

Chemopreventative Effect of Hochu-ekki-to (TJ-41) on Chemically
Induced Biliary Carcinogenesis in Hamsters

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Abstract

Background. Bilioenterostomy is a common surgical technique that is widely used. Recently, clinical studies have revealed that biliary carcinomas can occur after bilioenterostomy. The present study was designed to evaluate whether hochu-ekki-to (TJ-41), a Japanese herbal drug, could prevent chemically induced biliary carcinomas in bilioenterostomized hamsters.

Materials and methods. Syrian golden hamsters were subjected to choledochojejunostomy and then received subcutaneous injections of N-nitrosobis (2-oxopropyl) amine (BOP) every 2 weeks at a dose of 10 mg/kg. BOP administration was started 4 weeks after surgery. The animals were simultaneously orally administered TJ-41 in water every day at a dose of 1000 mg/kg (TJ-41 group). The control hamsters were administered water alone. The hamsters were sacrificed 22 weeks after surgery, and the development of biliary carcinomas, the presence and degree of cholangitis, and the cell kinetic status of the biliary epithelium were evaluated histologically.

Results. Intrahepatic bile duct carcinomas developed in 15/17 (88%) hamsters in the control group and in only 8/17 (47%) hamsters in the TJ-41 group ($p < 0.05$). The degree of cholangitis was not different between the two groups. However, the proliferating cell nuclear antigen labeling index (PCNA-LI) of the biliary epithelium in the TJ-41 group (6.46%) was significantly lower than the controls (9.67%) ($p < 0.05$). These findings indicated that TJ-41 reduced accelerated biliary epithelial cell kinetics after bilioenterostomy, resulting in the prevention of carcinogenesis.

Conclusion. TJ-41 has a preventive effect on chemically induced carcinoma of the biliary tract after bilioenterostomy.

Introduction

Bilioenterostomy is a common surgical technique that is widely used in the field of hepatobiliary pancreatic surgery. Reflux cholangitis (1-3), biliary stones (3,4), and liver abscess (2) are the well-known complications after bilioenterostomy. Recently, clinical studies have revealed that biliary carcinomas can occur as a delayed complication of bilioenterostomy for benign disease (5-8), and we have demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through an activation of biliary epithelial cell kinetics in hamsters (9, 10).

Hochu-ekki-to (TJ-41), a Japanese herbal drug, is known to reduce the degree of side effects such as leucopenia and intestinal damage occurring as a result of radiation or chemotherapy for malignant tumors (11, 12). On the other hand, TJ-41 has recently been reported to activate macrophages and natural killer (NK) cells (13-15). Moreover, some studies have demonstrated that TJ-41 inhibits experimental liver metastasis (16) and also exerts an anti-neoplastic effect in several kinds of malignant tumors, including skin cancer, hepatoma, ovarian cancer, and uterine cancer (17-20).

In the present study, we investigated whether TJ-41 could prevent biliary carcinogenesis in bilioenterostomized hamsters. We used Syrian golden hamsters because the anatomical structure of their pancreaticobiliary ductal system and the bile acid composition and pancreatic juice components in this species are similar to those of humans (21-23), and we concluded that TJ-41 is a possible agent for the prevention of biliary carcinogenesis in bilioenterostomized hamsters. To the best of our knowledge, this is the first successful *in vivo* study on the chemoprevention of biliary carcinogenesis by means of TJ-41.

Materials and Methods

Animals

Seven-week-old female Syrian golden hamsters (SLC, Inc., Shizuoka, Japan) were housed, one per plastic cage, on sawdust bedding. They were kept at $24 \pm 2^\circ\text{C}$ and $50 \pm 20\%$ humidity with a 12-h light/12-h dark cycle, fed a CE-2 pelleted diet (Clea Japan, Inc., Tokyo, Japan), and provided drinking water *ad libitum*. The animals were checked daily and

weighed every 2 weeks throughout the experiments. All experiments were conducted according to the Guidelines for Animal Experimentation of Nagasaki University.

