

EVALUATION OF EFFECTS OF CHITOSAN IN PREVENTING HEMORRHAGIC CYSTITIS IN RATS INDUCED BY CYCLOPHOSPHAMIDE

From the Department of Urology, Nagoya City University Medical School

Takehiko Okamura

From the Laboratory of Pathology, Aichi Cancer Center Research Institute

Tsuneo Masui

*From the Department of Pathology and Microbiology, and the Eppley Institute for
Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska, USA*

Margaret K. St. John and Samuel M. Cohen

*From Division of Urology, in the Department of Surgery, University
of Nebraska Medical Center, Omaha, Nebraska, USA*

Rodney J. Taylor

Hemorrhagic cystitis is a common problem following cyclophosphamide or radiation therapy. Chitosan has been shown to be an effective hemostatic agent and promoter of wound healing in animal experiments. We evaluated the safety and efficacy of intravesical chitosan in an animal model of cyclophosphamide cystitis. Hemorrhagic cystitis was induced in female F344 rats by intraperitoneal cyclophosphamide, 100mg/kg. Chitosan solution (0.3ml) was instilled intravesically on day 1 (Group 1), on days 1, 3, and 5 (Group 2), or 1 hour after the administration of cyclophosphamide (Group 3). The rats in group 4 were treated with chitosan diluent on day 1 after cyclophosphamide, and the rats in group 5 received intravesical chitosan without cyclophosphamide. Sequential examination revealed decreased mortality and lower incidences of severe bladder bleeding, necrosis and inflammation in Group 3. Treatment delayed until after the appearance of the cystitis, especially repeated treatments, appeared to make the cyclophosphamide-induced changes worse. Used within 1 hour of cyclophosphamide administration, before the cystitis develops, chitosan seemed to have the possibility to inhibit the appearance of hemorrhagic cystitis. In addition to the changes in the bladder, severe changes occurred in the kidneys secondary to cyclophosphamide. (Acta Urol. Jpn. 41: 289-296, 1995)

Key Words: Chitosan, cyclophosphamide, hemorrhagic cystitis, bladder instillation.

INTRODUCTION

Chitosan is a collective term applied to chitin in various stages of deacetylation and depolymerization. It is composed of poly-N-acetyl glycosamine units. This material has been shown, when mixed with whole blood, to form a tenacious coagulum¹⁾. Taking note of this chemical, several investigators reported that chitosan was a good hemostatic agent in animal experiments¹⁻³⁾.

Hemorrhagic cystitis is a result of

diffuse capillary fragility and bleeding within the damaged urothelial lining of the bladder. This clinical entity is a frequent result of cyclophosphamide therapy or radiation therapy⁴⁾. Current therapeutic modalities for treating this entity include withholding the causative agent, catheter drainage, bladder irrigation with normal saline, formalin or silver nitrate bladder instillations to cauterize the bladder, transurethral electrocautery, and occasionally, cystectomy and urinary diversion in order to control severe hem-

orrhage.

In this study we evaluated the safety and efficacy of intravesical chitosan in an animal model of cyclophosphamide-induced cystitis.

MATERIALS AND METHODS

Test chemicals

Chitosan was kindly supplied by Hoechst-Roussel Pharmaceuticals, Inc., (Somerville, NJ). Sterile chitosan solution contained 2 mg/ml chitosan and 2 mg/ml acetic acid. For a placebo, sterile acetic acid solution (2mg/ml) was also supplied by Hoechst-Roussel Pharmaceuticals, Inc., Cyclophosphamide was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ) and administered dissolved sterile water.

Animals

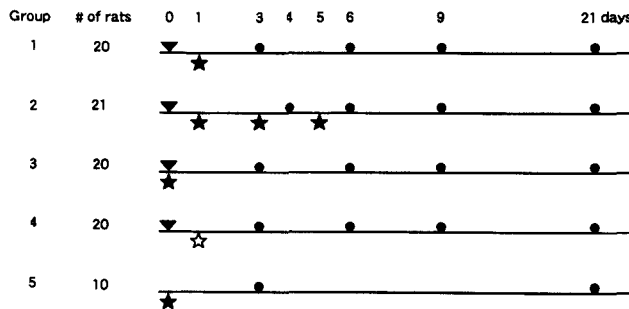
Ninety-one weanling (4 weeks old), female F344 rats were obtained from Charles River Breeding Laboratories, Inc.. (Kingston, NY). Upon arrival, the rats were weighed and randomly assigned to an experimental group by a weight stratification method⁵⁾. They were kept in quarantine on their respective control diets for 5 days prior to study. The rats were housed 5 or 6 per cage on dry corn-cob bedding in polycarbonate cages (16 × 18 × 20 inches) with stainless steel wire bar covers (Lab Products, Inc., Maywood, NJ). Animal rooms were maintained at a temperature of 22+3°C and 50+20%

humidity on a 12-h light/12-h dark cycle. Food and water were available ad libitum. Prolab 3000 diet was purchased from Agway, Inc. (St. Mary's, OH).

