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ORIGINAL ARTICLE

Histological changes in bladders of patients submitted to ifosfamide chemotherapy even with mesna prophylaxis

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Abstract
Purpose Hemorrhagic cystitis (HC) is a limiting side effect of chemotherapy with ifosfamide (IFS). Mesna is the drug of choice for prevention of HC. In this study, we analyzed cystoscopic and histological changes present in bladders of patients using IFS with mesna prophylaxis.
Methods Thirty-three patients selected for IFS plus three doses of mesna chemotherapy regime were assigned at random to two groups: Group I or reference group consisted of 18 patients yet untreated. Group II consisted of 15 patients in whom urinalysis and cystoscopy plus vesical biopsy were performed only 24 h after receiving the last dose of IFS. The cystoscopic and histological findings were used as parameters for evaluating the results. For the former the criterion adopted was macroscopic vesical changes in accordance with Gray's criteria. Histological analyses were performed by evaluation method especially adapted to this study.
Results Even under treatment with three doses of mesna, 66.7% of patients presented cystoscopic alterations and 100% showed bladder mucosa microscopic alterations such as edema, exocytosis, and hemorrhage.
Conclusions The standard protocol used for prevention of IFS-induced HC with three doses of mesna does not completely prevent bladder damage. The histopathological criteria used in this study for observation of inflammatory events allowed staging the intensity of IFS-induced urothelial and mucosal injury.

Keywords Ifosfamide · Hemorrhagic cystitis · Mesna · Histopathology

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Introduction

Ifosfamide (IFS) is an oxazaphosphorine-alkylating agent with a broad spectrum of antineoplastic activity in a variety of disseminated refractory solid tumors that do not traditionally respond to conventional alkylating agent therapy, specifically refractory germ cell tumors, soft tissue sarcomas, and malignant lymphomas [10]. IFS is a pro-drug metabolized in the liver by cytochrome P450 mixed-function oxidase enzymes to isofosforamide mustard, the active alkylating compound [5].

Chemotherapy of malignant tumors with oxazaphosphorines, cyclophosphamide, or IFS is often limited by the occurrence of urotoxicity. It has been reported that IFS produces hemorrhagic cystitis (HC) more commonly than cyclophosphamide [16]. In the absence of adequate uroprotection, HC becomes dose limiting, with an average incidence which ranges from 18 to 40%. The overall reported incidence of HC has varied widely among studies due to lack of agreement upon diagnostic criteria, uncertainty regarding the relative contribution of factors such as thrombocytopenia, concurrent or previous chemotherapy or radiotherapy, and viral infections [9].

Such toxicity is attributed to the renal excretion of acrolein, a urotoxic metabolite of ifosfamide. Prophylactic mesna has been used as a standard protocol for prevention of HC. Mesna (2-mercaptoethanesulfonic acid), a thiol compound, entered clinical trial as a systemic uroprotective agent in the late 1970s and became the drug of choice for preventing HC [2]. It is suggested that the sulfhydryl group present in this drug binds to acrolein within the urinary collecting system and detoxifies it [21]. Before the introduction of mesna, the incidence of hemorrhagic cystitis was as high as 68% with a mortality rate of 4% for severe cases [3, 13]. It has been proposed that urothelial damage occurs by direct contact with acrolein, which causes edema, ulceration, neovascularization, hemorrhage, and necrosis [4]. Prophylaxis is not totally capable of blocking inflammatory events both in clinical trial and in experimental investigations [6, 15, 17, 21].

Our group has shown that cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) were crucial mediators involved in inflammatory events and in urothelial damage and hemorrhage involved in HC [8, 18]. Furthermore, it was demonstrated that nitric oxide (NO) is the final mediator of urothelial damage and hemorrhage in HC, as drugs that inhibit the synthesis of NO reduced in a dose-dependent manner the edema and hemorrhage [18]. The administration of anti-TNF-α or anti-IL-1β sera significantly decreased the cyclophosphamide and ifosfamide-induced vesical inflammatory events as well as the rise in inducible nitric oxide synthase (iNOS) expression and activity [18, 20].

