weeks study:

3.4.2.1. Water and food consumption, body and gain weight and organ weights

Water consumption was significantly increased ($p < 0.05$) in the NaS group when compared to the diuron and control groups. The food consumption was significantly decreased ($p <0.05$) in the diuron group when compared to the control and NaS groups (Table 5-6, Figure 8-9). Similar to the seven day study, the mean body weight and weight gain of the NaS and 2500 ppm diuron groups were lower ($p <0.05$) compared to the control group (Table 7-8, Figure 10).

The mean absolute liver weights were lower ($p<0,05$*)* in the NaS and diuron groups compared to control however, the relative weights were significantly higher ($p<0,05$) in the diuron group compared to the control and NaS group. The mean absolute kidney weights were significantly decreased and the relative weights significantly increased in the diuron group when compared to the control and NaS groups ($p<0,05$). The absolute and relative spleen weights were significantly

increased (p<0,05) in the diuron group when compared to the control and NaS groups (Table 9). This increase has been widely recognized as a diuron-induced effect (APVMA, 2005; Cardoso et al.,2013

3.4.2.2. Histological analyses

The incidence of lesions identified by light microscopy in the urinary bladders was significantly increased (p< 0.05) in the NaS and diuron groups (5/14 and 8/13, respectively) when compared to control. The presence of simple hyperplasia in the kidney pelvis was also significantly higher (p< 0.05) in the NaS and diuron animals compared to control (Table 10).

Although visualization and classification of histological lesions was possible with both fixatives, the Bouin's fixative better preserved the cells compared to glutaraldehyde, which caused distortion and flattening of the cells and over staining (Figure 11).

3.4.2.3. Scanning electron microscopy

Similar to the seven day study, the incidence of severe urothelial alterations (Classes 4-5) characterized by areas of exfoliation, necrosis and cell hyperplasia, were significantly increased (p<0,01) in the diuron group (7/13) when compared to control. However, no swollen cells were observed on the urothelial surface of bladders from these animals. Although not significant, the incidence of severe lesions in NaS group was higher compared to the control group (6/14) (p<0,08) (Table 11 and figure 12).

The fixative or drying process used did not affect the quality of the samples for SEM examination of the urothelial surface.

3.5. DISCUSSION

This study documented by SEM the presence of swollen superficial cells in the urinary bladder as an early change after seven days of exposure to 2500 ppm diuron independent of the SEM processing methods used. This observation supports the hypothesis postulated by da Rocha et al. (2012) in which the MOA of diuron-induced rat urinary bladder carcinogenesis begins with degenerative prenecrotic swollen cells on the urothelial surface followed by necrosis, exfoliation and consequent sustained regenerative urothelial cell proliferation. The 15 week study conducted with treatment groups and SEM processing methods similar to the seven day study, showed severe cytotoxic changes including necrosis, exfoliation and simple hyperplasia on the luminal urinary bladder surface, however, swollen cells were not detected in these animals.It is well established that rats fed 2500 ppm diuron for 15 weeks or longer have increased necrosis and regenerative cell proliferation in the bladder urothelium (Nascimento et al., 2006; da Rocha et al., 2010; Cardoso et al., 2013). However, there was no conclusive evidence documenting the early urothelial changes induced by short-term exposure to the carcinogenic dose of 2500 ppm diuron. Da Rocha et al. (2012) reported the observation by SEM of the early occurrence of swollen superficial cells in the urinary bladders of rats exposed to 2500 ppm diuron that were fixed with Bouin's fixative and dried with HMDS. However, in a previous study conducted in our laboratory in which urinary bladders were fixed in Bouin's fixative and dried by CPD prior to examination by SEM, there were no such changes on the urinary bladder surface after exposure to 2500 ppm diuron for 7 days (Ihlaseh, 2011). Initially, it was hypothesized that these differing results were due to different methods used to process the urinary bladders prior to examination by SEM. In the present study, the two processing methods used allowed for a more thorough determination of the location and classification of swollen superficial cells (3 /13 animals) observed by SEM in diuron treated animals. Because these cells appear only in some animals and seem to be a very early initial event, it is possible that the lack of

detection of these cells by Ihlaseh, (2011) may be due to the use of only five animals per group in that study.

Glutaraldehyde fixative has been recommended for tissues which will be examined by SEM, but not for tissues which will be used for immunohistochemical detection of BrdU (Cohen et al., 2007). In this study, the BrdU labeling index could not be performed on glutaraldehyde fixed tissue, due to artifacts and the absence of labeling. Some authors described that the use of glutaraldehyde fixative produces changes in the tertiary structure of the antigen preventing antigen-antibody binding and consequently the immunohistochemical staining (Bozzola & Russell, 1992). In addition, in this study, the use of this fixative for the tissues examined by light microscopy also induced artifacts such as flattening of the cells and excessive dye impregnation, making histological analysis difficult.

The Bouin´s fixative is considered an excellent fixative for preservation of tissues which will be analyzed by SEM, light microscopy and immunohistochemistry (Cohen, 2002). In this study, this fixative also showed good performance without producing artifacts for any of the analyses.

Specimen drying for SEM is a critical step because done improperly, it can result in substantial and irreversible deformation of tissue structures (Buravkov et al.,2011). The CPD drying performed using instrument is the conventional preparation method for SEM analysis and usually does not induce artifacts (Postek et al.,1980) The HMDS, a chemical drying method, has been used as an alternative method with the advantages of low cost and easy implementation (Bray et al., 1993; Araújo et al., 2003). However, since some reports have indicated that HMDS can induce artifacts of retraction in samples (Slízová et al.,2003), it was thought that the presence of swollen cells described by da Rocha et al. (2012) could be an artifact of HMDS processing and not an early diuron induced event. Our findings confirm that the presence of prenecrotic swollen cells in the urothelium is an early event in the process of the cytotoxicity induced by diuron and it is not related to processing methods used for SEM. Although the use of different fixatives and drying methods allowed the detection by SEM of early proliferative changes in the urinary bladder in the seven day study, the

Bouin´s fixative used together with the CPD or HMDS drying methods better preserved the tissues allowing us perform histological, SEM and cell proliferation analyses on the same samples

Finally, the identification of early urothelial lesions associated with the carcinogenic process of chemicals can be used to characterize treatment doses without adverse effect (NOAEL). Therefore, it is essential to distinguish between treatment induced lesions and lesions which are artifacts of analytical procedures in order to elucidate the critical doses, key events and the real impact of these substances on human health.

3.6. CONCLUSION

The present data shows that the two fixatives and drying methods used in this study did not influence the detection of pre-necrotic swollen cells in the urinary bladders of rats fed 2500 ppm diuron for seven days. Regardless of fixative or drying method used, swollen cells were not observed in the bladders of animals exposed to diuron for 15 weeks. Our findings confirm the presence of prenecrotic swollen superficial cells in the urothelium as an early event in the process of the cytotoxicity induced by diuron and is not related to the tissue processing methods used for SEM

Although the use of either fixative or drying method allowed the detection of early proliferative changes in the urinary bladder by SEM in the seven day study, the use of Bouin's fixative better preserved the tissues, allowing histological, SEM and cell proliferation analyses to be performed on a single sample. CPD and HMDS drying methods produce good quality samples for SEM examination; however, HMDS is faster and easier to perform in comparison to CPD

3.7. . AKNOWLEDGEMENTS

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3.8. REFERENCES

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

Australian Pesticides and Veterinary Medicine Authority (APVMA), 2011. Review of the Mammalian Toxicology and Metabolism/Toxicokinetics of Diuron. Diuron Human Health Assessment. Australian Government (ok mi)

Araújo, J.C., Téran, F.C., Oliveira, R.A., Nour, E.A., Montenegro, M.A., Campos, J.R., Vazoller, R.F. (2003) Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. *J Electron Microsc* **52**, 429–433

Bozzola, J. J., Russell, D.L. Electron microscopy: Principles and techniques for biologists. 1ed. Local: Jones and Bartlett Publihers, 1992, p.41 -63 , 234-251.

