

Research Paper

Anti-inflammatory, anti-proliferative and antioxidant profiles of selective cyclooxygenase-2 inhibition as chemoprevention for rat bladder carcinogenesis

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BW, body weight; CEL, celecoxib; CIS, carcinoma in situ; COX, cyclooxygenase; CRP, C reactive protein; FRAP, ferric reducing antioxidant potential; HCT, haematocrit; HGB, hemoglobin; H&E, hematoxylin & eosin; IL-1 β , interleukin-1 β ; MDA, malondialdehyde; NSAIDs, nonsteroidal anti-inflammatory drugs; PCT, plaquetocrit; PLT, platelet count; PPARs, peroxisome proliferator-activated receptors; RBCs, red blood cell count; RET, reticulocyte count; ROS, reactive oxygen species; TAS, total antioxidant status; TBARs, thiobarbituric acid reactive-species; TCC, transitional cell carcinomas; TGF β 1, transforming growth factor β -1; TNF α , tumor necrosis factor α ; WBC, white blood cells

Key words: bladder cancer, chemoprevention, cyclooxygenase-2 inhibition, inflammation, proliferation, oxidative stress, rat model

Purpose: To evaluate the efficacy of a selective cyclooxygenase-2 (COX-2) inhibitor in rat bladder cancer chemoprevention, as well as to assess the relevance of inflammation, proliferation and oxidative stress in tumor growth and in its prevention.

Results: The main findings were: (I) the incidence of carcinoma was: control: 0% (0/8); BBN: 65% (13/20); CEL: 0% (0/8) and BBN + CEL: 12.5% (1/8); (II) the mean tumor volume per rat with tumor and per tumor were significantly lower in the BBN + CEL group (21.2 and 5.3 \pm 0.4 mm³) vs. BBN (138.5 \pm 7.5 and 112.5 \pm 6.4 mm³); (III) the incidence of pre-neoplastic (hyperplasia and dysplasia) and neoplastic (papillary tumors and carcinoma in situ—CIS) lesions were notoriously reduced in the CEL + BBN treatment; (IV) CEL significantly reduced serum TGF β 1 and CRP and increase TNF α and IL-1 β ($p < 0.001$); (V) CEL reduced MDA formation in serum ($p < 0.001$) and liver ($p < 0.05$) and also showed a trend to reduction in kidney.

Methods: Drug treatments were performed during the first 8 w, followed by 12 w for tumor expression/prevention, in the following groups: control—vehicle; carcinogen—0.05% of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN); celecoxib

(CEL)—10 mg/kg/day and preventive CEL + BBN. The bladders were analyzed for number and volume of tumor and nature of urothelium lesions. Serum was assessed for markers of inflammation, proliferation and redox status.

Conclusions: Celecoxib has demonstrated an outstanding inhibitory effect on bladder cancer chemoprevention, which might be due to its expected anti-inflammatory actions, as well as by anti-proliferatory and antioxidant actions. This data supports a pivotal role of cancer chemoprevention strategies based on COX-2 inhibition.

Introduction

Bladder cancer remains a huge concern for the medical community because of its malignancy, as well as high mortality, incidence and prevalence rates.^{1,2} Furthermore, recurrence rates are also high, explaining that prevalence exceeds the primary incidence.^{3,4} It is the fourth most common tumor in men and the eighth in women, accounting for 5–10% of all malignancies in Western countries.² Excepting for superficial forms, bladder cancer prognosis is poor, particularly when lately diagnosed or inadequately treated, and is associated with high socioeconomic cost.^{1,5,6} Despite the advances in medical care, the conventional methods of surgery, chemotherapy and radiotherapy have not impacted greatly on the general morbidity and mortality.^{7,8} Thus, preventive strategies are crucial for the management and treatment of bladder cancer, which will depend on a better elucidation of the mechanisms underlying cancer lesions development. Exogenous factors, such as cigarette smoking, which accounts for a huge percentage of cases, as well as

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occupational carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons, are important determinants of the disease appearance.^{9,10} However, apart from the genetic features already characterized,¹¹ the cellular and molecular mechanisms might involve inflammatory, proliferative and oxidative stress phenomenon that should be better elucidated.

The carcinogen-induced model of bladder cancer with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) in rats is a valuable experimental tool to study human cancer development, namely due to similar histological features with the human transitional cell carcinoma.¹² Urothelial transitional cell carcinoma is the predominant pathological lesion. The malignant transformation is a continuous process that includes dysplastic and proliferative epithelial abnormalities, pre-neoplastic changes and malignant lesions (papiloma and carcinoma).^{2,13,14} This model should also be important to evaluate the efficacy of modern therapeutic strategies.¹⁵ An early treatment with drugs that reverse these molecular and morphological pathways might hypothetically prevent the bladder cancer. Pre-clinical studies using green tea polyphenols, soy products, vitamins, selenium and nonsteroidal anti-inflammatory drugs (NSAIDs) demonstrate that bladder cancer is responsive to prevention strategies.^{16,17}

