Chemotherapy-induced hemorrhagic cystitis: pathogenesis, pharmacological approaches and new insights

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Abstract

Chemotherapy-induced hemorrhagic cystitis (HC) remains a common and life-threatening clinical complication, mainly due to the increasing usage of alkylating agents during conditioning regimen for hematopoietic cell transplantation.

Currently, mesna and hyperhydration are the two more employed preventive measures. However, these prophylactic approaches have been proven not to be completely effective, since cystoscopic and histopathologic bladder damage are evidenced. Therefore, understanding the pathogenesis of HC must be the cornerstone for the development of novel therapeutic strategies.

The purpose of this review is to examine the current knowledge regarding the pathogenesis of HC, describing the importance of transcription factors (nuclear factor kappaB), cytokines (tumor necrosis factor-alpha, interleukin-1β, -4, -6, and -8), enzymes (inducible nitric oxide synthase and cyclooxygenase-2), among other mediators, for the bladder injury. We also discuss the currently available animal models and future perspectives on the management of HC.

Key words: Cancer; Chemotherapy; Hemorrhagic cystitis; Pathogenesis

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Introduction

Cystitis is a common clinical occurrence in cancer patients. It can be separated into three broad categories: cystitis consequent to bladder cancer or adjacent cancers that encroach upon the bladder from the prostate, uterus, cervix, or rectum; infectious cystitis that occurs in immune-compromised cancer patients; and chemotherapy- or radiotherapy-induced cystitis [1].

Whatever the cause, patients often have complaints typical of cystitis, which can be grouped into a cluster of symptoms called “LUTS” (lower urinary tract symptoms): dysuria, frequency, urgency, nocturia, suprapubic pain, and microscopic or gross hematuria [1].

In this context, hemorrhagic cystitis (HC) is considered to be the most severe form in the spectrum of the chemotherapy-induced bladder injuries and is defined as the inflammation of the urinary bladder leading to irritative LUTS up to severe hemorrhage [1-3]. In patients with mild HC, hematuria can cause iron deficiency anemia, but in severe cases, it may lead to hemodynamic instability due to hypovolemic shock. In addition to the somatic symptoms, we hypothesize that HC may also lead to a burden of suffering, depression, limitations and a worse quality of life, although there has been a complete absence of studies in this area.

Although busulfan, adenovirus, BK virus, cytomegalovirus, graft-versus-host disease and radiation are causative factors of HC, it most often occurs following chemotherapy, mainly after regimens with oxazaphosphorines (cyclophosphamide or ifosfamide) [4]. Cyclophosphamide and ifosfamide are alkylating agents with therapeutic efficacies in multiple malignancies, including testicular cancer, small-cell and non small-cell lung cancer, soft tissue sarcoma, gynecological cancer, bladder cancer, non-Hodgkin’s lymphoma, advanced breast cancer, and high-dose cyclophosphamide is often used as part of a conditioning regimen for hematopoietic cell transplantation [5].

The incidence of HC is variable and appears to be dependent on the type of treatment given. Before the use of prophylactic regimens, HC occurred in
10-40% of the patients exposed to high-dose cyclophosphamide chemotherapy for solid tumors [6] and up to 70% of patients following bone marrow transplantation [7]. The incidence has been reduced to 6-50% using prophylactic measures, such as hyperhydration, bladder irrigation and 2-mercapto-ethane-sodium sulphonate (mesna) [7-9]. Recently, Hadjibabaie et al performed a non-randomized controlled clinical study and showed that continuous bladder irrigation with mesna treatment and hyperhydration can additionally decrease the incidence of cyclophosphamide-induced HC (by 32 to 50%) and its relative complications in a conditioning regimen for allo-geneic hematopoietic cell transplantation [10].

Despite the prophylactic approaches, HC remains a common and life-threatening complication of high-dose chemotherapy during bone marrow transplantation, leading to prolonged hospitalization [10, 11] and sometimes death [12]. It seems that the standard preventive protocol does not completely protect from the chemotherapy-induced bladder injury, even when clinical signs and symptoms are not reported. In a randomized controlled clinical study of ifosfamide-based chemotherapy recipients, we demonstrated that even with classical prophylaxis with three doses of mesna, 66.7% of patients presented with gross cystoscopic injuries, and 100% had urothelial damage, such as edema, exocytosis, and hemorrhage [13], possibly revealing a novel concept of “subclinical hemorrhagic cystitis.” It remains unknown if this clinical occurrence may have any negative consequences.

Thus, HC remains a clinical problem, and both therapeutic and preventive measures have been tested. Various therapeutic interventions are utilized to produce hemostasis, such as continuous bladder irrigation with isotonic saline, clot evacuation by cystoscopy, electrocoagulation of bleeding vessels, intravesical therapy with chemicals (formalin, aluminum, phenol, silver nitrate, and prostaglandin), and hyperbaric oxygen therapy. In more severe cases, the embolization or ligation of the vesical [14] or internal iliac arteries, urinary diversion, and cystectomy are used, although they are usually reserved for refractory hemorrhage [15].

