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ORIGINAL PAPER

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Inducible nitric oxide synthase inhibition in cyclophosphamide induced hemorrhagic cystitis in rats

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Abstract Cyclophosphamide (CP) is an antineoplastic agent used alone or in combination with other chemotherapeutic agents for the treatment of many neoplastic diseases. Hemorrhagic cystitis (HC) is a major potential toxicity and dose limiting side effect of CP. Recently, it has been shown that endogenous inflammatory mediators are involved in cystitis by increasing nitric oxide (NO) production in target tissue. The aim of this study was to evaluate the relationship between NO and CP induced hemorrhagic cystitis HC in rats. A total of 30 female Spraque-Dawley rats were divided into 4 groups. Group 1 served as control, three groups received single dose of CP (100 mg/kg) intraperitoneally (i.p.): group 2 received CP only. Group 3 received the NO precursor L-arginine (80 mg/kg/day), and group 4 received the selective inducible NO synthase (iNOS) inhibitor S-methylisothiourea (SMT; 20 mg/kg/day) before and the day after cyclophosphamide injection. CP injection resulted in severe cystitis. SMT but not L-arginine produced marked inhibition of CP induced bladder damage. We concluded that NO produced by iNOS, is an important mediator in the pathogenesis of CP induced cystitis.

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Introduction

Cyclophosphamide (CP) is an alkylating antineoplastic chemotherapeutic agent in the nitrogen mustard group. It is used alone or in combination with other chemotherapeutic agents for the treatment of many neoplastic diseases. Hemorrhagic cystitis (HC) is a major potential toxicity and dose limiting side effect of CP and ifosfamide, a synthetic analog of CP [1]. The incidence of this side effect is related to dosage and can be as high as 75% in patients receiving intravenous CP. Its clinical symptoms vary from transient irritative voiding symptoms, including urinary frequency, dysuria, urgency, suprapubic discomfort, and strangury with microhematuria, to life-threatening HC. Bladder fibrosis, necrosis, contracture, vesicoureteral reflux, and a 4% mortality rate among patients with massive bladder hemorrhage have also been reported [2].

The urotoxicty of these nitrogen mustard group cytostatics is not based on a direct alkylating activity in the urinary system but on the formation of renally excreted 4-hydroxy metabolites, in particular acrolein, which is formed from hepatic microsomal enzymatic hydroxylation [3]. Prevention is the best way to reduce the side effect of CP. Among various prophylactic measures to treat CP-induced HC, such as mesna (2-mercaptoethane sulfonate), high fluid intake, diuretics, forced diuresis, and urine alkalinization, mesna has shown the most promising results. It contains a sulfhydryl compound which binds acrolein within the urinary collecting system and detoxifies it; the resultant inert thioether is passed innocuously in urine and does not induce any damage to the uroepithelium [4]. Although mesna has been widely used as an effective agent against CP-induced cystitis, significant HC, defined as an episode of symptomatic (burning, frequency, dysuria) microscopic or macroscopic hematuria, has still been encountered clinically [5].

Recently, it has been shown that endogenous inflammatory mediators such as platelet activated factor (PAF),

tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are involved in cystitis by increasing nitric oxide (NO) production in target tissue [6, 7, 8]. NO is a free radical gas that regulates a number of important physiological and pathophysiological process including vascular tone, polymorphonuclear leukocytes (PMNs), adhesion, and inflammation. NO is synthesized from the amino acid L-arginine by the enzyme NO synthase (NOS). There are three subtypes of NOS; endothelial NOS (eNOS) found in endothelial cells and fibroblasts which is mainly responsible for vasodilation, neuronal NOS (nNOS) found in the nervous system where it acts as an important signalling molecule, and inducible NOS (iNOS) which can be up-regulated in many more cells, such as PMNs and macrophages. iNOS activation produces a significantly greater amount of NO than eNOS. There is evidence suggesting that NO produced by iNOS is toxic, since in animal models selective iNOS inhibition improves outcome and decreases inflammatory events [9].