Experimental procedure

A preliminary study demonstrated that a single dose of cyclophosphamide, 100 mg/kg, i.p., produced hemorrhagic cystitis in 100% of the rats.

Figure 1 shows the experimental design Group 1 consisted of 20 rats treated with a single intraperitoneal injection of cyclophosphamide at a dose of 100mg/kg body weight. After 24 hours, transurethral intravesical instillation of 0.3 ml chitosan was performed through a 23 G catheter (Argyl, Medicut "R", Sherwood Medical Industries, St. Louis, MO) under Nembutal (Abbott Laboratories, North Chicago, IL.) anesthesia. Immediately prior to and one hour after instillation, the bladder contents were emptied by light abdominal massage so that the duration of exposure to instilled compounds was consistent. Group 2 consisted of 21 rats injected with cyclophosphamide as in group 1. Intravesical instillation of 0.3ml chitosan was performed after 24 hours, and after 3 and 5 days. Group 3 contained 20 rats injected with cyclophosphamide as in group 1. Intravesical instillation of 0.3 ml chitosan was performed one hour after injection of cyclophosphamide. Group 4 contained 20 rats injected with cyclophosphamide as in



▼ : cyclophosphamide 100mg/kg I.P.
 ★ : chitosan 0.3 ml (2mg/ml) bladder instillation (nembutal anesthesia).
 ☆ : acetic acid 0.3 ml (2 mg/ml) bladder instillation (nembutal anesthesia).
 ● : sacrifice 5/group/time.

Fig. 1. Experimental design

group 1. After 24 hours, 0.3ml (mg/ml) of acetic acid was instilled into the bladder instead of chitosan. A fifth group of 10 rats was used as a negative control group they were treated with intravesical instillation of 0.3 ml chitosan, but without prior cyclophosphamide injection.

The rats were sequentially killed as indicated in Fig. 1. Two to five rats each from groups 1, 3 and 4 were killed 3, 6 and 9 days post-cyclophosphamide injection. In group 2, 2 to 5 animals were killed 4, 6, and 9 days post-cyclophosphamide treatment. Five animals in group 5 were killed on day 3 post-cyclophosphamide treatment. As occasion demanded, moribund rats were killed. The last day the rats were killed was 21 days post-cyclophosphamide treatment.

At death, the liver and each kidney were observed grossly, then carefully removed and weighed. These organs were placed directly into formalin fixative. The bladders were inflated in situ with fixative, and a day later were bisected sagittally and each half cut into 3 to 4 longitudinal strips. All tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined histopathologically.

Experimental data were evaluated statistically on an IBM 3090 mainframe operating system VM using the Wilcoxon rank test (for mortality), and Fisher's exact test 1 tail (for macroscopic and microscopic findings) from the Statistical Analysis System software package (SAS Institute, Inc., Cary, NC).

RESULTS

About 25% of the rats died before they were scheduled to because of cyclophosphamide toxicity excessive anesthesia. The rats were killed or found dead as shown in Table 1. The mortality in each group was (including moribund killed) 7/20 (35%) in group 1, 8/21 (38%) in group 2, 3/20 (15%) in group 3, 5/20 (25%) in group 4, 0/10 (0%) in group 5. Groups 1 and 2 were significantly different from group 5 at $p < 0.05$ (Wilcoxon rank test). In the groups administered cyclophosphamide, the mortality was the lowest in group 3 (however, no significant difference from the other groups).

Table 2 summarizes the macroscopic findings. In groups 1, 2 and 4, white nodules, 1 mm to 5 mm in diameter, were ob-

Table 1. Numbers of rats killed or found dead at different times after cyclophosphamide injection.

