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ORIGINAL ARTICLE

Amifostine and glutathione prevent ifosfamide- and acrolein-induced hemorrhagic cystitis

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Abstract *Introduction*: Ifosfamide (IFS) is an antineoplastic alkylating agent whose major side effect is hemorrhagic cystitis (HC). This toxicity is attributed to the renal excretion of acrolein (ACR), a highly urotoxic IFS metabolite. Despite the clinical use of mesna to prevent HC, a significant percent (~33%) of patients present with at last one feature of HC, mainly hematuria. *Aim*: To investigate the use of two antioxidants—amifostine and glutathione—for the prevention of experimental IFS- and ACR-induced HC. *Materials and methods*: Male Swiss mice were treated intraperitoneal (i.p.) with saline (control), glutathione (125, 250 or 500 mg/kg) or amifostine (25, 50 or 100 mg/kg), and 30 min later they received a single i.p. injection of IFS at a dose of 400 mg/kg. To investigate the systemic effects of the antioxidants on ACR-induced HC, the animals were treated with saline, amifostine (50 mg/kg, i.p.) or glutathione (500 mg/kg, i.p.), and 30 min afterward with 75 μg ACR intravesically (i.ve.). In another set of experiments, the antioxidants were injected directly into the bladder, where the mice received a single i.ve injection of ACR (75 μg) plus amifostine (1.5 mg/kg) or glutathione (2 mg/kg). HC was measured 3 h after IFS or ACR injection according to bladder wet weight, macroscopic (edema and hemorrhage) and microscopic changes, i.e., edema, hemorrhage, cellular infiltration, fibrin deposition and urothelial desquamation. *Results*: Pretreatments with amifostine or glutathione prevented IFS-induced HC in a dose-dependent manner. Furthermore, ACR-induced HC was also prevented by systemic (i.p.) or local (i.ve.) pretreatment with glutathione or amifostine. The greatest protective effect was seen with local amifostine treatment (2 mg/kg i.ve.) ($P < 0.05$). *Conclusions*: Glutathione and amifostine show a beneficial effect in experimental IFS- and ACR-induced HC. Thus, they should be investigated as an alternative treatment to prevent HC observed in patients undergoing IFS treatment.

Keywords Amifostine · Glutathione · Uroprotection · Hemorrhagic cystitis · Ifosfamide · Acrolein

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Introduction

Amifostine (Ethyol®), Walter-Reed (WR)-2721, is a wide spectrum cytoprotective agent chemically known as ethylphosforothioic acid S-2-[(3-aminopropyl) amino] used in clinical practice for post radiotherapy

xerostomy, neuro/nephrotoxicity induced by cisplatin and bone marrow suppression induced by cyclophosphamide [2]. It is an inactive phosphorylated pro-drug that is dephosphorylated to WR-1065, its active form which is a free sulfhydryl-containing compound. Amifostine is dephosphorylated by alkaline phosphatase present on the cell membrane, mainly in non-tumor cells. Therefore, amifostine selectively protects non-tumor tissues from the toxic effects of chemo/radiotherapy [12].

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is a constitutive endogenous tripeptide which also contains a sulfhydryl group located on the amino acid cysteine. Its terminal thiol, -SH, has been implicated in the natural defense against reactive oxygen- or nitrogen-species and other free radicals [12]. Normal tissues do not take up exogenous reduced glutathione, except for those that have high levels of γ-glutamyl-transferase, such as the kidney. Tissues maintain the level of reduced glutathione through de novo synthesis and/or reduction of the oxidized glutathione [12]. Despite the inability of cells to transport reduced glutathione to the intracellular compartment, it has been used to prevent the deleterious effects of extracellular free radicals released during the inflammatory process [8].