Bray, D.F., Bagu, J., Koegler, P. (1993). Comparison of hexamethyldisilazane (HMDS), Peldri ll, and critical-point drying methods for scanning electron microscopy of biological specimens.*Microsc Res Tech* **26**, 489-495

Buravkov, S.V., Chernikov, V.P., Buravkova, L.B. (2011) Simple Method of Specimen Preparation for Scanning Electron Microscopy. *Bull Exp Biol Med* **151**,378-382

Cano, M., Suzuki, T., Cohen, S.M. (1993).Application of scanning electron microscopy and X-ray analysis to urinary tract cancer in animals and humans.*Scanning Microsc* **7**, 363-370

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

Cohen, S. M. (1983). Pathology of experimental bladder cancer in rodents. In: The Pathology of Bladder Cancer (S.M. Cohen and G.T. Bryan, eds.), CRC Press, Boca Raton, FL, vol. II, pp. 1-40

Cohen, S.M., Fisher, M.J., Sakata, T. (1990). Comparative analysis of the proliferative response of the rat urinary bladder to sodium saccharin by light and scanning electron microscopy and autoradiography.*Scanning Microsc* **4**, 135-142

Cohen, S.M., Garland, E.M., Cano, M.,St John, M.K., Khachab, M., Wehner, J.M., Arnold, L.L. (1995). Effects of sodium ascorbate, sodium saccharin and ammonium chloride on male rat urinary bladder. *Carcinogenesis* **16**, 2743-2750.

Cohen, S.M., Arnold, L.L., Uzvolgyi, E., Cano, M., St John, M., Yamamoto, S., Lu, X., Le, X.C. (2002). Possible role of dimethylarsinous acid in dimethylarsinic acid-induced urothelial toxity and regeneration in the rat. *Chem Res* Toxicol **15**, 1150-57.

Cohen, S.M., Ohnishi, T., Clark, N.M., He, J., Arnold, L.L. (2007). Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. *Toxicol Pathol* **35**, 337-347

Falconi, M., Teti, G., Zago, M., Pelotti, S., Gobbi, P., Breschi, L.,Mazzotti, G. (2007) Effect of fixative on chromatin structure and DNA detection. Microsc Res and Tech **70**, 599-606

Garland, E.M., Shapiro, R., Wehner, J.M., Johnson, L.S., Mattson, B.J., Khachab, M., Asamoto, M, Cohen, S.M.,(1993). Effects of dietary iron and folate supplementation on the physiological changes produced in weanling rats by sodium saccharin exposure. *Food Chem. Toxicol*. **31**, 689-699

Ihlaseh, S.M. Estudo dose-resposta e perfil de expressão gênica do herbicida diuron [3,(3,4-diclofenil)-1,1-dimetiluréia] em bexiga urinária de ratos Wistar machos. 2011. 110 folhas. Tese (Doutorado em Patologia) – Universidade Estadual Paulista Júlio de Mesquita Filho. Botucatu, 2011

Ito, N., Fukushima, S.(1989). Promotion of urinary bladder carcinogenesis in experimental animals. Exp Pathol. **36**, 1-15

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

de Oliveira, M. L. C. S. (1998). Efeitos de sais de sódio, potássio e cálcio sobre a composição da urina sobre o epitélio da bexiga de ratos machos F344. Faculdade de Medicina, Universidade Estadual Paulista, Botucatu, tese (Doutorado) (in Portuguese).

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

Postek, T.M., Howard, K.S., Johnson, A.H., M^{c}Michael, K.L. Scanning electron microscopy: A student´s handbook. 1ed. Local: Ladd Research Industries, 1980, p.115-238.

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

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

da Rocha, M.S., Arnold, L.L., Dodmane, P.R., Pennington, K.L., Qiu, F., de Camargo, J.L., Cohen, S.M. (2013). Diuron matabolites and urothelial cytotoxicity: In vivo, in vitro and molecular approaches. *Toxicology* **314**, 238-246

Slízová, D., Krs,O., Pospísilová, B. (2003) Alternative method of rapid drying vascular specimens for Scanning Electron Microscopy. *J EndovascTher* **10**, 285-287

USEPA (1997). Carcinogenicity peer review of diuron: Office of Prevention, Pesticides and Toxic substances. [Memorandum]. Cincinnati, 22p

3.9. TABLES:

Table 1: Mean body weights and food and water consumption for rats exposed to NaS (7.1%) or diuron (2500 ppm) for seven days.

Groups		Body weight[a] (b.w.) (g)			Consumption	
	N◦	Initial	Final	Gain	Food (g/rat/day)	Water (ml/rat/day)
Control	14	232.28±26.42	284.57±28.05	52.28±6.74	24.98	29.52
NaS (7.1%)	14	233.58±22.78	**251.28±27.60**[b]	**17.92±12.40**[b]	17.98	**36.23**[c]
Diuron (2500 ppm)	14	238.50±36.60	**249.00±19.98**[b]	**10.42±26.18**[b]	20.75	**21.14**[d]

[a]Values are expressed as mean ±S.D.; N = number of animals, one-way ANOVA, Tukey´s test ($p<0.05$),
[b]Significantly different from control ($p<0,05$)
[c]Significantly different from diuron and control groups ($p<0,05$)
[d]Significantly different from NaS and control groups ($p<0,05$)

Table 2: Final body and tissue weights of male Wistar rats exposed to NaS (7.1%) or diuron (2500 ppm) for seven days

Groups	N◦	Body weight (g)[a]	Absolute weight [a] (g)			Relative weight [a] (g/kg b.w.)		
		Final	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Control	14	284.57 ±28.05	11.19 ±0.98	1.88± 0.17	0.93± 0.10	3.95 ± 0.34	0.664 ± 0.03	0.330 ± 0.03
NaS (7.1%)	14	**251.28 ± 27.60** [b]	**8.44 ± 2.57** [c]	2.34 ±2.15	0.83 ± 0.11	3.34 ± 0.99	**0.965 ± 1.00**[b]	0.335 ± 0.04
Diuron (2500ppm)	14	**249.00 ± 19.98** [b]	10.86 ±1.39	1.80 ± 0.20	**1.37 ± 0.28** [d]	**4.37 ± 0.48** [e]	**0.724 ± 0.04** [b]	**0.558 ± 0.13**[d]

[a]Values are expressed as mean ± S.D.; N = number of animals, one-way ANOVA, Tukey´s test (p<0.05)
[b]Significantly different from control (p<0.05)
[c]Significantly different from control and diuron groups (p<0.05)
[d]Significantly different from control and NaS groups (p<0.05)
[e]Significantly different from NaS group (p<0.05)

Table 3: Incidence of proliferative changes in the urinary bladders fixed with Bouin's or glutaraldehyde fixative and in the kidney pelvis fixed in 10% buffered formalin of rats exposed to NaS (7.1%) or diuron (2500 ppm) for seven days