Gray et al. [12] proposed some criteria for staging the severity of HC induced by oxazaphosphorines in animal models. For macroscopic evaluation, edema was considered severe (3+) when fluid was seen externally and internally in the bladder walls, moderate (2+) when confined to the internal mucosa, mild (1+) between normal and moderate, and normal (0) when no edema was observed. Hemorrhage was scored as follows: (3+) intravesical clots; (2+) mucosal hematomas; (1+) telangiectasia or dilatation of the bladder vessels; and (0) normal. Gray's criteria also scored microscopic alterations as follows: normal epithelium and absence of inflammatory cell infiltration and ulceration (0); mild changes involving reduction of epithelial cells, flattening with submucosal edema, mild hemorrhage, and few ulcerations (1+); and severe changes including mucosal erosion, inflammatory cell infiltration, fibrin deposition, hemorrhage, and multiple ulcerations (2+).

Although Gray's criteria are effective in staging the severity of experimental hemorrhagic cystitis they seem to be of little value when used for microscopic evaluation in IFS-treated patients. The inadequacy of the latter is explained by the fact that it is best used in experimental studies in which animals' bladders may be examined *in totum*; thus, Gray's criteria are insufficient as observation parameter of inflammatory events in samples consisting of tiny vesical specimens. In the present study we padronized scores for bladder histopathological evaluations based on: urothelial changes (dematuration, atypia, exocytosis, edema, erosion, regeneration) and lamina propria changes (edema, exocytosis, hemorrhage, hyalinization, or sclerosis and congestion). In addition we investigated the effectiveness of mesna in preventing microscopic IFS-induced damage of bladder mucosa.

Materials and methods

Patients

A total of 33 patients diagnosed with solid tumors potentially treated with IFS such as sarcoma, germinative tumors of the testis, primitive neuroectodermic tumor (PNET), and lung cancer (Table 1) were consentingly informed about the study respecting some exclusion parameters: Karnosfsky score under 40, previous treatment with IFS, cyclophosphamide, or corticosteroids on the last 4 weeks, previous pelvic radiotherapy, other neoplasias, or any urinary tract illness. Ceará Cancer Institute Ethics Committee for Human Experiments had approved the experimental protocol and it is in accordance with the guidelines approved by the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

Drugs and dose

Ifosfamide (Holoxane® 1 g) and mesna (Mitexan® 400 and 600 mg) were from ASTA Medica-AG (Germany). Ifosfamide and mesna were dissolved in 0.9% sterile saline.

Table 1 Baseline characteristics of the patients

Characteristic	Reference group (n = 18)	IFS + MESNA group (n = 15)
Age (year)		
Median	34	23
Range	17–68	17–68
Sex (% of patients)		
Male	55.5	46.7
Female	44.5	53.3
Pathological diagnostic		
Sarcomas	13	10
PNET	2	1
Germinative tumors	1	1
NSCLC	1	–
Malignant fibrohistiocytoma	1	1
Schwanoma	–	1
Renal nefroblastoma	–	1

Cystoscopy

A cystoscopy device labeled KARL STORZ 19-27026D; 30°-27005 BA-27068 D was used according to the technique described above [7].

Experimental protocol

The patients that agreed to participate were assigned at random to one of two treatment groups: Group I consisted of 18 patients selected for treatment with IFS but not treated yet. Patients from this group were submitted to an interview and physical examination with the clinical oncologist, and before chemotherapy treatment with IFS was performed, urinalysis and cystoscopy with biopsy were performed by a urologist to create the reference group. Subsequently, two small fragments (0.2 × 0.1 cm^2 each) were fixed in formalin and processed for hematoxylin and eosin staining, and histopathological analysis was developed by a single-blinded pathologist. All patients included in the "reference group" were naive in respect to chemotherapy treatment. However, after the initial cystoscopic analysis and biopsy, they received appropriate treatment, but only some of them were included in "IFS + MESNA group". Group II consisted of 15 patients in whom cystoscopy and vesical biopsy were performed 24 h after the last day of chemotherapy cycle. The average ifosfamide dose was 1.9 g/m^2/day with the lowest dose 1.8 g/m^2/day and the highest 2.5 g/m^2/day ranging from 3 to 5 days. These patients received the classical treatment with three doses of mesna: the first dose i.v. (20% of IFS dose) immediately before ifosfamide administration, the others p.o. (40% of IFS dose) at 2 and 6 h after IFS injection) and 18 were assigned to create the reference group [9].

