CYTOCHEMICAL STUDIES ON THE EFFECT OF INTRAPERITONEAL AND ORAL ADMINISTRATION OF A TRADITIONAL CHINESE MEDICINE (SHO-SAIKO-TO) ON THE D-GALACTOSAMINE-INDUCED HEPATIC INJURY OF RATS*

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The effect of "Sho-saiko-to" (TJ-9), a traditional Chinese medicine, on the D-galactosamine (D-Gal)-induced hepatic injury of rats was estimated from morphological and cytochemical aspects, comparing the methods of administration of TJ-9.

A single administration of D-Gal to rats resulted in a hepatic injury with some morphological and histo-cytochemical changes. Both light and electron microscopy showed a disorder in the hepatic lobular structure and configuration of hepatic cords, retention of lipid droplets in hepatocytes, infiltration of inflammatory cells, a decrease in the number of sinusoidal microvilli, dilatation of bile canaliculi, etc. Cytochemical observation showed a remarkable decline of activities of 5'-nucleotidase (5'Nase), a plasma membranous enzyme, and glucose-6-phosphatase (G6Pase), an endoplasmic membranous enzyme, in the liver cells injured by D-Gal. However, in the rats receiving prophylaxis or therapeutic treatment with TJ-9 by intraperitoneal or oral administration, neither severe morphological changes nor a remarkable decline of the enzyme activities was observed. These results suggest that the prophylaxis with TJ-9 prevents the occurrence of hepatic injury, and therapeutic treatment improves the pathological changes induced by D-Gal. Comparing the methods of TJ-9 administration to the rats, intraperitoneal administration was more effective than oral on the hepatic injury.

Enzyme cytochemical changes in the liver could be regarded as a useful hall mark for estimating the effect of a drug and the degree of hepatic dysfunction, as well as structural changes in the liver tissue. The present study provided morphological and cytochemical evidence supporting the fact that TJ-9 is efficacious in the treatment of human hepatitis.

Sho-saiko-to (Tsumura, TJ-9) is a traditional Chinese medicine which has been used by clinicians for a number of years as a treatment for human chronic hepatitis.


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The effect of TJ-9 on hepatitis is highly valued by both Chinese and Japanese clinicians from a practical viewpoint; however, the data of comprehensive basal studies on the basis of modern bioscientific methods have not been sufficient, since traditional Chinese medicine has been established on the basis of clinical practice and experience for thousands of years. In order to understand the exact action mode of traditional Chinese medicine, many approaches to the traditional medicine from some biomedical fields must be required.

The level of academic interest in the traditional Chinese medicine has therefore been deepened in the general population. Recently, some investigators evidenced that TJ-9 might have several effects by biochemical and pharmacologic studies (1-4, 6, 13, 18, 19, 25, 26). Since the effect of TJ-9 is not simple but multiple, it is difficult to elucidate all of them. In previous studies from our laboratory, we reported that TJ-9 was able to prevent D-Gal-induced hepatic injury in rats by intraperitoneal administration, by means of both light and electron microscopy (5, 24). However, the effect of TJ-9 should be examined not only by intraperitoneal administration but also by oral administration because of its close relationship with clinical treatment. In the present study, a cytochemical and ultrastructural examination of the preventive and curative effects of TJ-9 on the D-Gal-induced hepatic injury in rats was performed using both intraperitoneal and oral administration of TJ-9, with the hope of shedding some light on the mysteries of the traditional medicine.

MATERIALS AND METHODS

About one hundred female adult Wistar rats weighing 130-150 g were used for the elucidation of the effect of TJ-9. TJ-9 was used as a solution of crude powder extract (Tsumura, TJ-9 “Sho-sai-kō-to”). The experimental hepatic injury in rats was produced by a single intraperitoneal injection of D-galactosamine hydrochloride (D-Gal) at a dose of 1,500 mg/kg body weight. The hepatic injury in rats produced by D-Gal is well-established as a model of human hepatitis, and its action mode which is linked to depletion of uridine nucleotides within hepatocytes has been considerably clarified by many investigations (7-11, 14-17).