Group	1	2	3	4	5	6	7	8	9	16	21 days
S ^a			5			3			3		2
1 M ^b											
F ^c					2	4				1	
S				5		4			2		2
2 M											
F					8						
S			5			5			3		4
3 M							1	2			
F											
S			5			5			2		3
4 M				1							
F					4						
S			5								5
5 M											
F											

S^a scheduled death
M^b moribund death
F^c found dead

Table 2. Macroscopic findings of kidney and bladder^a

Group	Treatment	# of Rats	Kidney		Bladder	
			Nodule	Hydronephrosis	Bleeding	Adhesion
1	CP ^b →Chitosan Day 1	20	6 (30%) ^d	2 (10%)	9 (45%)	2 (10%)
2	CP→Chitosan Day 1, 3, 5	21	6 (29%) ^d	3 (14%)	17 (81%) ^{c,f,h}	8 (38%) ^{c,d,g}
3	CP→Chitosan Day 0	20	0 (0%)	1 (5%)	4 (20%)	1 (5%)
4	CP→Acetic Acid Day 1	20	7 (35%) ^e	2 (10%)	8 (40%)	1 (5%)
5	Chitosan Day 0	10	0 (0%)	1 (10%)	0 (0%)	0 (0%)

^a Statistical analyses were performed using Fisher's exact test (1-tail).

^b CP: cyclophosphamide.

^c Significantly different from Group 1, $p < 0.05$.

^d Significantly different from Group 3, $p < 0.05$.

^e Significantly different from Group 3, $p < 0.005$.

^f Significantly different from Group 3, $p < 0.001$.

^g Significantly different from Group 4, $p < 0.05$.

^h Significantly different from Group 4, $p < 0.01$.

Table 3. Histopathological findings of kidney and bladder^a

Group	Treatment	# of Rats	Kidney (%)			Bladder (%)			
			Necrotizing Papillitis	Severe Hyperplasia	Severe Bleeding	Erosion or Ulceration	Severe Necrosis or Inflammation	Simple Hyperplasia	Papillary or Nodular Hyperplasia
1	CP ^b →Chitosan Day 1	20 ^c	10 (50%) ^g	5 (25%)	3 (17%)	12 (67%)	3 (17%)	13 (72%) ⁱ	2 (11%)
2	CP→Chitosan Day 1, 3, 5	21	11 (52%) ^g	8 (38%)	11 (52%) ^{d,e,i}	17 (81%) ^f	11 (52%) ^{d,h,k}	15 (71%) ⁱ	3 (14%)
3	CP→Chitosan Day 0	20	1 (5%)	3 (15%)	3 (15%)	8 (40%)	0 (0%)	11 (55%)	0 (0%)
4	CP→Acetic Acid Day 1	20	12 (60%) ^h	5 (25%)	0 (0%)	13 (65%)	1 (5%)	6 (30%)	0 (0%)
5	Chitosan Day 0	10	0 (0%)	0 (0%)	0 (0%)	1 (10%)	0 (0%)	2 (20%)	1 (10%)

^a Statistical analyses were performed using Fisher's exact test (1-tail).

^b CP: cyclophosphamide.

^c 18 rats for bladder.

^d Significantly different from Group 1, $p < 0.05$.

^e Significantly different from Group 3, $p < 0.05$.

^f Significantly different from Group 3, $p < 0.01$.

^g Significantly different from Group 3, $p < 0.005$.

^h Significantly different from Group 3, $p < 0.001$.

ⁱ Significantly different from Group 4, $p < 0.05$.

^j Significantly different from Group 4, $p < 0.01$.

^k Significantly different from Group 4, $p < 0.005$.

^l Significantly different from Group 4, $p < 0.001$.

served in the kidneys in 29 to 35% of the rats. The nodules were homogeneous and not encapsulated. A few cases of hydronephrosis were observed in each group. Almost all of these cases involved adhesions of the bladder. Various grades of bladder hemorrhage were observed in the cyclophosphamide administered groups. The incidence was highest in group 2 (81%) ($p < 0.05$, $p < 0.001$, and $p < 0.01$, compared to

groups 1, 3, 4, respectively) and lowest in group 3 (20%). The incidence was similar in groups 1 and 4 (45% and 40%, respectively). Some rats had adhesions caused by severe inflammation, and this was the most common in group 2 (38%) ($p < 0.05$, compared to the other groups). Macroscopically, no significant findings were found in the liver.

Table 3 summarizes the histopathologi-



Fig. 2. Severe hemorrhage of bladder in rat from group 2, involving the entire thickness of the wall. Epithelial surface is at the top. H&E, $\times 40$.