Therefore, understanding the pathogenesis of oxazaphosphorine-induced HC must be the cornerstone for the development of novel effective preventive and therapeutic strategies. For that reason, animal models of HC have been created recently to aid in the studies of HC pathogenesis. The most recent hypothesis implicates a number of transcription factors, cytokines, and inducible enzymes. This review will describe in detail the ultimate mediators and pathways involved in the generation of HC and will also discuss the currently available animal models and future perspectives on the management of HC.

Animal models for understanding HC pathogenesis

Animal models have been developed to focus on the morphological changes in the bladder and on the pathogenic processes involved. The choice of an animal model depends on various factors, such as the accessibility to the animal, cost, administration route, and the question under investigation. In summary, there are two major models of oxazaphosphorine-induced HC in rats or mice: the systemic injections of cyclophosphamide or ifosfamide and the intravesical injections of acrolein (Fig.1).

Figure 1.
Experimental hemorrhagic cystitis. Routes of administration commonly used for the induction of experimental hemorrhagic cystitis. (a) Intravesical injection of acrolein; (b) intraperitoneal injection of cyclophosphamide or ifosfamide; (c) normal bladder; (d) bladder presenting hemorrhagic cystitis; (e) histopathologic aspect of normal and (f) injured bladder.
Cyclophosphamide-induced HC

Experimental cyclophosphamide-induced bladder damage has previously been shown in rats, dogs [16] and mice [17]. However, the first rat model was generated by Tolley and Castro in 1975 [18]. The animals received cyclophosphamide by intraperitoneal or intravenous injections (50, 100, 150, 200 and 250 mg/kg). At different times thereafter (from 2 h to 16 days), the animals were killed, and their bladders were removed and analyzed by histopathology. In this system, HC occurred in a dose-dependent manner and was measured by bladder wall edema, polymorphonuclear infiltration and submucosal hemorrhage, mainly identified 8 h following a single intravenous injection of 100 mg/kg [18]. Furthermore, Gray et al defined a system for the score evaluation of macroscopic edema and bleeding and for histological changes [19]. Souza-Filho et al assessed bladder edema by measuring the bladder wet weight and vascular permeability by Evans blue extravasation, with greater damage occurring 24 h after the cyclophosphamide injection [20].

Ifosfamide-induced HC

Based on the evidence that HC is observed more often in patients receiving ifosfamide than in those receiving cyclophosphamide [21], our group developed a model to study the ifosfamide-induced HC in Swiss mice [22]. HC was induced by the intraperitoneal injection of 100, 200 and 400 mg/kg ifosfamide. At various times thereafter (6, 12, 24, 36 and 48 h), the animals were euthanized, and their bladders were removed, emptied of urine and evaluated for vesical edema (bladder wet weight), macroscopic and microscopic Gray’s criteria [19]. Ifosfamide-induced vesical edema was greatest at 12 h after ifosfamide administration. The microscopic analysis revealed vascular congestion, edema, hemorrhage, fibrin deposition, neutrophil infiltration, and epithelial denudation [22]. In a similar study with rats, our group induced HC with an intraperitoneal injection of 400 mg/kg ifosfamide, and the bladders were removed 24 h later [23].

Acrolein-induced HC

In 1985, Chaviano et al administered acrolein into rat bladders and observed HC similar to that found with cyclophosphamide injected intraperitoneally [24]. Conversely, our group showed for the first time a mouse model of acrolein-induced HC using a pharmacological dose-response curve (25, 75 and 225 μg/bladder) [25]. This work showed that the intravesical administration of acrolein induced a dose- and time-dependent (3, 6, 12, 24 h) increase in the vascular permeability and bladder wet weight as confirmed by histopathological Gray’s criteria, and pretreatment with mesna inhibited all the changes induced by acrolein [25]. The injury was greatest at 12 h. This model has a potential advantage over the systemic administration of cyclophosphamide or ifosfamide because it does not require the hepatic metabolism to acrolein and could be a simple and useful model for the evaluation of HC pathogenesis and new uroprotective drugs.

Hemorrhagic cystitis pathogenesis

For a long time, the mechanisms involved in the pathogenesis of HC were largely unknown and were a matter of considerable interest for researchers. Over the last two decades, several studies have tried to elucidate these pathways. Most of the published articles concentrated on the cyclophosphamide-induced injury, and only a few are related to ifosfamide; however, the underlying mechanisms seem to be equivalent.

The first description of symptomatic bladder damage with macro- and microscopic hematuria after cyclophosphamide administration was described shortly after its introduction as an antineoplastic agent in 1958 [26, 27]. In addition, cyclophosphamide was shown to induce bladder lesions in rats and dogs [16].