Ifosfamide (IFS) is an alkylating agent from the oxazaphosphorine group with a broad spectrum of antineoplastic activity, and is generally used for the treatment of malignant tumors such as refractory germ cell tumors, soft tissue sarcomas, hematological lymphoproliferative diseases or for myelosuppression in rheumatoid arthritis, for example [9]. In the absence of adequate uroprotection, patients treated with IFS may develop hemorrhagic cystitis (HC), a dose-limiting and high morbidity side effect. Such toxicity is attributed to the ACR renal excretion, a highly urotoxic IFS metabolite, but not chloroacetaldeyde, that is more implicated in nephro- and neurotoxicity [6]. It has been proposed that urothelial damage occurs by the action of ACR on bladder tissue, causing edema, ulceration, neovascularization, hemorrhage and necrosis [4]. Recently, we developed a model of ACR-induced HC in mice. The intravesical (i.ve.) administration of ACR induces dose-dependent cystitis which peaks after 3 h [1].

Despite the preventive use of mesna for alkylating agent-induced HC, the occurrence of HC has been observed at different stages in a significant percent of patients [18]. Furthermore, we have demonstrated that 33.3% of animals submitted to the experimental protocol to induce HC with IFS and treated with mesna still had microscopic alterations [23]. These facts increase the importance of studies aimed at determining the mechanisms involved in bladder lesions resulting from alkylating agent therapy. In this context, it has been demonstrated that the antioxidants melatonin [21] and flavonoid [13] also have a protective role in alkylating agent-induced HC.

We had previously shown that pro-infflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and also PAF are involved in the pathogenesis of experimental oxazaphosphorine-induced HC. It seems that these mediators stimulate the expression of inducible nitric oxide (NO) synthase, which produces a high amount of NO, a key mediator of the urothelial lesion and hemorrhage [15, 19]. In fact, treatment of IFS-injected mice with NOS inhibitors diminishes bladder damage in a dose-dependent manner [19]. Besides the involvement of nitric oxide, reactive oxygen species and peroxynitrite seem to be also involved in HC [11, 21].

In the present study, we investigated whether amifostine and glutathione, well-known antioxidant agents, have a protective role in two models of HC: the classical IFS model and ACR i.ve. injection, a model developed in our laboratory.

Materials and methods

Animals

Male Swiss mice (25–35 g) were kept in appropriate cages in temperature-controlled rooms, receiving food ad libitum, but without access to water during the final 18 h before the experiments with ACR but not with IFS. The experimental protocol was in accordance with the guidelines approved by the Council of the American Psychological Society for use of experimental animals.

Drugs

Acrolein was obtained from Sigma Chemical Co. (UK), IFS (Holoxane®) and mesna (Mitexan®) from Asta Medica (São Paulo, Brazil), glutathione from Sigma Chemical Co. (USA) and amifostine (Ethyol®) from Schering-Plough. All other reagents were from Sigma Chemical Co. (USA).

Effect of glutathione and amifostine on IFS-induced HC

Groups of animals (six–eight mice) received sterile saline intraperitoneal (i.p.) or glutathione (GSH, 125, 250 or 500 mg/kg, i.p.) or amifostine (AMF, 25, 50 or

100 mg/kg, i.p.) or mesna (2-mercaptoethane–sulfonic acid) in the classic protocol (20% of IFS dose, 80 mg/kg, i.p., 5 min before, 4 and 8 h after IFS administration) 1 h before IFS (IFO, 400 mg/kg, i.p.) administration. The control group received only sterile saline. The animals were sacrificed 12 h after the administration of IFO, and their bladders were removed by careful dissection for evaluation.

Effect of glutathione and amifostine on ACR-induced HC

Animals were anesthetized with chloral hydrate (4%, 1 ml/100 g). A small midline abdominal incision was made, the bladder was exposed and ACR (75 μg) was injected into the bladder lumen (i.ve.), together with sterile saline or mesna (2 mg/bladder, i.ve.) or glutathione (2 mg/bladder, i.ve.) or amifostine (1.5 mg/bladder, i.ve.). In another set of experiments, the animals were treated with sterile saline or mesna (80 mg/kg, i.p.) or glutathione (500 mg/kg, i.p.) or amifostine (50 mg/kg, i.p.) 1 h before ACR (75 μg, i.ve.) injection. The negative control group received an i.ve. injection of sterile saline. Three hours after ACR administration, the animals were euthanized, and their bladders were removed by careful dissection, emptied of urine, and used to determine the different parameters described below.