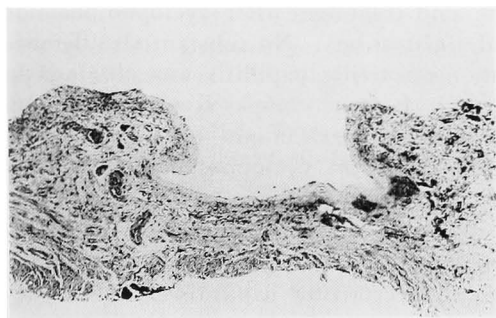


Fig. 3. Erosion and ulceration of bladder in rat from group 2, H&E, $\times 20$

cal findings. In group 2, the incidence of severe bleeding (52%) ($p < 0.05$, $p < 0.05$, and $p < 0.001$, compared to groups 1, 3, 4, respectively) (Fig. 2), erosion or ulceration (81%) ($p < 0.01$, compared to group 3) (Fig. 3), and severe necrosis or inflammation (52%) ($p < 0.05$, $p < 0.001$, and $p < 0.005$, compared to groups 1, 3, 4, respectively) of the bladder was higher than that in the other three groups injected with cyclophosphamide. None of the group 3 animals had severe necrosis or inflammation. Simple hyperplasia of the bladder epithelium was seen in all groups. Papillary or nodular hyperplasia was seen in groups 1, 2, and 5 (11%, 11%, and 10%, respectively) on day 9 or later.

Interestingly, necrotizing papillitis of the kidneys was seen in only 5% of the rats in group 3, in contrast to 50 to 60% incidences in groups 1, 2, and 4 ($p < 0.005$, $p < 0.005$, and $p < 0.001$, respectively). The

white nodules in the kidneys were composed of inflammatory granulation tissue by microscopic examination. Severe hyperplasia of the renal pelvis was seen in 15 to 38% of the rats in the cyclophosphamide-administered groups, and it was highest in group 2 (38%) and lowest in group 3 (15%).

Because of advanced autolytic changes, two bladders in group 1 were excluded from histopathological examination. In group 5, one rat had multiple stones composed of calcium phosphate.

DISCUSSION AND CONCLUSIONS

Chitosan is a unique hemostatic agent. Chitosan-induced coagulum does not depend on the normal clotting cascade mechanism, and the resulting "clot" does not undergo retraction like a normal clot. Furthermore, reaction does not occur with albumin, globulin, or platelets. However, the same coagulum occurs with heparinized blood, washed red cells, and defibrinated blood²¹. Mallette et al.²² have shown in studies involving dacron grafts placed into dogs that the chitosan coagulum remains in place around the graft until replaced by ingrowth of normal smooth muscle, vasovascularum, and nerve fibrils. This is in direct contrast to the extensive fibrotic reaction and fibrosis that occurs around untreated dacron grafts^{1,22}. In another study involving the effects of chitosan on capillary bleeding, Brandenberg et al.³ showed it to be an effective topical hemostatic agent when applied to surgically created central nervous system wounds in cats. In a recent study, Bartone and Adickes⁶ demonstrated the effect of chitosan on wounds of the genitourinary system in dogs. Wounds were made in the kidney, ureter and penile foreskin, with decreased fibrosis observed with chitosan in all tissues studied.

Hemorrhagic cystitis is a fairly common clinical problem. It is frequently caused by cyclophosphamide therapy or radiation therapy, but may be associated with vesical malignancy, bladder amyloidosis, or viral infection. Current therapeutic modalities are directed at controlling the bleeding

until healing occurs. Use of caustic agents, such as 1% formalin or silver nitrate, to control the acute bleeding often results in further damage to the urothelium⁷⁾. Patients in whom this entity occurs are frequently immunosuppressed, chronically ill cancer patients in whom normal healing is retarded at best and for whom the risk of infection and persistent bleeding is much greater. In addition, patients are known to be at greater risk to develop bladder cancer following successful treatment with cyclophosphamide^{4,7-9)}. A further problem involved with hemorrhagic cystitis regardless of the cause is that agents currently used to treat the hemorrhagic cystitis (i.e. formalin or silver nitrate) are caustic in nature and do not infrequently result in scarring of the bladder with a significant decrease in the pliability and volume of the bladder. Such patients may develop severe urinary problems after treatment related to a poorly functioning bladder. Furthermore, the risk of repeated bleeding episodes is significant.

In this study, we evaluated whether chitosan, an agent of proven value in controlling capillary bleeding, can, when used either prophylactically or therapeutically, decrease the incidence and sequelae of hemorrhagic cystitis in an animal model.

Chitosan was not therapeutic as it did not improve the hemorrhagic cystitis once it had already developed. Surprisingly, in the group treated with three instillations, the bleeding and inflammation of the bladder were increased in severity. The chitosan solution has very high viscosity, which may cause it to be adherent and irritating. Once inflammation has occurred, chitosan might have the potential to make it worse. The chitosan diluent, acetic acid, may also have irritating properties because of its acidity, which might make the inflammation worse. Furthermore, intravesical instillation itself may produce inflammation. In the group treated only with chitosan instillation without cyclophosphamide, one rat had severe inflammation with stones, providing some support for this suggestion.