As previously suggested, bladder cancer development might be linked with inflammatory stimuli.¹⁸ The prostaglandin G/H synthase classe, which include the cyclooxygenase-1 (COX-1) and COX-2 isoforms, are key enzymes in the synthesis of prostaglandins from arachidonic acid. COX-1 is expressed in nearly all tissues and is thought to be responsible for the low levels of prostaglandins needed for tissue maintenance and homeostasis. On the contrary, COX-2 is not expressed in most tissues under normal conditions, but expression is rapidly induced by growth factors or agents that cause tissue irritation or inflammation.¹⁹ The role of COX-2 in acute inflammatory processes contribute at multiple points throughout tumorigenesis, including premalignant hyperproliferation, transformation, maintenance of tumor viability, growth, invasion and metastatic spread.²⁰

According to several studies, the modulation of mice COX-2 levels and/or activity, by genetic deletion or chemical inhibition, has decreased tumors development. Therefore, the use of selective COX-2 inhibitors (Coxibs) for chemoprevention was already tested in other types of cancer,²¹⁻²³ but its efficacy in bladder cancer has been poorly investigated.

This work intend to discuss the hypothesis that aberrant induction of COX-2 and upregulation of the prostaglandin cascade play a significant role in carcinogenesis, a process know as "inflammogenesis of cancer." In order to do so, we have used a rat model of bladder cancer to test the chemopreventive effect of a selective COX-2 inhibitor (Celecoxib), as well as to assess the relevance of inflammation, proliferation and oxidative stress in tumor growth and in its potential prevention.

Results

Tumor chemoprevention data. Body weight and beverage consumption. No relevant changes were obtained between the groups during the study concerning body weight and beverage

consumption. However, there was a reduction in beverage consumption (data not shown) in the CEL + BBN rats vs. the BBN animals between the Ti and the Tf (8–20 w), which cannot explain per se the CEL efficacy on cancer chemoprevention because during that period all the animals were only under water consumption (BBN treatment was performed between the T0 and the Ti).

Macroscopic evaluation. All formaldehyde pre-fixed bladders were opened and analyzed macroscopically for wall (urothelium) texture, thickness and vascularization (Fig. 1). The percentage of rats with tumors in each group, the nr of tumors per rat with tumor, as well as the mean tumor volume per rat and per tumor were then evaluated (Table 1). All the bladders from the control animals have revealed a pattern of normality, with absence of any type of malignity. The wall texture, thickness and vascularization were normal (Fig. 1A). Similar profile was found for the CEL group, with limpid, translucent and tiny bladders, without presence of any abnormal mass or vascularization (Fig. 1B). In the BBN group, however, 65.0% (13 in 20) of the rats had bladder tumors, almost all easily seen by macroscopic analysis. The bladder walls were thicker, with new or enlarged small vessels, suggesting neo-angiogenesis, and there was unequivocal formation of tumor, some of them of relevant volume, also visible in the whole-bladder (Fig. 1C and D). In the CEL + BBN group, excepting one case, all bladder shown a similar pattern to those seen for the control or CEL rats, with macroscopically unrecognized signs of abnormality (Fig. 1E and F).

Quantitative evaluation. In the control group, as expected, no rat developed bladder cancer. The same profile was found for the CEL animals. In the BBN group (n = 20 rats), the percentage of rats with bladder cancer was 65.0% (13 in 20), with a mean of 1.2 ± 0.1 tumors per rat with tumor. Furthermore, the mean tumor volume per rat with tumor (in 13 rats) was 138.5 ± 7.5 mm³ and the mean tumor volume per tumor (in 16 tumors) was 112.5 ± 6.4 mm³ (Table 1). In the CEL + BBN group, there was notorious bladder cancer prevention, with a percentage of tumors in the group of 12.5% (1 in 8), which corresponds to only one rat with cancer in the group universe of eight animals. In agreement, the mean tumor volume per tumor (in four tumors of only one rat) was 5.3 ± 0.4 mm³ and the mean tumor volume per tumor was 21.2 mm³ (Table 1).

Qualitative evaluation. Concerning the microscopic analysis of urothelium layer and urothelial tumors, the control and the CEL rats have shown normal patterns (Fig. 2A and B, respectively). The bladder from control animals had no signs of pre-neoplastic lesions (neither hyperplasia nor dysplasia), as well as those from the CEL rats (Table 1). In the carcinogen (BBN) group, there was evident malignant transformation, including hyperplasia (100%) and dysplasia (100%), present in all the animals, including those without tumor formation. Furthermore, there were also malignant lesions, papillary, infiltrative and CIS (Table 1 and Fig. 2C and D). The use of CEL (group CEL + BBN) has notoriously prevented bladder cancer development. Therefore, as above mentioned, only one bladder of the eight rats from the group have shown cancer development (12.5% of the rats from the group) (Table 1). Apart from the rat with tumors (all papillary), whose urothelium has demonstrated hyperplasia and dysplasia, without infiltrative or CIS (Table 1 and Fig. 2E), all the other rats have presented a normal