L-arginine is the precursor to nitrite-nitrate formation and NO is an intermediate in this reaction. Inhibition of NOS reduces the production of nitrite-nitrate. These findings show that the oxidation of NO is a major source of nitrite-nitrate, the plasma levels of which provide useful information on NO production [10].

Since detoxifying acrolein with mesna can not remove HC symptoms completely, and inflammatory mediators have been shown to be involved in pathogenesis, CP induced HC may not only be due to direct contact of acrolein with the bladder mucosa, but may also be related to increased NO production leading to inflammatory events. In order to examine this hypothesis, we investigated the changes of CP induced bladder damage, with or without iNOS inhibition, in an animal model.

Materials and methods

Animals

A total of 30 female Spraque-Dawley rats weighing 180–210 g were divided into four groups (Table 1) which had free access to food and water. The experimental protocol was approved by the Gulhane Military Medical Academy Animal Care and Use Committee.

Drug administration

A dose of 100 mg/kg CYP was used for cystitis induction in groups 2–4. The NO substrate L-arginine (80 mg/kg/day) and the selective iNOS inhibitor S-methylisothiourea (SMT, 20 mg/kg/day) were administered twice daily for a total of six doses in group 3 and 4, respectively. All drug administrations were performed intraperitoneally (i.p.).

Table 1 Cyclophosphamide, L-arginine and SMT treatment schedule. PS physiological saline instead of cyclophosphamide i.p., CP 100 mg/kg cyclophosphamide i.p., L-arg 2×40 mg/kg L-arginine i.p. SMT 2×40 mg/kg S-methylisothiourea i.p.

Groups	Day 1	Day 2	Day 3
1. Control ($n=7$)	-	PS	-
2. CP($n=7$)	-	CP	-
3. L-arg + CP ($n=8$)	L-arg	CP + L-arg	L-arg
4. SMT + CP ($n=8$)	SMT	CP + SMT	SMT

Tissue preparation

After 48 h of cystitis induction, rats were anesthetized using ketamine HCl (85 mg/kg) and xylazine HCl (12.5 mg/kg) i.p. Blood samples (6–8 ml) were taken from the vena cava inferior to analyze nitrite-nitrate levels. Bladders were taken intact and weighed to determine whether edema was present after the residual urine was removed. They were then fixed for 24 h in 10% buffered formalin. Tissue taken from the trigone region was embedded in paraffin and stained with hematoxylin-eosin. A pathologist, blinded to the study groups, rated for mean histological damage, including edema, hemorrhage and inflammation on a scale of 1 (normal) to 4 (severe changes). Mucosal ulceration was scored as 1 (normal), 2 (epithelial denuding), 3 (focal ulceration), and 5 (widespread epithelial ulceration). The measurement of plasma nitrite-nitrate was performed as described by Tracey et al. [11].

Statistics

The rate of histopathological damage is given as a median (min-max), nitrite-nitrate levels and bladder/body weight ratios are expressed as mean ± SEM; $P<0.05$ was considered to be significant. All of the numerical data were first analyzed using the nonparametric Kruskal-Wallis test to determine whether there were differences between groups and then the Mann-Whitney U-test was performed to analyze each pair of groups.

Results

All histological parameters, bladder/body weight ratios and nitrite-nitrate levels are summarized in Table 2. Control animals had histologically normal bladders with assigned scores of 1 for all parameters (Fig. 1). CP showed severe histological changes (Fig. 2) and macroscopic hematuria continued at the end of the study. Hematuria disappeared in the SMT group at 48 h after CP administration, but continued in the L-arginine group. No significant effect on bladder damage was observed in the L-arginine administrated group (Fig. 3). SMT showed nearly complete histological improvement (Fig. 4). CYP caused an increase of approximately 2.5-

Table 2 Comparison of the scores of histological damage in rat bladder [median (min-max)]. * Severe bladder damage as compared with control group ($P<0.01$ for all), ** Statistically significant improvement in bladder histology when compared with CP group ($P<0.05$ for edema, $P<0.01$ for hemorrhage, inflammation, and ulceration)