The preventive effect of intraperitoneal administration of TJ-9 to rats has already been described (24). The rats were divided into four groups as follows; (I) D-Gal control group which was administered only D-Gal (1,500 mg/kg body weight) by a single intraperitoneal injection, (II) TJ-9 intraperitoneal (i.p.) post-treatment group which was intraperitoneally administered TJ-9 at a daily dose of 20 mg/kg body weight for 7 consecutive days from one day after the D-Gal injection, (III) TJ-9 oral pre-treatment group which was orally administered TJ-9 at a dose of 2.0 g/kg body weight by using stomach sonde for 4 consecutive days before the D-Gal injection, and (IV) TJ-9 oral post-treatment group which was orally administered TJ-9 at a daily dose of 2.0 g/kg body weight by using stomach sonde for 7 consecutive days from one day after the D-Gal injection. These groups of rats were sacrificed 2, 3, 4 or 7 days after D-Gal injection, to be compared with each other by the observation of their morphological and cytochemical changes.

Each experimental group was examined using the following three procedures; 1) conventional light microscopic observation with hematoxyline and eosin staining and Sudan IV staining, 2) ultrastructural observation by conventional electron
microscopy, 3) histo- and cytochemical detection of 5'-nucleotidase (5'Nase) and glucose-6-phosphatase (G6Pase) activities by both light and electron microscopy.

For electron microscopy and cytochemical examination, under anesthesia by sodium pentobarbital, the livers were fixed by perfusion via portal vein with 0.5% glutaraldehyde in 30 mM PIPES-NaOH buffer, pH 7.2, containing 8% sucrose at 4°C for 5 min. These liver tissues were trimmed into 1 mm cubic blocks for conventional electron microscopy. The frozen-10 μm and nonfrozen-40 μm thick sections were prepared by a freezing microtome or Microslicer for light and electron microscopic cytochemistry, respectively. Detection of 5'Nase activity was performed by the lead nitrate method of Uusitalo and Karnovsky (21) for light microscopy and by the cerium method of Robinson and Karnovsky (20) for electron microscopy. G6Pase activity was detected by the lead method of Wachstein and Meisel (22).

For electron microscopy, these tissues were post-fixed with buffered 1% osmium tetroxide for 30 min, dehydrated and embedded in Spurr's resin. Ultrathin sections were prepared with LKB Ultrrotome, and stained with uranyl acetate and lead citrate, and observed under JEOL 100CX or 1200EX electron microscopes. Mainly the centrolobular areas, which were selected by the observation of Toluidine blue stained semithin sections, were examined under electron microscopes.

RESULTS

In the D-Gal control group of rats receiving only D-Gal injection, at 2 to 4 days after the D-Gal injection, severe morphological changes showing acute liver injury were revealed at both the light and electron microscopic level. However, this liver injury was reversed at 7 days after the D-Gal injection as shown in our previous report (24), because this hepatic injury is not chronic but reversible. Therefore, the liver tissues from each group at 2 to 4 days after the D-Gal injection were mainly compared with each other.

Light microscopic observation

In the greater part of the liver of the D-Gal control group, the normal architecture of hepatic lobules and hepatic cords was lost 2 days after the D-Gal injection. The degree of structural changes in the perilobular zones was more severe than in centrolobular zones. Light microscopy of the liver stained with Sudan IV revealed numerous lipid droplets distributed throughout the cytoplasm of all hepatocytes, which was similar to fatty liver (Fig. 1a). Cytochemically, the activity of 5'Nase localized on the bile canalicular and sinusoidal membranes was considerably diminished. In particular, the bile canalicular activity had largely disappeared (Fig. 1b). Though the activity of G6Pase in normal rat hepatic tissues reveals a homogeneous staining pattern in the hepatic lobules, the D-Gal-injured hepatic tissues showed varying intensities and distribution patterns from cell to cell (Fig. 1c).

In the TJ-9 i.p. post-treatment group of rats 2 to 4 days after the D-Gal injection, the configuration of hepatic tissues had a close resemblance to a normal appearance. Accumulation of lipid droplets in the hepatocytes became much less than that in the D-Gal control group (Fig. 2a). The activity of 5'Nase returned to a distribution pattern similar to normal hepatic tissues which have intense activity on both bile canalicular and sinusoidal membranes (Fig. 2b). G6Pase activity was also demonstrated to be recovered to a nearly normal level. Intense activity was observed
homogeneously in the cytoplasm of all hepatocytes (Fig. 2c).

In the TJ-9 oral pre-treatment group of rats, no conspicuous changes were caused by the D-Gal injection. Although a few lipid droplets were seen in the cytoplasm of hepatocytes (Fig. 3a), the disturbance of the architecture in the hepatic tissue was not recognized by light microscopic observation. Histochemically, 5′Nase activity was detected on both bile canalicular and sinusoidal membranes (Fig. 3b). G6Pase also showed intense activity in all hepatocytes (Fig. 3c).