By contrast, treatment with chitosan before the cystitis developed seemed to protect the bladder. The causative agent of hemorrhagic cystitis following cyclophosphamide injection has been identified as acrolein that is generated in the urine^{10,11)}. If chitosan really protects the bladder, protection by chitosan might be caused either by adhesion to the bladder surface, preventing contact with acrolein, or by a direct interaction with acrolein, inactivating it. The incidence of severe bleeding, severe necrosis or inflammation, simple hyperplasia in the urinary bladder was significantly less than the chitosan-treated groups (Group 1 and 2) in Group 4 (acetic acid treatment after cyclophosphamide administration). No substantial difference but necrotizing papillitis was obtained in Group 4 when compared with Group 3. Acetic acid itself might have the preventing effect on cyclophosphamide-induced hemorrhagic cystitis.

In the group (Group 3) involving prophylactic intravesical administration of chitosan, necrotizing papillitis of the kidney was almost completely inhibited. The mechanism of protection is unknown but perhaps occurred by reflux of the chitosan, protecting the kidney pelvis like the bladder, or by preventing reabsorption and renal excretion of the active metabolites of cyclophosphamide and preventing bladder damage.

The finding of necrotizing papillitis was an unexpected complication of cyclophosphamide. Necrotizing papillitis is rarely a clinical problem, and it is usually observed only in patients with diabetes mellitus, pyelonephritis, or dehydration¹²⁾. The exact mechanism by which necrotizing papillitis develops remains uncertain^{12,13)}. In animal experiments, Molland¹⁴⁾ showed that aspirin or phenacetin administration induced necrotizing papillitis, and ischemia and dehydration exacerbated it. Animals may have become dehydrated due to the toxicity of cyclophosphamide, resulting in ischemia of the kidney and necrotizing papillitis. More likely, cyclophosphamide in the female rat produced severe, ulcera-

ting inflammation secondary to toxicity in the kidney similar to that in the bladder.

In conclusion, chitosan does not have an active therapeutic effect for hemorrhagic cystitis once it develops, but it may act as a prophylactic measure against the development of hemorrhagic cystitis and necrotizing papillitis secondary to cyclophosphamide in a rat model. Additional studies are required to further evaluate the mechanism involved and the potential usefulness, especially for patients receiving very high doses, such as those in bone marrow transplantation protocols for the treatment of various malignancies.

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和文抄録

ラット Cyclophosphamide 誘発出血性膀胱炎に対する
Chitosan 膀胱内注入効果の検討

名古屋市立大学医学部泌尿器科学教室 (主任: 郡 健二郎教授)

岡 村 武 彦

愛知県がんセンター研究所病理学第一部 (部長: 立松生衛)

増 井 恒 夫

From the Department of Pathology and Microbiology, and the Eppley Institute for
Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska USA

Margaret K. St. John and Samuel M. Cohen

From Division of Urology in the Department of Surgery, University
of Nebraska Medical Center, Omaha, Nebraska, USA

Rodney J. Taylor

メスラット cyclophosphamide 誘発出血性膀胱炎に対して, chitin の一種でユニークな止血作用を有する chitosan の膀胱内注入を行い, その効果について検討を行った. F344 メスラットを用い, 100 mg/kg の cyclophosphamide を腹腔内投与することにより, 出血性膀胱炎を誘発し, chitosan (0.3 ml) を以下のように膀胱内注入した. 第1日目 (グループ1), 第1・3・5日目 (グループ2), cyclophosphamide 投与1時間後 (グループ3). グループ4は第1日目に chitosan 溶解液のみを膀胱内注入し, グループ5は cyclophosphamide を投与せず, chitosan のみを

膀胱内注入した. 経時的に屠殺し, 肉眼的, 病理組織学的に検討した結果, グループ3において死亡率の減少と, 肉眼的膀胱出血, 壊死および炎症の発生率の低下が認められた. 膀胱炎発生後の chitosan 膀胱内注入は, 特に繰り返し施行した場合, cyclophosphamide による変化を悪化させる傾向がみられた. cyclophosphamide 投与1時間後の投与群では, chitosan は出血性膀胱炎の発生を抑制する可能性が示唆された. さらに, これら膀胱の変化以外に, cyclophosphamide による腎の変化も認められた.

(泌尿紀要 41: 289-296, 1995)