DNA Content Analysis, Expression of Ki-67 and p53 in Rat Urothelial Lesions Induced by *N*-Butyl-*N*-(4-Hydroxybutyl) Nitrosamine and Treated with Mitomycin C and Bacillus Calmette-Guérin

PAULA A. OLIVEIRA[1], CARLOS PALMEIRA[2,3], AURA COLAÇO[1], LUIS F. DE LA CRUZ P[4] and CARLOS LOPES[5,6]

[1]Department of Veterinary Sciences, CECAV, University of Trás-os-Montes e Alto Douro; Departments of [2]Immunology and [5]Pathology Portuguese Institute of Oncology, 4200-072, Porto; [3]Fernando Pessoa University, Porto, Portugal; [4]Department of Physiology, Veterinary Faculty, University of Santiago de Compostela, Lugo, Spain; [6]Department of Molecular Biology and Immunology, ICBAS, Porto University, Porto, Portugal

Abstract. *N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced urothelial carcinogenesis is a useful model for studying urothelial carcinogenesis. Here, the DNA content and the expression of Ki-67 and p53 in urothelial lesions induced by BBN and treated with mitomycin C (MMC) and Bacillus Calmette-Guérin (BCG) were investigated. Female Fisher 344 rats were distributed into five groups treated with 0.05% BBN in their drinking water for 20 weeks. Ten animals were used as negative control. Intravesical instillations were performed with MMC, BCG and physiological saline solution (PSS), once per week, for 6 weeks. The animals were sacrificed 1 week after the last intravesical instillation. DNA ploidy analysis was carried out by static cytometry. Ki-67 and p53 were analysed immunohistochemically in paraffin-embedded tissue. The incidence of lesions developed in rats with PSS was greater than in rats instilled with MMC and BCG. The incidence of aneuploidy was lower in tumours treated with MMC and BCG. Low- and high-grade papillary carcinoma treated with MMC and BCG showed a decrease in labelling index and an increase of apoptotic index. The proliferative index was correlated with the apoptotic index ($r=0.438$, $p<0.01$). Significant correlations were also found between the proliferative index and lesion, and the apoptotic index and lesion ($r=0.425$, $p<0.01$ and $r=0.275$, $p<0.01$), respectively. A significant correlation was found between ploidy and the apoptotic index ($r=0.245$, $p<0.05$). Our results provide information on the biological behaviour of chemically-induced bladder tumours treated with MMC and BCG.*

Correspondence to: Paula A. Oliveira, Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal. e-mail: pamo@utad.pt

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Bladder cancer is the ninth most common cancer worldwide (1), but is superficial at presentation in 70 to 80% of patients. Due to the excellent prognosis, initial treatment is usually by transurethral resection (2). However, further treatment is necessary either to eradicate residual malignancy or to lessen the likelihood of recurrence and progression (3). Intravesical chemotherapy with mitomycin C (MMC) and intravesical immunotherapy with Bacillus Calmette-Guérin (BCG) have been used intravesically to treat bladder cancer (3, 4).

Rodent carcinogenesis models featuring the production of urinary bladder carcinomas by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) are well established (5, 6). BBN is a genotoxic compound with potent and selective organotropic carcinogenic activity, inducing the development of neoplastic lesions in the urothelium of several animals (7-9). This substance, administered in drinking water to rats, induces urothelial tumours that morphologically resemble their human counterparts (10).

In recent years, many techniques have been used to predict the behaviour of urothelial tumours. The combination of DNA image cytometry with immunohistochemistry could lead increased knowledge of the efficacy of BCG and MMC in the treatment of chemically-induced bladder cancer. DNA image cytometry measurement of DNA ploidy has provided useful information regarding the biological behaviour of a vast range of human and animal neoplastic and preneoplastic lesions (11, 12). Different authors have investigated the influence of distinct antigens in bladder cancer (13). One of

the most common markers used is the Ki-67 labelling index. Ki-67 is a nuclear protein present during the G1-, S-, G2- and M-phases of cycling cells (14). Several studies have consistently shown that the labelling index, measured by Ki-67 immunoexpression, correlates with the growth of many tumours, including bladder cancer (15). The p53 tumour suppressor gene encodes for a protein that plays an important role in cell cycle regulation and apoptosis mechanisms (16). Gene mutations in this tumour suppressor gene result in the production of an altered protein, which does not function correctly or have a prolonged half-life (17). Immunohistochemical p53 detection is correlated with the amount of mutated p53 gene (16).

The aims of the current study were: a) to examine the effects of intravesical instillations of MMC and BCG on the development of bladder tumours induced in rats by BBN; b) to study the DNA content in lesions induced by BBN and treated with BCG and MMC; c) to characterise the expressions of Ki-67 and p53 in the urothelial tumours induced by BBN and treated with BCG and MMC.