In the TJ-9 oral post-treatment group of rats 2 to 4 days after the D-Gal injection, though some lesions remained, the tendency toward recovery was apparent. Though a small number of lipid droplets were seen in the cytoplasm of hepatocytes, the volume of lipid droplets remarkably decreased in comparison with that of the D-Gal control group (Fig. 4a). The localization and intensity of the enzyme activities seemed to be not so different from normal (Figs. 4b, c).

*Electron microscopic observation*

At an ultrastructural level, in the hepatocytes from D-Gal control group of rats 2 to 4 days after the D-Gal injection, several structural changes were observed. Many hepatocytes were enlarged and had become rounder. The lipid droplets were often seen in the cytoplasm of the hepatocytes. The intracellular organelles such as

Figs. 1–4. Light microscopy of the liver tissues from each group. (a) Sudan IV staining for lipid, (b) 5′-nucleotidase (5′Nase) activity detected by the method of Uusitalo and Karnovsky (21), (c) Glucose-6-phosphatase (G6Pase) activity detected by the method of Wachstein and Meisel (22).

Figs. 1a–c. Light micrographs of the liver tissues from the D-Gal control group of rats 2 days after the D-Gal injection.

(a) Sudan IV staining shows that numerous lipid droplets are accumulated in the cytoplasm of hepatocytes. It seems that the pathological change in the perilobular area is more severe than that in the centrolobular area. ×140

(b) The activity of 5′Nase in the bile canalicular and sinusoidal regions was markedly decreased by the D-Gal hepatic injury. ×140

(c) Although the activity of G6Pase is observed in the cytoplasm of some hepatocytes, the activity in most hepatocytes is weaker than that of normal liver tissues. ×280

Figs. 2a–c. Light micrographs of the liver tissues from the TJ-9 i.p. post-treatment group of rats 3 days after the D-Gal injection.

(a) Sudan IV staining shows that this liver tissue is free from lipid droplet accumulation. ×140

(b) 5′Nase activity in the bile canaliculi and sinusoidal regions has returned to a nearly normal level by the TJ-9 treatments. ×140

(c) The intense activity of G6Pase is demonstrated in all hepatocytes. ×280

Figs. 3a–c. Light micrographs of the liver tissues from the TJ-9 oral pre-treatment group of rats 2 days after the D-Gal injection.

(a) Only a small number of lipid droplets are seen in some hepatocytes by Sudan IV staining. ×280

(b) 5′Nase activity in the bile canaliculi is well preserved. ×280

(c) G6Pase activity is observed in the cytoplasm of all hepatocytes. It appears to be normal and free from D-Gal injury. ×140

Figs. 4a–c. Light micrographs of the liver tissues from the TJ-9 oral post-treatment group of rats 3 days after the D-Gal injection.

(a) Although a few lipid droplets are present in some hepatocytes, no other remarkable changes are noticed. ×280

(b) No decrease of 5′Nase activity is observed in the bile canalicular region. ×280

(c) All hepatocytes show almost normal activity of G6Pase in the cytoplasm. ×140
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1a  2a

1b  2b

1c  2c
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5a

5b

5c
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Figs. 5–8. Electron microscopy of the hepatocytes from each group. (a) Conventional electron micrographs of the liver tissues, (b) 5’Nase activity demonstrated by the cerium method of Robinson and Karnovsky (20), (c) G6Pase activity demonstrated by the method of Wachstein and Meisel (22).

The following symbols are used: N, nucleus; B, bile canaliculi; S, sinusoid; Li, lipid droplets; E, endothelial cells.

Figs. 5a–c. Electron micrographs of the liver tissues from the D-Gal control group of rats 2 days after D-Gal injection.

(a) Electron micrograph showing ultrastructural changes of centrolobular hepatocytes in which the damage by D-Gal is advanced. The bile canaliculi (B) are markedly dilated with partial loss of microvilli. This finding may imply dysfunction of plasma membranes altered by the D-Gal. The intracellular organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus are also unusual, though the changes considerably varied from cell to cell. ×10,000

(b) 5’Nase activity of the hepatocytes injured by D-Gal. A few reaction products are localized on the dilated bile canalicular plasma membrane and sinusoidal membrane with loss of microvilli. The reaction products of 5’Nase activity are less than those in normal hepatocytes. Some lipid droplets (Li) can be seen in perisinusoidal cytoplasm of this hepatocyte. ×8,500

(c) Many smooth and rough endoplasmic reticulum in the hepatocyte exhibit weak or no activity of G6Pase, although activity is still present normally in some places of endoplasmic reticulum. The arrangement of the rough endoplasmic reticulum is partially disordered. ×11,000

Figs. 6a–c. Electron micrographs of hepatocytes from the TJ-9 i.p. post-treatment group of rats 3 days after D-Gal injection.