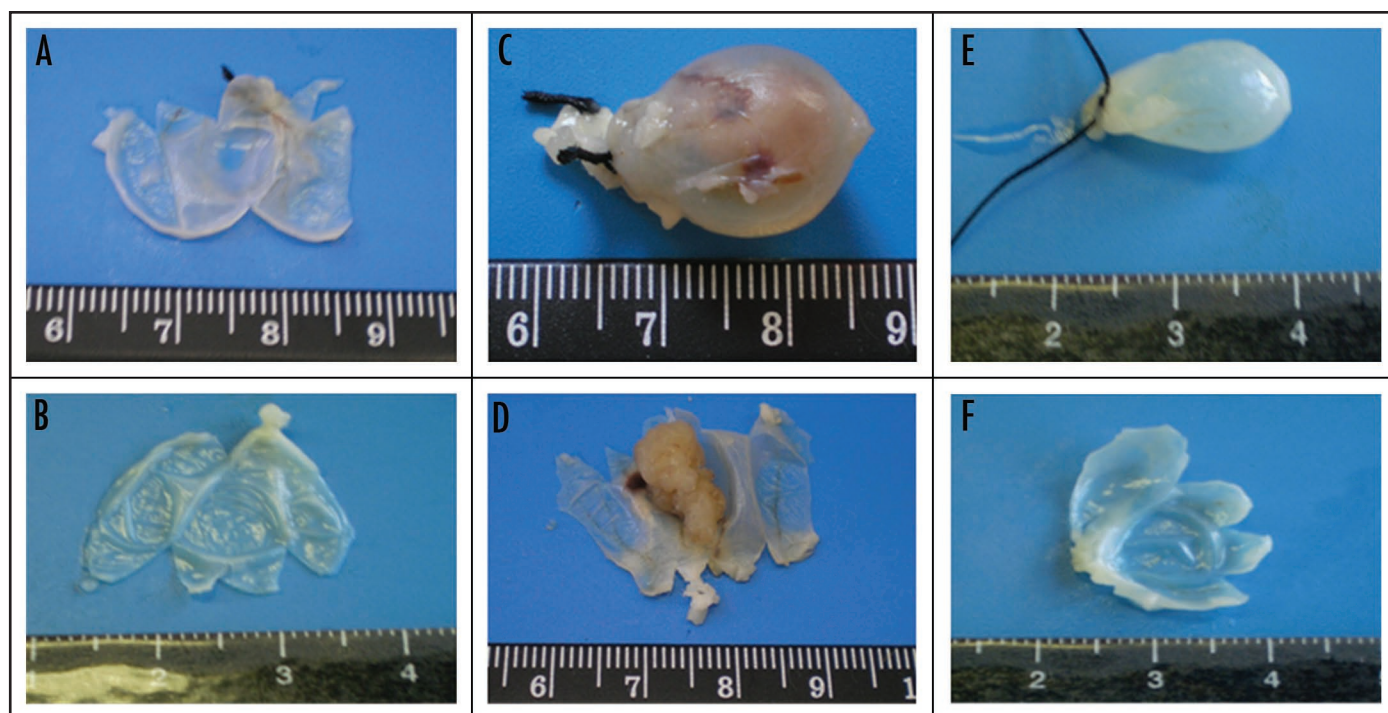


Figure 1. Macroscopic histomorphology. All the bladders from the control (A) and CEL (B) treated-rats have revealed a pattern of total normality. In the BBN group (C and D), 65.0% of the rats had bladder tumors, almost all easily seen by macroscopic analysis. The bladder walls were thicker, with new or enlarged small vessels, suggesting neo-angiogenesis and there was unequivocal formation of tumor. In the CEL + BBN group (E and F), all bladders, excepting one case, shown a macroscopic normal pattern.

Table 1 The effects of a selective cyclooxygenase-2 inhibitor on bladder cancer chemoprevention: Macroscopic (quantitative) and microscopic (qualitative) evaluation of urothelium lesions

Macroscopy (quantitative)	Control (n = 8)	BBN (n = 20)		CEL (n = 8)	CEL + BBN (n = 8)	
Tumor number						
% rats with tumor	0	(13 in 20) 65.0%		0	(1 in 8) 12.5%	
Nr tumors/rat	0	1.2 ± 0.1 (16 in 13)		0	4.0 [†] (4 in 1)	
Tumor volume (mm ³)						
Mean/rat	0	138.5 ± 7.5 (in 13)		0	21.2 [†] (in 1)	
Mean/tumor	0	112.5 ± 6.4 (in 16)		0	5.3 ± 0.4 (in 4)	
Microscopy (qualitative)	Control	BBN		CEL	CEL + BBN	
		Tumor group	Total group		Tumor group	Total group
Pre-neoplastic lesions						
Hyperplasia	0	100 (13/13)	100 (20/20)	0	100 (1/1)	37.5 (3/8)
High-Grade Dysplasia	0	100 (13/13)	75 (15/20)	0	0 (0/1)	0 (0/8)
Low-Grade Dysplasia	0	0 (0/13)	25 (5/20)	0	100 (1/1)	12.5 (1/8)
Malignant lesions						
Papillary tumor	0	100 (13/13)	65 (13/20)	0	100 (1/1)	12.5 (1/8)
Infiltrative tumor	0	15 (2/13)	10 (2/20)	0	0 (0/1)	0 (0/8)
Carcinoma In Situ	0	31 (4/13)	20 (4/20)	0	0 (0/1)	0 (0/8)

urothelium profile (there was only low-grade hypertrophy in two of those seven animals) (Table 1 and Fig. 2F).