Surgical techniques

Cholechojejunostomy using a Roux-en-Y procedure was performed on all hamsters. The schema of the completed cholechojejunostomy surgical procedure is illustrated in Figure 1. Following anesthesia with sodium pentobarbital (50 mg/kg of body weight), an upper abdominal midline incision was made, and the distal end of the common bile duct was double-ligated with 6-0 nylon and divided. Following ligation of the cystic duct, the gallbladder was removed. The jejunum was double-ligated with 6-0 nylon and cut 7 cm distal to the pyloric ring of the stomach. About 4 cm of the anal side of the jejunum was used for the Roux-en-Y anastomosis, and an intestinal anastomosis was made in a side-to-side manner with 7-0 nylon. A 20G needle was inserted into the elevated jejunal wall approximately 10 mm distal to the jejunal stump, and the tied common bile duct was then inserted into the hole that had been made by the needle. The common bile duct was then cut about halfway

towards the tied end for bile drainage, taken back into the jejunum, and fixed to the jejunal wall (9).

Chemoprevention protocol

All hamsters that were operated on were given subcutaneous injections of a chemical carcinogen, N-nitrosobis (2-oxopropyl) amine (BOP) (Nakarai Tesque, Kyoto, Japan), every 2 weeks at a dose of 10 mg/kg body weight (24, 25). BOP administration was started 4 weeks after surgery and continued for 18 weeks (9, 25). The animals were randomly divided into 2 groups according to the different regimens. Twenty hamsters were provided with an oral administration of TJ-41 (Tsumura Co., Ltd., Tokyo, Japan) every day at a dose of 1000 mg/kg body weight in water, starting 4 weeks after surgery and continuing for 18 weeks (TJ-41 group). TJ-41 was added to the water, and the hamsters ingested the water *ad-libitum*. In the control group, twenty hamsters were provided with just water. At postoperative week 22, all hamsters were sacrificed.

Morphological and biochemical analyses

At autopsy, the maximum diameter of the extrahepatic bile duct was measured. Blood samples from the vena cava were collected in ice-chilled

tubes containing heparin and centrifuged (3,000 r.p.m) for 10 min, and then serum samples were collected in new ice-chilled tubes. The serum levels of total bilirubin (T-Bil), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured.

Histological studies

The liver, biliary system, and pancreas were removed *en bloc* at autopsy. After fixation in 10% neutral formalin, the specimens were cut into five blocks so that four sections contained the liver and one section contained the hepatic duct, and the specimens were embedded in paraffin. The histological sections were stained with hematoxylin and eosin (HE) and then examined by a pathologist who was blinded to the treatment allocation of the sections. The number of histologically verified carcinomas was counted. Carcinoma was diagnosed on the basis of the WHO classification of tumors of the hamster (26).

Inflammatory changes

To evaluate the relationship between cholangitis and biliary carcinogenesis, we scored the grade of cholangitis in accordance with the

infiltration of inflammatory cells and the fibrous change of Glisson as follows: grade 0, no cholangitis; grade 1, mild invasion of inflammatory cells around the bile duct without fibrous change of Glisson; grade 2, severe invasion of inflammatory cells around the bile duct and/or fibrous change of Glisson; grade 3, abscess formation in the liver (9).

Cell kinetic studies

Proliferating cell nuclear antigen (PCNA) was used as a marker of biliary epithelial cell kinetics. Tissue sections were cut at 4 μ m, mounted on glass slides coated with 5-aminoprophyltriethoxy saline, and dewaxed in xylene. The sections were treated with microwave heating for 5 min in phosphate-buffered saline (PBS) at 500 W. After the blocking of endogenous peroxidase, the sections were incubated with mouse monoclonal antibodies against PCNA (clone-PC 10; DAKO, Kyoto, Japan) at a dilution of 1:100. The cell nuclei were counterstained with hematoxylin. The proportion of labeled nuclei (labeling index; LI) was determined by counting the labeled nuclei in >1000 non-neoplastic epithelial cells of the intrahepatic bile ducts (27).

Adverse effects of TJ-41 on vital state

The side effects of TJ-41, such as pseudo aldosteronism, liver dysfunction, and myopathy, may have adverse effects on the vital state of hamsters. Accordingly, the animals were checked daily and weighed every 2 weeks throughout the experiments.

Statistical analyses

The incidence of carcinomas developed and the grade of cholangitis were analyzed using the χ^2 exact test. The Mann-Whitney U test was also used for statistical analyses of the diameter of the extrahepatic bile duct, number of tumors per animal, laboratory data on serum, PCNA-LI, and PGE₂ production. Differences of $p < 0.05$ were considered statistically significant.