Group	Histology[a]								Labeling index (%) BrdU[b]
			Bouin's fixative		Glutaraldehyde fixative		Kidney pelvis		Bouin's fixative[c]
	N	Incidence of HS[d]	Normal	SH	Normal	SH	Normal	SH	
Control	13	1	6	1	6	0	8	6	0.01±0.01
NaS (7.1%)	14	1	6	1	7	0	3	11	0.06±0.04
Diuron (2500 ppm)	14	2	6	1	6	1	3	11	0.07±0.04

[a] Values expressed per animal, Fisher's Exact test (2-tail)
[b] Values are expressed per animal ± Standard Error (S.E.),Ducan's test, N: number of animals.
[c] The BrdU labeling index was performed only in urinary bladders fixed with Bouin's.
[d] SH – simple hyperplasia

Table 4: Incidence of urothelial lesions in the urinary bladders of Wistar rats exposed to NaS (7,1%) or diuron (2500 ppm) for seven days and processed by various methods for SEM examination

Groups	N	Incidence of lesions[a]		Processing methods/ SEM classification[a]							
				Bouin's fixative (CPD)		Bouin's fixative (HMDS		Glutaraldehyde fixative (CPD)		Glutaraldehyde fixative (HMDS)	
		1-3	4-5	1-3	4-5	1-3	4-5	1-3	4-5	1-3	4-5
Control	12	12	0	4	0	6	0	5	1	5	1
NaS (7.1%)	14	12	2	4	1	6	0	6	1	6	0
Diuron (2500 ppm)	13	**8**[b] **(3)**[c]	5	5(1)[d]	2	4(1)[d]	3	4(1)[e](1)[f]	2	4(1)[e]	2

[a]Values expressed per animal, N: number of animals, Fisher's Exact test (2-tail), CPD (critical point drying), HMDS (hexamethyldisilazane),Classes 1-3 (normal) and 4-5 (severe changes) (Cohen et al.,2007)
[b]Significantly different from control and NaS groups ($p<0.05$)
[c]Presence of swollen prenecrotic cells in three bladders
[d] Detection of swollen prenecrotic cells in the bladder of the same animal by different processing methods
[e] Detection of swollen prenecrotic cells in the bladder of the same animal by different processing methods
[f] Detection of swollen prenecrotic cells in one bladder

Table 5: Water consumption for rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks (ml/rat/day)

Groups (n=14)	Weeks							
	1	**3**	**5**	**7**	**9**	**11**	**13**	**15**
Control	30.14	34.95	34.68	34.75	31.09	26.18	29.82	29.54
NaS (7.1%)	**36.82**[a]	**51.55**[a]	**55.46**[a]	**51.45**[a]	**51.23**[a]	**49.61**[a]	**52.86**[a]	**53.14**[a]
Diuron (2500 ppm)	**23.41**[b]	29.68	29.50	29.91	24.25	27.17	28.21	28.88

Values are expressed as mean, one-way ANOVA, Tukey´s test (p<0,05)
[a] Significantly different from control and diuron groups (p<0.05)
[b] Significantly different from control and NaS groups (p<0.05)

Table 6: Food consumption for rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks (g/rat/day)

Groups (n=14)	Weeks							
	1	**3**	**5**	**7**	**9**	**11**	**13**	**15**
Control	25.54	27.79	22.05	25.91	22.93	21.23	24.66	24.39
NaS (7.1%)	**18.11**[a]	27.93	28.38	25.89	26.55	24.98	27.20	26.89
Diuron (2500 ppm)	**19.91**[a]	**22.45**[b]	**20.68**[b]	**20.70**[b]	**17.63**[b]	**19.25**[c]	**21.75**[b]	**20.00**[b]

Values are expressed as mean., One-way ANOVA, Tukey´s test *(p<0,05)*
[a] Significantly different from control (p<0.05)
[b] Significantly different from control and Nas groups (p<0.05)
[c] Significantly different from NaS group but not from control group (p<0.05)

Table 7: Mean body weights for rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks.

Groups (n=14)	Weeks							
	1	**3**	**5**	**7**	**9**	**11**	**13**	**15**
Control	232.14 ± 19.51	323.71 ± 21.68	386.50± 22.39	440.43± 24.63	469.21± 21.89	476.07± 32.81	484.21± 37.13	501.07± 29.80
NaS (7,1%)	234.36± 22.08	**296.79 ± 26.62**[a]	**350.86± 29.30**[b]	**385.14± 28.59**[b]	**426.07± 29.38**[b]	442.07± 27.58	**452.50± 25.60**[b]	**473.00± 26.85**[b]
Diuron (2500 ppm)	230.71 ±19.04	**278.14 ± 18.80** [a]	**317.43 ± 29.09**[c]	**342.29± 29.17**[c]	**359.93± 32.31**[c]	**349.79± 56.81**[c]	**377.54± 38.74**[c]	**378.08± 34.26**[c]

Values are expressed as mean ± S.D., one-way ANOVA, Tukey´s test *(p<0,05)*

[a] Significantly different from control (p<0.05)

[b] Significantly different from control and diuron groups (p<0.05)

[c] Significantly different from control and Nas groups (p<0.05)

Table 8: Mean body weights by rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks

Groups	N	Body Weight (g)[a]		
		Initial	**Final**	**Gain**
Control	14	232.14± 19.51	505.57 ±30.82	273.42 ±28.86
NaS (7.1%)	14	234.35±22.07	**475.21± 27.59**[b]	**240.85± 28.21**[b]
Diuron (2500 ppm)	14	230.71± 19.04	**375.53± 36.81**[c]	**143.23 ±29.66**[c]

[a] Values are expressed as mean ± S.D., One-way ANOVA, Tukey´s test *(p<0,05)*
[b] Significantly different from control (p<0.05)
[c] Significantly different from control and NaS (p<0.05)

Table 9: Final body and tissue weights of male Wistar rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks

Groups	N	Body Weight (g) [a]	Absolute Weight (g)[a]			Relative Weight (g/Kg b.w.)[a]		
		Final	Liver	Kidneys	Spleen	Liver	Kidneys	Spleen
Control	**14**	505.57 ±30.82	13.93 ±1.93	2.96± 0.28	1.21± 0.15	2.75 ± 0.34	0.58 ± 0.05	0.24 ± 0.02
Nas (7,1%)	**14**	**475.21 ± 27.59** [b]	**12.46 ±1.20** [b]	2.85 ±0.29	**1.00 ± 0.10** [b]	2.62 ± 0.16	0.60 ± 0.04	0.21 ± 0.02
Diuron (2,500ppm)	**14**	**375.53 ± 36.81** [c]	**12.26 ±0.83** [b]	**2.45 ± 0.23** [c]	**1.95 ± 0.33** [c]	**3.29 ± 0.40** [c]	**0.65 ± 0.06** [c]	**0.52 ± 0.08** [c]

[a] Values are expressed as mean ± S.D., one-way ANOVA, Tukey´s test (p<0,05)
[b] Significantly different from control (p<0.05)
[c] Significantly different from control and NaS groups (p<0.05)

Table 10: Incidence of simple hyperplasia in urinary bladders fixed with Bouin's or glutaraldehyde and in kidney pelvis fixed in 10% buffered formalin of rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks

Group	Histology[a]							
			Bouin		Glutaraldehyde		Kidney Pelvis	
	N	Incidence of SH	Normal	SH	Normal	SH	Normal	SH
Control	14	0	7	0	7	0	13	1
NaS (7.1%)	14	**5**[b]	4	3	5	2	6	**8**[b]
Diuron (2500 ppm)	13	**8**[b]	1	6	4	2	1	**12**[b]

