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# Inhibition by Xiao-chai-hu-tang (TJ-9) of Development of Hepatic Foci Induced by N-Nitrosomorpholine in Sprague-Dawley Rats

Masaharu Tatsuta,[1] Hiroyasu Iishi, Miyako Baba, Akihiko Nakaizumi and Hiroyuki Uehara

*Department of Gastrointestinal Oncology, The Center for Adult Diseases, Osaka, 3-3, Nakamichi 1-chome, Higashinari-ku, Osaka 537*

**The effect of Xiao-chai-hu-tang (TJ-9) on hepatocarcinogenesis induced by N-nitrosomorpholine (NNM) was investigated in male Sprague-Dawley rats. Rats were given normal chow pellets containing 0.5% or 1.0% TJ-9 until the end of the experiment, and drinking water containing NNM for 8 weeks. Pre-neoplastic and neoplastic lesions staining for γ-glutamyl transpeptidase (GGT) or the placental type of glutathione-*S*-transferase (GST-P) were examined histochemically. In Week 15, quantitative histological analysis showed that prolonged treatment with 0.5% TJ-9 significantly reduced the number and volume of GGT-positive and GST-P-positive hepatic lesions. Treatment with 1.0% TJ-9 inhibited the development of GGT-positive and GST-P-positive lesions, but was less effective than 0.5% TJ-9. Administration of 0.5% TJ-9 also caused a significant increase in the proportion of helper T lymphocytes and a significant decrease in the labeling index of pre-neoplastic lesions. These findings indicate that TJ-9 inhibits the development of hepatic foci.**

Key words: Xiao-chai-hu-tang (TJ-9) — Hepatocarcinogenesis — Immunomodulation

The traditional Chinese herbal medicine, Xiao-chai-hu-tang (TJ-9),[2] has recently been administered orally for treatment of chronic hepatitis, and found to have clear clinical effects.[1–3] The antitumor activities of TJ-9 and its components have been reported.[4–7] Kumazawa *et al.*[6] found that i.p. administration of TJ-9 to mice resulted in marked activation of macrophages. Polysaccharide separated from these drugs exhibits antitumor activities at least partially through an increase in the immune response with macrophage involvement. Macrophages participate as important effector cells in the host defense system to eliminate microbial invaders and malignancies. Itoh and Shimura[8] found that TJ-9 not only inhibited the growth of Lewis lung carcinoma, but also reduced the number of metastatic nodules in the lung. These findings suggest that treatment with TJ-9 might inhibit hepatocarcinogenesis. Yamamoto *et al.*[9] recently performed a controlled prospective study for evaluation of the potential of TJ-9 for prevention of hepatocellular carcinoma, and found that TJ-9 prevents or delays the emergence of latent hepatocellular carcinoma in patients with liver cirrhosis. However, few studies have been done on the inhibitory effect of TJ-9 on hepatocarcinogenesis in experimental animals. Therefore, in the present work, we investigated the effect of administration of TJ-9 to rats on the development of enzyme-altered lesions of the liver induced by NNM.

## MATERIALS AND METHODS

**Animals** Sixty young (6-week-old) male Sprague-Dawley rats were purchased from SLC, Japan (Shizuoka). The animals were housed in suspended, wire-bottomed metal cages in animal quarters with controlled temperature (21–22°C), humidity (30–50%), and lighting (12-h cycle).

**Carcinogen and treatment** Animals were randomly divided into three groups of 20 rats each and treated as follows: Group 1 was given regular chow pellets (Oriental Yeast, Tokyo) until the end of the experiment. From the beginning of the experiment, animals were also given drinking water containing 250 mg/liter of NNM (Sigma, St. Louis, MO) for 8 weeks. The NNM was dissolved in distilled water at a concentration of 50 g/liter, and stored in a cool place. The stock solution was diluted to 250 mg/liter with tap water just before use, and supplied to rats from bottles. The solution was renewed every other day. From Week 9 onward, rats were given normal tap water only until the end of the experiment. Group 2 was given chow pellets containing 0.5% TJ-9 (Tsumura, Tokyo) throughout the experiment, and drinking water containing 250 mg/liter of NNM for 8 weeks. Group 3 was given chow pellets containing 1.0% TJ-9 throughout the experiment and drinking water containing 250 mg/liter of NNM for 8 weeks.

[1] To whom correspondence should be addressed.

[2] Abbreviations: TJ-9, Xiao-chai-hu-tang (Sho-saiko-to); NNM, N-nitrosomorpholine; GGT, γ-glutamyl transpeptidase; GST-P, glutathione-*S*-transferase placental type; BrdU, bromodeoxyuridine.