Results

Morphological and biochemical changes

The morphological and biochemical changes in the hepatobiliary system of hamsters are summarized in Table 1. The total number of hamsters examined was 17 in both the control and TJ-41 groups, because 3 hamsters

in each group died of liver abscess and/or obstructive jaundice before sacrifice. There was no significant difference between the two groups in the average diameter of the extrahepatic bile duct. However, the serum levels of AST and ALP were significantly higher in the control group than in the TJ-41 group ($p < 0.05$).

Occurrence of biliary carcinomas

Figure 2 shows HE staining of the typical intrahepatic bile duct cancer developed in the hamster. Biliary carcinomas were observed in hamsters of both groups (Table 2). In the control group, intrahepatic bile duct carcinoma developed in 88% of the hamsters, and the average number of carcinomas per animal was 11.4. In the TJ-41 group, only 47% of the hamsters developed intrahepatic bile duct carcinoma, and the average number of carcinomas per animal was 3.9. Both the incidence of carcinoma and the average number of carcinomas per animal were significantly lower in the TJ-41 group than in the control group ($p < 0.05$).

Cholangitis, biliary epithelial cell kinetics, PGE₂ production, and biliary carcinogenesis

Cholangitis was recognized in all hamsters in the control group and

in 82% of the hamsters in the TJ-41 group (Table 3). There was no significant difference in the degree of cholangitis between the two groups (P=0.06).

Figure 3 shows PCNA staining of the biliary epithelium. PCNA-LI of the biliary epithelium in the control group was significantly higher than that of the TJ-41 group ($p < 0.05$).

Change of body weight

Figure 4 shows the transition curves of the body weight of hamsters in each group during the experiment. In the control group, the average body weight of the hamsters increased until the 15th week of the study and then gradually decreased thereafter. Contrarily, the body weight of the hamsters in the TJ-41 group increased throughout the study.

Discussion

Despite recent advances in diagnostic modalities and surgical techniques, the clinical course of patients with carcinoma of the biliary tract

remains dismal, even in patients who undergo a curative resection. Both intra- and extrahepatic biliary carcinomas tolerate traditional cytotoxic chemo- and radiotherapeutic approaches. Cancer chemoprevention is, thus, expected to be a new approach in the management of biliary carcinoma.

Chronic infection and inflammation involving the biliary tree, such as mechanical irritation by means of cholelithiasis (28), chronic intrahepatic cholangitis with hepatolithiasis (29), bile stasis and bacterial infection (30), and primary sclerosing cholangitis (PSC) (31-33), are the risk factors for the development of biliary carcinoma. We have obtained evidence that persistent cholangitis after bilioenterostomy in hamsters accelerates the development of biliary carcinoma through an increase in the proliferative activity of the biliary epithelium in accordance with the severity of cholangitis (9,10) and that etodolac, a selective COX-2 inhibitor, reduces both the occurrence of severe cholangitis and the acceleration of biliary epithelial cell kinetics after bilioenterostomy in hamsters, resulting in the prevention of BOP-induced biliary carcinogenesis (34). Recently, some studies have proved that TJ-41 reduced the development of several kinds of cancer (17-20) and activities of inflammatory cytokines (35-37).

Therefore, we examined a chemopreventative effect of TJ-41 on biliary carcinogenesis in bilioenterostomized hamsters.

The present study results clearly demonstrated the preventative effect of TJ-41 on BOP-induced biliary carcinogenesis in hamsters undergoing bilioenterostomy, although TJ-41 failed to inhibit the occurrence of reflux cholangitis. Meanwhile, TJ-41 down-regulated the cell kinetic activity of the biliary epithelium. The authors of several recent studies have reported that TJ-41 has several cancer-preventing mechanisms, such as the activation of NK cells/T-cells (17), a blocking effect on the cell cycle (18), and inhibition of the expression of c-jun, tumor necrosis factor (TNF)-alpha, and estrogen receptor (ER)-alpha/beta (20). Although the inhibitory effect of TJ-41 on biliary epithelial cell kinetics may participate in the prevention of biliary carcinoma in our hamster model with persistent reflux cholangitis, further studies should be carried out to clarify the cancer-preventing mechanism of TJ-41.