[a] Values expressed per animal Fisher's Exact test (2-tail), SH: simple hyperplasia, N: number of animals
[b] Significantly different from control ($p<0.05$)

Table 11: Incidence of urothelial lesions in the urinary bladders of Wistar rats exposed to NaS (7.1%) or diuron (2500 ppm) for 15 weeks and processed by various methods for SEM

Groups	N	Incidence of lesions[a]		Processing methods/ SEM classification[a]							
				Bouin's (CPD)[c]		Bouin's (HMDS)[c]		Glutaraldehyde (CPD)[c]		Glutaraldehyde (HMDS)[c]	
		1-3	4-5	1-3	4-5	1-3	4-5	1-3	4-5	1-3	4-5
Control	14	13	1	7	0	6	1	7	0	7	0
NaS (71%)	14	8	6	5	2	4	3	5	2	6	1
Diuron (2500 ppm)	13	6	**7**[b]	3	2	5	2	4	2	4	2

[a]Values expressed per animal- Fisher's Exact test ($p<0.01$), CPD (Critical Point Drying), HMDS (Hexamethyldisilazane), Classes 1-3 (normal) and 4-5 (severe changes) Cohen et al 2007
[b]Significantly different from control
[c]Detection of severe alterations in the bladder by differents processing methods

3.10. FIGURES:

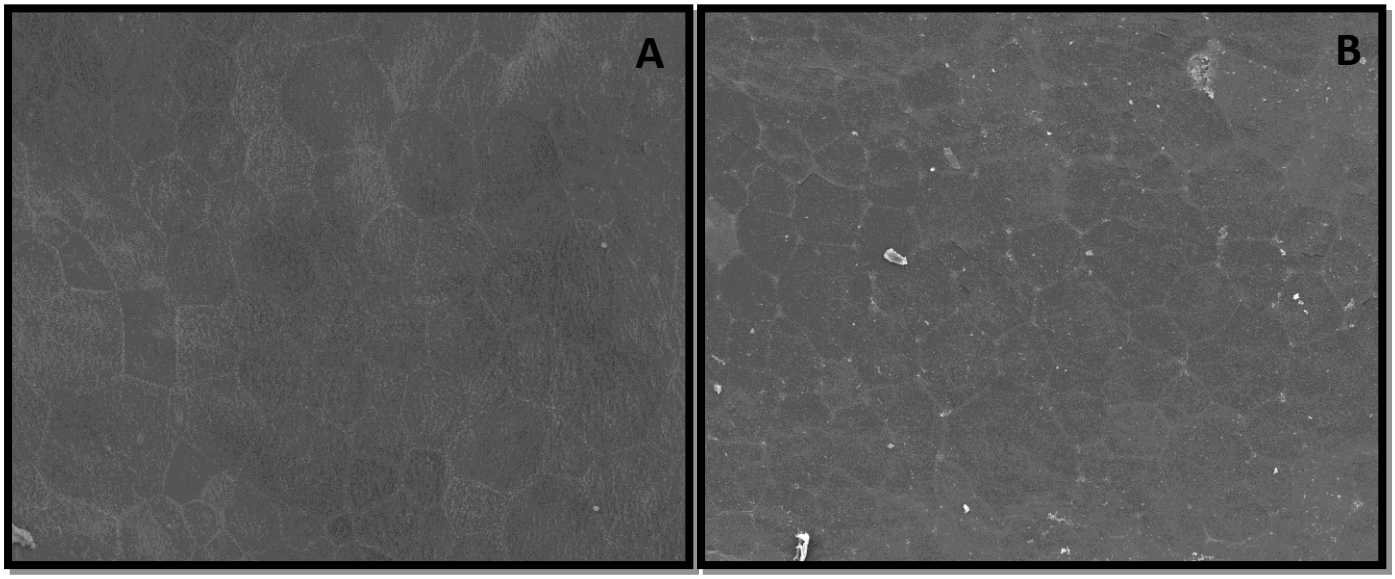

Figure 1: SEM of Control group A) glutaraldehyde and processing with CPD (600X), B) (400x) Bouin and processing with HMDS, showing urothelial cells with polygonal and flat aspect.

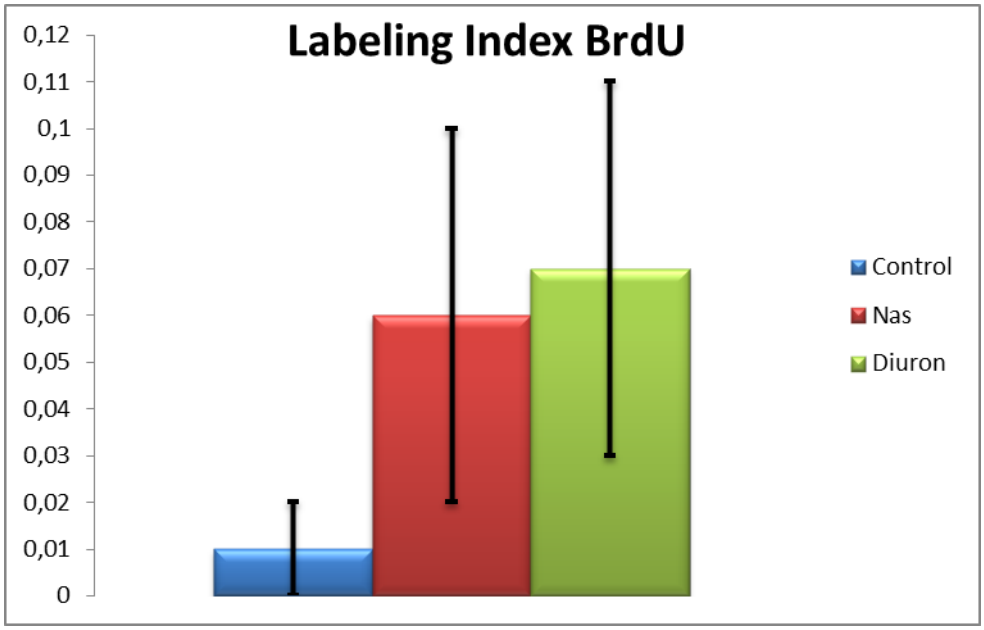

Figure 2: Labeling index of animals fed NaS (7.1%) or diuron (2500ppm)

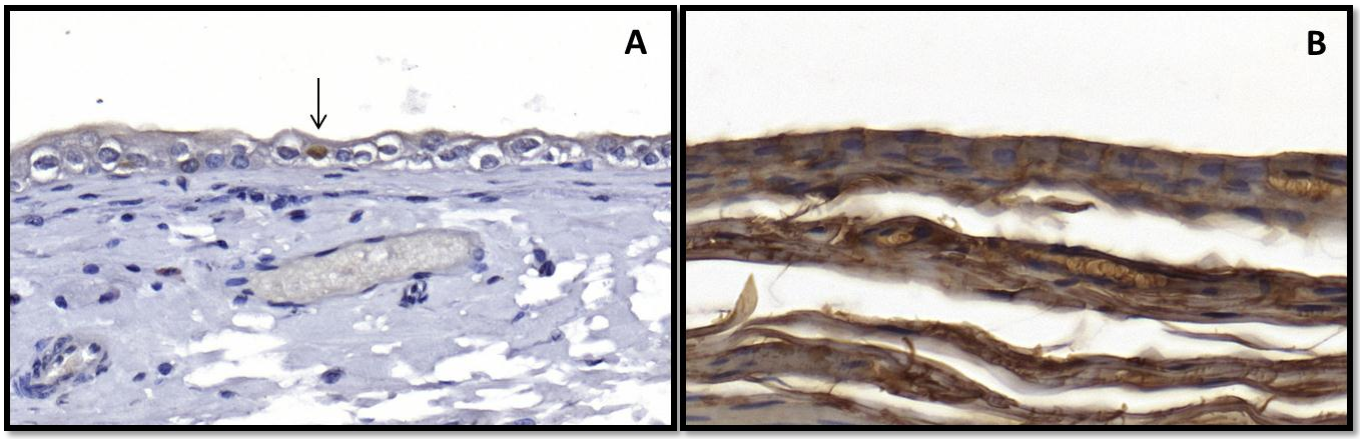

Figure 3: Immunohistochemistry of urinary bladder (400x) A) Fixation with Bouin showing well preserved tissue BrdU-labeled nucleus can be seen (diuron group, seven days); B) Fixation with 2.5% glutaraldehyde showing intense background, tissue distortion and absence of labeling (NaS group, seven days).