Urinalysis and cystoscopy with biopsy were evaluated 24 h after the IFS treatment, based on experimental data showing that increase in bladder wet weight of rats injected with IFS was maximum between 24 and 48 h [18]. After 48 h the bladder wet weight drops significantly. We therefore chose 24 h to access ifosfamide-induced HC. During the study, none of the specialists had access to patients' records, avoiding any possible bias in results. Finally, at the end of the study, all results were obtained and plotted together.

Macroscopic evaluation

The macroscopic findings were evaluated using Gray's criteria on direct visualization of bladder by cystoscopy as follows: severe (3+) when fluid was seen internally in the bladder walls, moderate (2+) when confined to the internal mucosa, mild (1+) between normal and moderate, and normal (0) when no edema was observed. Hemorrhage was scored as follows: (3+) intravesical clots; (2+) mucosal hematomas; (1+) telangiectasia or dilatation of the bladder vessels; and (0) normal.

Histopathological analysis

Gray's criteria have little value when assessing microscopic alterations in human bladders, despite the different methods used to analyze experimental HC. These criteria do not contemplate independent inflammatory events, chronic or acute alterations, or early signs of tissue repair. Hence, we elaborated a new quantitative morphometric analysis capable of making inferences on dimensions, extension, volumes, proportions of tissue components, comparisons among individuals, and especially mediating independent inflammatory events. In each case, microscopic analysis was considered to be the final score (the sum) of changes seen in urothelium known to play a role in inflammatory process, either by triggering usually toxic or ischemic epithelial necrosis or by leading to adaptive or hyperplastic regeneration. In the stroma, especially in lamina propria, degrees (1–3) of congestion, edema, hemorrhage, exudates, hyalinization, and sclerosis were analyzed and a cellularity-based assessment of the quality of the exudates (acute or chronic) was performed. Thus, the maximum score obtained was 26, as shown below:

1. Urothelial changes:
   a. Dematuration: polarity loss, absence of differentiation at the superficial layer. Score 0, absent; 1, present.

b. Atypia: anomalous mitoses in parabasal or superficial layer. 0, absent; 1, present.
c. Exocytosis: leukocytes interposed on urothelium. 0, inexistent; 1, one focus; 2, two or three foci; 3, diffuse or more than three foci.
d. Edema. Scores: 0, absent; 1, mild (cell separation); 2, strong (disruption of urothelial continuity and vesicle).
e. Erosion: loss of urothelial lining: 0, none; 1, loss only of superficial layer; 2, loss of superficial and parabasal layer with basal layer unaffected; 3, ulceration with lamina propria exposure.
f. Regeneration: typical mitoses and enlargement of basal layer. 0, inexistent; 1, normal; 2, highly hyperplasic; 3, if methaplasic intestinal tissue was found.

2. Lamina propria changes:

a. Edema. 0, absent; 1, mild; 2, strong.
b. Exocytosis (exudate). 0, none; 1, one focus; 2, two or three foci; 3, diffuse or more than three foci.
c. Hemorrhage. 0, none; 1, one focus; 2, two or three foci; 3, four or more foci.
d. Hyalinization or sclerosis. 0, none; 1, hyalinization only; 2, sclerosis only; 3, hyalinization and sclerosis.
e. Congestion: increase of vascularization, marked by the presence of a large number of ecstatic capillary or vessels. 0, absent; 1, mild; 2, strong.

Statistical analysis

The number of patients was calculated using confidence interval of 95% and statistical power of 80%. Results were reported as median values (macroscopic and histopathological data), then comparisons were drawn among groups. Macroscopic and microscopic data were considered to be of statistical significance ($P < 0.05$) assessed by Fisher's test analysis based on "cut-off" point. The cut-off point was defined as the median of reference group obtained according to all scores from both cystoscopic and microscopic analysis.