The lungs, stomach, liver, kidneys and intestines were normal on gross inspection and on histological examination.

Proliferation, inflammation and redox status profiles. In the BBN group there was a significant increment in serum TGFβ1 (transforming growth factor β-1) ($p < 0.01$) and C-reactive protein (CRP) ($p < 0.001$) when compared with the control group

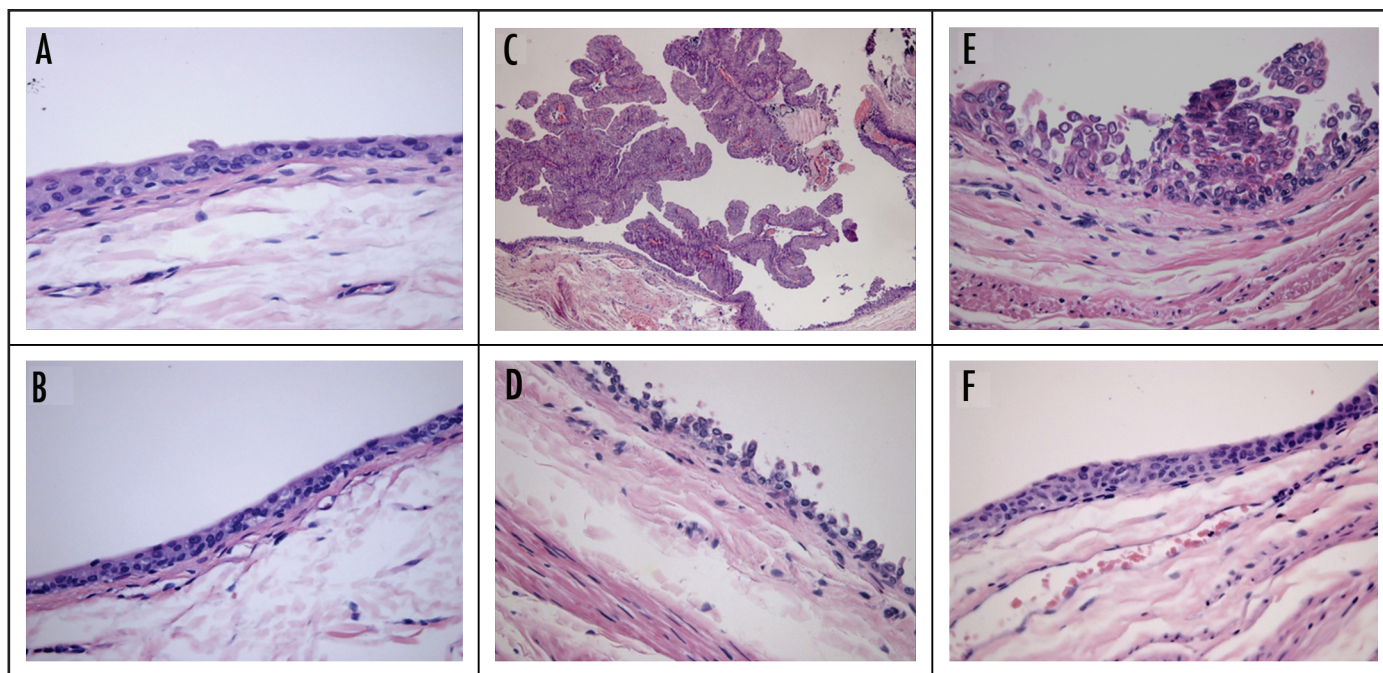


Figure 2. Microscopic histomorphology (H&E). The bladder from control (A) and CEL (B) treated-rats had no signs of pre-neoplastic lesions or gross tumour formation. In the carcinogen group (C and D), the bladder from all the animals presented hyperplasia and high-grade dysplasia, including those without tumor formation, as well as malignant lesions, such as papillary tumors (C) and CIS (D). In the CEL + BBN group (E and F), only one bladder of the eight rats presented tumor formation with dysplasia (E), while all the other rats have presented a normal urothelium profile (F).

(Fig. 3A and C, respectively). Serum TNF α (tumor necrosis factor α) and IL-1 β (interleukin 1 β) contents were unchanged between BBN and control rats (Fig. 3B and D, respectively). The CEL rats showed unchanged values of TGF β 1, increased levels of TNF α ($p < 0.01$) and IL-1 β ($p < 0.05$) and significantly decreased contents of CRP ($p < 0.001$) (Fig. 3). When CEL was given to prevent the bladder cancer, the BBN-induced increment in TNF α and IL-1 β levels was prevented ($p < 0.01$ and $p < 0.001$, respectively), while the other BBN-unchanged markers remained similar to those of the CEL group (Fig. 3).

The liver and kidney MDA (malondialdehyde) content, a lipidic peroxidation marker, was unchanged between the control and the BBN groups (Fig. 4). However, in the CEL rats there was a significant decrease in both liver ($p < 0.05$) and kidney ($p < 0.001$) MDA content. Similar profile was encountered for the CEL + BBN rats (Fig. 4). Serum MDA concentration showed a trend to higher values in the BBN group when compared with the control one. This value was accompanied by a significant increase ($p < 0.05$) in serum total antioxidant status (TAS). In agreement, serum MDA/TAS ratio, a marker of oxidative imbalance, was lower (trend to) than in the control rats. In the CEL animals, serum MDA content was reduced ($p < 0.001$) when compared with the control, TAS was unchanged and, accordingly, MDA/TAS ratio was significantly ($p < 0.05$) reduced. Similar serum antioxidant protection was shown for the CEL + BBN group vs. BBN ($p < 0.001$) (Fig. 4).