Measurement of vesical edema

Vesical edema was determined by an increase in bladder wet weight (BWW; mg/20 g body weight), reported as the mean ± SEM. as compared to the saline group (S, treated with ACR or IFS and saline).

Macroscopic and microscopic evaluation

Bladders were excised, freed from surrounding connective tissue, and examined grossly for edema and hemorrhage. Histological examination was performed by a pathologist in a single-blind fashion. Edema, bleeding, and histological changes were assessed according to the criteria of Gray et al. [7] as follows: edema was considered severe (3+) when fluid was seen externally and internally on the walls of the bladder, moderate (2+) when confined to the internal mucosa, mild (1+) when normal to moderate, or absent (0). Hemorrhage was scored as follows: 3+, i.ve. clots; 2+, mucosal hematomas; 1+, telangiectasia or dilatation of the bladder vessels; 0, normal. Histopathology was scored as follows: 0, normal epithelium and absence of inflammatory cell infiltration and ulceration; 1, mild changes involving reduction of epithelial cells, flattening with submucosal edema, mild hemorrhage, and few ulcerations; 2, severe changes including mucosal erosion, inflammatory cell infiltration, fibrin deposition, hemorrhage, and multiple ulcerations. The macroscopic and microscopic observations were reported as medians and range.

Statistical analysis

Evaluation of the BWW data was carried out by analysis of variance (ANOVA) followed by Bonferroni's test when appropriate. For macroscopic and microscopic analysis, we used the non-parametric Kruskal–Wallis test, followed by Dunn's test when necessary, to compare medians. $P < 0.05$ was considered as statistically significance.

Results

Protective effect of amifostine and glutathione against IFS-induced HC

Intraperitoneal injection of IFO 400 mg/kg induced a marked increase (193% $P < 0.05$) in bladder wet weight (BWW) at 12 h compared to the control group. IFO-evoked increase in BWW was significantly inhibited by pretreatment with glutathione at doses of 250 and 500 mg/kg (63 and 78% reduction, respectively, $P < 0.05$, Fig. 1a), and with amifostine at doses of 25, 50 and 100 mg/kg (75, 93 and 97% reduction, respectively, $P < 0.05$; Fig. 1b). Pretreatment with the classic mesna protocol similarly inhibited the increase in BWW (88% reduction, $P < 0.05$).

Table 1 shows the scores of the macroscopic analysis of the HC 12 h after IFO administration. It was characterized by the presence of severe edema, receiving a score of 3 (3–3), and by marked hemorrhage with mucosal hematomas and i.ve. clots, receiving a score of 2 (2–3), being significantly ($P < 0.05$) different from the control group which received a score of 0 (0–0) for edema and hemorrhage. Treatment with glutathione (500 mg/kg) or amifostine (50 or 100 mg/kg) or mesna in the classic protocol reduced the intensity of HC scores ($P < 0.05$).

According to Gray's histopathological criteria, 12 h after IFO administration there was histological finding of HC: extensive mucosal erosion with ulceration, fibrin deposition, hemorrhage, edema, and leukocyte infiltration, receiving a score of 2 (2–2) (Fig. 2b). These alterations were almost abolished ($P < 0.05$) by the pretreatment with glutathione 500 mg/kg (Fig. 2c) or