Early studies focused on implicating the alkylating agent itself. However, Philips et al demonstrated that the effects were caused by the metabolic products excreted into the urine [16]. They also found that the urine from the dogs treated with cyclophosphamide could reproduce cystitis when transplanted into the bladder of control dogs [16]. Ten years later, acrolein was characterized as a component present in the urine after cyclophosphamide metabolism [28]. In 1979, subsequent work by Cox definitively identified acrolein as the causative agent of HC lesions [29].

Another important hallmark was the first clinical description of the preventive use of mesna in the late 1970s [30]. Despite this knowledge, the first demonstration of the involvement of pro-inflammatory mediators, such as cytokines and nitric oxide (NO), in the pathogenesis of oxazaphosphorine-induced HC was described more than fifteen years later by our group [20, 31]. Since then, remarkable progress has been made.

Based on the current information on the pathophysiology of HC and taking into account the four-stage model proposed by Sonis for alimentary

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tract mucositis [32], we suggest a four-stage model to describe the phases involved in HC (Fig.2). The first stage (initiation phase) occurs after acrolein accumulates in the bladder, leading to urothelial damage. The following stage (inflammatory phase) is marked by the up-regulation of transcription factors, such as nuclear factor kappaB (NF-κB) [33, 34], and the local release of inflammatory cytokines by the epithelial and conjunctive resident cells, such as macrophages [22, 31]. The amplified response is marked by large amounts of cytokines, reactive oxygen species and the expression of inflammatory enzymes, such as the inducible nitric oxide synthase (iNOS) isoform and cyclooxygenase-2 (COX-2) [20, 22, 31, 35-37]. The combination of NO and superoxide radicals can lead to the production of the over-reactive free radical peroxynitrite [38]. The third stage (symptomatic phase) involves the urothelial denudation and ulcer formation, also leading to visceral pain and bladder dysfunction [39]. The fourth stage (healing phase) involves the tissue repair with possible signaling from the fibroblasts and the local release of growth factors, such as keratinocyte growth factor [40]. In the following sections, we attempt to describe the main pathological aspects of chemotherapy-induced HC.

Figure 2. Proposed sequential phases of the development of hemorrhagic cystitis.

The role of transcription factors and inflammatory mediators in the pathogenesis of hemorrhagic cystitis

Along with other groups, our laboratory has investigated the role of inflammatory mediators in the pathogenesis of alkylating agent-induced HC. Beginning with the demonstration of the participation of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [31] and through the latest studies, the involvement of several cytokines and other inflammatory mediators has been suggested [20, 22, 35-39, 41-43]. Each of these mediators can be up-regulated by the activation of the NF-κB pathway in response to inflammatory damage, which makes this transcription factor a possible target for the prevention of chemotherapy-induced HC [44].

Nuclear factor kappaB

The transcription factor NF-κB is one of the key regulators of the genes involved in the immune and inflammatory response [45]. Under most circumstances, NF-κB homodimers or heterodimers are bound to IκB inhibitory proteins in the cytoplasm of unstimulated cells. Many cytokines promote the classical NF-κB pathway, in which the serine phosphorylation and degradation of the IκB proteins leads to the dissociation of the inactive cytosolic NF-κB/IκB complexes and the subsequent NF-κB translocation to the nucleus for DNA binding. Upon translocation to the nucleus, the NF-κB dimer binds to the promoters of the NF-κB-dependent genes and facilitates their transcription [46]. NF-κB induces the expression of the cytokines TNF-α and IL-1β, the acute phase proteins iNOS and COX-2, and adhesion molecules [34]. The inappropriate activation of NF-κB can lead to diseases, such as HC.

The first experimental evidence for the role of NF-κB in the pathogenesis of HC was assessed by Kiuchi and co-workers [34] through the use of a potent NF-κB inhibitor, parthenolide [47]. These authors clearly showed by western blotting that parthenolide prevents the phosphorylation of NF-κB p65 and also blocks IκBα phosphorylation/degradation and NF-κB nuclear translocation [34]. They also demonstrated that parthenolide causes the marked inhibition of COX-2 expression in the bladders of cyclophosphamide-treated animals and inflammatory urothelial cells. These in vitro and in vivo anti-inflammatory effects of parthenolide were suggested to be dependent on the inhibition of NF-κB activity. NF-κB activity in the cyclophos-
phamide-treated animals contributes to the activation of downstream inflammatory genes, leading to bladder hyperactivity and HC [34].

**Tumor necrosis factor-α**

TNF-α regulates a number of cell functions, including cell proliferation, survival, differentiation, and apoptosis. TNF-α has been shown to play a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases. Because of this role as a key regulator of inflammatory cytokine production, it has been proposed as a therapeutic target for a number of diseases [48]. Aberrant TNF-α production and TNF receptor signaling have been associated with the pathogenesis of several diseases, including rheumatoid arthritis, psoriasis [49], Crohn’s disease [50], sepsis [51] and diabetes [52]. The receptors for TNF-α are either constitutively expressed in most mammalian tissues (TNF-R1) or are highly regulated and expressed in the cells of the immune system [53]. TNF, through its interaction with TNF-R1, causes various cellular events, including the activation of the caspase cascade that leads to apoptosis. The interaction between TNF and TNF-R1 also leads to the activation of NF-κB. TNF-R2 is known not to possess a death domain and therefore cannot directly cause apoptosis [53]. Depending on the cell type, TNF-R1 and TNF-R2 may have both distinct and overlapping roles in signal transduction and gene expression [48].

