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INVOLVEMENT OF NITRIC OXIDE AND HYPERBARIC OXYGEN IN THE PATHOGENESIS OF CYCLOPHOSPHAMIDE INDUCED HEMORRHAGIC CYSTITIS IN RATS

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ABSTRACT

Purpose: We evaluated the relationship between nitric oxide and hyperbaric oxygenation in the pathogenesis and treatment of cyclophosphamide induced hemorrhagic cystitis in rats.

Materials and Methods: Cyclophosphamide (100 mg/kg) was injected in male Sprague-Dawley rats for cystitis induction. Animals were treated before and the day after cyclophosphamide injection with 100 mg/kg of the nitric oxide substrate L-arginine, 20 mg/kg of the nonselective nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester and 20 mg/kg of the selective inducible nitric oxide synthase inhibitor S-methylisothiourea. Animals were exposed to hyperbaric oxygen (2.8 atmospheres absolute for 90 minutes twice daily) with or without the administration of L-arginine and nitric oxide synthase inhibitors.

Results: Cyclophosphamide injection resulted in severe cystitis. S-methylisothiourea produced marked inhibition of cyclophosphamide induced bladder tissue damage. L-arginine and L-N^G-nitroarginine methyl ester failed to a show meaningful protective effect. Hyperbaric oxygen protected the bladder only against ulceration. Moreover, hyperbaric oxygen did not contribute to the protective effects of L-arginine, L-N^G-nitroarginine methyl ester or S-methylisothiourea.

Conclusions: Nitric oxide produced by inducible nitric oxide synthase is an important mediator in the pathogenesis of cyclophosphamide induced cystitis. Hyperbaric oxygen has a beneficial effect on repairing bladder damage rather than on bladder protection.

KEY WORDS: bladder; nitric oxide; hyperbaric oxygenation; cystitis; rats, Sprague-Dawley

Cyclophosphamide (CYP) is widely used for treating many neoplastic diseases. Hemorrhagic cystitis has been recognized as a common and dose limiting side effect after using CYP and ifosfamide, a synthetic analogue of CYP.[1] The urotoxicity of CYP is mainly attributable to the renal excretion of acrolein, a urotoxic metabolite of CYP.[2] It has been proposed that urothelial damage occurs at direct contact with acrolein, which causes edema, hemorrhage, ulceration, leukocyte infiltration and necrosis. Recently it was shown that endogenous inflammatory mediators, such as platelet activated factor, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are involved in cystitis.[3,4] Prevention is the best way to decrease the side effects of CYP. Of preventive agents mesna (2-mercaptoethane sulfonate) has been widely used as an effective agent but bladder protection is not always achieved.[5]

Nitric oxide (NO) is a free radical gas that regulates a number of important physiological and pathophysiological processes, including vascular tone, polymorph nuclear leukocyte (PMNL) adhesion and inflammation. NO is synthesized from the amino acid L-arginine by the enzyme NO synthase (NOS).[6] There are 3 subtypes of NOS. Endothelial NOS (eNOS) is found in endothelial cells and fibroblasts, and it is mainly responsible for vasodilatation. NO is produced by neuronal NOS (nNOS) in the nervous system. It acts as an important signaling molecule. Inducible NOS (iNOS) can be up-regulated in many more cells than PMNL and macrophage iNOS activation produces a significantly greater amount of NO that eNOS does. There is evidence suggesting that NO produced iNOS is toxic since in animal models selective iNOS inhibition improved the outcome and decreased inflammatory events.[6] The NOS enzyme catalyzes the reaction of L-arginine to citrulline and NO, and the NO produced is oxidized to nitrate and nitrite. NOS inhibition decreases the production of nitrite-nitrate.[7]

Hyperbaric oxygen (HBO_2) is a therapy method used for many urological diseases, including CYP induced cystitis.[8] It has been shown experimentally and clinically that HBO_2 may help protect the bladder against acrolein damage.[8,9] HBO_2 induces the healing of tissue damage, and decreases edema, necrosis and leukocyte infiltration. Several studies have also shown the beneficial effect of HBO_2 in preserving cellular homeostasis with respect to adenosine triphosphate levels and maintaining normal cellular osmolarity.[10] Although it had beneficial effects, HBO_2 alone did not provide complete protection against CYP induced cystitis. However, when combined with mesna, much better results were obtained.[11] Consequently it is likely that HBO_2 is more effective in repairing tissue damage rather than in preventing cystitis.