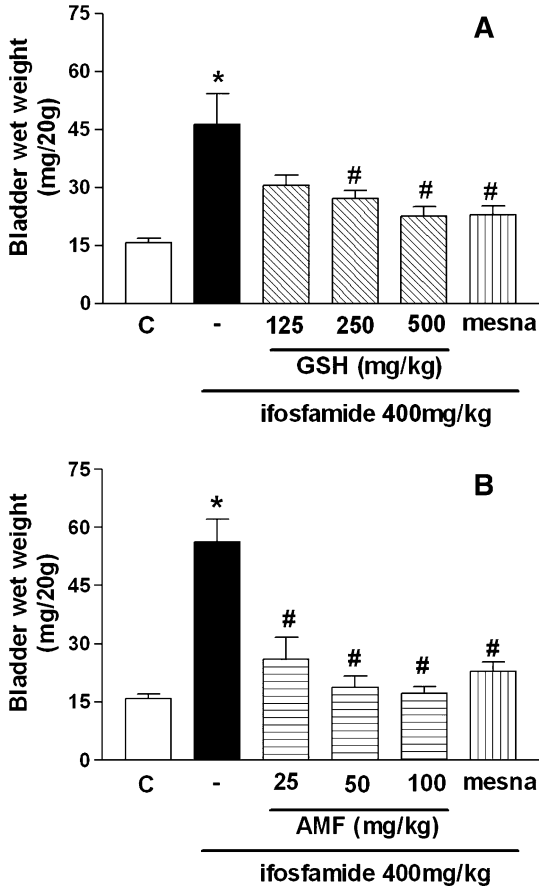

Fig. 1 Protective effect of glutathione (**a**) or amifostine (**b**) against bladder wet weight increase in ifosfamide (*IFS*)-induced hemorrhagic cystitis (*HC*). **a** Ifosfamide-induced increase in bladder wet weight was inhibited by glutathione (*GSH*) at 250 and 500 mg/kg, and **b** by amifostine (*AMF*) at 25, 50 and 100 mg/kg. Mesna in the classic protocol also inhibited the BWW increase. The results are reported as means ± SEM ($n = 6$). $^{*}P < 0.05$ compared to the control group (*C*) which received only saline. $^{\#}P < 0.05$ compared to the group that received only IFS 400 mg/kg plus vehicle. ANOVA followed by Bonferroni's test

amifostine 50 or 100 mg/kg (Fig. 2d) or mesna. The scores of Gray's parameters (medians and range) are shown in Table 1.

Protective effect of amifostine and glutathione on ACR-induced HC

Acrolein evoked a significant BWW intensification observed 3 h after its i.ve. injection (193% increase, $P < 0.05$) when compared to the control group. Systemic (500 mg/kg, i.p.) or i.ve. (2 mg/bladder, i.ve.) treatments with glutathione inhibited the BWW increase (76 and 100% reduction, respectively, $P < 0.05$; Fig. 3a). Similarly, systemic (50 mg/kg, i.p.) or i.ve. (1.5 mg/bladder, i.ve.) treatments with amifostine also

Table 1 Effect of glutathione and amifostine on ifosfamide (IFS)-induced macroscopic and microscopic alterations

Groups	Macroscopic analysis (edema)	Macroscopic analysis (hemorrhage)	Microscopic analysis
C	0 (0–0)	0 (0–0)	0 (0–0)
IFO	3 (3–3)	2 (2–3)	2 (2–2)
GSH 125	1 (1–2)	1 (0–2)	2 (1–2)
GSH 250	1 (0–1)*	0 (0–1)	1 (1–2)
GSH 500	1 (0–1)*	0 (0–0)*	1 (0–1)*
AMF 25	0 (0–2)*	0 (0–1)*	0 (0–2)*
AMF 50	0 (0–1)*	0 (0–1)*	0 (0–1)*
AMF 100	0 (0–1)*	0 (0–1)*	0 (0–1)*
Mesna	0 (0–2)*	0 (0–2)*	0.5 (0–2)

Ifosfamide (400 mg/kg)-induced macroscopic and microscopic alterations were evaluated at 12 h after its administration. Glutathione (125, 250, 500 mg/kg) or amifostine (25, 50, 100 mg/kg) or mesna in the classic protocol-treated animals were evaluated at 12 h after IFS injection. The results are reported as medians and range ($n = 6$)

$^{*}P < 0.05$, compared to the IFS group (IFO, treated with IFS and saline). Kruskal–Wallis and Dunn's test

inhibited BWW increase (52 and 87%, respectively; Fig. 3b). We evidenced a statistical difference between systemic and local pretreatments with amifostine ($P < 0.05$). Mesna inhibited BWW increase in systemic (80 mg/kg, i.p.) or i.ve. (2 mg/bladder, i.ve.) treatments (74 and 78%, respectively, $P < 0.05$, data not shown).

