*Findings of microarray analysis*

The selected results of microarray analysis are shown in Table 2. In BN rats of the PS-treated group, many genes related to cellular markers (CD74 antigen, Fc receptor, MHC class II alpha chain RT1.D alpha, MHC class II antigen RT1.B-1 beta-chain, RT1.D beta chain, RT1.Ma), inflammation (orosomucoid 1, T-kininogen),

Table 2
Changes is the gene expression profile in BN and Wistar rats of the PS-treated group

| Gene | BN | | Wistar | | Accession No. |
|---|---|---|---|---|---|
| | 2PS | 4PS | 2PS | 4PS | |
| Amino acid metabolism | | | | | |
| ornithine aminotransferase (Oat) | −1.35 | | | | NM_022521.1 |
| Apoptosis | | | | | |
| metallothionein 1 | | | 1.6 | | AF411318.1 |
| Basal metabolism | | | | | |
| hepcidin antimicrobial peptide (Hamp), mRNA. | −1.9 | | | | NM_053469.1 |
| serine dehydratase (Sds) | | 2.05 | | | NM_053962.1 |
| Bile acid metabolism | | | | | |
| alpha-methylacyl-CoA racemase (Amacr), mRNA | | | | 1.7 | NM_012816.1 |
| Carbohydrate metabolism | | | | | |
| glucokinase (Gck) | | | | −1.3 | NM_012565.1 |
| Cellular marker | | | | | |
| anti-acetylcholine receptor antibody gene | | | 1.65 | | L22655.1 |
| cadherin 17 (Cdh 17) | | | −1.55 | | NM_053977.1 |
| CD74 antigen (invariant polpypeptide of MHC class II antigen-associated) (Cd74) | 1.25 | | | 1.25 | NM_013069.1 |
| D site albumin promoter binding protein | −1.05 | | −1.15 | | AI230048 |
| Fc receptor, IgG, low affinity II (Fcgr3) | 2.35 | | | | NM_053843.1 |
| MHC class II alpha chain (RT1.D) alpha (a). | 1.85 | | | 1.45 | Y00480.1 |
| MHC class II antigen RT1.B-1 beta-chain | 1.15 | | | 1.45 | AI715202 |
| RT1.D beta chain | 1.35 | | | | BI279526 |
| RT1.Ma | 1.6 | | | | BI301490 |
| Cell proliferation | | | | | |
| growth response protein (CL-6) (LOC64194) | 1.45 | | | | NM_022392.1 |
| Channel | | | | | |
| amino acid transporter system A (ATAZ) | | 1.05 | | | AF249673.1 |
| Choresterol metabolism | | | | | |
| 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1) | 2.55 | | | | NM_017268.1 |
| Inflammation | | | | | |
| orosomucoid 1 (Orm1) | 1.6 | | | 1 | NM_053288.1 |
| T-kininogen, see also D1 1Eh1 and D11MitB (Kng) | 1.65 | | | | NM_012696.1 |
| Oxidation/Reduction | | | | | |
| GPI-anchored ceruloplasmin | 1.15 | | | | AF202115.1 |
| Secretory products | | | | | |
| alpha-2-macroglobulin (A2m) | 3.05 | | | | NM_012488.1 |
| Ig active lambda2-like chain | | 1.6 | | | BG374683 |
| macrophage migration inhibitory factor (Mif) | −1.15 | | | | NM_031051.1 |
| Stress/Cell damage | | | | | |
| lipopolysaccharide binding protein | 3.25 | | | | BF289368 |
| Transcription | | | | | |
| hepatocyte nuclear factor 3 gamma (Hnf3g) | | −1.05 | | | NM_017077.1 |
| L-type pyruvate kinase | −1.25 | | | | MI7685.1 |
| nuclear receptor subfamily 0, group B, member 2 (Nr0b2) | | | −1 | | NM_057133.1 |

stress/cell damage (lipopolysaccharide binding protein), secretory products (alpha-2-macroglobulin), and growth response protein (CL-6) showed an enhanced expression at 2 weeks (Table 2). At 4 weeks, instead of the abovementioned genes, the expression of genes coding serine dehydratase, amino acid transporter system, and Ig active lambda2-like chain increased. On the other hand, in Wistar rats of the PS-treated group, the expression of genes coding metallothionein 1 and anti-acetylcholine receptor antibody was elevated at 2 weeks. At 4 weeks, the expression of three genes coding cellular markers (CD74 antigen, MHC class II alpha chain RT1.D alpha, MHC class II antigen RT1.B-1 beta-chain), as well as those coding alpha-methylacyl-CoA racemase and orosomucoid 1, increased (Table 2).

On the other hand, the expression of genes coding ornithine aminotransferase, hepcidin antimicrobial peptide, and macrophage migration inhibitory factor decreased in BN rats of the PS-treated group at 2 weeks. In addition, in the PS-treated groups of both strains, genes coding D site albumin promoter binding protein and transcription were down regulated.