Many studies have shown that HBO_2 is a therapeutic agent that increases dissolved oxygen in plasma and also affects the inflammatory process. HBO_2 significantly decreases PMNL adhesion and rolling[12] by down-regulating intercellular adhesion molecule-1 expression.[13] HBO_2 is also known to increase the NO production, and eNOS and iNOS expression. It is plausible that the beneficial effects of HBO_2 are at least in part mediated by NO.[10] In our study we examined changes in NO in CYP induced bladder damage and the benefit of HBO_2 as hemorrhagic cystitis treatment.

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MATERIALS AND METHODS

Animals. A total of 90 male Sprague-Dawley rats weighing 150 to 180 gm were divided into 9 groups (table 1). They had free access to food and water.

Drug and HBO_2 administration. The NO substrate L-arginine (100 mg/kg), the nonselective NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) (20 mg/kg) and the selective iNOS inhibitor S-methylisothiourea (SMT) (20 mg/kg) were administered twice daily for a total of 6 doses. CYP (100 mg/kg) was used for cystitis induction. All drug administrations were performed intraperitoneally. A steel animal hyperbaric oxygen chamber was flushed with 100% oxygen at the beginning and chamber pressure was increased to 2.8 atmospheres absolute (ATA) in 10 minutes. Decompression to normobaric air at the end of the session was completed gradually in 5 minutes. A temperature of 22C to 26C and airflow of 15 l. per minute were maintained in the medium for a session of 90 minutes.

Tissue preparations. After 48 hours of cystitis induction rats were anesthetized using of ketamine HCl (85 mg/kg) and xylazine HCl (12.5 mg/kg). The bladder was removed intact and residual urine was removed. The bladder was weighed to determine if edema was present and fixed for 24 hours in 10% buffered formalin. Tissues were embedded with paraffin and stained with hematoxylin and eosin. A pathologist blinded to the study group rated 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. Measurement of plasma nitrite-nitrate was performed as described previously.[14]

Statistics. Results are expressed as the median and range with $p < 0.05$ considered statistically significant. All numerical data were first analyzed using the nonparametric Kruskal-Wallis test to determine whether there was a difference between groups. The Mann-Whitney U test was then performed to analyze 2 groups consecutively.

RESULTS

Table 2 lists all histological parameters, bladder-to-body weight (BLW/BW) ratios and nitrite-nitrate levels. Control animals had histologically normal bladders with assigned scores of 1 for all parameters (fig. 1). CYP caused severe histological changes (fig. 2). Macroscopic hematuria continued to the end of the study. Although L-NAME decreased nitrite-nitrate levels ($p < 0.01$), no significant effect on bladder damage was observed due to L-NAME or L-arginine administration (fig. 3). SMT decreased each ratio significantly ($p < 0.05$ and < 0.01, respectively, fig. 4, *A*). CYP caused an approximately 2.5-fold increase in BLW/BW ratios and a 3.5-fold increase in nitrite-nitrate levels (fig. 5). HBO_2 affected neither the BLW/BW nor the nitrite-nitrate ratios caused by CYP (figs. 5 and 6).

HBO_2 protected against necrosis but not other histological parameters (fig. 7). It did not have an additive effect on the other groups. Hematuria disappeared in the SMT group 48 hours after CYP administration but continued in the L-NAME, L-arginine and HBO_2 groups.

DISCUSSION

Experimental studies indicate that CYP induced cystitis is due not only to direct contact of acrolein with bladder mucosa, but also to inflammatory mediators. It was shown that TNF-α, IL-1β and platelet activated factor mediated endogenous NO are involved in the inflammatory events leading to cystitis.[3] It was demonstrated that the cytokines TNF-α and IL-1β mediate the production of NO in ifosfamide induced cystitis.[4] Studies suggest that L-NAME treatment almost abolishes histopathological alterations caused by cyclophosphamide and ifosfamide. However, Alfieri et al failed to note histological improvement using 7-nitroindazole[15] (inhibitory potency, nNOS = eNOS = iNOS[16]) and the selective iNOS inhibitor SMT produced marked inhibition (greater than 90%) of CYP induced bladder tissue inflammatory changes.