The role of TNF-α in the inflammatory cascade of HC was previously investigated by Gomes et al [31]. These authors clearly demonstrated that anti-TNF-α serum inhibits the histopathologic parameters of cyclophosphamide-induced tissue damage, such as mucosal erosion, hemorrhage, edema, leukocyte migration, fibrin deposition and ulcerations. The gains in both the bladder wet weight and vascular permeability were prevented in the anti-TNF-α serum-treated mice. These results are supported by the fact that thalidomide, which has been reported to inhibit the production of TNF-α by increasing its mRNA degradation [54], also prevented the ifosfamide-induced bladder injury [22]. Therefore, considering that anti-TNF-α therapy has been shown to effectively manage several malignances [55-58], clinical trials are needed to investigate the efficacy of specific TNF-modulating agents to treat HC.

**Interleukin-1β**

IL-1β is a highly inflammatory cytokine that affects nearly every cell type and often works in concert with other cytokines or small mediator molecules. The IL-1 receptor family (IL-1R) includes coreceptors, decoy receptors, binding proteins, and inhibitory receptors [59]. IL-1β interacts with two IL-1 receptors, type I (IL-1RI), which transduces cell signaling, and type II (IL-1RII), which lacks the signaling-competent cytosolic domain and is a decoy receptor [60, 61]. The extracellular IL-1 receptor chains, also known as soluble IL-1RI (sIL-1RI), sIL-1RII, and sIL-1RACp, are inducible negative regulators of IL-1β signaling in the extracellular space [61].

IL-1β first binds to the ligand-binding chain, IL-1RI, and then the coreceptor chain, termed the accessory protein (IL-1RACp), is recruited. The signal is initiated with the recruitment of the adaptors MyD88 to the Toll-IL-1 receptor (TIR) domain [62]. Subsequently, several kinases are phosphorylated, NF-κB translocates to the nucleus, and several inflammatory genes are expressed [62].

IL-1β is a pivotal pro-inflammatory cytokine implicated in the pathogenesis of stroke, diabetes and rheumatoid arthritis [62, 63]. Agents that reduce the production and/or activity of IL-1 are likely to impact the clinical management of pathologies such as HC.

Gomes et al evaluated the effect of anti-IL-1β serum on the animal model of hemorrhagic cystitis [31]. This modulating approach was beneficial against the cyclophosphamide-induced bladder damage and vesical wet weight gain but to a milder degree than the anti-TNF serum. In addition, pentoxifylline exerts multiple beneficial immunomodulatory effects by down-regulating IL-1β and TNF-α synthesis, decreasing interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor and IL-6 secretion, and attenuating the cell surface expression of the IL-2 receptor [64] and was also shown to inhibit the vesical edema and microscopic alterations induced by ifosfamide administration [22]. In fact, IL-1β is highly expressed in the epithelial and subepithelial bladder cells of the ifosfamide-injected mice as demonstrated by immunohistochemistry [43].

Considering the ability of IL-1β to orchestrate the inflammatory process and the protective effect achieved by its inhibition, the selective inhibition of IL-1 function could also present interesting clinical outcomes.

**Interleukin-4**

IL-4 is a cytokine that acts as an anti-inflammatory compound under certain conditions and is therefore responsible for the homeostasis of
the immune system. It has a marked inhibitory effect on the expression and release of pro-inflammatory mediators, such as the monocyte-derived cytokines TNF-α and IL-1β [65] and a modulating effect on the expression of IL-1 receptor antagonist (IL-1ra) [66]. In addition to the inhibition of the production of inflammatory cytokines, IL-4 has been reported to inhibit the induction of COX-2, with a consequent reduction in the production of prostaglandins [67].

Macedo et al explored the anti-inflammatory potential of IL-4 in the model of ifosfamide-induced HC [43]. Interestingly, IL-4 production is enhanced after ifosfamide administration. However, the administration of exogenous IL-4 inhibits inflammatory parameters, such as the expression of pro-inflammatory cytokines (TNF-α and IL-1β) and enzymes (iNOS and COX-2) [43]. In addition, it was demonstrated that both IL-4 antiserum-treated and genetically deficient mice have exacerbated HC.