(a) Ultrastructurally no remarkable changes are observed. The bile canaliculi (B) are not dilated. The sinusoidal membrane and microvilli seem to be well. Other subcellular organelles are not appreciably altered. ×11,000

(b) 5’Nase activity is localized on the whole of plasma membranes of hepatocytes. The enzyme activity of sinusoidal surface (S) and bile canaliculi (B) are preserved as well as the ultrastructure. ×7,300

(c) G6Pase activity in endoplasmic reticulum and nuclear envelope is also demonstrated little less than normal. The lamellarr arrangement of endoplasmic reticulum shows a normal appearance. ×9,700

Figs. 7a–c. Electron micrographs of hepatocytes from the TJ-9 oral pre-treatment group of rats 2 days after the D-Gal injection.

(a) No noteworthy changes are seen in the bile canaliculi (B), sinusoidal membrane and other organelles. ×12,000

(b) 5’Nase activity is seen on the bile canalicular, sinusoidal (S) and lateral membrane in the hepatocytes. ×13,000

(c) G6Pase activity is seen in all of endoplasmic reticulum and nuclear envelope. ×11,000

Figs. 8a–c. Electron micrographs of hepatocytes from the TJ-9 oral post-treatment group of rats 3 days after the D-Gal injection.

(a) The ultrastructure of sinusoidal regions of the hepatocytes keeps normal appearance. The hepatocytes have many microvilli on the sinusoidal surface. ×14,000

(b) 5’Nase activity is normally demonstrated on the bile canalicular, sinusoidal and lateral membranes of the hepatocytes. ×10,000

(c) G6Pase activity in endoplasmic reticulum and nuclear envelope appears to be normal localization and intensity. ×11,000
mitochondria, Golgi apparatus, and endoplasmic reticulum (ER) also seemed to be damaged somewhat, though these findings varied considerably in degree from cell to cell. Dilated bile canaliculi with transformations of their walls were the characteristic feature of D-Gal-injured hepatic tissues (Fig. 5a). In some lesions, the necrosis of hepatocytes and the inflammatory infiltrate consisted largely of neutrophils and phagocytic cells were observed. Cytochemical electron microscopy revealed a marked decrease of 5'Nase activity on the dilated bile canaliculi with loss of microvilli and transformation of the canalicular wall. The reduction in number and length of microvilli on the sinusoidal membrane seemed to result in the decrease of 5'Nase activity on the sinusoidal membrane (Fig. 5b). Decrease of G6Pase activity in the rough ER in the damaged cells and a partial loss of the parallel lamellar arrangement of rough ER were also clearly observed by cytochemical electron microscopy (Fig. 5c). These ultrastructural and cytochemical changes induced by D-Gal were essentially identical with those reported previously (8, 15-17, 24).

In the TJ-9 i.p. post-treatment group of rats 2 to 4 days after the D-Gal injection, such conspicuous ultrastructural changes were largely improved by the TJ-9 treatment. No noteworthy changes were seen in their intracellular organelles and other ultrastructures (Fig. 6a). 5'Nase activity was localized on the whole of the plasma membrane in the hepatocytes no less than in normal hepatocytes (Fig. 6b). The activity of G6Pase was observed on nuclear envelope membranes, smooth ER and rough ER showing a parallel lamellar arrangement (Fig. 6c).

Electron microscopy of the hepatocytes from the TJ-9 oral pre-treatment group of rats confirmed the light microscopic observation. The ultrastructure and enzyme activities of 5'Nase and G6Pase were not remarkably changed by the D-Gal injection (Figs. 7a-c). This preventive effect of TJ-9 by oral administration was similar, but a little less effective than the intraperitoneal administration which was previously reported in our publication (24).

Electron micrographs from the TJ-9 oral post-treatment group of rats also revealed that the ultrastructural changes in D-Gal-injured hepatocytes were reduced by the oral TJ-9 treatment. Although minor pathological changes such as lipid droplet accumulation in the cytoplasm were observed in some places in the liver, no severe alterations of the bile canaliculi and other organelles were noticed (Fig. 8a). Enzyme activities of 5'Nase and G6Pase tended to recover to normal intensity and localization, being accompanied by the ultrastructural recovery of the places on which the enzymes localized (Figs. 8b-c).