Results

Of 37 patients initially included in this study, four were excluded during the study due to: one, reports of discomfort during cystoscopy; two others, showing desires to quit because of nauseas and emesis induced by chemotherapy. The last was excluded due to impossibility of accomplishing the cystoscopy in view of a urethral stenosis. Of the remainder, 15 were randomized for classical treatment with three doses of mesna (the first dose i.v. immediately before ifosfamide administration, the others p.o. at 2 and 6 h after IFS injection) and 18 were assigned to make up the reference group.

Demographic data

A total of 82% were sarcomas followed by PNET (9%), germinative tumors of the testis (6%) and one case of lung tumor (3%); 51.5% were male and 48.5% female. The mean age was 34.09 years with the youngest being 17 years and the oldest 68 (see Table 1).

Ifosfamide regimen and doses

In 15 patients treated, ifosfamide was used in association with adriamycin in 60%. In the others, ifosfamide was used in association with VP-16 (26.7%). The average ifosfamide dose was 1.89 g/m^2/day with the lowest dose 1.8 g/m^2/day and the highest 2.5 g/ m^2/day ranging from 3 to 5 days.

Urinalysis

Red cells: 15 patients (83.3%) of the reference group presented less than 5 red cells per field, while 1 (5.6%) and 2 (11.1%) patients obtained mild hematuria with 6–10 or 10–20 red cells per field, respectively. Five or less red cells per field were detected in 14 patients (93.3%) treated with three doses of mesna. There was no significant difference ($P > 0.05$) between the IFS plus mesna-treated group and the reference group. *Proteins*: There was no significant difference between the groups ($P > 0.05$). *Leukocytes*: In reference group, 13 patients (72.7%) presented less than four leukocytes per field in contrast to 13 (86.6%) in the treated group, with no statistical difference ($P > 0.05$).

Macroscopic evaluation

Cystitis observed 24 h after chemotherapy administration (IFS and three doses of mesna for HC prevention) was characterized macroscopically according to Gray's criteria by the presence of mild edema, receiving a median score of 1 (1–2), and by hemorrhage with mucosal hematomas and telangiectasia, receiving a median score of 2 (1–3), in 66.7% patients, being significantly ($P < 0.05$) different from the reference group which received a median score of 0 (0–1) for edema and hemorrhage (Fig. 1). Only 11.7% patients reported urinary symptoms 24 h after chemotherapy, such as dysuria and frequency.

Histopathological analysis

Twenty-four hours after IFS administration, even with treatment with mesna, urothelial edema was reported as minimum in 73.3% and intense in 6.7% of patients. Only in 20%, edema was reported as absent. In the reference group (patients before IFS administration) edema was minimal in 33.3% and in 66.6% there was no evidence of edema (Table 2 and Fig. 2). In those treated with IFS and mesna, the presence of edema in the lamina propria was 93.3% (read as 86.7% minimum and 6.6% intense). There was no edema in the lamina propria in 6.7% of patients treated. In the reference group, edema was absent in 27.8% and minimum in 72.2% ($P < 0.05$ when comparing reference group and IFS-treated patients) (Table 2 and Fig. 2).

Erosion was seen in 93.4% after IFS and mesna administration and 61.2% in the reference group, most being superficial in the latter, reaching a significant statistical difference between groups ($P < 0.05$) (Table 2 and Figs. 2, 3). Exocytosis was seen in 48.9% in the reference group, while it was 84.7% 24 h after IFS and mesna administration. Hemorrhage was absent in 77.8% of patients in reference group and only 26.7% in IFS and mesna administration (Table 2 and Figs. 2, 3). The majority of those patients did not even show microscopic hematuria; however, these alterations were very significant in a high number of patients.

Hyalinization and sclerosis, resulting in tissue repair of inflammatory damage, were very high ($P < 0.05$) in patients treated with both ifosfamide and mesna (80%), when compared with reference group (43.8%) (Table 2 and Fig. 3).