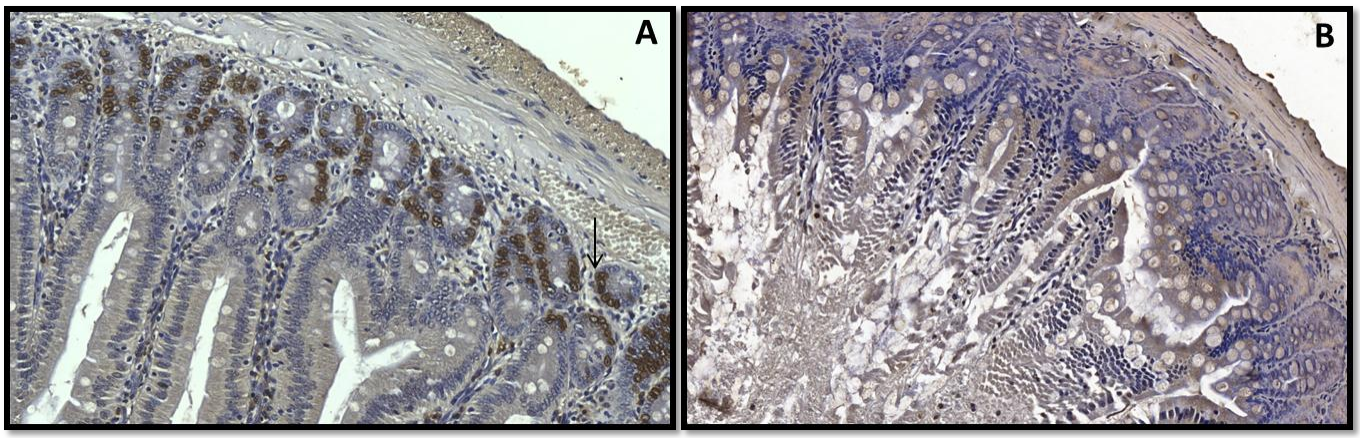

Figure 4: Immunohistochemistry of duodenum (positive control)(200x) A) Fixation with Bouin showing BrdU labeled nuclei and good tissue preservation (diuron group, seven days); B) Fixation with 2.5% glutaraldehyde showing tissue distortion, background and absence of labeling (NaS group, seven days).

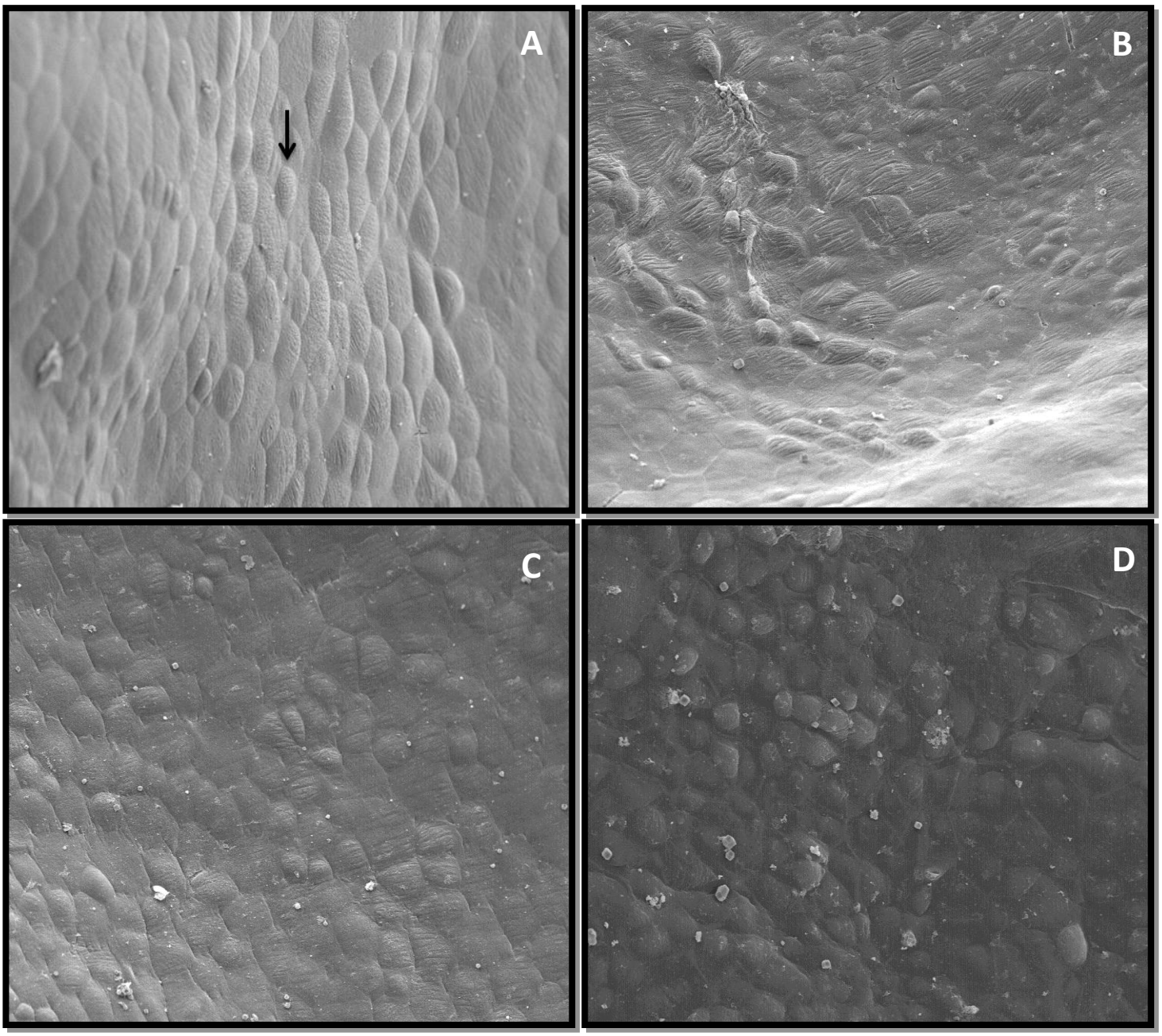

Figure 5 :SEM 2500ppm diuron group, seven days: Samples fixed with 2.5% glutaraldehyde (600x) A) CPD processing and B) HMDS processing and fixed with Bouin fixative C) CPD (600x) and D) HMDS processing (1200x). Showing prenecrotic swollen cells in the urothelium