Two grams of TJ-9 preparation contains extracts of 7.0 g of *Bupleuri radix*, 5.0 g of *Pinelliae tuber*, 3.0 g of *Scutellariae radix*, 3.0 g of *Zizyphi fructus*, 3.0 g of *Ginseng radix*, 2.0 g of *Glycyrrhizae radix*, and 1.0 g of *Zingiberis rhizoma*.

**Histological and histochemical observations of hepatic lesions** In Week 15, all surviving rats (non-starved) were killed by ether anesthesia. The liver was promptly excised and 2- or 3-mm-thick liver sections obtained from the left and middle lobes were fixed in cold acetone (0–4°C) for 6 h, and embedded in paraffin. Serial sections of 3 μm thickness were stained with hematoxylin and eosin, for examination of GGT activity as described by Ruttenberg *et al.*,[10] and for examination of GST-P by an immunohistochemical PAP method[11] using anti-rat GST-P rabbit serum (Bio Prep Medlabs, Stillogan, CD).

**Volumetric analysis** Serial sections were scored for GGT-positive lesions and GST-P-positive lesions without knowledge of their group of origin. Only pre-neoplastic or neoplastic lesions of 0.2 mm or more in longest diameter in the plane of section were counted, because reproducible evaluation of lesions of less than 0.2 mm in diameter was impossible. The transectional area of the lesions in the plane of the tissue section and the area of the entire liver section were measured with an LA-500 Personal Image Analyzer System (Pias, Tokyo). From the measured areas of transected lesions, the number of lesions per unit volume was estimated by the method of Pugh *et al.*,[12] and the mean volume of the lesions per unit liver volume was calculated by the method of Campbell *et al.*[13]

**Immunological examination** The localization of helper T lymphocytes as defined by the monoclonal antibody CL003A[14] was determined by an indirect immunoperoxidase technique on cryostat sections of the liver[15] in Week 15. For this, tissues were removed from the left lobe of the liver of each group, promptly embedded in OCT embedding compound (Raymond A. Lamb, London), and stored at −70°C. Cryostat sections of 7 μm thickness were air-dried for 1 h, fixed in absolute ethanol at 0–4°C for 10 min and washed with phosphate-saline buffer. A mouse $IgG_1$ antibody CL003A against rat T helper cells (diluted 1:50; Cedarlane, Ontario, Canada) was layered over the sections for 2 h at 4–6°C. The sections were then stained by the avidin-biotin-peroxidase complex method[16] using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The percentage of helper T cells in lymphocytes was determined by counting the labeled cells among lymphocytes in five areas in or surrounding the pre-neoplastic or neoplastic lesions.

**Labeling indices of the enzyme-altered lesions and the surrounding liver** The labeling indices of the enzyme-altered lesions and the surrounding liver were examined in Week 15. The labeling index was measured with an immunohistochemical analysis kit for assaying BrdU incorporation[17, 18] (Becton-Dickinson, Mountain View, CA), by a modification of the method described by Tada *et al.*[19] Briefly, five non-starved rats in each group received an i.p. injection of 20 mg/kg of BrdU, and 1 h later they were killed with ether. For determining the labeling index, the numbers of BrdU-labeled cells were counted among 500 cells in the surrounding liver and in GGT-positive lesions of 0.7–1.2 mm longest diameter. The labeling index was expressed as the percentage of labeled cells among the cells examined.

**Statistical analysis** Data on body and liver weights, labeling index, % helper T cells and number, size and volume of enzyme-altered lesions and hepatocellular carcinomas were analyzed by one-way analysis of variance with Dunn's multiple comparison (by Bonferroni's inequality),[20, 21] because the data were multiple sample means. Data are shown as means±SE. The chi-square test[22] was used to analyze the incidence of hepatocellular carcinomas. "Significant" indicates a calculated $P$ value of less than 0.05.

## RESULTS

**Body and liver weights** The body and liver weights of the NNM-treated rats are summarized in Table I. In Week 15, the rats treated with TJ-9 at either dosage had

Table I. Body and Liver Weights of NNM-treated Rats

| Group no. | Treatment[a] | Body weight (g) | | Effective no. of rats | Liver weight (g) |
|---|---|---|---|---|---|
| | | Initial | Week 15 | | |
| 1 | Control | 126±1 | 347±7 | 19 | 14.8±0.4 |
| 2 | 0.5% TJ-9 | 124±1 | 320±7[b] | 20 | 13.3±0.3[b] |
| 3 | 1.0% TJ-9 | 124±2 | 323±5[b] | 20 | 13.7±0.4 |

*a*) Treatment: Rats were given drinking water containing NNM for 8 weeks, and fed normal chow pellets (Group 1) or chow pellets containing 0.5% (Group 2) or 1.0% (Group 3) TJ-9 (Xiao-chai-hu-tang).
*b*) Significantly different from the value for Group 1 at $P<0.05$.