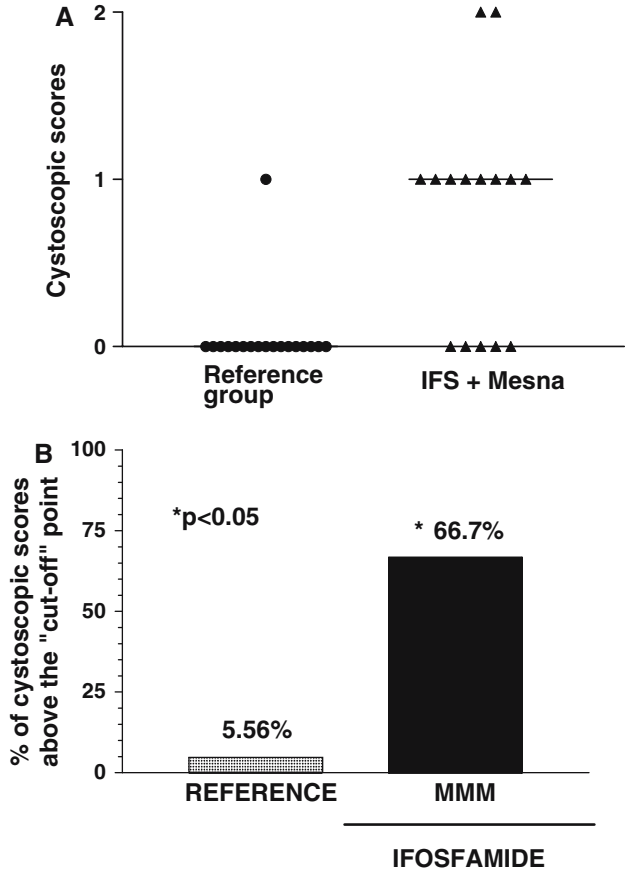

Fig. 1 Effect of standard therapy with three doses of mesna (*MMM*) for hemorrhagic cystitis prevention on macroscopic cystoscopic score. **a** Dot plot of patient cystoscopic scores. **b** The columns represent the percentage of patients presenting edema or hemorrhage with mucosal hematomas and telangiectasia with total cystoscopic scores above the "cut-off" point. *$P < 0.05$ when compared to the reference group by Fisher's exact test

Table 2 Histological findings for bladders of untreated patients (Group I) and patients treated with ifosfamide associated with three doses of mesna (Group II)

	Urothelium		Lamina propria	
	Group I	Group II	Group I	Group II
Edema				
Absent	12 (66.6%)	3 (20.0%)*	5 (27.8%)	1 (6.7%)
Mild	6 (33.3%)	11 (73.3%)*	13 (72.2%)	13 (86.6%)
Strong		1 (6.7%)		1 (6.7%)
Erosion				
Absent	38.80%	1 (6.7%)*		
Superficial	61.20%	9 (60%)		
Superficial and parabasal		4 (26.6%)*		
Ulceration		1 (6.7%)		
Hemorrhage				
Present			4 (22.2%)	11 (73.3%)*
Absent			16 (77.8%)	4 (26.7%)*
Repair				
Absent			10 (56.3%)	3 (20.0%)*
Hyalinization			6 (31.3%)	1 (6.7%)*
Sclerosis			2 (12.5%)	11 (73.3%)*
Cellularity				
Absent			11 (61.1%)	2 (13.3%)*
Granulocytes			1 (5.6%)	1 (6.7%)
Non-granulocytes			3 (16.7%)	4 (26.7%)
Mixed cellularity			3 (16.7%)	8 (53.3%)*

Ifosfamide-induced microscopic alterations were evaluated 24 h after its administration plus three doses of mesna (Group II). Numbers outside brackets indicate absolute number of patients in each group, while numbers inside indicate the percentage

*$P < 0.05$ when compared to the reference group (Group I, not yet treated with ifosfamide) by Fisher's exact test

Discussion

In the present study, the preventive effect of intravenous/oral mesna in ifosfamide-induced bladder dam-