TJ-41 has some side effects, such as hypercalcemia, liver dysfunction, allergy, and digestive dysfunction, although the incidences are very low. In the present study, the body weight of hamsters in the TJ-41

group was well maintained throughout the experiment, in contrast to the hamsters in the control group. This may have been due to the suppression of developing biliary carcinoma in the TJ-41 group and also suggested that TJ-41 had no critical adverse effects. The Japanese herbal drug including TJ-41 has a cost benefit over other medical drugs, and thus long-term administration of TJ-41 should be feasible and convenient for cancer prevention.

In conclusion, TJ-41 inhibited BOP-induced biliary carcinogenesis in hamsters undergoing choledochojejunostomy, and suppression of the proliferative activity of the biliary epithelial cells was considered to be the possible mechanism of cancer prevention in this hamster model. Clinical trials should be performed to assess the utility of TJ-41 in the prevention of biliary carcinogenesis in patients undergoing biliary reconstruction with bilioenterostomy.

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Figure Legends

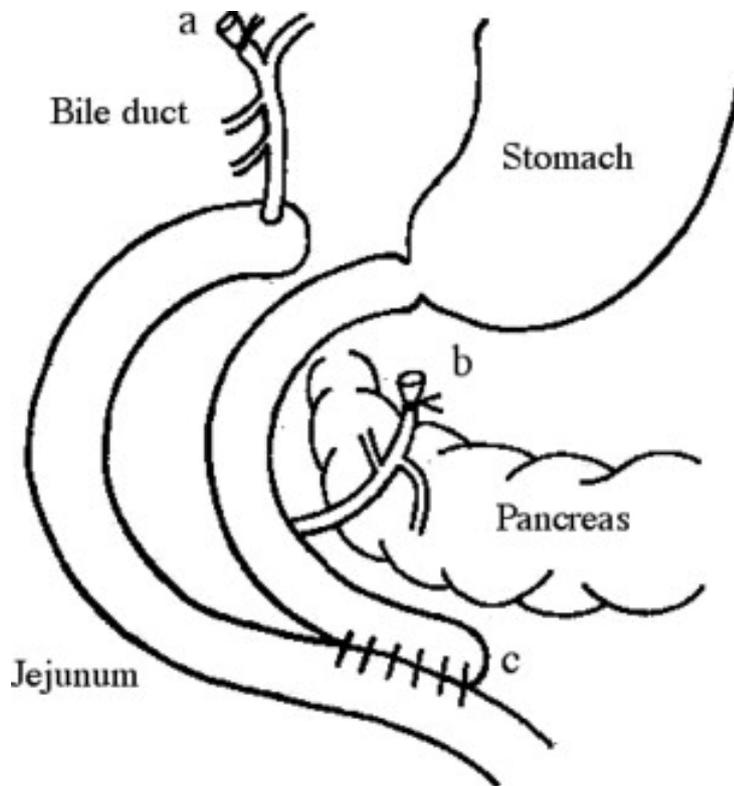


Figure 1. Operating scheme: choledochojejunostomy in a hamster using the Roux-en-Y procedure. The jejunal limb was 4 cm long.

a: The gallbladder was removed.

b: The common bile duct was transected at the distal end.

c: Side-to-side intestinal anastomosis, 7 cm from the pyloric ring.