Materials and Methods

Animals, diet, carcinogen and experimental design. The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direcção Geral de Veterinária, Approval no. 520/000/000/2003). Sixty-seven female Fisher 344 rats were obtained at the age of 5 weeks from Harlan (Amsterdam, Netherlands). The rats were used in this study after a week of acclimatization. They were randomly housed 5 to 7 in a plastic cage, with hard wood chips for bedding and were maintained on a basal diet (Harlan Teklad, Global Diet). The room temperature and the relative humidity were controlled at 22±3˚C and 60±10%, respectively. Fluorescent lighting was provided in a 12-h light/dark cycle. BBN was purchased from Tokyo Kasey Kogyo (Japan) and administered in the drinking water, in light impermeable bottles, at a concentration of 0.05% to groups 1, 3, 4, 5 and 6. Group 2 served as the control without any chemical supplement. The experimental design is presented in Figure 1. The animals were observed daily. Body weights, as well as food consumption, were measured weekly. The drinking solution was changed twice per week and the volume that had been drunk was recorded. To evaluate histological changes induced in the urothelium by BBN treatment alone, the rats from group 1 were sacrificed 20 weeks after initial exposure. At the same time, animals from the control group were also sacrificed. The rats were sacrificed with 0.4% sodium pentobarbital (1 ml/Kg, intraperitoneal). The time of BBN exposure was based on previous studies (18). Group 6 was exposed to BBN for 20 weeks and was maintained with normal tap water until the end of the experiment. Groups 3, 4 and 5 were then maintained without any treatment for 1 week after which began intravesical instillations, once a week for 6 weeks, with MMC, BCG and physiological saline solution (PSS), respectively. Briefly, the animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). Before and 1 h after the instillation, micturition of the rats was induced by light abdominal massage so that the duration of exposure was constant. The

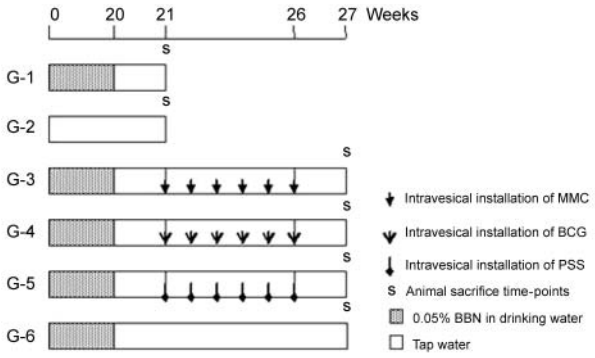

Figure 1. *Experimental design (G, group; MMC, mitomycin C; BCG, Bacillus Calmette-Guérin; PSS, physiological saline solution).*

bladder was catheterized *via* the urethra with an 18-gauge plastic intravenous cannula. All the experiments described herein were carried out under aseptic conditions. The rats were turned 90 degrees every 15 min to facilitate whole bladder exposure to the substances intravesically instilled. Body temperature was maintained with a homoeothermic bandage. After recovery from the anaesthesia, the rats were placed in individual standard cages for 12 h and were monitored daily for haematuria.

For intravesical immunotherapy, lyophilised Connaught BCG strain was kindly provided by INIBSA (Portugal), each vial containing 81 mg of lyophilised Connaught BCG with at least $1.8x10^8$ colony-forming units. Companhia Portuguesa de Higiene (Portugal) kindly provided the MMC (Mitomicina-C Kyowa). Both compounds were reconstituted with PSS, according to the manufacturer's instructions. Dosing schedules were based on those commonly used in clinical work; 300 μl of MMC solution (1 mg/1 ml) and 300 μl of BCG solution (81 mg/3 ml) (19, 20) were used.

All surviving animals were sacrificed 1 week after the last instillation by intraperitoneal administration of sodium pentobarbital. Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated *in situ* by injection of 10% phosphate-buffered formalin (1 ml), ligated around the neck to maintain proper distension and then were immersed in the same solution for 12 h. After fixation, the formalin was removed; the urinary bladder was weighed and cut into four strips and was routinely processed for haematoxylin and eosin staining.

Histopathological analysis. All sections were reviewed by two researchers and the urothelial lesions staged by the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (21). The urothelial lesions were categorized as simple hyperplasia, nodular hyperplasia, dysplasia, carcinoma *in situ* (CIS), papillary tumours, papillary neoplasm of low malignant potential, low-grade papillary carcinoma, high-grade papillary carcinoma, invasive carcinoma, spinocellular carcinoma or squamous metaplasia.

Quantification of DNA content. The ICM methods and the DNA histogram analysis used were previously described in detail (12) and are summarized here. For each lesion, the G0/G1-peak visually

Table I. *Average body weight, food and water intake for female Fisher 344 rats exposed to BBN followed by MMC, BCG and PSS intravesical instillation.*

Group	Initial body weight (g)	Final body weight (g)	Initial food intake (g/rat/day)	Final food intake (g/rat/day)	Initial water intake (g/rat/day)	Final water intake (g/rat/day)
1	130.57±5.09[a]	215.23±9.28	11.87	10.56	18.97	19.77
2	131.29±5.39	209.71±7.15	11.92	9.96	18.76	19.78
3	132.20±5.83	203.70±20.66	11.82	8.69	22.75	19.59
4	132.36±10.27	196.23±15.44*	11.87	8.80	21.16	18.92
5	130.17±8.48	188.33±16.70**	10.67	7.78	17.63	17.21
6	132.25±11.14	217.48±10.59	9.97	10.98	17.50	18.54