Groups	Edema	Hemorrhage	Inflammation	Ulceration
1. Control ($n=7$)	1	1	1	1
2. CP($n=7$)	4 (3–4)*	4 (3–5)*	4 (3–5)*	5 (4–5)*
3. L-arg + CP ($n=8$)	4 (3–4)*	4 (3–5)*	3.5 (3–4)*	4 (3–5)*
4. SMT + CP ($n=8$)	2.5 (2–4)**	2 (1–4)**	1.5 (1–2)**	1 (1–2)**

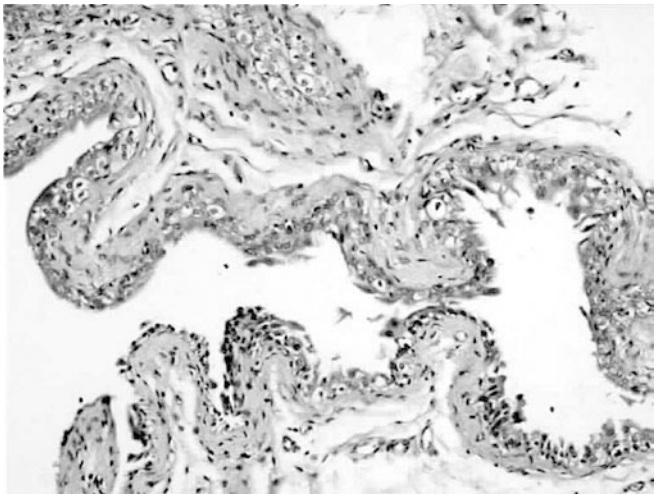

Fig. 1 Control animal: scores 1 for all parameters (edema, hemorrhage, inflammation, and ulceration). (H and E, approx. ×50)

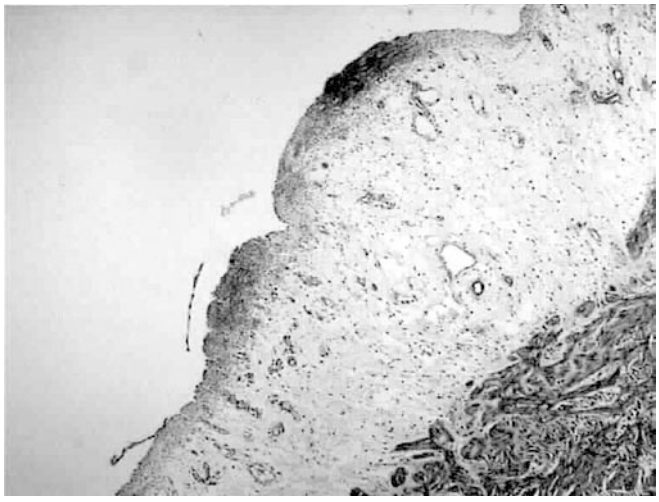

Fig. 3 Group 3: no significant effect with L-arginine on CP-induced bladder damage with a score of 4 for edema and hemorrhage, and 3 for inflammation and ulceration. (H and E, approx. ×50)

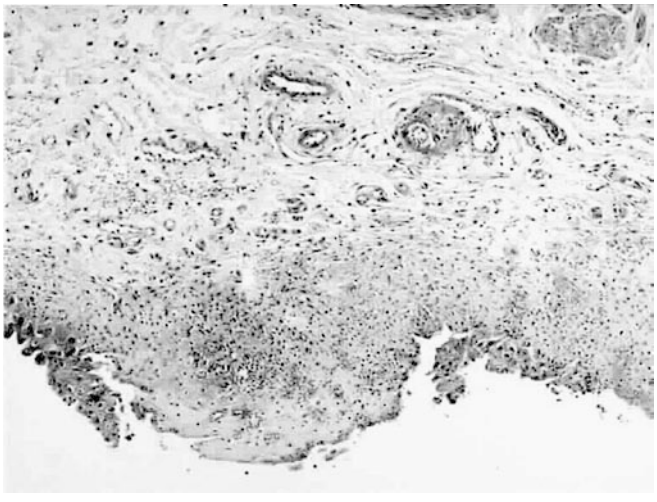

Fig. 2 Severe histopathological changes in group 2 induced by CP; score of 4 for edema, hemorrhage, and inflammation, 5 for ulceration. (H and E, approx. ×50)