TABLE 1. *Cyclophosphamide, L-arginine, L-NAME, SMT and HBO_2 treatment schedule*

Groups (mg/kg)	Drugs + HBO_2 (exposure)			
	Day 1	Day 2	Day 3	Day 4
Control	—	—	Saline (2 ml)	—
CYP	—	—	CYP (100 mg/kg)	—
HBO_2/CYP	HBO_2 (2 × 2 ATA, 90 mins)	HBO_2 (2 × 2 ATA, 90 mins)	CYP (100 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	HBO_2 (2 × 2 ATA, 90 mins)
L-arginine/CYP	—	L-arginine (2 × 20 mg/kg)	CYP (100 mg/kg) + L-arginine (2 × 20 mg/kg)	L-arginine (2 × 20 mg/kg)
HBO_2/L-arginine/CYP	HBO_2 (2 × 2 ATA, 90 mins)	L-arginine (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	CYP (100 mg/kg), L-arginine (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	L-arginine (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)
L-NAME/CYP	—	L-NAME (2 × 20 mg/kg)	CYP (100 mg/kg) + L-NAME (2 × 20 mg/kg)	L-NAME (2 × 20 mg/kg)
HBO_2/L-NAME/CYP	HBO_2 (2 × 2 ATA, 90 mins)	L-NAME (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	CYP (100 mg/kg), L-NAME (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	L-NAME (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)
SMT/CYP	—	SMT (2 × 20 mg/kg)	CYP (100 mg/kg) + SMT (2 × 20 mg/kg)	SMT (2 × 20 mg/kg)
HBO_2/SMT/CYP	HBO_2 (2 × 2 ATA, 90 mins)	SMT (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	CYP (100 mg/kg), SMT (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)	SMT (2 × 20 mg/kg) + HBO_2 (2 × 2 ATA, 90 mins)

TABLE 2. *Rat histological damage scores and BLW/BW ratios, and rat bladder plasma nitrite-nitrate*

Groups	Median Edema (range)	Median Hemorrhage (range)	Median Inflammation (range)	Median Ulceration (range)	Median BLW/BW (range)	Median Mmol/Ml Nitrite-Nitrate (range)
Control	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–1)	0.28 (0.25–0.33)	0.042 (0.025–0.056)
CYP	4 (4–4)	3 (1–4)	2 (2–3)	4 (2–5)	0.76 (0.69–0.95)	0.148 (0.113–0.172)
HBO_2/CYP	4 (3–4)	3 (1–4)	2 (1–3)	1 (1–2)*	0.78 (0.63–1.05)	0.146 (0.127–0.171)
L-arginine/CYP	4 (2–4)	4 (3–4)	3 (2–4)	3 (2–5)	0.76 (0.65–1.23)	0.138 (0.117–0.156)
HBO_2/L-arginine/CYP	3 (3–4)	4 (2–4)	3 (2–4)	3 (2–5)	0.78 (0.54–1.21)	0.178 (0.121–0.202)
L-NAME/CYP	3 (2–4)	2 (1–4)	2 (1–4)	2 (1–5)	0.58 (0.39–1.23)	0.077 (0.062–0.094)*
HBO_2/L-NAME/CYP	4 (3–4)	2 (1–4)	2 (1–3)	3 (1–5)	0.74 (0.48–1.21)	0.091 (0.069–0.107)*
SMT/CYP	2 (1–3)*	1 (1–1)*	2 (1–2)*	1 (1–1)*	0.49 (0.39–0.63)*	0.077 (0.062–0.084)*
HBO_2/SMT/CYP	3 (2–4)*	1 (1–2)*	2 (1–3)	1 (1–2)*	0.61 (0.52–0.91)	0.081 (0.069–0.095)*

* Vs CYP $p < 0.05$.

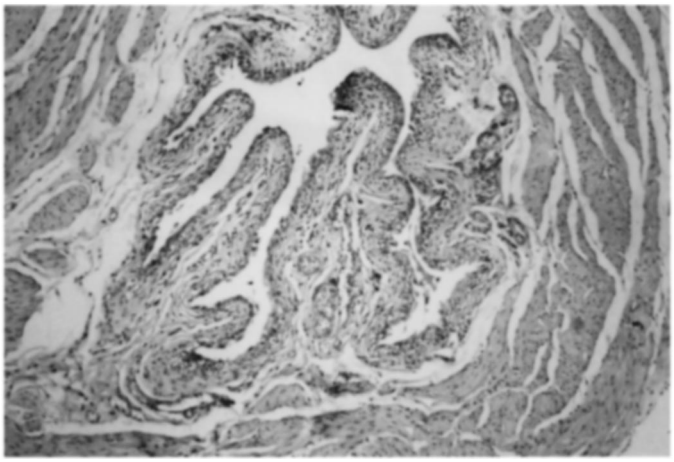

FIG. 1. Normal control rat bladder histology with score of 1 for edema, hemorrhage, inflammation and ulceration. H & E, reduced from ×25.