[a]Values are mean values ± SEM(standard errors of the mean).*,**Statistically different from group 6 ($p<0.05$).

identified, mean, standard deviation (SD) and the coefficient of variation (CV) values were calculated. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of the mean DNA content of the urothelial G0/G1-peak divided by the mean DNA content of the resting diploid lymphocyte G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values above 5 n. Lesions were considered aneuploid only if a separate G0/G1-peak was distinguishable on the histogram and if it differed from the reference lymphocyte population by more than 2 SD, (two standard deviations). A DNA diploid lesion showed a single distinct G0/G1-peak with a DI within 2 SD from the control lymphocytes and usually with less than 1% of 5cER.

Immunohistochemistry. The expression of markers was detected using the three-step streptavidin-biotin immunoperoxidase method. The mouse monoclonal antibody Ki-67 (1:20, M 7248, DakoCytomation) was used to identify proliferative activity. The p53 expression was performed using AB-1 monoclonal antibody (1:50; MS-104-PO, Neomarkers-Labvision). Tissue sections from paraffin-embedded tissue were deparaffinised in xylene, rehydrated through a down-graded alcohol series and washed with phosphate-buffered saline (PBS). To improve antigen detection, the sections were pre-treated in a microwave oven (700 W) for 20 min in a 10 mM citrate buffer pH 6.0 or EDTA buffer pH 8.0, for Ki-67 and p53, respectively. After cooling, the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distilled water for 30 min to block endogenous peroxidase activity. Non-specific staining was eliminated by 30-min incubation with normal rabbit serum (X 0902, DakoCytomation). Excess normal serum was removed, replaced by the primary antibodies used and incubated overnight (4˚C) in a humidified chamber. After washing the slides, the sections were incubated with a 1:400 dilution of biotin-labelled anti-mouse (E 0354, DakoCytomation) secondary antibody followed by the streptavidin-biotin-complex (TS-125-HR, Labvision Corporation) for 10 min at room temperature. Subsequently, the colour was developed with 3,3'-diaminobenzidine tetrahydrochloride with H_2O_2 in PBS buffer for 7 min. Slides were counterstained with Gill's haematoxylin, were dehydrated and mounted. Primary antibodies and biotinylated secondary antibodies were diluted in PBS. Negative controls were carried out by replacing the primary antibody with PBS. Paraffin sections from colon and breast cancer with known immunoreactivity to p53 and Ki-67 antigens, respectively, were used as positive controls.

For each case, positively-stained tumour cells within five microscopic fields with the highest immunoreactivity ("hot spot" areas) were counted at high magnification (400 X) using a 10x10 grid. The presence and location of the lesions were carefully controlled to guarantee that the Ki-67 and p53 sections contained the same lesion. The Ki-67 labelling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 1000 cells per lesion were examined. The reaction for p53 was considered positive when more than 10% of tumour cells exhibited strong diffuse immunostaining (22). The apoptotic index (AI) was calculated as the percentage of positive nuclei, based on an average of 1000 nuclei counted per lesion. All sections were blind analysed twice.

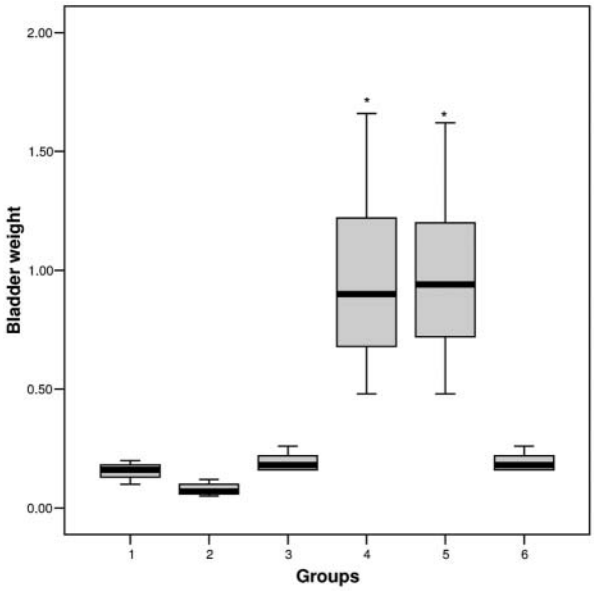

Figure 2. *Bladder weights in the different animal groups. *Statistically different from groups 3 and 6 ($p<0.05$).*

Analysis and statistics. A descriptive study was performed for all variables included. The statistical analysis was done using the SPSS 12.0 statistical package for Windows. The differences between the mean values of body weight, relative bladder weight, LI and AI were evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. The differences in the incidence of lesions between

Table II. *Incidence of urothelial lesions in female Fisher 344 rats exposed to BBN and treated with MMC, BCG and PSS.*