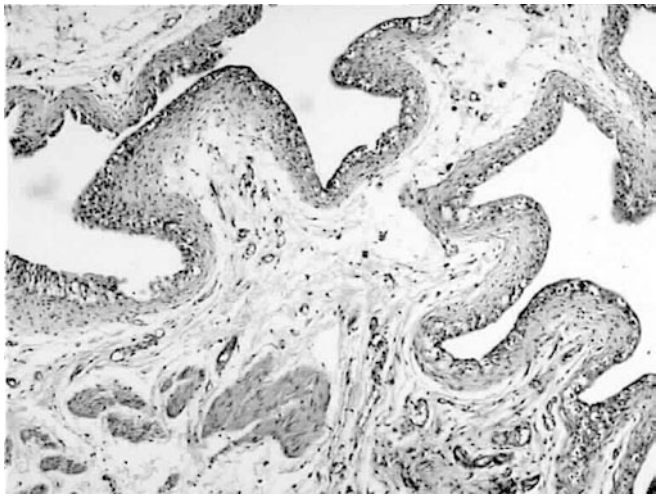

Fig. 4 Nearly complete histological improvement with SMT; score 2 for edema and inflammation, and 1 for hemorrhage and ulceration. (H and E, approx.×50)

fold in bladder/body weight ratios and 3.5-fold in nitrite-nitrate levels (Fig. 5, 6). SMT decreased both findings significantly ($P < 0.01$ for both).

No side effects were observed.

Discussion

CP, an antineoplastic alkylating agent, is used in more than 200,000 patients annually in the United States to treat neoplastic, immune mediated and transplant related diseases, and its use is likely to increase as new applications are discovered. HC, a major therapy-limiting side effect of CP, is thought to be induced by acrolein, a cytotoxic metobolite of CP which is excreted in the urine [1]. The main futures of HC are urothelial

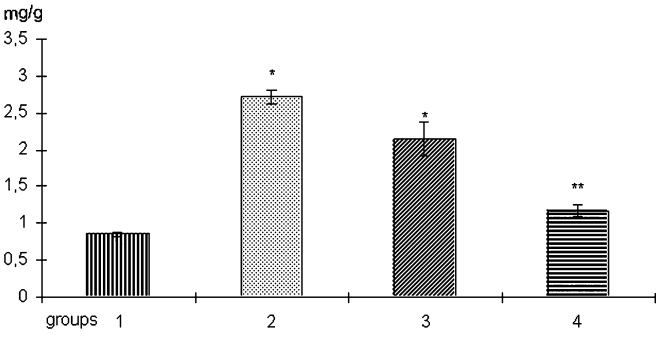

Fig. 5 Bladder/body weight ratios expressed as mg/g (mean ± SEM). An *asterisk* indicates significantly increased compared with control animals ($P < 0.01$). A *double asterisk* indicates significantly decreased if compared with the CP group ($P < 0.01$)

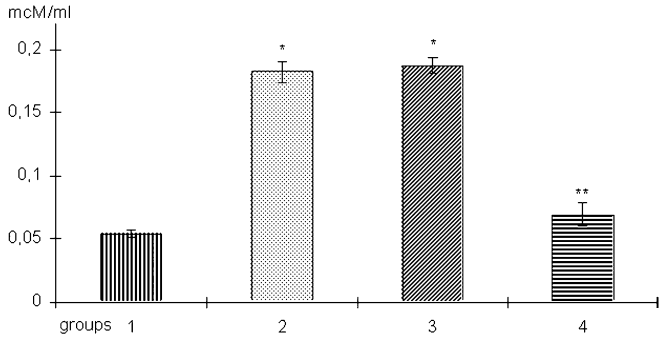

Fig. 6 Plasma nitrite-nitrate levels expressed as mmoles/ml (mean ± SEM). An *asterisk* indicates significantly increased when compared with control animals ($P < 0.01$). A *double asterisk* indicates significantly decreased if compared with CP group ($P < 0.01$)