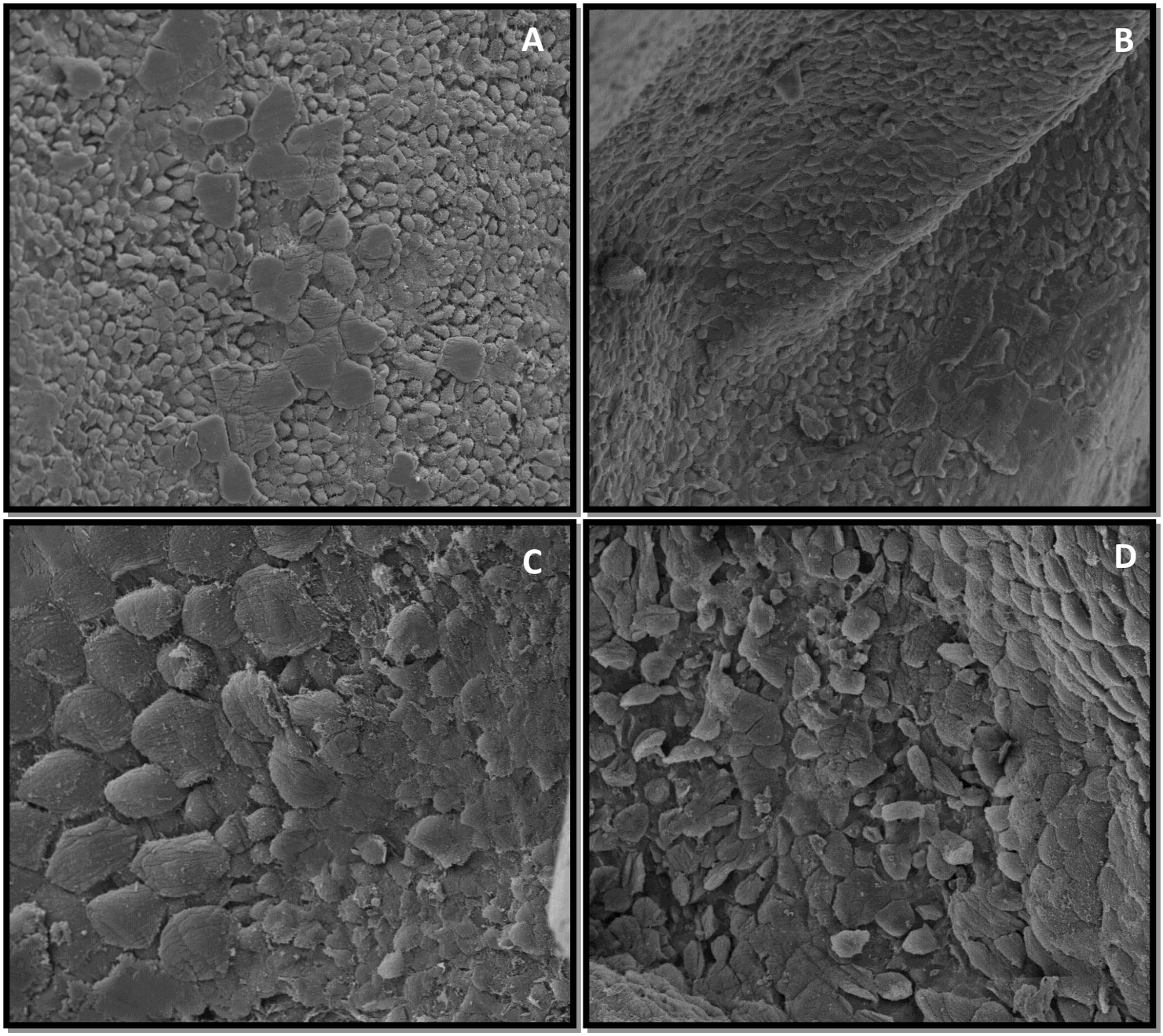

Figure 6: SEM 2500ppm diuron group, seven days: Samples fixed with glutaraldehyde (600x) A) CPD processing and B) HMDS processing fixed with Bouin fixative C) CPD processing (1000x) and D) HMDS processing (600x). Show extensive necrosis and exfoliation with piling up of small rounded cells (urothelial hyperplasia) in the urothelium.

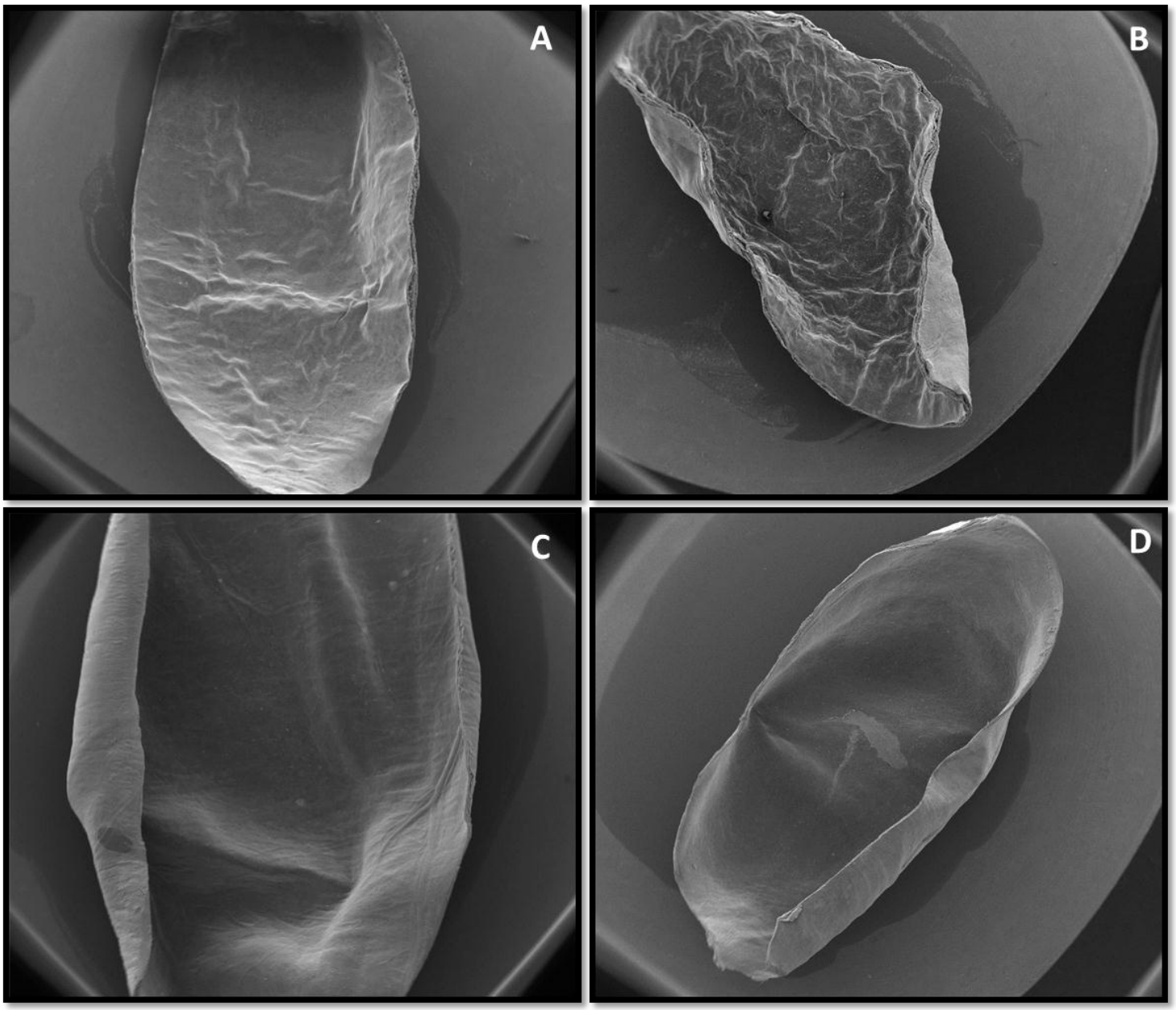

Figure 7: SEM 2500ppm diuron group, seven days: urinary bladders fixed with glutaraldehyde 2.5% A) CPD processing showing mild artefacts and shrinkage in the surface of urinary bladder (24x) and B) HMDS processing showing artefacts and shrinkage in the surface of urinary bladder (23x) Urinary bladders fixed with Bouin fixative C) CPD processing showing good tissue preservation (36x) and D) HMDS processing showing good tissue preservation (24x)