Number of animals (%)												
Group (n)	Urothelium	Simple hyperplasia	Nodular hyperplasia	Dysplasia	CIS	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma	Invasive carcinoma	Spinocellular carcinoma	Squamous metaplasia
1 (12)	0	2(16.6)	10(87.3)	10(87.3)	0	4(33.3)	4(33.3)	10(83.3)	2(16.6)	2(16.6)	1(8,3)	6(50)
2 (10)	10(100)	0	0	0	0	0	0	0	0	0	0	0
3 (10)	0	1(10)	9(90)	10(100)	7(70)	0	3(30)	9(90)	8(80)	2(20)	0	5(50)
4 (8)	0	3(37.5)	8(100)	8(100)	0	1(12.5)	4(50)	7(87.5)	7(87.5)	2(25)	0	7(70)
5 (11)	0	1(9)	11(100)	11(100)	0	0	4(36.7)	11(100)	11(100)	5(45.4)	3(27.3)	10(91)
6 (10)	0	2(20)	10(100)	10(100)	0	3(30)	6(60)	10(100)	9(90)	1(10)	0	8(80)

PNLMP, papillary neoplasm of low malignant potential; CIS, carcinoma *in situ;*
BBN, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine; MMC, mitomycin C; BCG, Bacillus Calmette-Guérin; PSS, physiological saline solution.

the groups were assessed by Fisher's exact probability test or the Chi-square test. The Pearson correlation was used to evaluate the association of markers with each other, with the lesion and with ploidy. A $p<0.05$ value was accepted as statistically significant.

Results

General animal condition. Six rats from groups 3, 4 and 5 died during the instillation therapy and were therefore excluded from analysis. Animals surviving the 27 weeks were included in the effective numbers for histopathological examination. The initial and final average body weights, food and water intakes are summarised in Table I. The body weights of the rats in the groups treated with BCG and PSS (groups 4 and 5) were less than those of group 6. The mean food and water consumption were less in animals treated with PSS.

The relative bladder weights are summarized in Figure 2. The bladder weights in group 1 did not differ from those of group 2. The urinary bladder weights were significantly higher in groups 4 and 5 than those in groups 3 and 6.

Macroscopic and microscopic evaluation. Greyish-white urinary bladder masses, varying in size from less than 1 mm to masses that filled the entire lumen, were observed, those in groups 4 and 5 being the largest. The lesions were pedunculated with irregular surfaces and areas of necrosis, haemorrhage and focal ulcerations. The lesions were distributed randomly throughout the entire urinary bladder. Stone formation in the urinary bladder was not observed in any rat. No bladder mucosal lesions were seen that could be attributed to mechanical damage during intravesical instillation. The incidence of histopathological lesions in each group is shown in Table II. No histopathological changes in urothelial cells were observed in the control group. Simple and nodular hyperplasia and dysplasia were observed in all the bladders of the BBN-treated rats and the incidence of these lesions was approximately equal in the different groups. Seven animals in the group treated with MMC (group 3) had carcinoma *in situ* at the time of sacrifice. The incidence of tumours in rats treated with PSS was higher than in groups treated with MMC and BCG, although no statistical significance was observed. The incidence of both low- and high-grade papillary carcinoma decreased in groups 3 and 4, but there was no statistically significant difference between them and that in groups 5 and 6. The histopathological findings are shown in Figure 3. Histopathological inflammatory reaction, mainly lymphocytic aggregates, were observed in the submucosal layer of the groups 1, 3, 4, 5 and 6. Mast cells were present in all the animals from groups 1, 3, 4, 5 and 6. Populations of eosinophils and neutrophils were observed in the animals treated with BCG. No macroscopic or microscopic changes were seen in the liver, lung, kidney or gastrointestinal tract.

DNA content. ICM DNA analysis was successfully performed on 155 lesions and ten normal urothelia. The results for the DNA content obtained by image cytometry analysis are presented in Table III. The degree of aneuploidy in rats treated with PSS was greater than in those treated with MMC and BCG.

Two representative histograms obtained by ICM are shown in Figure 4. Despite the period of observation and the kind of treatment, the frequency of the DNA aneuploid pattern increased with the degree of tissue transformation ($p<0.01$) between high-grade papillary carcinoma when compared to normal urothelium, simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, PNLMP and low-grade papillary carcinoma (Figure 5).

Immunohistochemistry. The normal urothelium in the control groups was uniformly negative for p53 and Ki-67