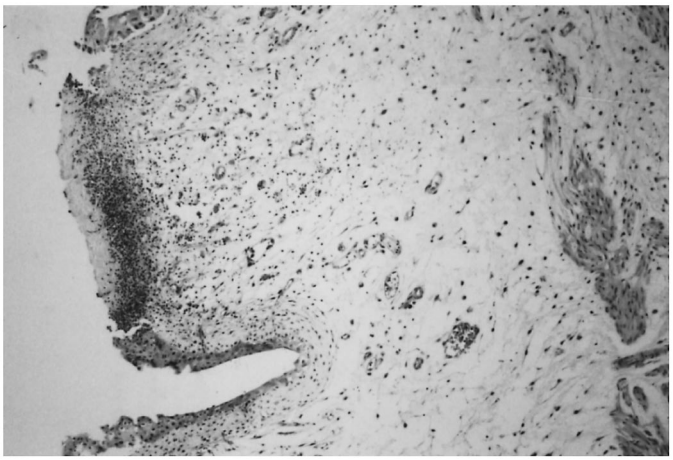

FIG. 2. Severe cyclophosphamide induced cystitis with mean histological score of 3 for hemorrhage, inflammation and ulceration, and 4 for edema. H & E, reduced from ×100.

In the current study we also observed histological improvement using SMT but not L-NAME. Inhibition of eNOS along with iNOS may be harmful to tissues because physiological demands are lacking (for example vasoregulation and intercellular communication).[6]

Many studies indicate that HBO_2 has anti-inflammatory properties. Yamashita and Yamashita recently reported that HBO_2 attenuates the induction of cytokines, such as TNF-α and IL-6, after massive hemorrhage.[17] Zymosan is known to induce inflammation by causing the production of various cytokines and pro-inflammatory mediators. It was shown that HBO_2 decreased TNF-α concentrations in a zymosan induced shock model.[18] Similarly Weisz et al concluded that decreased IL-1, IL-6 and TNF-α secretion is due to HBO_2 treatment in patients with perianal Crohn's disease.[19] It is well known that iNOS synthesis is strongly induced by IL-1 and TNF-α, and inhibition of these inflammatory mediators decreases iNOS expression. HBO_2 also down-regulates intracellular adhesion molecule-1 expression through the induction of eNOS but not iNOS, leading to decreased PMNL rolling on the microvasculature.[13] Given these effects HBO_2 may have beneficial impact on inflammation in CYP induced cystitis.

In our study HBO_2 did not show anti-inflammatory properties. Histological findings in the CYP group treated with HBO_2 were similar to those in the group treated with CYP alone except HBO_2 decreased necrosis ($p < 0.01$). HBO_2 probably could not prevent cyclophosphamide induced cystitis but it accelerated tissue repairing. This result correlates with a previous study showing HBO_2 alone could not completely protect the bladder against acrolein insult.[11]

Using direct acrolein intravesically it was shown that HBO_2 significantly decreased tissue damage resulting from acrolein.[9] Interestingly Xu et al reported that exposure to TNF-α and interferon-δ produced a marked increase in the expression of iNOS and NO production in culture medium.[20] However, exposure to CYP or its metabolite acrolein did not increase iNOS or NO metabolite levels in primary culture of rat bladder smooth muscle cells. On the other hand; incubation of primary cell cultures with plasma from rats treated with CYP produced a marked increase in iNOS expression and NO production. That group decided that NO has an important role in the pathogenesis of CYP induced cystitis in rats and some factors may be released in CYP treated rat plasma that stimulate iNOS expression. As a result, if intravesical acrolein is used for cystitis induction,[9] no inflammatory changes occur and HBO_2 may only accelerate the effects of tissue healing without having anti-inflammatory properties.