Table II. Number and Size of GGT-positive Lesions and GST-P-positive Lesions of the Liver in NNM-treated Rats

| Enzyme-altered lesions | GGT-positive lesions | | | GST-P-positive lesions | | |
|---|---|---|---|---|---|---|
| Group no. | 1 | 2 | 3 | 1 | 2 | 3 |
| Treatment[a] | Control | 0.5% TJ-9 | 1.0% TJ-9 | Control | 0.5% TJ-9 | 1.0% TJ-9 |
| Observed transectional data on lesions | | | | | | |
| No./cm$^2$ | 61±4 | 36±4[d] | 41±4[c] | 95±4 | 69±4[d] | 78±5[b] |
| Mean area (mm$^2$) | 0.33±0.05 | 0.23±0.04 | 0.23±0.03 | 0.30±0.03 | 0.25±0.03 | 0.26±0.03 |
| Calculated volumetric data on lesions | | | | | | |
| No./cm$^3$ | 1182±87 | 828±85[b] | 902±82 | 2040±121 | 1562±74[c] | 1696±114 |
| Mean volume (mm$^3$) | 0.20±0.04 | 0.10±0.02[b] | 0.11±0.02 | 0.16±0.02 | 0.12±0.02 | 0.14±0.02 |
| Volume as % of parenchyma | 20.4±3.5 | 7.8±1.1[c] | 10.3±2.2 | 29.0±3.0 | 18.1±2.4[b] | 21.0±2.7 |

*a*) For explanation of treatments, see Table I.
*b–d*) Significantly different from the value for Group 1: *b*) $P<0.05$, *c*) $P<0.01$, *d*) $P<0.001$.

Table III. Incidence, Number, Size, and Volume of Hepatocellular Carcinomas in NNM-treated Rats

| Group no. | 1 | 2 | 3 |
|---|---|---|---|
| Treatment[a] | Control | 0.5% TJ-9 | 1.0% TJ-9 |
| Effective no. of rats | 19 | 20 | 20 |
| No. of rats with hepatocellular carcinoma (%) | 9 (47) | 2 (10)[b] | 3 (15) |
| Observed transectional data on lesions | | | |
| No./cm$^2$ | 0.2±0.1 | 0.1±0.0 | 0.1±0.1 |
| Mean area (mm$^2$) | 1.38±0.63 | 0.12±0.07 | 0.15±0.12 |
| Calculated volumetric data on lesions | | | |
| No./cm$^3$ | 1.2±0.4 | 0.9±0.5 | 0.7±0.4 |
| Mean volume (mm$^3$) | 0.83±0.34 | 0.01±0.01[b] | 0.01±0.00[b] |
| Volume as % of parenchyma | 0.8±0.3 | 0.1±0.0[b] | 0.1±0.1 |

*a*) For explanation of treatments, see Table I.
*b*) Significantly different from the value for Group 1 at $P<0.05$.

significantly lower body weights than the controls. Treatment with 0.5% TJ-9 significantly reduced the liver weight.

**Number, size, and volume of enzyme-altered lesions in the liver** Table II summarizes the numbers, sizes, and volumes of GGT-positive lesions and GST-P-positive lesions in NNM-treated rats. The two-dimensional data showed that GGT-positive lesions and GST-P-positive lesions were significantly fewer in Group 2 (0.5% TJ-9) than in Group 1 (control). Statistical analysis of the calculated volumetric data showed significantly lower values in Group 2 than in Group 1 for the number of lesions per cm$^3$, the mean volume, and the volume as a percentage of parenchyma of GGT-positive lesions, and the number of lesions per cm$^3$ and the volume as a percentage of parenchyma of GST-P-positive lesions. Table II shows that treatment with 1.0% TJ-9 (Group 3) also significantly inhibited the development of GGT- and GST-P-positive lesions, but that its effect was less than that of 0.5% TJ-9 (Group 2).

**Incidence, number, size, and volume of hepatocellular carcinomas** Table III summarizes the incidences, num-

Table IV. Labeling Indices of the Enzyme-altered Lesions and Adjacent Normal Liver, and % Helper T Cells

| Group no. | Treatment[a] | Labeling index (%) | | Helper T cells | |
|---|---|---|---|---|---|
| | | GGT-posilive lesion | Adjacent liver | No. of tumor-infiltrating lymphocytes examined | % Helper T cells |
| 1 | Control | 3.6±0.4 | 1.0±0.3 | 2470 | 10.2±1.5 |
| 2 | 0.5% TJ-9 | 2.3±0.9[c] | 0.8±0.4 | 2166 | 17.5±6.8[b] |
| 3 | 1.0% TJ-9 | 3.0±0.2 | 1.0±0.5 | 1798 | 17.0±3.1 |