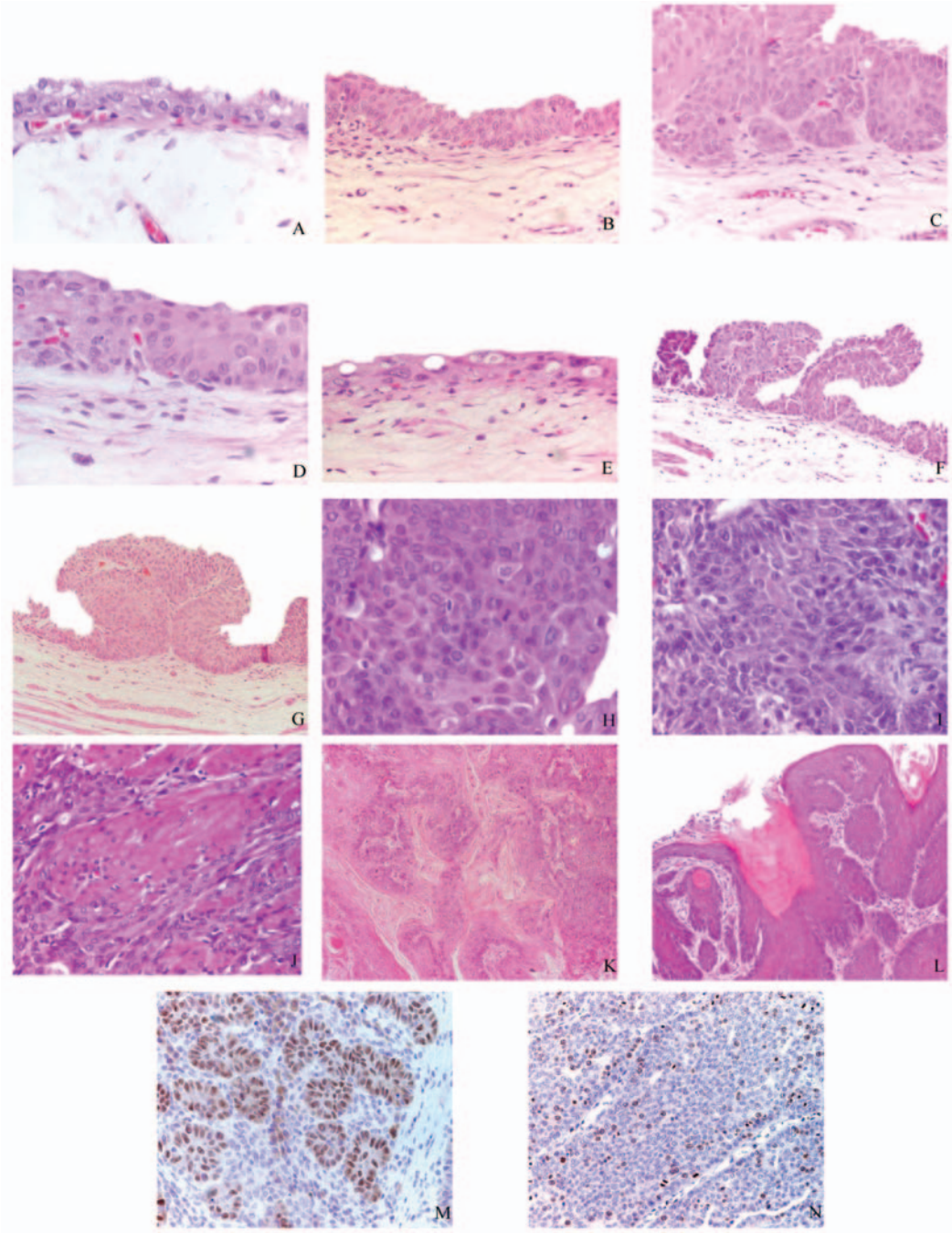

Figure 3. *Histopathological representation of lesions identified in rat urothelial carcinogenesis. A) Normal rat urothelium (Control group, H&E, 400x); B) simple hyperplasia (H&E, 400x); C) nodular hyperplasia (H&E, 400x); D) dysplasia (H&E, 600x); E) carcinoma in situ (H&E, 400x); F) papilloma (H&E, 200x); G) papillary neoplasms of low malignant potential (H&E, 200x) H) low-grade papillary carcinoma (H&E, 200x); I) high-grade papillary carcinoma (H&E, 600x); J) invasive carcinoma (H&E, 400x); K) spinocellular carcinoma (H&E, 200x); L) squamous metaplasia (H&E, 200x); M) p53 expression on low-grade papillary carcinoma (400x); N) Ki-67 expression on low-grade papillary carcinoma (200x).*

expressions. In terms of the Ki-67 and p53 immunoreactivity, 124 lesions showed positive immunoreactivity for both antibodies. Ki-67 immunostaining was evident as diffuse or dot-like nuclear and nucleolar staining (Figure 3N). p53 protein staining was confined to the nuclei (Figure 3M). The LI and AI were different within the various groups, showing the lowest values in group 1 and the highest in the group treated with PSS. The mean values of

Table III. *DNA content results regarding the histological pattern and animal group.*