**Interleukin-6 and interleukin-8**

IL-6 is a member of a cytokine family sharing a common signal transducer (gp130), which also includes IL-11, IL-27, IL-31, leukemia inhibitory factor, ciliary neurotrophic factor and cardiotoxin-I [68]. Some studies have reported an increased local expression of IL-6 in the bladder or in the serum after cyclophosphamide challenge [69-71]. Nishii et al [69] showed that IL-6 gene expression is significantly up-regulated in the submucosal layer of the bladder after cyclophosphamide administration in mice, peaking at 6 h and declining thereafter. In addition, Girard et al also showed that IL-6, IL-6 receptor α subunit (IL-6Ra) and gp130 (β subunit) are highly expressed in the urothelium and detrusor following cyclophosphamide treatment [71]. In contrast, IL-8 expression and its role in HC have never been investigated. IL-8 is a critical chemotactic agent responsible for leukocyte recruitment, cancer proliferation, and angiogenesis [72]. IL-8 has been implicated in the pathogenesis of diseases, including acute lung injury and acute respiratory distress syndrome [73], osteoarthritis [74] and cystic fibrosis [75].

We showed that IL-6 and IL-8 are involved in the pathogenesis of HC. Mice intraperitoneally injected with 200 mg/kg cyclophosphamide had significant increases in bladder wet weight and vascular permeability when compared to the saline-treated group. Furthermore, treatment with anti-IL-6 and anti-IL-8 sera (25 or 50 μL/animal) before the cyclophosphamide injection markedly prevented the development of edema (unpublished data).

As far as we know, this is the first report of an experimental modulation of IL-6 and IL-8 and the pathological contribution of these cytokines to the cyclophosphamide-induced edema in HC. Further studies are needed to precisely define the role of these mediators in the inflammatory cascade involved in this pathology.

**Bradykinin**

Bradykinin and its homologues, known collectively as kinins, are produced by the action of kininase enzymes [76]. The kinins are blood-derived, local-acting peptides that have broad effects mediated by two related G-protein-coupled receptors, the B1 and B2 bradykinin receptors. The endogenous kinin-kinin system controls blood circulation and kidney function and promotes inflammation and pain in pathological conditions [77], including sepsis and cancer [78].

Maggi and co-workers demonstrated the participation of bradykinin via B2 receptors in the genesis of detrusor hyperreflexia during cyclophosphamide-induced cystitis [79]. In addition, a study published by Chopra and colleagues showed that the B2 receptor, but not the B1 receptor, is expressed in the urothelium and detrusor smooth muscle of bladders from control animals [80]. However, the expression of B1 receptors is highly increased during cyclophosphamide-induced HC. Convincingly, these authors demonstrated that the urothelial expression of bradykinin receptors is plastic and altered by pathology, indicating a role of both bradykinin receptors in the cyclophosphamide-induced bladder hyperactivity via the extracellular release of urothelial-derived factors, such as ATP, which act on the pelvic nerve afferents [80].

The participation of the B2 receptors in cyclophosphamide-induced bladder edema formation was also investigated. Rats had a significant increase in their bladder wet weight and vascular permeability 48 h after the administration of cyclophosphamide (100 mg/kg) when compared to the saline-treated group. Animals pre-treated with Hoe-140 (2 mg/kg), a B2 receptor antagonist, plus cyclophosphamide markedly ameliorated both the vesical wet weight and vascular permeability in comparison with the cyclophosphamide-injected rats (unpublished data).

Despite the knowledge concerning the inflammatory importance of bradykinin, only one bradykinin receptor antagonist, icatibant (Hoe-140),
has reached the market for the treatment of hereditary angioedema [81].

Cyclooxygenases

COX is a group of bifunctional enzymes that have both cyclooxygenase and peroxidase activities. COXs catalyze the complex conversion of arachidonic acid to prostaglandins and thromboxanes, which act as autacoids with autocrine and paracrine biological effects to trigger many physiological and pathophysiological responses [82, 83]. Three isoforms of COX have been described to date: COX-1, -2, and -3. COX-2 is an isofrom whose expression is commonly upregulated during inflammatory processes. It plays an important role in pain [84], tumor activation [83], inflammation and neurodegenerative diseases [85]. The expression of COX-2 was already demonstrated during cyclophosphamide- [86], ifosfamide- [36] and acrolein- [37] induced HC.

Our group assessed the role of COX-2 on the pathogenesis of ifosfamide-related HC with a selective inhibitor of COX-2, etoricoxib, and a non-selective inhibitor, indomethacin. We observed that COX-2 is highly expressed 24 h after a single injection of ifosfamide to induce HC mainly in myofibroblasts and, to a lesser extent, in mast cells [36].

Hu et al also provided evidence for COX-2 mRNA expression due to cyclophosphamide injection [86]. The demonstration that COX-2 is observed a few hours after the induction of HC suggests the participation of other mediators in the first hours following ifosfamide administration [20, 22]. We verified this hypothesis by the use of non-selective cytokine inhibitors, thalidomide and pentoxifylline [36]. It was shown that these agents prevent some of the inflammatory parameters when compared to the groups treated only with ifosfamide, which is in accordance with the data published by Ribeiro et al [22]. In addition, a lower expression of COX-2 was observed when the animals were given thalidomide and pentoxifylline before ifosfamide administration, which supports a role for cytokines on COX-2 expression in this animal model of HC [26].