Biochemical and hematological data. In the BBN group, there were a trend to lower values of urea and higher of uric acid, when compared with the control group, without significant changes of

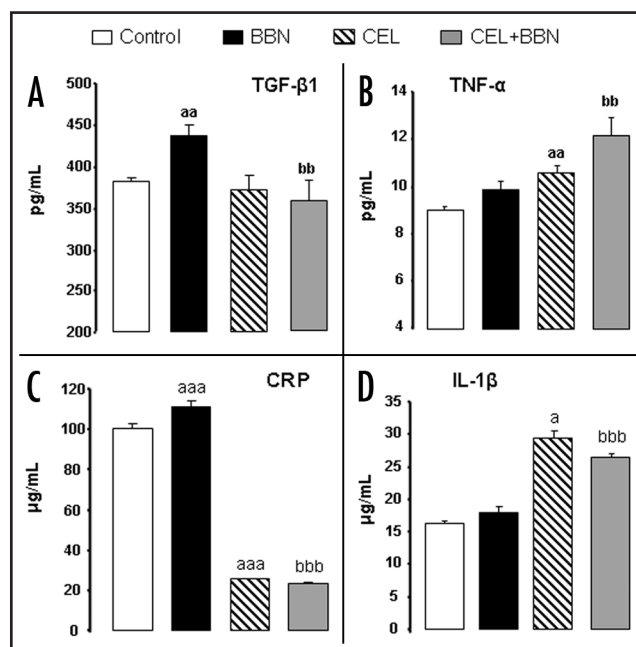


Figure 3. Serum markers of proliferation and inflammation: TGF β 1 (A), TNF α (B), CRP (C) and IL-1 β (D). Values are mean \pm s.e.m. ^a $p < 0.05$, ^{aa} $p < 0.01$ and ^{aaa} $p < 0.001$ vs. the control group; ^b $p < 0.05$, ^{bb} $p < 0.01$ and ^{bbb} $p < 0.001$ vs. the BBN group.

creatinine and kidney weight. An inverse profile was found for the CEL group. In the group under CEL + BBN treatment, all biochemical renal function parameters (creatinine, urea and uric acid)

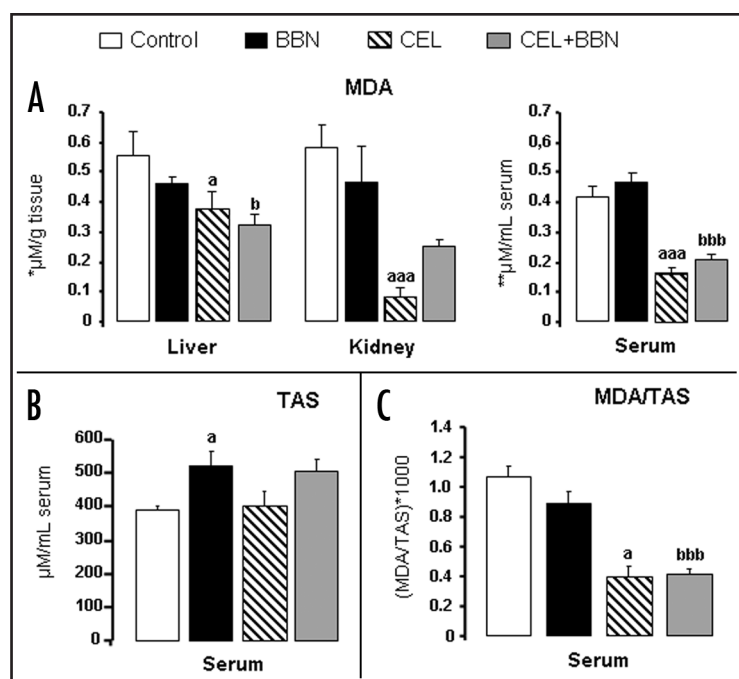


Figure 4. Redox status markers: lipidic peroxidation (MDA content) in liver, kidney and serum (A); serum TAS (B) content and serum MDA/TAS ratio (C). Values are mean \pm s.e.m. ^a $p < 0.05$, ^{aa} $p < 0.01$ and ^{aaa} $p < 0.001$ vs. the control group; ^b $p < 0.05$, ^{bb} $p < 0.01$ and ^{bbb} $p < 0.001$ vs. the BBN group.

were significantly ($p < 0.01$) higher than in the BBN group, as well as the kidney/body weights ratio ($p > 0.001$) (Table 2). Concerning liver function, AST and ALT for the BBN rats were higher ($p > 0.05$) than in the control animals, as well as liver weight and liver/body ratio ($p > 0.05$), contrasting with the profile of the CEL group, identical to the control. In the CEL + BBN group, all parameters were substantially lower than those of the BBN rats, mimicking those of the CEL rats (Table 2). Concerning haematology, in the BBN rats there was a higher RBC count value ($p < 0.05$) than in the control ones, a trend to elevated levels of HCT and a significantly lower value of WBCs ($p < 0.05$). In the CEL group, however, despite non-statistically relevance, there was a trend to reduced values in all the parameters for RBCs, platelets and WBCs. The values of the CEL + BBN rats were identical to those of the control animals; that is, CEL treatment in the BBN rats decreased the BBN-induced changes on haematological parameters (Table 2).