*a*) For explanation of treatments, see Table I.
*b, c*) Significantly different from the value for Group 1: *b*) $P<0.05$, *c*) $P<0.01$.

bers, sizes, and volumes of hepatocellular carcinomas in NNM-treated rats. The incidence of hepatocellular carcinomas was significantly less in Group 2 (0.5% TJ-9) than in Group 1 (control). Hepatocellular carcinomas were less frequent in Group 3 (1.0% TJ-9), but the difference was not statistically significant ($\chi^2=3.39$, $0.05<P<0.1$). The calculated volumetric data show that the mean volume and volume as a percentage of parenchyma were significantly less in Group 2 than in Group 1. Treatment with 1.0% TJ-9 (Group 3) significantly reduced the mean volume of the carcinomas.

**Labeling index of enzyme-altered lesions and % helper T cells** Table IV summarizes data on the labeling indices of enzyme-altered lesions and adjacent normal liver and % helper T cells among the tumor-infiltrating lymphocytes. Treatment with 0.5% TJ-9 (Group 2) significantly decreased the labeling index of pre-neoplastic lesions, but not adjacent normal liver, and significantly increased the proportion of helper T cells among the tumor-infiltrating lymphocytes. The labeling index of pre-neoplastic lesions and the proportion of helper T cells among the tumor-infiltrating lymphocytes were increased and decreased, respectively, but the differences were not statistically significant.

## DISCUSSION

In this study, oral treatment with TJ-9 resulted in significant inhibition of the development of GGT-positive and GST-P-positive lesions of the liver. The mechanism(s) of this effect is unknown, but at least three possible explanations can be considered.

The first may involve decrease in caloric intake. Klurfeld *et al.*[23] found that rats treated with 7,12-dimethylbenz[*a*]anthracene and subjected to caloric restriction weighed 40% less and had a significantly lower incidence of mammary and colon tumors than rats fed *ad libitum*. Albanes[24] also found that total caloric intake is an important determinant of tumorigenesis in mice, and that body weight may be a sensitive indicator of this effect. In the present work, the body weights of rats treated with both dosages of TJ-9 were significantly lower than those of rats given normal chow pellets without TJ-9. This difference reflected lower food or calorie consumption in the rats treated with TJ-9. Thus the smaller number and size of enzyme-altered hepatic lesions in TJ-9-treated rats could by explained by caloric restriction.

A second possibility is inhibition by TJ-9 of free radical processes. TJ-9 has a high content of flavonoids, which are scavengers of superoxide anions.[25] We have reported that the incidence of gastric cancers induced by N-methyl-N′-nitro-N-nitrosoguanidine was significantly reduced by prolonged administration of a radical scavenger, butylated hydroxytoluene.[26] Moreover, Nakamura *et al.*[27] found that TJ-9 suppressed active oxygen-induced hepatic injury by paraquat.

A third possibility is an effect of TJ-9 on the immune system.[1] Kumazawa *et al.*[6] found that i.p. administration of TJ-9 to mice resulted in significant macrophage accumulation in the peritoneal cavity and increased lysosomal enzyme activities, and concluded that TJ-9 is a potent macrophage activator. Itoh and Shimura[8] observed that the number of peritoneal macrophages and the degree of binding of C3 cleavage products (C3b) to macrophages were enhanced in mice treated with TJ-9. Furthermore, Kakumu and Fuji[28] examined whether TJ-9 could induce immune interferon-$\gamma$ production by peripheral blood mononuclear cells from healthy individuals, and also whether treatment with TJ-9 could enhance immune interferon-$\gamma$ production in patients with chronic active hepatitis. From their results, they concluded that TJ-9 enhances immune interferon-$\gamma$ production by activating both monocytes and T lymphocytes. In the present work, we found that administration of TJ-9 at a low dosage significantly increased the proportion of helper T cells among tumor-infiltrating lymphocytes.

Use of the traditional Chinese medicine TJ-9 has been systematized by clinical experience accumulated over thousands of years in China. Recently, Yamamoto *et al.*[9] performed a controlled prospective study for evaluation of the potential of TJ-9 for prevention of hepatocellular carcinoma, and found that TJ-9 prevents or delays the emergence of latent hepatocellular carcinoma in patients with liver cirrhosis. However, TJ-9 is not evaluated as highly as western drugs. The main reason for this is that TJ-9 is a mixture of natural crude drugs. The structures of the main components have been determined, but the pharmacological actions of many unknown minor components have still to be examined. Moreover, the effective principles contained in plant materials are variable depending on the site and time of the collection or weather conditions.

Although it is necessary to use more than two samples and to confirm whether the results obtained are reproducible or not, our present results show that TJ-9 inhibits the development of hepatic foci by NNM. The mechanism of this effect is unknown, and further investigations are required on this subject.

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