It is known that HC can arise as a complication of both cyclophosphamide and ifosfamide treatment and that it is caused by the urinary metabolite acrolein. Our group showed in mice that acrolein can cause severe edema and hemorrhage, similar to cyclophosphamide and ifosfamide [25].

In another study published by our group, acrolein injected into the rat bladder induced a dose- and time-dependent HC, and the resulting lesion is partially mediated by the increased expression of COX-2 [37]. However, this study also showed that even 3 and 6 h after the acrolein instillation into the bladder, COX-2 expression is almost absent, despite the presence of severe edema and hemorrhage.

Macedo et al reported that these observations suggest the participation of other inflammatory mediators in the changes observed within the first hours of acrolein-induced HC [37], as demonstrated previously for ifosfamide- and cyclophosphamide-induced HC [22, 31]. Macedo et al showed that between 3 and 6 h, there is not sufficient expression of the COX-2 enzyme to affect the increase of bladder wet weight in animals injected with acrolein [37]. The anti-inflammatory effect of etoricoxib was only detected 12 h post-acrolein injection.

Hu et al demonstrated that conscious cystometry in rats injected with cyclophosphamide and the COX-2 inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl2(5H)-furanone (DFU) showed increased micturition intervals 4 and 48 h after cyclophosphamide treatment and decreased intravesical pressures during filling and micturition compared with rats administered cyclophosphamide [86]. This finding clearly suggested an involvement of the urinary bladder COX-2 and its metabolites in the altered micturition reflexes during cyclophosphamide-induced cystitis.

Additionally, a very recent article published by our group showed that the HC experimentally induced by ifosfamide promotes detrimental changes in the motor functions of the rat lower urinary tract, interferes with the urinary bladder emptying process in anesthetized rats, and alters the contractile behavior as assessed by in vitro methods [39]. We also suggested that this phenomenon involves the inflammatory events mediated by the COX-2 expression within the urinary bladder with a consequent rise in the plasma levels of PGE2 [39]. In addition, the selective pharmacological inhibition of COX-2 activity partially ameliorated these functional changes. We concluded that the prevention of the inflammatory process ameliorates the consequences of the ifosfamide-induced cystitis.

Maggi [87] reviewed the role of prostanoids, such as PGE2, as activators of bladder afferents, which changes the micturition reflex threshold. The mechanism of PGE2-induced normal bladder hyperactivity was investigated by Ishizuka and colleagues [88], who showed that intravesical PGE2 might stimulate micturition by releasing
tachykinins from the nerves in and/or immediately below the urothelium. These tachykinins, in turn, initiate a micturition reflex by stimulating the NK1 and NK2 receptors, contributing to both urge and the bladder hyperactivity observed in the inflammatory conditions of the lower urinary tract. However, the precise mechanisms involved in the detrimental effects of ifosfamide in HC deserve further investigation. Possible explanations involve the modification of the synaptic mechanisms in the urinary bladder, e.g., those related to the muscarinic receptor response to agonists, which still needs to be proven.

Nitric oxide

NO is synthesized by a family of enzymes known as nitric oxide synthases (NOSs). Three distinct isoforms of NOS, namely endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), have been described based on the cells in which they were first identified, isolated, purified, and cloned [89]. Over the past two decades, NO has gained increasing recognition as an important neurotransmitter and cell mediator with a broad range of functions in the lower urinary tract.

A convincing first report on the participation of NO in the pathogenesis of chemotherapy-induced HC was published by our group [20]. This study showed that the injection of cyclophosphamide increases iNOS activity. Additionally, two NOS inhibitors, L-NAME and L-NOARG, dose-dependently inhibited the cyclophosphamide-induced plasma protein extravasation and bladder wet weight, which was reversed by L-arginine, a NO precursor. Such inhibitory actions on NO activation critically prevented bladder damage, as shown both macroscopically and microscopically by the reduced mucosal damage. Another study performed by Oter and colleagues evaluated the role of iNOS-derived NO on bladder injury due to cyclophosphamide injection [35]. In that study, the administration of S-methylisothiourea, a selective iNOS inhibitor, led to similar results as those previously reported.

We also investigated the role of constitutive NOS enzymes and found that NOS activity in the normal bladder was mainly due to the cNOS (calcium-dependent) isoform (>95%). Cyclophosphamide administration significantly increased the calcium-independent activity of iNOS, which was evident 6 h after the injection and remained elevated for up to 48 h. In contrast, cNOS decreased over a similar time course in the presence of cyclophosphamide [20]. Consistent with these findings, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), a marker for NOS-containing cells, was expressed in the urothelium and in the lamina propria of control rats. This expression decreased dramatically 12 h following cyclophosphamide administration and coincided with an intense sloughing of the urothelium. Alferi et al also explored the effect of the selective inhibition of nNOS, which did not result in a protective effect [41]. These observations indicate that inducible, but not constitutive, NOS-derived NO exerts a fundamental role in the pathogenesis of HC.