Discussion

Bladder cancer management remains a difficult mission, particularly because of the limited efficacy of the current conventional surgical techniques and pharmacological therapeutic options, especially in the control of recurrences and progressions.^{1,8} Thus, preventive strategies are needed to ameliorate bladder cancer management, but the exact mechanisms accounting for the cancer appearance and progression are yet poorly elucidate.

The carcinogen-induced bladder cancer with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) in rats has been used as a valuable model to study bladder cancer pathophysiology and

therapeutics effects, particularly due to the histological similarities it presents with the human transitional cell carcinoma.¹²⁻¹⁴ Previous studies have shown a positive response to prevention strategies.¹⁶ Thus, in this study we intended to evaluate, using a carcinogen (BBN)-induced rat carcinogenesis model, the efficacy of a selective COX-2 inhibitor on chemoprevention for tumor development and progression. Our results are in agreement with previous data from this model, concerning both % incidence of tumors and the type of lesions in the BBN rats,²⁴ confirming the credibility and value of this model to evaluate chemopreventive efficacy of drugs.

It is largely accepted that the mechanisms underlying cancer appearance and progression are multifactorial. Inflammation, through its mediators (cytokines and other growth factors), seems to be one of the main contributors for cancer growth.²⁵ One of the mechanisms more closely linked to inflammation is the pathway of COX enzymes, in particular COX-2,¹⁹ whose levels and activity have been reported to be elevated in several types of cancers.²⁰ Furthermore, a key role for COX-2 in carcinogenesis has been given by the positive effect of its downregulation on tumors incidence both in clinical and experimental studies for distinct types of tumors, including the bladder cancer.^{17,18,21-23,26}

A chemopreventive role for COX-2 inhibition in bladder cancer was previously reported in animal models,²⁶⁻²⁸ but the mechanisms by which these compounds are able to act on carcinogenesis remain to be elucidated. In our study, the COX-2 inhibitor (CEL) has promoted a remarkable chemopreventive effect on bladder cancer development. In fact, the incidence was only 12.5% (one in eight rats), with a reduced mean tumor volume per rat (21.2 mm³) and per tumor (5.3 \pm 0.4 mm³), contrasting with the data from the BBN rats: 65% of tumor incidence and 138.5 \pm 7.5 and 112.5 \pm 6.4 mm³ for mean tumor volume per rat and per tumor, respectively. Furthermore, and even more relevant, apart from the only rat with tumors (all papillary), whose urothelium has demonstrated hyperplasia and dysplasia of low-grade, without infiltrative or CIS, all the other rats (seven in eight) have presented a normal urothelium profile (there was only low-grade hypertrophy in two of those seven animals), which represents a clear chemopreventive effect against the BBN-induced carcinogenesis.

Several molecular mechanisms have been proposed by others to explain cancer development in different types of tumors.²⁹⁻³¹ Our data support the hypothesis that inflammation and proliferation are key "fuels" for cancer progression. According to other authors, bladder cancer development is linked with production of TGF α and VEGF as mitogenic/angiogenic factor.¹⁷ In our study, the cancer incidence and severity reduction instigated by the preventive use of a selective COX-2 inhibitor was accompanied by a total reduction of the acute phase protein (CRP) and of the transforming growth factor β (TGF β), both significantly elevated in the BBN group. The increment in TGF β in the BBN group, which exerts a dual effect on tumor growth, as an antiproliferative agent in the normal epithelium and pro-invasive in the tumor,³² is consistent with an invasive tumor phase. However, despite no

Table 2 Renal and liver function and hematological data

Parameters	Control (n = 8)	BBN (n = 20)	CEL (n = 8)	CEL + BBN (n = 8)
<i>Renal function</i>				
Creatinine ($\mu\text{mol/L}$)	51.27 \pm 1.77	54.81 \pm 1.77	53.04 \pm 1.77	64.53 \pm 2.65 ^{bb}
Urea (mmol/L)	13.70 \pm 0.67	13.50 \pm 0.47	14.71 \pm 1.36	18.55 \pm 0.71 ^{bb}
Uric Acid ($\mu\text{mol/L}$)	36.88 \pm 4.16	57.10 \pm 7.14	27.96 \pm 1.78	109.44 \pm 14.87 ^{bb}
<i>Liver function</i>				
AST (IU/L)	51.57 \pm 1.09	78.15 \pm 6.04 ^a	55.00 \pm 2.71	64.57 \pm 4.30 ^{bb}
ALT (IU/L)	30.86 \pm 1.75	38.15 \pm 2.92 ^a	28.25 \pm 2.02	30.14 \pm 2.58 ^{bb}
<i>Hematological data</i>				
RBC count ($10^{12}/\text{L}$)	7.58 \pm 0.29	8.072 \pm 0.11 ^a	7.34 \pm 0.08	7.66 \pm 0.10 ^{bb}
HCT (pp of 0.1)	0.40 \pm 0.02	0.42 \pm 0.04	0.38 \pm 0.04	0.40 \pm 0.06
HGB (g/L)	141.50 \pm 5.20	146.00 \pm 1.50	132.80 \pm 1.40	140.50 \pm 2.00
Ret (pp of 0.1)	0.054 \pm 0.002	0.052 \pm 0.004	0.042 \pm 0.004	0.044 \pm 0.005
PLT ($\times 10^9/\text{L}$)	990.67 \pm 46.21	1008.13 \pm 46.96	884.75 \pm 42.58	942.38 \pm 36.89
PCT (pp of 0.1)	0.0057 \pm 0.0002	0.0058 \pm 0.0002	0.0053 \pm 0.0002	0.0055 \pm 0.0002
WBC count ($10^9/\text{L}$)	4.60 \pm 0.56	3.52 \pm 0.26 ^a	2.85 \pm 0.85	4.50 \pm 0.44