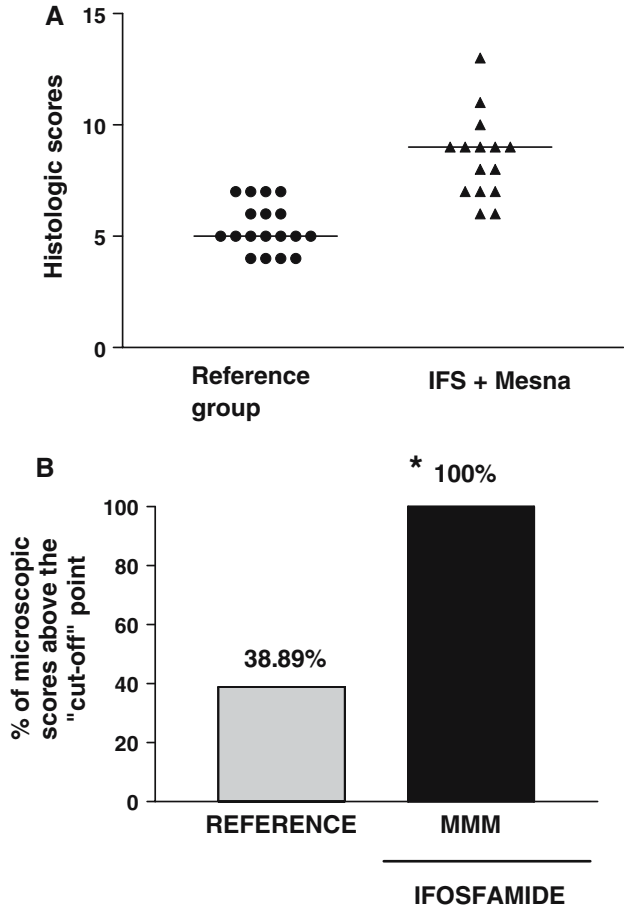

Fig. 2 Effect of standard therapy with three doses of mesna (*MMM*) for hemorrhagic cystitis prevention on microscopic scores (edema, erosion, cellularity, hemorrhage, and repair). **a** Dot plot of patient histological scores. **b** The columns represent the percentage of total microscopic scores above the "cut-off" point. $*P < 0.05$ when compared to the reference group by Fisher's exact test

age was investigated, using macroscopic and microscopic parameters and comparing these findings with the morphological appearance of the bladder of patients before IFS use. Our data demonstrated that even under classical mesna standard protocol the cystoscopy detected discrete macroscopic alterations such as mild edema and hemorrhage with mucosal hematomas and telangiectasia in a significant percentage of patients as compared with the reference cystoscopy, made before IFS treatment. However, these findings were not associated with clinical complaints or altered urinalysis parameters such as red cells, hematuria, leukocytes, or proteins.

Mesna (sodium-2-mercaptoethanesulfonate) is a thiol compound that functions as a regional detoxicant of the oxazaphosphorine metabolites. After oral or intravenous administration, mesna undergoes rapid oxidation in the plasma to dimesna. Both mesna and dimesna are very hydrophilic and are rapidly cleared by the kidneys. The free sulfhydryl (thiol) groups of mesna combine directly with a double bond of acrolein and with other urotoxic 4-hydroxy-oxazaphosphorine metabolites to form stable non-toxic compounds. Thus, mesna has been used as a systemic uroprotective agent, becoming the drug of choice for preventing HC [9]. The i.v./oral mesna regimen used here has been shown to be at least as uroprotective as the approved i.v. regimen [14]. Before the use of mesna, hematuria was reported in 20–40% of patients, limiting IFS use [1]. After mesna being routinely introduced, this frequency fell to about 5% [1]. Most studies use urinary complaints such as dysuria, hematuria and frequency, and urinalysis as parameters for evaluating IFS urotoxicity and mesna protective effect. Cystoscopy and bladder biopsy are not routinely performed.

In the current study, a detailed microscopic evaluation from bladder biopsy showed significant histopathological changes in bladders of patients treated with IFS and mesna as compared to those biopsy samples harvested before IFS administration, suggesting that mesna really protects from massive IFS-induced damage but does not confer full protection. These microscopic changes were mainly edema, erosion, exocytosis, hyalinization, and sclerosis. According to our data, it has been demonstrated that mesna is not capable of providing complete uroprotection in both experimental and clinical reports [6, 15, 19, 21]. However, none of the clinical studies has investigated the histopathological alterations in patients under IFS chemotherapy regime and mesna prophylaxis for HC, as they were based on clinical and urinalysis data.