TNF-α and IL-1β were shown to mediate the production of NO through iNOS induction in the pathogenesis of ifosfamide- and cyclophosphamide-induced HC [22, 31]. The induction of iNOS in the urothelium appeared to depend on the production of the cytokines IL-1β and TNF-α because antisera against these cytokines also reduced the expression of iNOS in the urothelium [31].

Peroxynitrite

The cellular damage that commonly occurs during conditions of oxidative stress has been observed following the formation of peroxynitrite (ONOO−), which originates from the reaction of NO with superoxide free radicals (O₂•−) [90]. An excessive formation of peroxynitrite represents an important mechanism contributing to DNA damage, the inactivation of metabolic enzymes, ionic pumps, and structural proteins, and the disruption of cell membranes. Peroxynitrite reacts with the tyrosine in proteins to create nitrotyrosines [91]. Because of its ability to oxidize biomolecules, peroxynitrite is implicated in an increasing number of diseases, including neurodegenerative and cardiovascular disorders, inflammation, pain, autoimmunity, cancer, and aging [92].

A first report published by Korkmaz et al showed that peroxynitrite might contribute to the pathogenesis of cyclophosphamide-induced HC [38]; Korkmaz et al reported that ebselen, which has potent scavenging properties and was shown to react with peroxynitrite [93], markedly protects against the development of HC. The results of this study suggest that the scavenging of peroxynitrite and the inhibition of iNOS have similar protective effects.

Platelet activating factor

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid mediator that acts largely by binding to its receptor (PAFR), a G-protein-coupled receptor
found on most cells, such as monocytes, neutrophils, B lymphocytes, and keratinocytes [94-96]. PAF is involved in many biological processes, including cellular activation, cytoskeletal reorganization and intracellular signaling, and is a potent mediator in many inflammatory processes, including inflammatory bowel disease [97], multiple sclerosis [98], and asthma [99].

In a study by Rickard et al, the combination of increased prostacyclin and PAF in the bladder circulation resulted in vasodilation and increased polymorphonuclear leukocyte adherence to the endothelium and facilitated the recruitment of polymorphonuclear leukocytes to the bladder wall of patients with interstitial cystitis [100]. In addition, we found that PAF is one of the inflammatory mediators contributing to the activation of the L-arginine–NO pathway in HC [20]. This conclusion was reached by the use of the PAF antagonist BN52021, which markedly protected rats from the cyclophosphamide-induced plasma extravasation and bladder wet weight gain as well as further prevented the increase in bladder iNOS activity without modifying cNOS activity. The maintenance of cNOS activity during the PAFR blockade implies that the integrity of the epithelial surface was also maintained, corroborating the deleterious effect of PAF in HC.

**Substance P**

Neurogenic inflammation, components of which include arteriolar vasodilation (flare) and edema caused by the extravasation of plasma from post-capillary venules, is triggered by the release of substances, such as the proinflammatory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), from sensory nerve terminals [101]. In the urinary tract, such responses include hyperemia, plasma protein leakage, contraction of the ureter and bladder and hyperactivity of the bladder.

Acting through the tachykinin NK1 receptors, SP may induce tissue inflammation [102] due to the increased production of NO [103]. Structurally unrelated antagonist of the NK1 receptors has been shown to ameliorate the increase in the plasma permeability extravasation and the histological damage of the bladder induced by cyclophosphamide both in rats and ferrets [104]. It is possible that SP released from the primary afferent capsaicin-sensitive fibers stimulated by cyclophosphamide or its metabolite acrolein may increase NO levels, thereby inducing inflammatory changes [41].

**Alpha1-adrenoceptor**

Sympathetic efferents are known to modulate the neurogenic inflammatory responses by interacting with primary afferent terminals. α1-Adrenoceptor antagonists have been successfully used for the treatment of the symptoms in patients with indwelling double-J urethral stents [105], ureteral colic [106], prostatitis and benign prostatic hyperplasia [107].

Trevisani et al investigated whether α1-adrenoceptors are expressed in primary sensory neurons and whether they contribute to the neurogenic inflammatory responses that originated in the urinary tract following cyclophosphamide treatment [108]. These authors suggested that the α1-adrenoceptors contribute to neurogenic inflammation in the bladder based on the finding that locally applied phenylephrine increases plasma extravasation in a manner abolished by alfuzosin, an α1-adrenoceptor antagonist, and by NK1 receptor blockade. In addition, these agents were equally effective at inhibiting the bladder plasma extravasation due to cyclophosphamide injection. However, alfuzosin failed to affect the plasma extravasation evoked by the direct stimulation of the endothelial NK1 receptor by \([\text{Sar}^{9},\text{Met(O)_{2}}^{11}]\text{-SP}\), indicating selectivity. These results suggest that α1-adrenoceptor activation at the terminals of primary sensory neurons releases SP, which in turn stimulates the vascular NK1 receptors, promoting plasma protein leakage in the bladder tissue [108].