pp, proportion. Values are mean \pm s.e.m. ^ap < 0.05, ^{aa}p < 0.01 and ^{aaa}p < 0.001 vs. the control group; ^bp < 0.05, ^{bb}p < 0.01 and ^{bbb}p < 0.001 vs. the BBN group.

changes in the carcinogen animals, both CEL groups (CEL and CEL + BBN) have promoted an increment in IL-1 β and TNF α , which might hypothetically be viewed as a counter-effect of those treatments, independent of the carcinogenesis process but related with the anti-inflammatory action. The increment of TNF α , a multifactorial cytokine that exhibits antitumor effects by several mechanisms, might be viewed as part of the antitumor effect of CEL.³³ According to other authors, CEL is able to induce apoptosis and reduce Bcl₂, independently of COX-2 expression, suggesting distinct pathways for COX-2 and Bcl₂.³⁴ Furthermore, they reported in the same work a 100% inhibition of proliferation of different doses of CEL.³⁴ Cell cycle arrest associated with apoptosis is another strong hypothesis for the antitumor effect of COX-2 inhibitors.³⁵ According to previous studies, the suppression of inflammation due to inhibition of peroxisome proliferator-activated receptors (PPARs) expression, which controls COX-2 expression, was associated with a good impact on bladder cancer therapy.^{36,37} In human bladder cancer cell lines, celecoxib exerted antitumor effects through mechanisms dependent and independent of COX-2.³⁸

In our study, apart from the above reported anti-inflammatory and anti-proliferative effects, the positive impact of the selective COX-2 inhibitor on bladder cancer chemoprevention seems also to be related with a beneficial influence on redox status. In the carcinogen group, the tumor appearance and/or progression seem to be independent of impairment between the formation of reactive species and the antioxidants content, because liver, kidney and serum markers of lipidic peroxidation (MDA) were unchanged when compared with the control animals and serum total antioxidant status was even positively impacted. This data seem to be in opposition with the increment in MDA values found in humans with transitional cell carcinoma (TCC), which are correlated with the tumor phase,³⁹ as well as with the general

idea of a key involvement of oxidative stress, together with chronic inflammation, in cancer development.²⁵ In our study, the BBN group showed an antioxidant effect, hypothetically as a counter-response against the formation of deleterious reactive oxygen species (ROS), which, thus, remained at normal levels. Anyway, further studies should analyze the early influence of drugs after the first phase of treatment (8 w) in order to elucidate if BBN is able to promote any relevant change (namely on COX-2 expression and activity) that could be used as earlier marker of bladder tumor and, furthermore, to elucidate if an earlier treatment with a selective COX-2 inhibitor is able to prevent it. Even though the less influence of ROS formation in the carcinogenesis process, the positive effect of CEL seem to be extended to the redox status, given by the remarkable shift to the antioxidant capacity in the liver, kidney and serum.

In a similar work performed by Grubbs et al.,²⁶ the effect of celecoxib was tested in rats under treatments in analogous conditions to ours but with high concentrations of the drug (200, 500 and 1,250 mg/kg). Since identical incidence of cancer was reported, our data, with a lower dose, achieves additional relevance. Therefore, few potential adverse effects were encountered, namely on the renal function, together even with a positive impact on liver function and hematological parameters. The increment in renal function markers might result from the effect of COX-2 inhibition on the urinary prostanoids balance. According to Gupta et al.⁴⁰ CEL treatment was able to promote tubulointerstitial nephritis, but using a dose three times higher than our. Therefore, Alhan et al.⁴¹ using a dose identical to that tested by us, did not find changes on renal function. In our study, when CEL was given alone, as a second control group, there was no relevant change on renal function markers, suggesting that the increment in the CEL + BBN group might be due to the combination of both treatments (carcinogen plus drug).