Hemorrhagic cystitis observed 3 h after ACR administration was characterized macroscopically by the presence of substantial edema, receiving a score of 2 (2–3) and by extensive hemorrhage, receiving a score of 3 (2–3), being significantly ($P < 0.05$) different from the control group which received a score of 0 (0–0) for edema and hemorrhage. Macroscopical analysis of the bladder 3 h after ACR injection revealed that glutathione (i.p. or i.ve.) or amifostine (i.p. or i.ve.) or mesna (i.p. or i.ve.) significantly reduced ($P < 0.05$) the parameters analyzed (Table 2). According to Gray's histopathological criteria, ACR-injected bladders received a score of 2 (1–2) and this alteration was almost abolished ($P < 0.05$) by treatment with glutathione or amifostine or mesna (i.p. and i.ve.; Table 2).

Discussion

In the present study, we demonstrated the protective effect of glutathione and amifostine against HC induced by IFS and its urotoxic metabolite ACR. Either glutathione or amifostine reversed, in a dose-dependent manner, the IFS-induced increase in bladder wet weight, edema and hemorrhage and histopathological

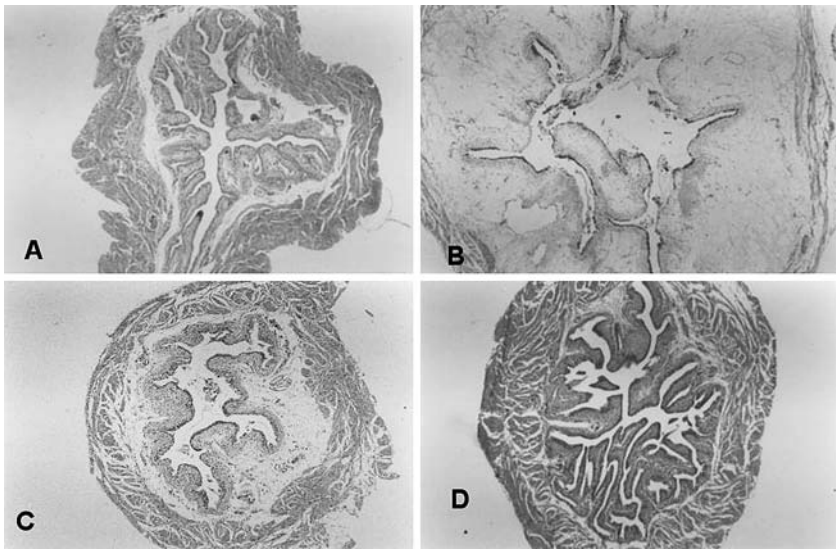

Fig. 2 Histological analysis of cross sections of representative bladder walls. **a** Normal bladder. **b** Hemorrhagic cystitis in mice treated 12 h prior with IFS [400 mg/kg, intraperitoneal (i.p.)] showing intense urothelial damage, edema, leukocyte infiltration, hemorrhage and fibrin deposition. **c** Bladder of mice treated with glutathione (500 mg/kg, i.p.). Observed were urothelium preservation and the absence of edema, hemorrhage, leukocyte infiltration and ulcerations. **d** Bladder of mice treated with amifostine (100 mg/kg, i.p.). Shown is the absence of microscopic alterations, as seen with glutathione treatment. Hematoxylin–eosin, ×40. *Bar* 250 μm

findings characterized by intense urothelial damage, edema, leukocyte infiltration, hemorrhage and fibrin deposition. Furthemore, glutathione and amifostine administered systemically or intravesically also protected against the urotoxicity induced by ACR.