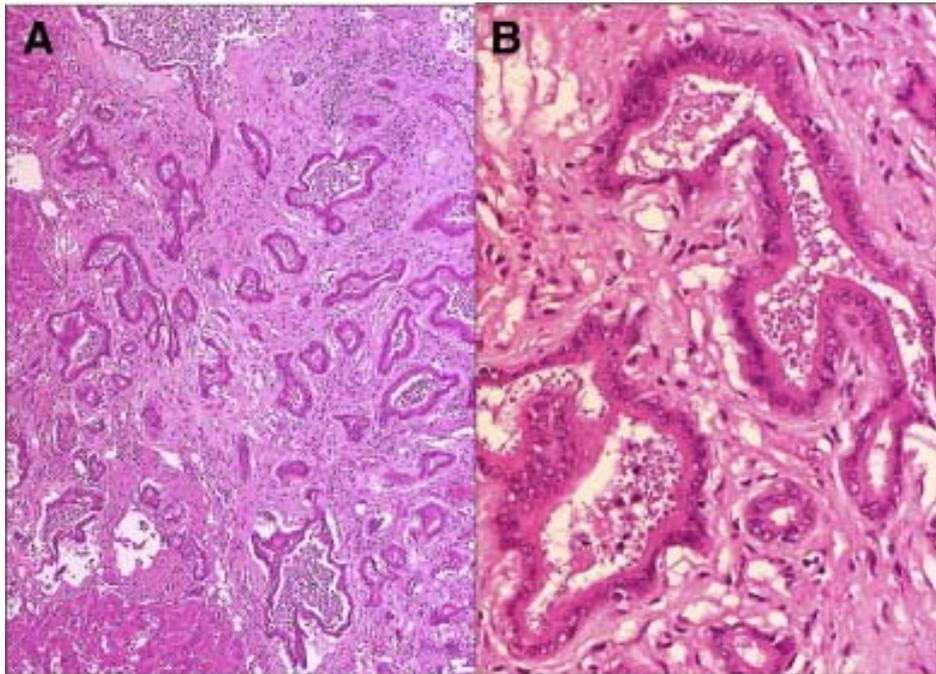


Figure 2.

HE staining of the typical intrahepatic bile duct cancer developed in the hamster.

(a) x 50. (b) x 100.

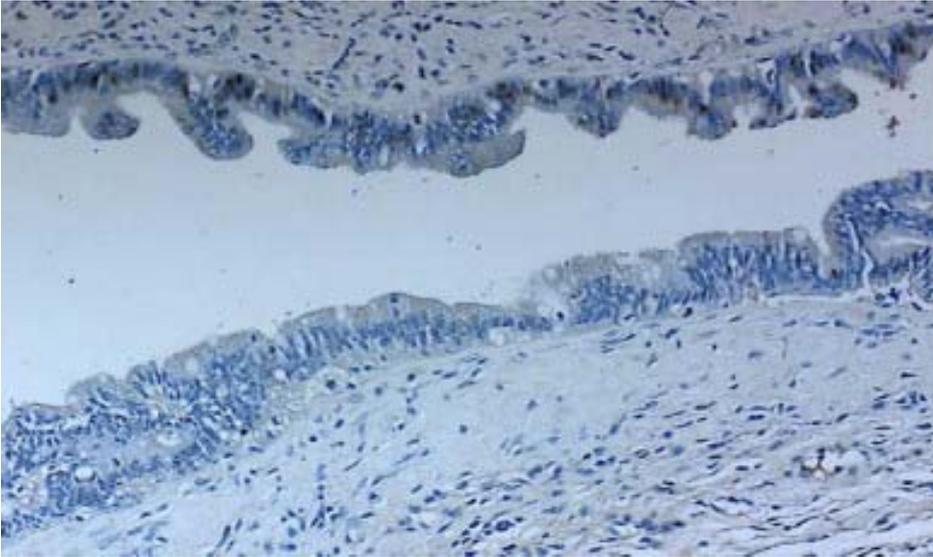


Figure 3.

PCNA staining of non-neoplastic epithelial cells of the intrahepatic bile ducts (x 50).