The multi-step nature of carcinogenesis provides opportunities for intervention with agents targeted at specific mechanisms involved in the initiation, promotion and progression stages of cancers.⁴² Inflammation, proliferation and oxidative stress seem to be important for bladder carcinogenesis. According to some authors, the formation of COX-2 are dependent on the degree of bladder tumor, having a pre-neoplastic action and being particularly elevated in more advanced phases.^{18,43,44} We have demonstrated here that the use of a selective COX-2 inhibitor in the stage of cancer initiation, or even before, as chemopreventive therapy, has a notable effect on cancer appearance and progression, which might be particularly useful for individuals of higher risk as well as for patients treated for an previous episode of bladder cancer and under high risk of recurrence.^{3,4} Further studies from our group will focus on testing its potential as therapy for reverse or retard cancer evolution, which will also be relevant for patients with bladder cancer with a poor prognosis considering the current surgical procedures and pharmacological treatments.

In conclusion, our study demonstrates that Celecoxib treatment has an outstanding inhibitory effect on bladder cancer chemoprevention in rat, which might be due to its expected anti-inflammatory actions, as well as by mechanisms not directly dependent of COX-2 inhibition, such as cellular proliferation control and improvement in antioxidant profile. This data supports a pivotal role of cancer chemoprevention strategies based on COX-2 inhibition.

Materials and Methods

Animals and groups. Male Wistar rats (Charles River Lab. Inc., Barcelona, Spain), 250–285 g, were maintained in an air conditioned room, subjected to 12 h dark/light cycles and given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Animal experiments were conducted according to the “Principles of laboratory animal care.” The animals were divided in four groups: control group (n = 8)—vehicle (orange juice); carcinogen (BBN) group (n = 20)—0.05% of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); Celecoxib (CEL) group (n = 8)—10 mg/kg/day of the selective COX-2 inhibitor Celebrex[®] (Laboratórios Pfizer, S.a., Lisbon, Portugal); Preventive Celecoxib—CEL + BBN groups (n = 8). Treatments were performed in a two-step protocol:—a first period of eight weeks for tumor induction and pharmacological treatment (orange juice, BBN and CEL) and a second one of 12 w for cancer expression/prevention. BBN was given in drinking water and orange juice (given to all groups) and CEL (prepared in vehicle) were given by an esophageal cannula. All the animals have completed the 20-w study protocol. Body weight and drink beverage were monitored during the experimental period.

Sample collection and preparation. Blood. At the end of treatments the rats were injected with intraperitoneal anesthesia with 2 mg/Kg BW of a 2:1 (v:v) 50 mg/mL Ketamine (Ketalar[®], Parke-Davis, Pfizer Laboratories Lda, Seixal, Portuga) solution in 2.5% chlorpromazine (Largatil[®], Rhône-Poulenc Rorer, Vitória laboratories, Amadora, Portugal). Blood samples were immediately

collected by venipuncture from the jugular vein in needles with no anticoagulant (for serum samples collection) or with appropriated anticoagulant: EDTA, heparin or a solution of Acid citrate-dextrose, were used.

Tissues. The rats were sacrificed by cervical dislocation, and the lungs, stomach, liver, kidneys and intestines were immediately removed, weighted and placed in ice-cold Krebs buffer or formaldehyde. Before removal, bladders were intra-luminally injected with a buffered formaldehyde solution as pre-fixation for histological analyses.

Tumor chemoprevention analysis. Quantitative analysis (number and volume of tumors). Each bladder pre-fixed in formaldehyde was carefully open, the lumen was inspected for grossly visible lesions and the number of tumors per rat and per group and the volume of each tumor were reported to further calculate the % of tumor per group and the mean volume per rat and tumor.

Qualitative analysis (bladder histology). The bladder was immersion-fixed in 4% buffered formaldehyde and processed for paraffin sectioning. Three slices from each bladder were embedded. Three micrometer thick sections were stained with haematoxylin & eosin (H&E) and examined histologically by one author (Cunha MF) unknowing the treatments.

Proliferation, inflammation and redox status markers. Serum levels of transforming growth factor β -1 (TGF β 1), tumor necrosis factor (TNF α) and interleukin-1 β (IL-1 β) were measured by using ultrasensitive Quantikine[®] ELISA kits (R&D Systems, Minneapolis, USA). Serum C-reactive protein (CRP) was measured by using an ELISA kit from Helica Biosystems, Inc., (Fullerton, CA USA). The thiobarbituric acid reactive-species (TBARs) assay was used to assess serum, kidney and liver lipid peroxidation, namely malondialdehyde (MDA).⁴⁵ The serum total antioxidant status (TAS) was measured through the ferric reducing antioxidant potential (FRAP) assay.⁴⁶

Biochemical and haematological data. Renal and liver function. Serum creatinine, urea and uric acid concentrations were used as renal function indexes and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assessed for hepatic evaluation, through automatic validated methods and equipments (Hitachi 717 analyser, Roche Diagnostics Inc., MA USA).

Hematological data. Red blood cell (RBC) count, haematocrit (HCT), hemoglobin concentration (HGB), reticulocyte count (RET), platelet count (PLT), plaquetocrit (PCT) and white blood cells (WBC) were assessed in EDTA whole-blood by using an automatic Coulter Counter[®] (Beckman Coulter Inc., CA USA).

Statistical analysis. The Statview 4.53 software from Abacus Concepts Inc., (Berkeley, CA USA) was used for data analysis. Results are means \pm S.E.M. Comparisons between groups were performed using one-way ANOVA and Fisher's test. Significance was accepted at *p* less than 0.05.

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