There are few studies describing the effects of sulfhydryl-containing compounds (such as amifostine and glutathione) on oxazaphosphorine-induced HC. Supporting our results, it was observed that the inhibition of glutathione synthase by buthionine sulfoximine increases [10] and L-cysteine, a glutathione precursor aminoacid, prevents [16] experimental, cyclophosphamide-induced cystitis. Moreover, in a recent study, the authors compared the effects of mesna, the standard drug used to prevent HC induced by oxazaphosphorines, and glutathione on cyclophosphamide-induced HC, concluding that the protective effects are similar [3]. Another previous study demonstrated the uroprotective effect of amifostine in cyclophosphamide-induced HC. However, that work did not test amifostine in ACR-induced HC [20].

We did not investigate in the present study the mechanism by which amifostine and glutathione protects against IFS or ACR-induced HC. However, the literature describes that the mechanisms of action of these two compounds are similar, but not exactly the same. Amifostine (WR-2721) needs to be metabolized to WR-1065, the free thiol compound, that has a sulfhydryl group (-SH) in its chemical structure, which has a cytoprotective effect. It binds to toxic metabolites of drugs and/or to free radicals, inactivating them. Besides these effects, WR-2721 is able to repair the cell DNA through the donation of hydrogen atoms [12, 14]. Furthermore, it is also described that amifostine increases glutathione blood levels, promoting an additional protection pathway [22]. On the other hand, glutathione is a physiological anti-oxidant compound in the mammalians. It contains a sulfhydryl group in L-cysteine residues, which is responsible for its protective effect against xenobiotics, reactive oxygen and nitrogen species and other types of oxidative stress [5].

In a previous study, we demonstrated that nitric oxide derived from inducible NOS is involved in oxazophosphorine-induced cystitis [15, 19]. The treatment of cyclophosphamide-injected mice with NOS inhibitors protected in dose-dependent manner the bladder lesions. The induction of inducible NOS seems to be mediated by pro-infllammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and also by platelet-activating factor (PAF) [15, 19]. In fact, the treatment of oxazophosphorine-injected animals with antibodies against IL-1β or TNF-α 14 [15], with thalidomide and pentoxifyline [15], drugs that inhibit the synthesis of those cytokines, or with BN52021, an anti-PAF receptor antagonist [19], reduces bladder lesions and also the expression of inducible NOS. The involvement of reactive oxygen