In this study, we described new parameters for histological evaluation of inflammatory events that occur in bladders of patients submitted to IFS-based chemotherapy. The rational basis for this evaluation is due to the fact that classical Gray's criteria, largely used in animal studies, provide a lack of information when used to quantify and qualify histopathological changes in human bladders, so an extensive accuracy method was imperative to establish a correlation between urothelial damage and the inflammatory process observed in such bladders. Furthermore, this detailed information on microscopic bladder damage induced by IFS, even under mesna prophylaxis, raises the importance of seeking new agents that would provide additional uroprotection, as microscopic inflammatory insult may be a risk factor for developing other complications such as neoplastic disease.

Mesna seems to be necessary for initial uroprotection through its neutralizing effect on urothelial damage initiated by acrolein. However, mesna is not an

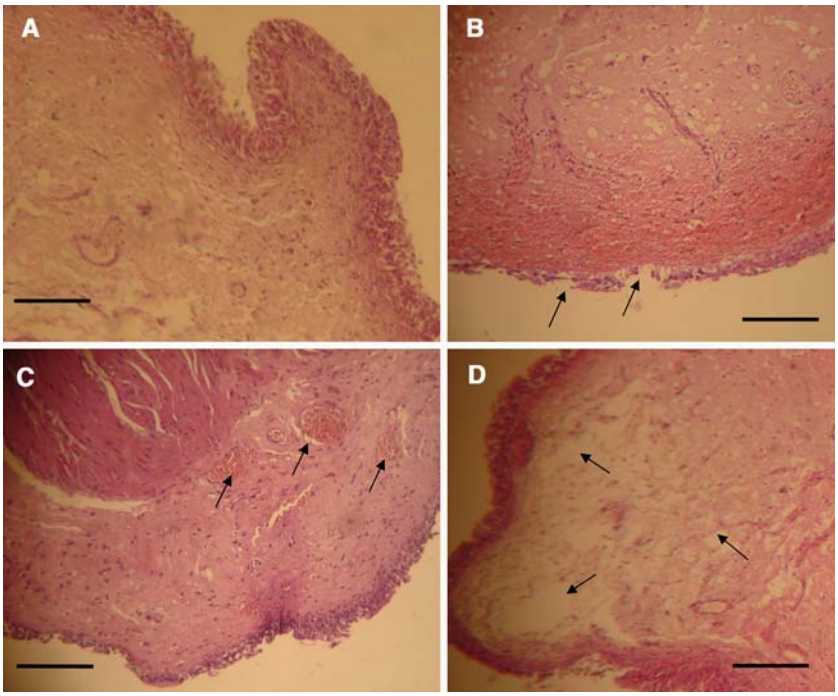

Fig. 3 Histological analysis of representative bladder walls in cross-section. **a** Normal bladder in a patient before ifosfamide chemotherapy (Group I). **b** Urothelium of a patient treated 24 h previously with ifosfamide (IFS) and three doses of mesna showing intense urothelial damage with ulceration indicated by the *arrows* (Group II). Severe sub-epithelial hemorrhage can be also seen. **c** Bladder of patient treated with IFS and three doses of mesna 24 h showing important congestion (*arrows*) and erosion (Group II). **d** Bladder of patient treated with IFS and three doses of mesna 24 h showing intense edema in lamina propria (*arrows*) (Group II). Hematoxylin–eosin, ×400. *Bar* 250 μm

anti-inflammatory drug and it did not block the inflammatory events reported in our study. The role of TNF-α [8], IL-1β [8], and iNOS [18] was demonstrated in animals and more recently the participation of COX-2 expression [11] in cyclophosphamide-induced hemorrhagic cystitis. It was demonstrated that the use of dexamethasone, a drug with potent anti-inflammatory properties, provided full protection when associated to mesna, avoiding all bladder mucosa inflammatory response that follows IFS use in experimental studies [22].

In conclusion, the data presented here show that the standard protocol used for prevention of IFS-induced HC with three doses of mesna does not completely prevent bladder damage. The histopathological criteria adopted in this study were efficient in staging the severity of IFS-induced urothelial and mucosal damage and could be useful as an important tool in evaluating new alternatives complementary to mesna for preventing clinical and sub-clinical hemorrhagic cystitis due to ifosfamide.

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