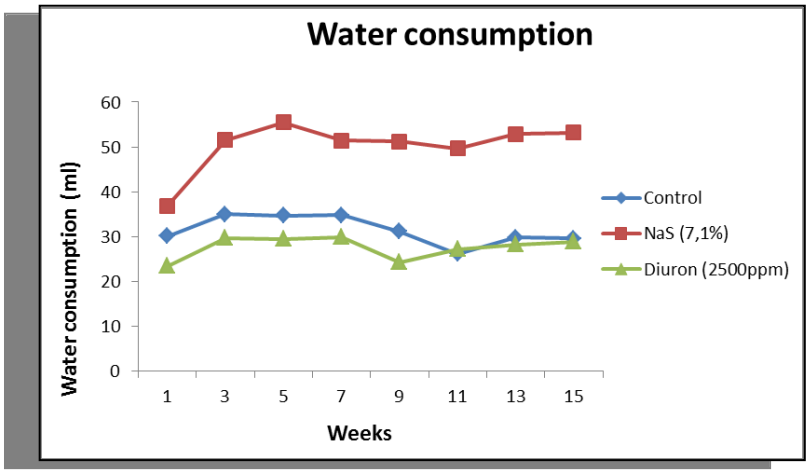

Figure 8: Water consumption of animals exposed to NaS (7,1%) or diuron (2500ppm) during 15 weeks

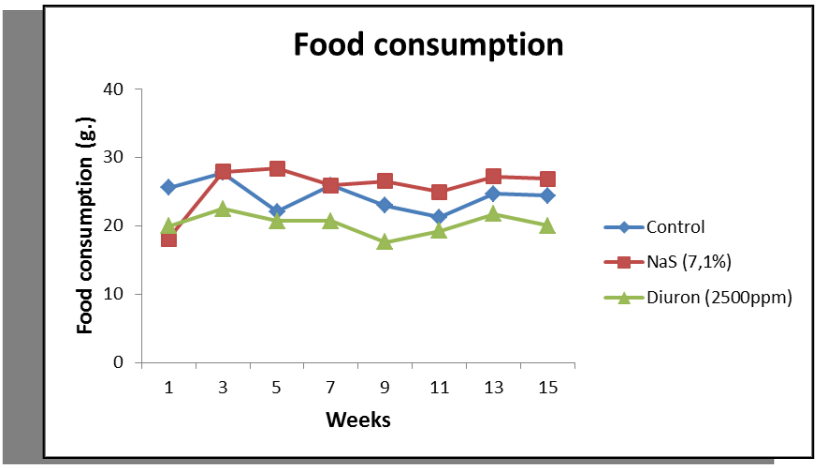

Figure 9: Food consumption of animals exposed to NaS (7,1%) or diuron (2500ppm) during 15 weeks

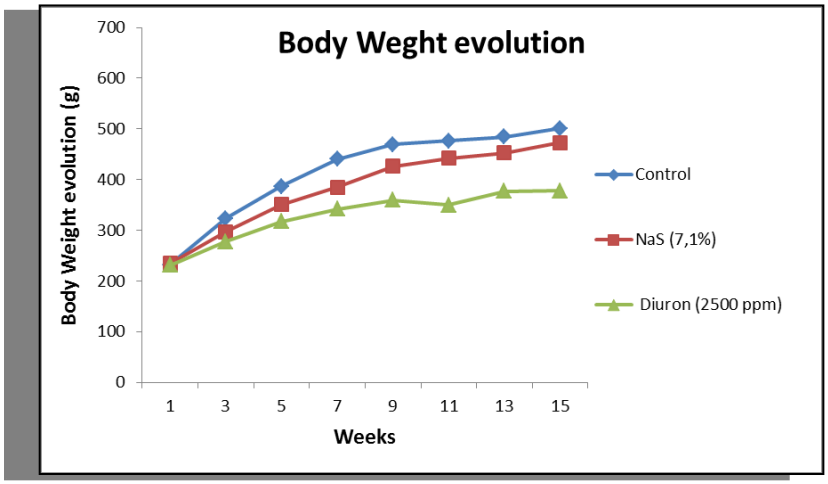

Figure 10 Body weight evolution of animals exposed to NaS (7,1%) or diuron (2500ppm)

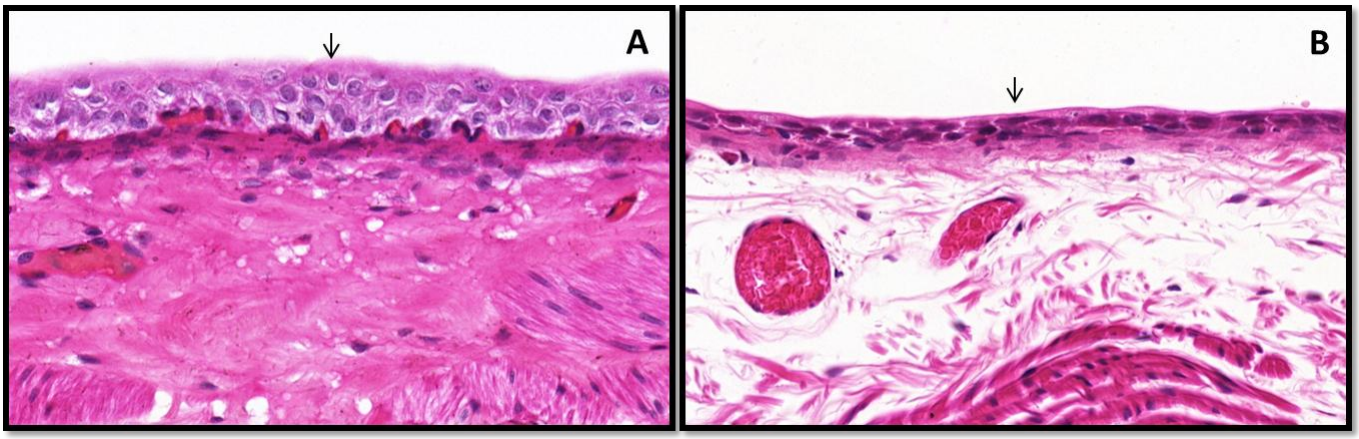

Figure 11: H&E Staining of urinary bladders of animals exposed to diuron 2500ppm for 15 weeks (100x) A) Fixation with Bouin showing good tissue preservation B) Fixation with 2.5 % glutaraldehyde showing artifacts of distortion, squashing and overstaining