In our experiment HBO_2 did not show a beneficial effect alone or with L-arginine and L-NAME. Moreover, HBO_2 did not improve the SMT group outcome. It is intriguing that all observed beneficial effects of HBO_2 treatment may be explained to date by NO. Further HBO_2 cystitis studies using NOS inhibitors and specific knockout animals could confirm the connection between HBO_2 and NO production as the

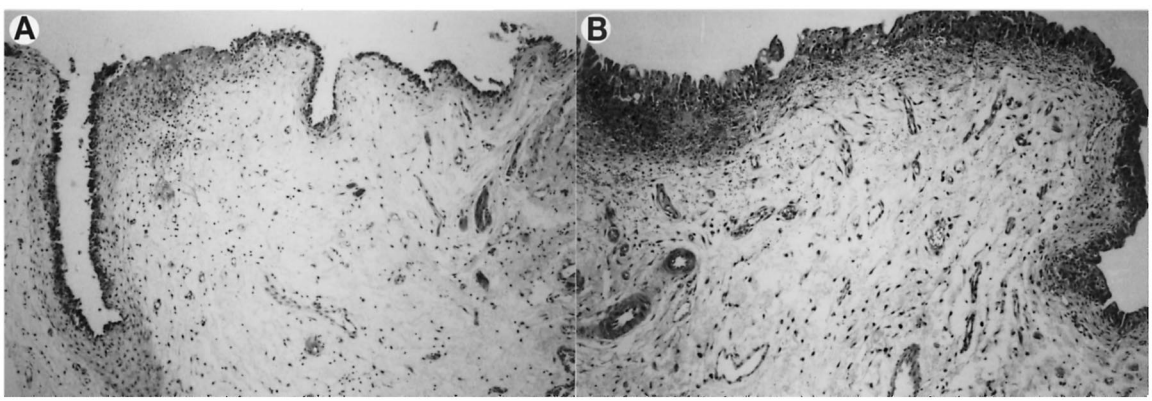

FIG. 3. There was similar histological damage in L-arginine and L-NAME groups with no meaningful contribution from HBO_2. *A*, L-arginine group with score of 3 for all parameters. H & E, reduced from ×50. *B*, L-NAME group with score of 3 for edema and 2 for other parameters. H & E, reduced from ×100.

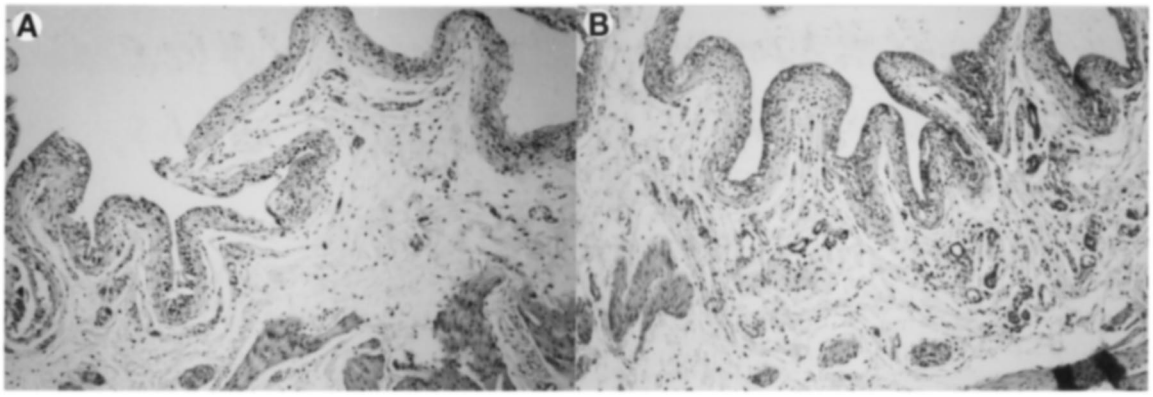

FIG. 4. SMT group. *A*, minimal edema and inflammatory cell infiltration with no hemorrhage or ulceration. *B*, HBO_2 slightly impeded effect of SMT. H & E, reduced from ×100.