Group	Histological pattern	Diploid n (%)	Aneuploid n (%)
	Simple hyperplasia (2)	2(100)	0
	Nodular hyperplasia (3)	2(66.7)	1(33.3)
	Dysplasia (6)	4(66.7)	2(33.3)
	Papilloma (4)	3(75)	1(25)
1	PNLMP (4)	2(50)	2(50)
	Low-grade papillary carcinoma (4)	3(75)	1(25)
	High-grade papillary carcinoma (2)	0	2(100)
	Spinocellular carcinoma (1)	1(100)	0
	Squamous metaplasia (2)	1(50)	1(50)
2	Normal urothelium (10)	10(100)	0
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (2)	2(100)	0
	Dysplasia (3)	2(66.7)	1(33.3)
	CIS (7)	0	7(100)
3	PNLMP (1)	0	1(100)
	Low-grade papillary carcinoma (3)	2(66.7)	1(33.3)
	High-grade papillary carcinoma (6)	0	6(100)
	Invasive carcinoma (1)	0	1(100)
	Simple hyperplasia (3)	3(100)	0
	Nodular hyperplasia (4)	4(100)	0
	Dysplasia (8)	3(37.5)	5(62.5)
	Papilloma (1)	1(100)	0
4	PNLMP (2)	1(50)	1(50)
	Low-grade papillary carcinoma (8)	7(87.5)	1(12.5)
	High-grade papillary carcinoma (7)	0	7(100)
	Invasive carcinoma (1)	0	1(100)
	Squamous metaplasia (2)	2(100)	0
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (6)	4(66.7)	2(33.3)
	Dysplasia (4)	1(25)	3(75)
5	PNLMP (2)	2(100)	0
	Low-grade papillary carcinoma (7)	4(57.1)	3(42.9)
	High-grade papillary carcinoma (7)	0	7(100)
	Invasive carcinoma (2)	0	2(100)
	Squamous metaplasia (5)	4(80)	1(20)
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (4)	4(100)	0
	Dysplasia (3)	2(66.7)	1(33.3)
6	PNLMP (4)	2(50)	2(50)
	Low-grade papillary carcinoma (10)	8 (80)	2(20)
	High-grade papillary carcinoma (8)	0	8(100)
	Squamous metaplasia (3)	3(100)	0

PNLMP, papillary neoplasm of low malignant potential; CIS, carcinoma *in situ*.

LI and AI for each lesion evaluated are summarised in Table IV. The measurements (performed twice) of LI and AI were very well correlated ($r=0.95$, $p<0.001$).

The mean values of LI and AI for each lesion, regardless of the kind of treatment, are summarised in Figure 6. The highest values of LI and AI were observed in high-grade papillary tumours. Statistically, the proliferation index was correlated with AI; the Pearson correlation of these two markers was $r=0.438$ ($p<0.01$). A significant correlation was found between both LI and histological lesion ($r=0.452$, $p<0.01$) and AI and histological lesion ($r=0.275$, $p<0.01$). The correlation between ploidy and AI was $r=0.245$ and $p<0.05$.

Discussion

The histopathogenesis of BBN chemically-induced urothelial tumours has been well delineated (7, 8, 10). However, relatively little is known regarding the effects of MMC and BCG in the development of rat urothelial carcinogenesis. In this study, animals treated with PSS had the lowest body weight; they also showed a lower water and food consumption compared to the other groups. The bladder weights in groups 4 and 5 were significantly higher than in groups 1, 2, 3 and 6. The difference in bladder weight seems to be due to the different development of the bladder tumours.

The results of the present study indicated that both MMC and BCG exerted therapeutic effects against BBN-induced urinary tumorigenesis in rats. Inhibition of tumour growth occurred in animals treated with MMC and BCG compared to animals treated with PSS. These results are similar to those observed in C3H/He mice (23). The vehicle-treated animals (PSS) showed an increased incidence of low- and high-grade papillary carcinoma, invasive carcinoma and spinocellular carcinoma. Other researchers (18, 24) reported the promoter activity of PSS in urothelial tumours induced by BBN.

Intravesical therapy is an important adjuvant therapy after transurethral resection in patients with superficial bladder cancer and for the treatment of superficial bladder tumours (3, 4). MMC and BCG are often used as intravesical therapy, because these drugs cause little systemic toxicity and result in high concentrations in the bladder, in direct contact with tumours (10). There are several reports that intravesical instillation of MMC and BCG is effective for eradicating tumours and for inhibiting their recurrence in experimental models (25-27).

In the present work, the histological pattern classified as CIS was only found in the animal group treated with MMC. Szende *et al.* also identified CIS in female rats treated with MMC (28). Some authors reported a co-carcinogenic activity of MMC on urothelial carcinogenesis (29, 30). In a previous study by our group, the mitogenic and

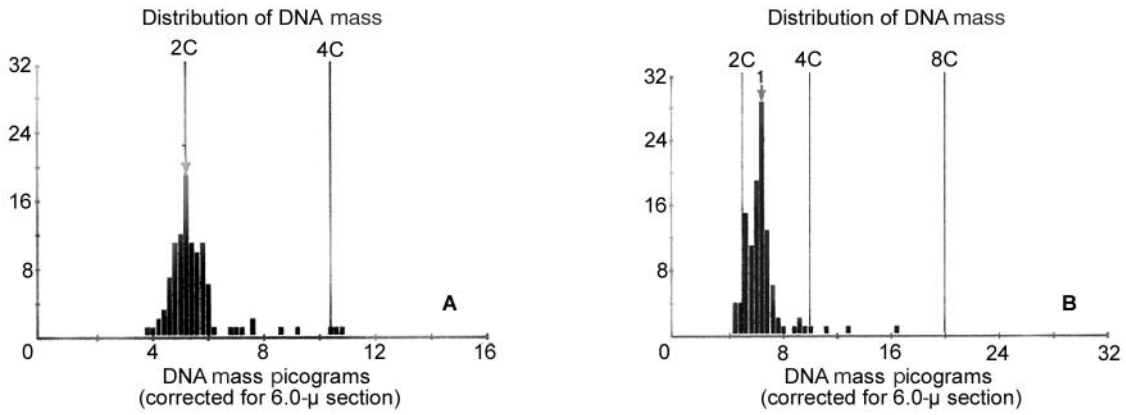

Figure 4. *(A) Image analysis DNA histogram showing a DNA diploid cell population. (B) Image analysis DNA histogram showing a DNA aneuploid cell population.*