damage, transmural edema, hemorrhage, mucosal ulceration and epithelial necrosis, which could be demonstrated within 24 h of a single dose. Mucosal sloughing has been frequently associated with acute and chronic hemorrhage. Within the first few hours, epithelial cells in the superficial mucosal layer of the bladder begin to degenerate and necrosis occurs. At 18 h p.i., most of the mucosal lining is eroded or ulcerated, and basal membrane damage is evident with subsequent damage to the surrounding capillaries. Thereafter, healing begins with evidence of mucosal hyperplasia and bizarre papillary proliferation. Neovascularisation and leukocyte infiltration may also be seen during the following days [4].

eNOS produced NO is secreted from endothelium, including some veins, arteries, and microvessels, in response to a variety of substances, such as acetylcholine, adenine nucleotides, thrombin, substance P, and bradykinin. Other stimuli, such as hypoxia, increased blood flow, and electrical stimulation, also cause NO production. This NO is released for short periods in response to receptor stimulation. The main mission of eNOS produced NO is vasoregulation and there is no evidence that it is associated with inflammatory process [9].

The cytotoxicity of activated macrophages and PMNs is dependent on the presence of L-arginine [10]. Thus, activated macrophages synthesize L-citrulline and nitrite from L-arginine and non-selective NOS inhibitors prevent the synthesis of both of these products as well as the expression of cytotoxicity. Following the demonstration of NO synthesis from L-arginine, it became apparent that NO was the most likely inorganic nitrogen oxide intermediate in the pathway of nitrite-nitrate synthesis in macrophages. iNOS in the macrophage differs from that in endothelial cells and the nervous system. This enzyme is inducible, and if once expressed it synthesizes NO in great amounts for long periods. It is well known that iNOS synthesis is strongly induced by PAF, TNF-α, and IL-1β, and inhibition of these inflammatory mediators decreases iNOS expression [9].

Experimental work indicates that CP induced HC is not only due to the direct contact of acrolein with the bladder mucosa, but also involves inflammatory mediators. PAF, TNF-α and IL-1β mediated endogenous NO plays a role in the inflammatory events leading to cystitis. In addition, the cytokines TNF-α and IL-1β mediate the production of NO in ifosfamide induced cystitis [6, 7, 8].

Xu et al. [12] reported that exposure to TNF-α and interferon gamma produced a marked increase in the expression of iNOS and NO production in bladder muscle cell culture medium. But exposure to CP or its metabolite acrolein did not increase iNOS or NO-metabolite levels in primary cultures of rat bladder smooth muscle cells. On the other hand, the incubation of primary cell cultures with plasma from CP treated rats produced a marked increase in iNOS expression and NO production. Taken together, the authors decided that NO plays an important role in the pathogenesis of CP-induced cystitis in rats, and some factors may be released in CP-treated rat plasma which stimulate iNOS expression [12].

In our experiment, we found that CP induction dramatically increased plasma nitrite-nitrate levels and caused severe bladder damage. Administration of L-arginine, the NOS substrate, showed similar results to the CP-only group. When iNOS was blocked selectively with SMT, confirmed with a decrease of plasma nitrite-nitrate levels, bladder damage disappeared. Moreover, in the CP and L-arginine, but not the SMT, groups, hematuria continued at the end of study.

iNOS induced NO production may play an important role in CP induced HC. Our data are consistent with previous studies performed with nonselective NOS inhibition [7] and in cell culture [12]. Similar results were also reported by Alfieri et al., suggesting that NO plays an important role in CP-induced cystitis and the increase of NO is partly due to tachykinin receptor activation [13, 14]. Further studies should focus on other selective iNOS inhibitors such as aminoguanidine [15], or use specific NOS knock-out animals to clarify the pathophysiological mechanism of CP induced HC.

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