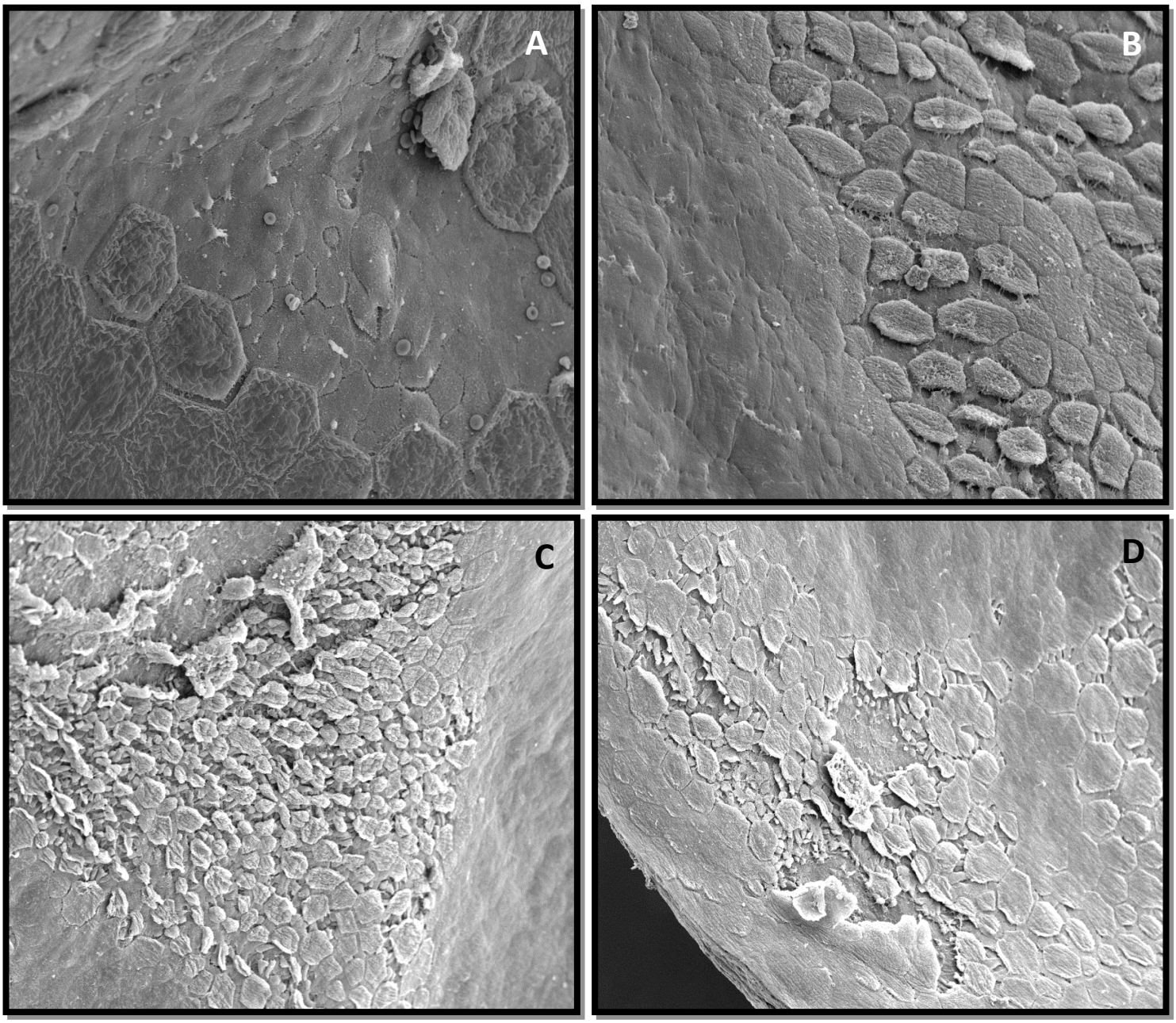

Figure 12: SEM 2500ppm diuron group,15 weeks, fixed with Bouin fixative A) CPD processing (1200x) B) HMDS processing (800x) showing a foci of exfoliation, C) ,D) HMDS processing (400x) Showing extensive necrosis and exfoliation with piling up of small rounded cells (urothelial hyperplasia) in the lumen at the urothelium.

Anexos

3.11. ANEXOS

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

FACULDADE DE MEDICINA FMB 1963 BOTUCATU

MUDANÇA DE TÍTULO EM PROJETO DE PESQUISA

Objetivo Acadêmico:

( ) Pós Doutorado
( ) Tese Doutorado
(x) Dissertação de Mestrado
( ) Trabalho científico
( ) Outros: Especificar

Título constante no parecer inicial de aprovação: Detecção de alterações proliferativas ultraestruturais induzidas quimicamente no urotélio vesical de ratos Wistar- A influência da fixação e ponto crítico de secagem

Titulo FINAL: Influência da fixação e de métodos de secagem na detecção de alterações proliferativas ultraestruturais induzidas pelo diuron no urotélio vesical de ratos wistar

Data da reunião do CEP que aprovou o parecer inicial: 29/03/2012

Declaro que o trabalho não sofreu alterações nos objetivos e/ou conteúdo metodológico da época de apresentação para análise do CEP.

Orientadora: Dra. Maria Luiza C. S. de Oliveira

Orientada: Rafaela Marono Fava

08 01 14
p/ Kleber M. Camargo

Faculdade de Medicina de Botucatu – Seção Técnica de Pós-graduação
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UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FMB BOTUCATU

CEEA

Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 26/04/99

Certificado

Certificamos que o **(Protocolo CEEA 928/2012)** Detecção de alterações proliferativas ultraestruturais induzidas quimicamente no urotélio vesical de ratos Wistar – a influência da fixação e ponto crítico de secagem, a ser conduzido por Rafaela Marono Fava, orientada pela Profª. Drª. Maria Luiza Cotrin Sartor de Oliveira, co-orientada por Merielen Garcia Nascimento, com a colaboração de Ana Paula Ferragut Cardoso, João Lauro Viana de Camargo, Mitscheli Sanches da Rocha, com o Apoio Técnico de Paulo César Georgete e Paulo Roberto Cardoso, está 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 atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 29/03/2012.

Profª Drª Maria Rosa Bet Moraes Silva
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone/Fax: (0xx14) 3811-6143 e-mail secretaria: capellup@fmb.unesp.br

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International Council For Laboratory Animal Science
ICLAS Network Member for Promotion of Animal Quality in Research
www.cemib.unicamp.br

Profª Maria Luiza Cotrim Sartor de Oliveira
CPF 039.004.168-85
UNESP
BOTUCATU -SP

Atestado de Saúde Animal

Atestamos que os Ratos (90 Machos) da linhagem **HanUnib:WH,** provenientes da Divisão de Produção de Animais S.P.F. (Specific Pathogen Free) deste Centro, pertencem à categoria sanitária S.P.F. e apresentam-se isentos dos agentes patogênicos pesquisados pelo laboratório de controle de qualidade sanitária. Informamos que os mesmos encontram-se livres de outros agentes infecciosos capazes de causarem riscos à saúde humana. A validade deste Atestado consta da data dos últimos testes do programa de monitorização sanitária, rotineiramente realizados pelo Laboratório de Controle de Qualidade Animal - C.Q.S.**(*)**.

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

(*) Data dos últimos testes de monitorização sanitária realizados em Dezembro de 2012.

Campinas, 03 de Janeiro de 2013.

Drª. Sônia Cano Montebelo Rachel
CRMV-SP: 4462 - Médica Veterinária
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Prof.Dr.Rovilson Gilioli
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