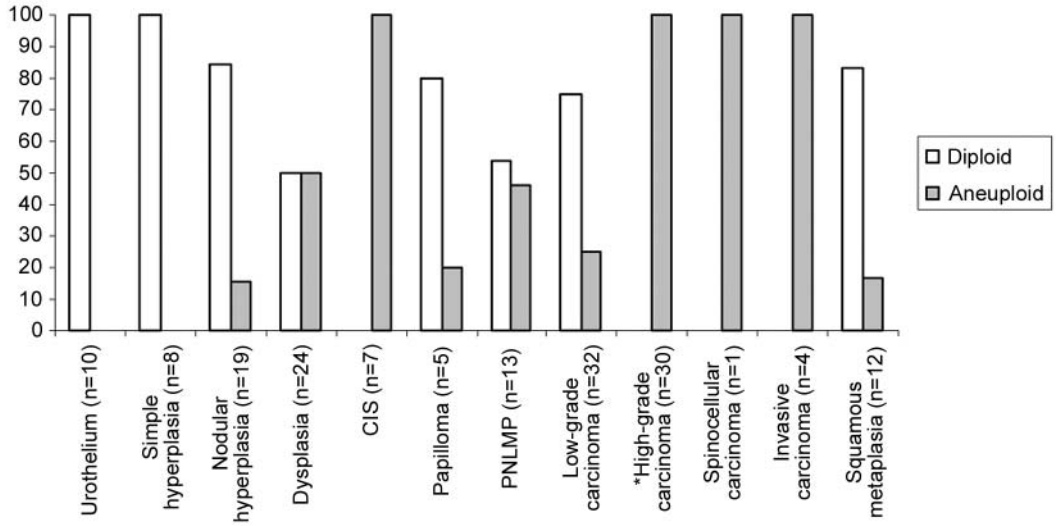

Figure 5. *DNA ploidy observed in the different histological patterns analysed. *Statistically different from normal urothelium, simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, PNLMP and low-grade papillary carcinoma ($p<0.01$). CIS, carcinoma in situ; PNLMP, papillary neoplasm of low malignant potential.*

aneuploidogenic effects of MMC in rat normal urothelium were reported. More studies should be done for better understanding the biological mechanism of MMC therapy (submitted for publication by Oliveira *et al.*, 2005).

The DNA content of the urinary bladder lesions identified after intravesical instillation with MMC, BCG and PSS exhibited differences. The incidence of aneuploidy in rats treated with PSS was greater than in those treated with MMC or BCG. Lesions with morphologically identical cells sometimes have different biological behaviours. The use of ICM, by an individual cell evaluation, is a suitable method to detect the biological aggressiveness of a given lesion. Our results demonstrated that ICM provides more reliable results than classic histological methods. In this study, the evaluation of DNA ploidy in relation to the histopathological classification showed an aneuploid pattern with increasing frequency from simple hyperplasia to invasive carcinoma, as previously described in humans and mice (11, 12). During the progression of urothelial cell carcinoma, a selection of those cells with gains of genetic material seems to occur, conferring the DNA aneuploid status (31). Several preneoplastic lesions were aneuploid and these lesions frequently co-exist with bladder cancer and are hardly visible in humans during cytoscopy or may be concealed in the normal-appearing bladder mucosa. However, these lesions are of considerable importance in the recurrence and progression rates of bladder cancer. The use of ICM as a biomarker is biologically interesting since it reflects the

Table IV. *Association between LI and AI in the different urothelial lesions and experimental groups.*

Histological pattern	Group 1	Group 3	Group 4	Group 5	Group 6
Nodular hyperplasia					
LI	23.72±12.96[a]	21.71±0.51	13.59±5,82	23.71±5.24	20.10±11.31[b]
AI	28.50±0.70	35.50±3.53	30.50±2.88[b,c]	33.60±11.10	32.00±0.1[b]
Dysplasia					
LI	9.40±1.98	21.31±3.76	14.00±2.94[b]	22.15±6.43	23.50±0.70
AI	35.50±9.19	41.00±7.00	30.50±2.88[b,c]	36.00±4.96	45.00±1.41[e]
PNLMP					
LI	17.93±3.44	18.55±0.97	15.00±1.41	15.25±5.86[b]	21.33±3.21[b]
AI	23.33±3.05	33.00±1.41[f]	28.00±1.41[b,c]	21.50±0.70[b]	28.00±2.00[b]
Low-grade papillary carcinoma					
LI	17.58±3.46	29.41±4.95	20.80±6.57[e]	30.21±8.27	28.00±3.34
AI	35.66±5.89	39.66±5.77	42.28±3.19[g]	36.66±8.81	35.00±3.98[b]
High-grade papillary carcinoma					
LI	–	38.07±9.91	28.50±8.11	35.28±4.26	34.62±2.05
AI	–	45.60±6.42	50.00±4.85[d]	39.57±10.32	44.00±3.80
Invasive carcinoma					
LI	–	16.88±1.33	17.98±2.51	23.94±0.55	–
AI	–	26.83±2.30	27.50±2.12[b,c]	38.85±0.21[g]	–
Squamous metaplasia					
LI	24.92±9.74	32.11±4.08	31.05±2.76	31.79±8.71	32.40±5.82
AI	28.00±6.73	36.50±0.70	47.00±2.82	40.72±3.36	41.80±7.46