**Pharmacological approaches to hemorrhagic cystitis**

Table 1 shows a broad review of the experimental tools, natural products, and clinically available drugs that have already been tested in acrolein/chemotherapy-induced HC models. Among them, mesna and hyperhydration are currently the only measures used clinically to prevent HC. From our point of view, the literature lacks studies that test the effectiveness of anti-cytokine therapy of HC. However, some anti-inflammatory cytokines, such as IL-4, IL-11 and keratinocyte growth factor, have been experimentally evaluated with important contributions.

**Interleukin-4**

As discussed above, exogenous IL-4 administration was able to prevent the development of HC. Additionally, IL-4 seems to play an important role in HC pathogenesis, possibly in counteracting the inflammatory pathways, because

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HC was exacerbated in IL-4-deficient mice and in anti-IL-4 serum-treated mice [43].

**Interleukin-11**

IL-11 is a pleiotropic 178-amino acid polypeptide with a molecular weight of 18 kDa and is secreted by osteoblasts, synoviocytes, fibroblasts, chondrocytes, trophoblasts, and many other cell types in culture [109]. Recombinant human IL-11 (rhIL-11, oprelvelkin) has a number of biological activities, including the inhibition of pro-inflammatory cytokines (TNF-α and IL-1β), IL-12, NO synthesis and apoptosis [110, 111] as well as the stimulation of cell proliferation, differentiation and connective tissue protection [111]. Several studies have shown a protective role for IL-11 in a variety of models of inflammatory diseases, such as inflammatory bowel disease, psoriasis, and autoimmune joint disease [112, 113].

IL-11 affects various stages of megakaryocytogenesis and thrombopoiesis, and its clinical use has been shown to shorten the duration of chemotherapy-induced thrombocytopenia [114]. Additionally, the potential anti-inflammatory modulating effect of IL-11 was further investigated in a study published by our group [115] using an animal model of ifosfamide-induced HC. This study showed that rhIL-11 inhibits ifosfamide-induced vascular permeability and edema formation in a dose-dependent manner, which was confirmed by macroscopic and microscopic analysis. These results indicate that the therapeutic manipulation of IL-11 signaling may provide clinical benefits for HC.

**Keratinocyte growth factor**

Keratinocyte growth factor (KGF), a heparin-binding fibroblast growth factor also known as FGF-7, has been suggested to play an important role in the mesenchymal stimulation of normal epithelial cell proliferation [116]. The administration of KGF has been shown to protect animals from various insults, such as radiation- and bleomycin-induced lung injury [117] and mucositis [118, 119]. Furthermore, Ulich et al showed that KGF protects against cyclophosphamide-induced HC with a marked trophic action on urothelial cells and minor histological changes, such as edema and acute inflammation of the urothelium and submucosa [40].
### Table 1. Pharmacological approaches in acrolein- or cyclophosphamide(CYP)/ifosfamide(IFO)-induced hemorrhagic cystitis

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Chemotherapy-induced HC was considered to be an unsolved problem in the oncology patient population. Currently, clinical studies with palifermin (recombinant KGF) have indicated that hematopoietic stem cell transplant patients treated with high-dose cytotoxic regimens present with a reduced severity of lesions in the oral mucosa [120]. Considering that mucosal ulceration seems to be a key event in the most symptomatic phase of HC, further studies are needed to better investigate the potential protective effect of KGF on this disease.

Corticosteroid therapy
Taking into consideration the unquestionable participation of various inflammatory mediators in the pathogenesis of HC, there is a question of whether corticosteroids would be effective at preventing HC-associated bladder damage. This
hypothesis was experimentally tested by our group [23, 121]. In these studies, we verified that dexamethasone in combination with mesna was efficient in attenuating cyclophosphamide- or ifosfamide-induced HC. However, the replacement of the last two doses of mesna with saline or all of the mesna doses with dexamethasone did not prevent HC. This is a clear demonstration that mesna is still needed to prevent the initial epithelial bladder damage, which is not achieved by dexamethasone alone. However, the corticosteroid inhibition of cytokine activation prevents the underlying inflammatory aspect of HC. Therefore, clinical trials are needed to prove the experimental findings using corticosteroids.

Conclusions
Hemorrhagic cystitis is an important clinical issue that has been recently brought to attention due to an augmented incidence mainly following the increase in treatment regimens of high-dose alkylating agents in the context of hematopoietic stem cell transplantation and cancer chemotherapy. Several inflammatory mediators have been implicated in the development of hemorrhagic cystitis due to chemotherapy agents (Fig.3). Despite the recent advances in the knowledge of the pathogenesis of this clinical condition, further investigation is needed to determine the precise inflammatory cascade involved. The enhanced understanding of the role played by these inflammatory pathways may allow the identification of novel pharmacologic targets for this pathology.

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