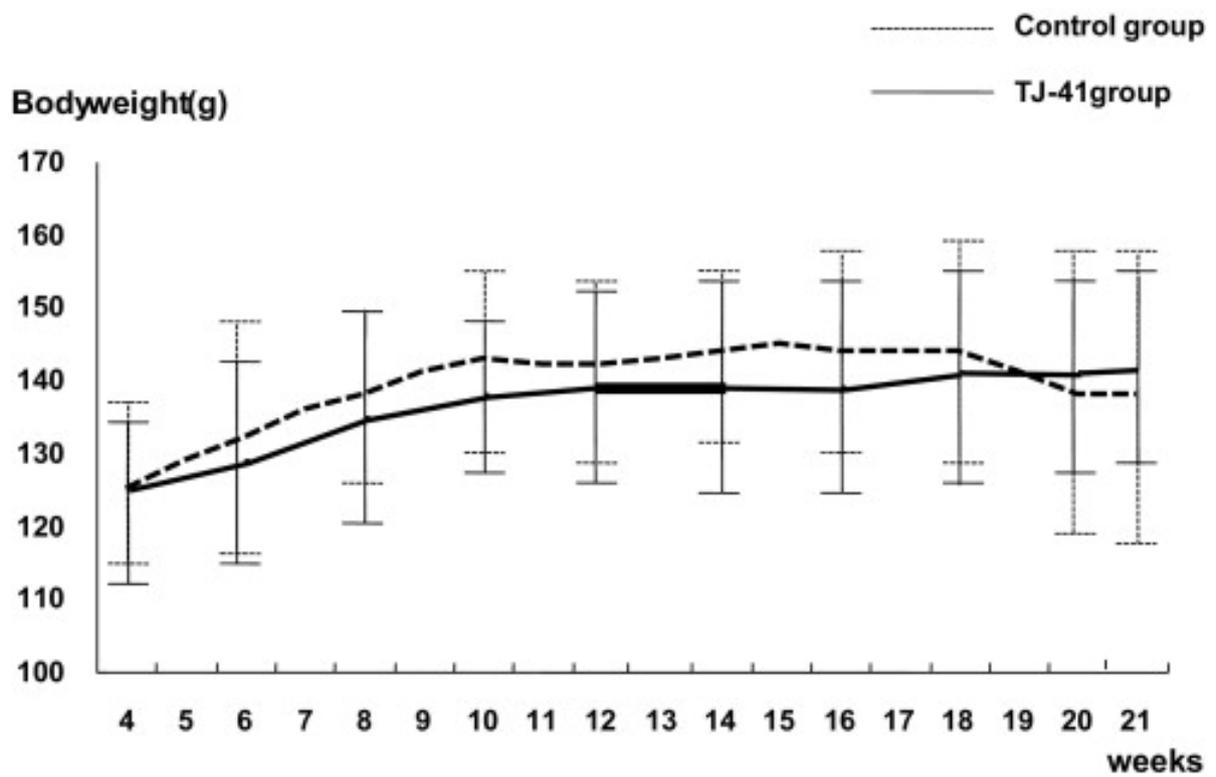


Figure 4. Transition curve of average body weight in hamsters after bilioenterostomy.

TJ-41 group; Control group

The bars demonstrate the standard error.

TABLE 1**Morphological and Biochemical Changes in the Hepatobiliary System of Hamsters After Bilioenterostomy**

Groups	No. of hamsters	Average diameter of the EBD (mm)*	Serum levels*			
			T.Bil (mg/L)	ALP (IU/L)	AST(IU/L)	ALT(IU/L)
Control	17	2.8 ± 1.9	1.9 ± 1.8	249.5 ± 278.1	187.2 ± 136.6	95.1 ± 47.5
TJ-41	17	3.3 ± 2.7	1.3 ± 1.3	95.2 ± 44.5**	109.5 ± 75.9**	78.6 ± 47.0

EBD = extrahepatic bile duct; T.Bil = total bilirubin; ALP = alkaline phosphatase; AST = asparatate aminotransferase; ALT = alanine aminotransferase.

* Mean ± SD.

** Significantly different from control group ($P < 0.05$).

TABLE 2**The Incidence and Number of Intrahepatic Bile Duct Carcinomas Developed in Hamsters After Bilioenterostomy**

Groups	No. of hamsters	No. (%) of hamsters with carcinoma	No. of carcinomas developed	Average no. of carcinomas per animal*
Control	17	15 (88)	193	11.4 ± 12.4
TJ-41	17	8 (47)**	67	3.9 ± 5.8**

* Mean ± SD.

** Significantly different from control group ($P < 0.05$).

TABLE 3**The Occurrence of Cholangitis and Changes in Biliary Epithelial Cell Kinetics and PGE₂ Products in Hamsters After Bilioenterostomy**

Groups	No. of hamsters	No. (%) of hamsters with cholangitis	Average of	
			Cholangitis score*	PCNA-LI (%)*
Control	17	17 (100)	2.06 ± 0.23	9.67 ± 5.90
TJ-41	17	14 (82)	1.47 ± 0.24	6.46 ± 4.82**

PCNA-LI = proliferating cell nuclear antigen labeling index.

* Mean ± SD.

** Significantly different from control group ($P < 0.05$).