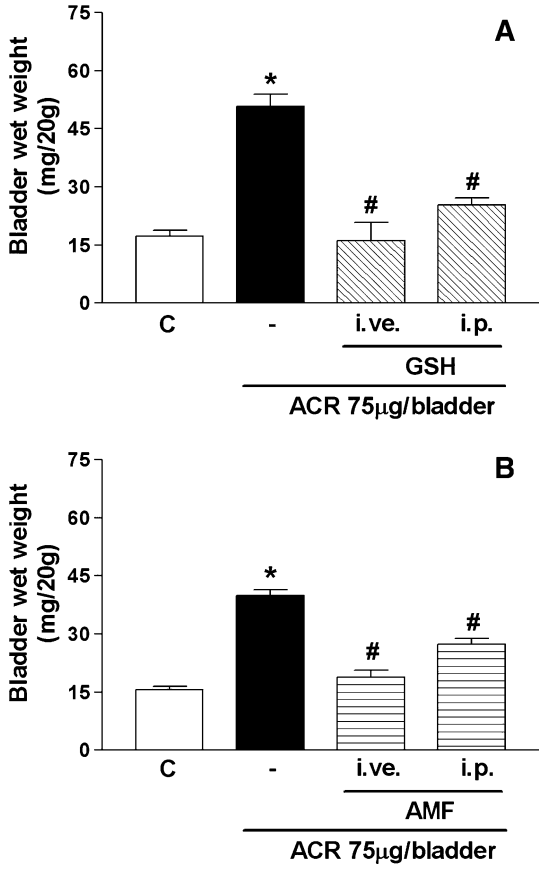

Fig. 3 Protective effect of glutathione (**a**) or amifostine (**b**) on bladder wet weight increase in acrolein (*ACR*)-induced HC. ACR (75 μg/bladder)-induced increase in bladder wet weight was inhibited by **a** glutathione (*GSH*) administration [intravesical (i.ve.)2 mg/bladder or i.p. 500 mg/kg], or **b** amifostine (*AMF*) (i.ve. 1.5 mg/bladder or i.p. 50 mg/kg), or Mesna 80 mg/kg i.p. or 2 mg/bladder i.ve. also inhibited the BWW increase. The results are reported as means ± SEM ($n = 6$). $^{*}P < 0.05$, compared to the group that received only saline. $^{\#}P < 0.05$, compared to the group that received only IFS 400 mg/kg plus vehicle. ANOVA followed by Bonferroni's test

species and peroxynitrite in oxazophosphorine-induced cystitis has also been suggested [11, 21]. Thus, it can be suggested that the protective effects of amifostine and glutathione in cystitis are, at least in part, a consequence of the inactivation of the free radicals released in the bladder due oxazophosphorine treatment.

The mechanism of action of mesna in alkylating agent-induced HC is related to its capacity to bind ACR, the final urotoxic metabolite, forming a non-urotoxic thioether, and thereby preventing the initiation of the inflammatory pathway [12]. Taking into account that mesna is also a sulfhydryl-containing drug, we could not discard the possibility that the urotrotective mechanism of amifostine and glutathione could be also due to an ACR binding or that mesna uroprotection could be due to a free-radical scavenging.

Table 2 Effect of glutathione [intravesical (i.ve.) and intraperitoneal (i.p.)] and amifostine (i.ve. and i.p.) on acrolein (ACR)-induced macroscopic and microscopic alterations

Groups	Macroscopic analysis (edema)	Macroscopic analysis (hemorrhage)	Microscopic analysis
C	0 (0–0)	0 (0–0)	0 (0–0)
ACR 75 μg	2 (2–3)	3 (2–3)	2 (1–2)
GSH i.ve.	0 (0–0)*	0 (0–1)*	0 (0–1)*
GSH i.p.	0 (0–0)*	0 (0–0)*	0 (0–1)*
AMF i.ve.	0 (0–1)*	0 (0–1)*	1 (1–2)*
AMF i.p.	1 (1–2)*	1 (0–2)*	1 (1–2)*
Mesna i.ve.	0 (0–1)*	0 (0–1)*	0 (0–1)*
Mesna i.p.	1 (0–1)*	0 (0–1)*	0 (0–2)*

Acrolein (75 μg/bladder)-induced macroscopic and microscopic alterations were evaluated at 3 h after its administration. Glutathione (i.ve. 2 mg/bladder or i.p. 500 mg/kg) or amifostine (i.ve. 1.5 mg/bladder or i.p. 50 mg/kg) or mesna (i.ve. 2 mg/bladder or i.p. 80 mg/kg)-treated animals were evaluated at 3 h after ACR injection. The results are reported as medians and range ($n = 6$)

$^{*}P < 0.05$, compared to the ACR group (ACR, treated with ACR and saline). Kruskal–Wallis and Dunn's tests

Studies of ischemia-reperfusion lesion suggest that mesna act as a free-radical scavenger [17].

In conclusion, the present study demonstrated that amifostine and glutathione, both antioxidant agents, are capable of protecting against IFS- and ACR-induced HC. The effects on the ACR-model were demonstrated by systemic (i.p.) as well local (i.ve.) administration. Taking into account that amifostine is already being used for the management of cancer patients, it is possible to propose a clinical trial to investigate its effect on human HC.

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