Comparing the effect of TJ-9 by each method of administration on the hepatic injury in the rats, the intraperitoneal administration was more effective than the oral administration. In the case of oral administration, individual differences in the effect of TJ-9 was observed more frequently than in a case of intraperitoneal administration.

**DISCUSSION**

TJ-9 is a blended herbal medicine which consists of seven kinds of herbs: *Bupleuri radix*, *Pinelliae tuber*, *Scutellariae radix*, *Zizyphi fructus*, *Glycyrrhizae radix*, *Ginseng radix* and *Zingiberis rhizoma*. The clinical constituent of each herb has been established from the viewpoint of the connection between the prescription and its clinical application with naturalistic and humanistic philosophy in its thousands of years of history. The com-
bining mechanism of the various herbs contained in the prescription is too complicated to resolve them all by any scientific technique. Therefore, basic scientific analysis in traditional Chinese medicine has been limited. However, recently we have had a better opinion of the traditional Chinese medicine because there is no adverse reaction, and the application of the prescription has become very popular in general hospitals and clinics. In particular TJ-9 is widely used for human hepatitis, since there is no chemical medicine based on Western medicine which has any conspicuous effect in human chronic hepatitis, while the number of hepatitis patients has been increasing. Recently active attempts of scientific analysis based on Western medicine have been started for traditional Chinese medicine. It is hoped that a better system of medicine will arise from the union of modern Western medicine and traditional Chinese medicine.

In our laboratory, we have been investigating the effect of TJ-9 on experimental hepatic injury in rats by various procedures including cytochemical, morphological and biochemical techniques with special reference to the enzyme activity and cell ultrastructure (5, 24). We have already demonstrated that intraperitoneal pre-treatment of TJ-9 is undoubtedly effective in preventing D-Gal-induced rat hepatic injury which shows ultrastructural changes and a remarkable decline of enzyme activities of 5′Nase, G6Pase and a slight decline of alkaline phosphatase activity in the liver, by means of both cytochemical detection and biochemical assay (24). The present study demonstrated with procedures similar to previous ones that TJ-9 is effective in hepatic injury not only by intraperitoneal pre-treatment but also by intraperitoneal post-treatment, oral pre-treatment and oral post-treatment. It is conceivable that the present result is important as regards its close relationship with the clinical application, since the prescription of TJ-9 is usually administered per os to patients with hepatitis.

Our study revealed that TJ-9 had preventive and curative effects on the decrease of 5′Nase activity, which is a marker enzyme of plasma membrane in the hepatocytes, by D-Gal hepatic dysfunction. The preventive effect of TJ-9 was more drastic than the curative effect. It may be suggested that TJ-9 directly affected the plasma membrane and increased resistance of the hepatocytes by a protecting effect on the plasma membrane. It has been reported that saikosaponin, a main essence of Bupiuri radix in TJ-9, has a protective effect on hepatocytes from various type hepatic injuries without relation to the pathogenesis including D-Gal, carbon tetrachloride intoxication and virus et al. (1, 4, 6, 18, 19, 25, 26). It can be considered that the plasma membrane of the hepatocytes is the initial or a very early site for the development of these liver injuries (7, 12, 15, 18, 23). Abe et al. revealed that the saikosaponins had a direct effect on the biological membrane by the relationship between the structures of saikosaponins and hemolytic activity (3) and the change of electron spin resonance spectra from spin-labeled erythrocytes and erythrocyte ghost membrane (2). Therefore, it is possible that the portion of the effect of TJ-9 on the hepatic injury may be owing to the effect of saikosaponins on the plasma membrane of hepatocytes. Furthermore, we have shown the effect of TJ-9 against a decline of G6Pase activity, a marker enzyme of endoplasmic reticulum where the main intracellular metabolic system is present. It strongly suggests that TJ-9 may act not only on the outer plasma membrane but also on endoplasmic membranes.

However, it has been proved that TJ-9 has other pharmacologic effects such as anti-inflammatory action, anti-allergic action, immuno-activating action and enhance-
ment action of inner steroid effect (6, 13, 18, 19). These actions also must be involved
in the effect of TJ-9 on the hepatic injury. It can be speculated that these actions are
brought about by the combining mechanism of the various herbs contained in the
prescription of TJ-9. Although the exact mechanisms by which TJ-9 acts on the
hepatic injury should be examined by further studies, the present study allowed us to
confirm the effect of TJ-9 objectively.

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