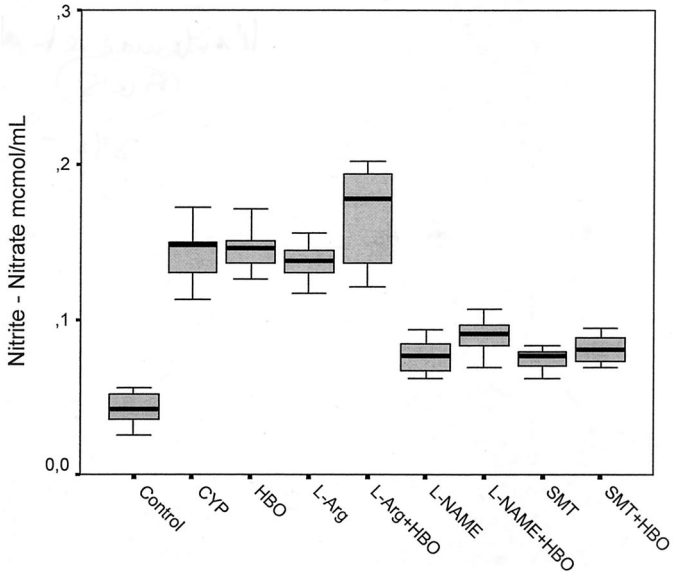

FIG. 5. CYP and L-arginine (*L-Arg*) increased but NOS inhibitors decreased nitrite-nitrate, while HBO_2 (*HBO*) did not affect nitrite-nitrate increase or decrease. Bold lines represent median.

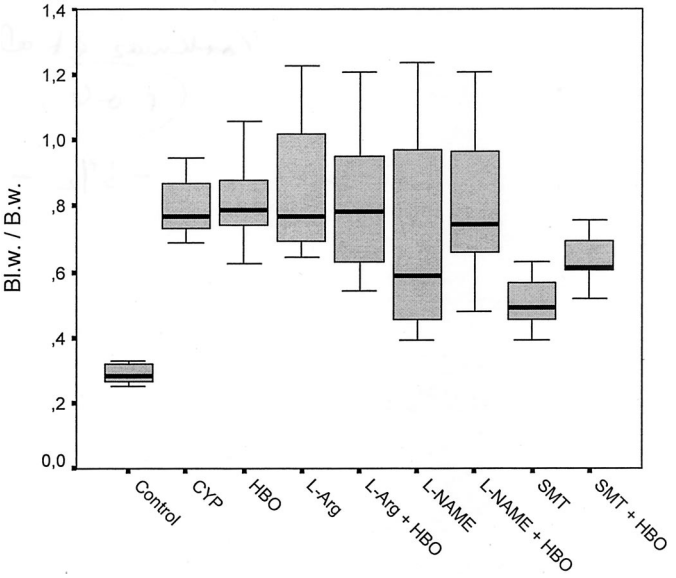

FIG. 6. CYP increased BLW/BW ratios approximately 2.5-fold, while only SMT administration decreased ratio (vs CYP $p < 0.05$). Bold lines represent median. *HBO*, HBO_2. *L-Arg*, L-arginine.

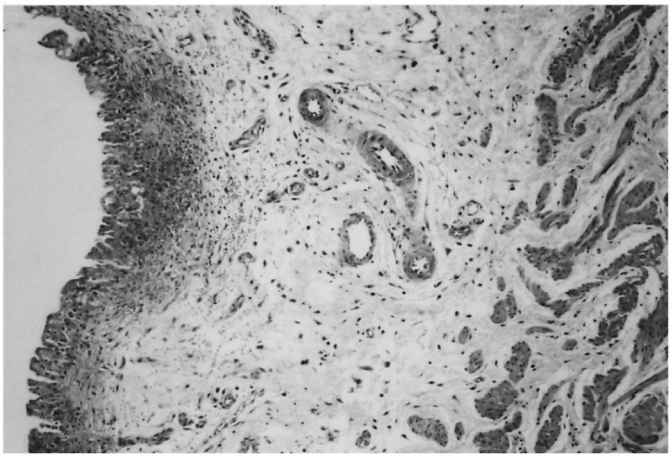

FIG. 7. HBO_2 group bladder histology with mean histological score of 3 for edema and hemorrhage, and 2 for inflammation and epithelial denuding. H & E, reduced from ×100.

primary mechanism responsible for protection and/or healing in cyclophosphamide induced cystitis.

CONCLUSIONS

Cyclophosphamide induced hemorrhagic cystitis is due not only to direct contact of acrolein with the bladder, but also to cytokine mediated endogenous NO production. HBO_2 has beneficial effects on bladder tissue healing rather than preventing acrolein insult. It can be concluded that there is no need to use HBO_2 for prophylaxis of CYP induced cystitis. Further studies should focus on selective NOS inhibitors, such as SMT, aminoguanidine and N-nitro-L-arginine,[16] and specific NOS knockout animals to clarify the pathophysiological mechanism of hemorrhagic cystitis.

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