[a]values are mean values ± SEM (Standard errors of the mean); LI, labelling index; AI, apoptotic index; PNLMP, papillary neoplasm of low malignant potential; PSS, physiological saline solution. [b]Statistically different from high-grade papillary carcinoma. [c]Statistically different from high-grade papillary carcinoma. [d]Statistically different from all other lesions. [e]Statistically different from PNLMP. [f]Statistically different from animals treated with PSS. [g]Statistically different from animals treated with BCG.

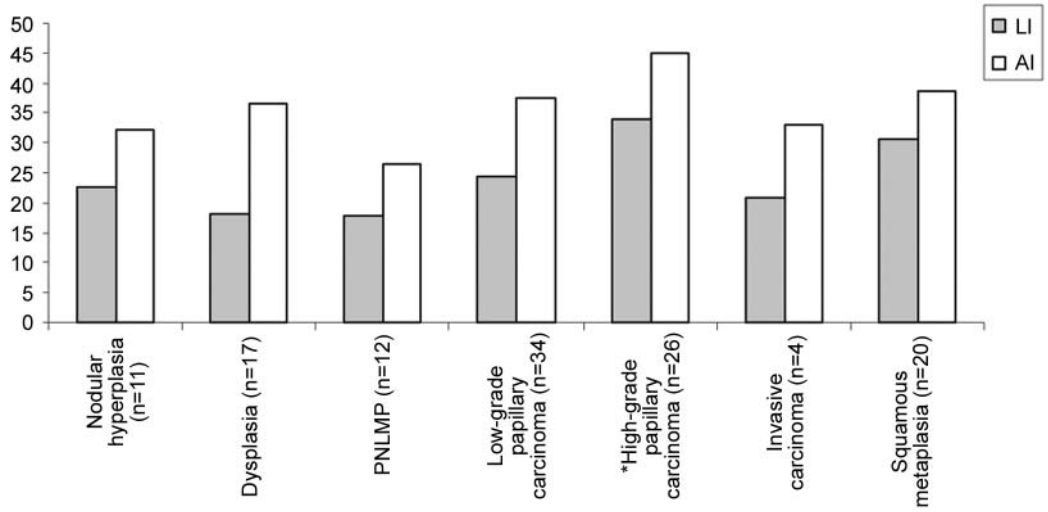

Figure 6. *Mean values of LI (labelling index) and AI (apoptotic index) in each lesion evaluated. *LI and AI statistically different from preneoplastic lesions and other tumours except squamous metaplasia ($p<0.05$). PNLMP-, papillary neoplasm of low malignant potential.*

phenotypic expression of malignancy, is reproducible and is easily applicable in routine practice (17, 32, 33).

It is interesting to note that exposure of the urothelium to MMC and BCG affected the expression of tumour markers. The immunoexpressions of Ki-67 and p53 differed between groups treated with BCG, MMC and PSS. In general, rats treated with MMC and BCG showed the lowest LI values compared to those instilled with the vehicle, suggesting that these compounds decrease cell proliferation. After MMC and BCG intravesical therapy, AI increased particularly in the high-grade papillary carcinoma. Thus, MMC and BCG intravesical therapy may act throughout the induction of

apoptosis and high-grade papillary carcinoma cells may be much more sensitive to this therapy.

We observed a statistically significant concomitant increase in Ki-67 and p53 expression patterns with the degree of tissue transformation. Ki-67 expression was studied in tumours of different organs and was shown to correlate with the histopathological pattern (34). p53 nuclear accumulation in bladder carcinomas has been reported to be correlated with the labelling index (35, 36). The correlation between apoptosis and proliferation in tumour tissues is important, since the rate of tumour cell accumulation is not only dependent on the rate of cell death, but also on the rate of tumour cell proliferation (22).

In conclusion, our results revealed that repeated instillation of MMC and BCG decreased tumour incidence in the urothelium of rats exposed to BBN. These lesions presented a low LI, a high AI and the incidence of DNA aneuploidy decreased. On the contrary, untreated animals showed a high incidence of bladder tumours with the highest LI and lowest AI and in this group the incidence of aneuploidy increased. The urothelium of rats fed BBN seemed to be in a phase of carcinogenesis sensitive to intravesical chemotherapy and immunotherapy.

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