### *Findings of RT-PCR*

The results of RT-PCR are shown in Figs. 2 and 3. In BN rats of the PS-treated group, the expression levels of all the CD74, MHC class II alpha chain, RT1.B-1 beta chain, and RT1.D mRNAs were significantly elevated at 2 weeks compared with the control group and, except for that of CD74 mRNA, they maintained higher levels until 4 weeks (Fig. 3). In Wistar rats of the PS-treated group, the expression level of CD74 mRNA was significantly lower at 3 and 4 weeks and significantly higher at 8 weeks compared with the control group (Fig. 3). In addition, the expression level of RT1.D mRNA was significantly lower until 4 weeks and that of RT1.B-1 beta mRNA was significantly higher at 8 weeks compared with the control group (Fig. 3).

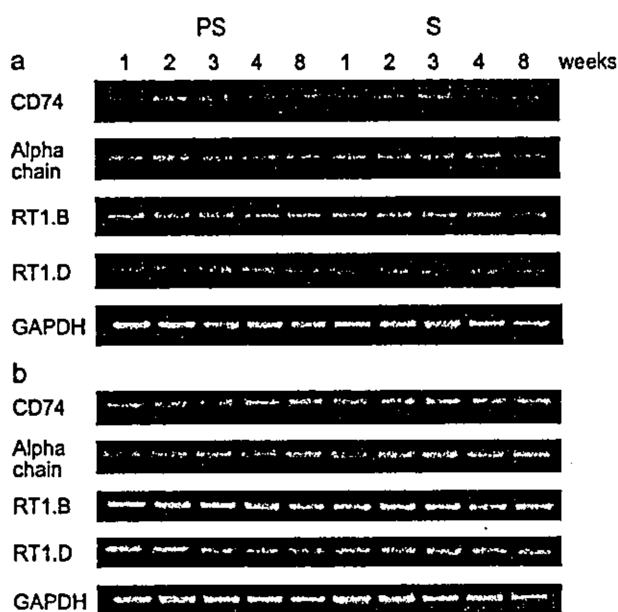



Fig. 2. The expression of MHC class II-related genes mRNAs in the liver of BN (a) and Wistar rats (b) by semiquantitative RT-PCR.

## Discussion

In this study, genes, especially MHC class II-related genes, expression profiles were examined in PS-induced hepatic fibrosis in BN and Wistar rats. Histological changes and their sequences were similar to those described in our previous report (Baba et al., 2004). Namely, inflammatory cell infiltration and hepatic fibrosis occurred earlier in BN rats than in Wistar rats.

In microarray analysis done at 2 and 4 weeks, MHC class II- and inflammation-related genes significantly increased at 2 weeks in BN rats. In Wistar rats, some of the MHC class II-related genes detected in BN rats as mentioned before showed an increased expression at 4 weeks. The results suggest that the initiation of PS-induced hepatic fibrosis may have a certain relation to an antigen presentation through MHC class II.

Based on the results of microarray analysis, RT-PCR was performed focusing on the expression of MHC class II-related genes (CD74, MHC class II alpha chain RT1.D alpha, MHC class II antigen RT1.B-1 beta chain, and RT1.D beta chain) mRNAs. In BN rats, the expression of these four gene mRNAs kept higher levels in the PS-treated group than in the control group from 2 to 4 weeks, especially at 2 weeks. These results of RT-PCR corresponded to those of microarray analysis. On the other hand, in Wistar rats of the PS-treated group, the expression levels of these genes mRNAs were somewhat lower than those of the control group until 4 weeks, and those of CD74 and MHC class II antigen RT1.B-1 beta chain mRNAs were significantly lower at 8 weeks. Thus, in the case of Wistar rats, there were differences between the results of microarray analysis and RT-PCR, although the reason is obscure.

Orosomucoid (Dowton and Colten, 1988), T-kininogen (Kageyama et al., 1985), and lipopolysaccharide (Geller et al., 1993) genes that showed an increased expression in BN rats at 2 weeks are inflammation-induced factors in the acute phase. Orosomucoid is reported as a hepatic fibrosis accelerator (Ozeki et al., 1986), and T-kininogen (Lukjan et al., 1975) and lipopolysaccharide (Su et al., 1998) are reported to increase in patients and animals of hepatic fibrosis.

The expression of anti-acetylcholine receptor antibody-coding gene was elevated at 2 weeks in Wistar rats, and the expression of genes coding Fc receptor and Ig active lambda2-like chain increased at 2 and 4 weeks in BN rats. Anti-acetylcholine receptor antibody is said to be involved in autoimmune diseases such as primary biliary cirrhosis (Sundewall et al., 1985), chronic active hepatitis, systematic

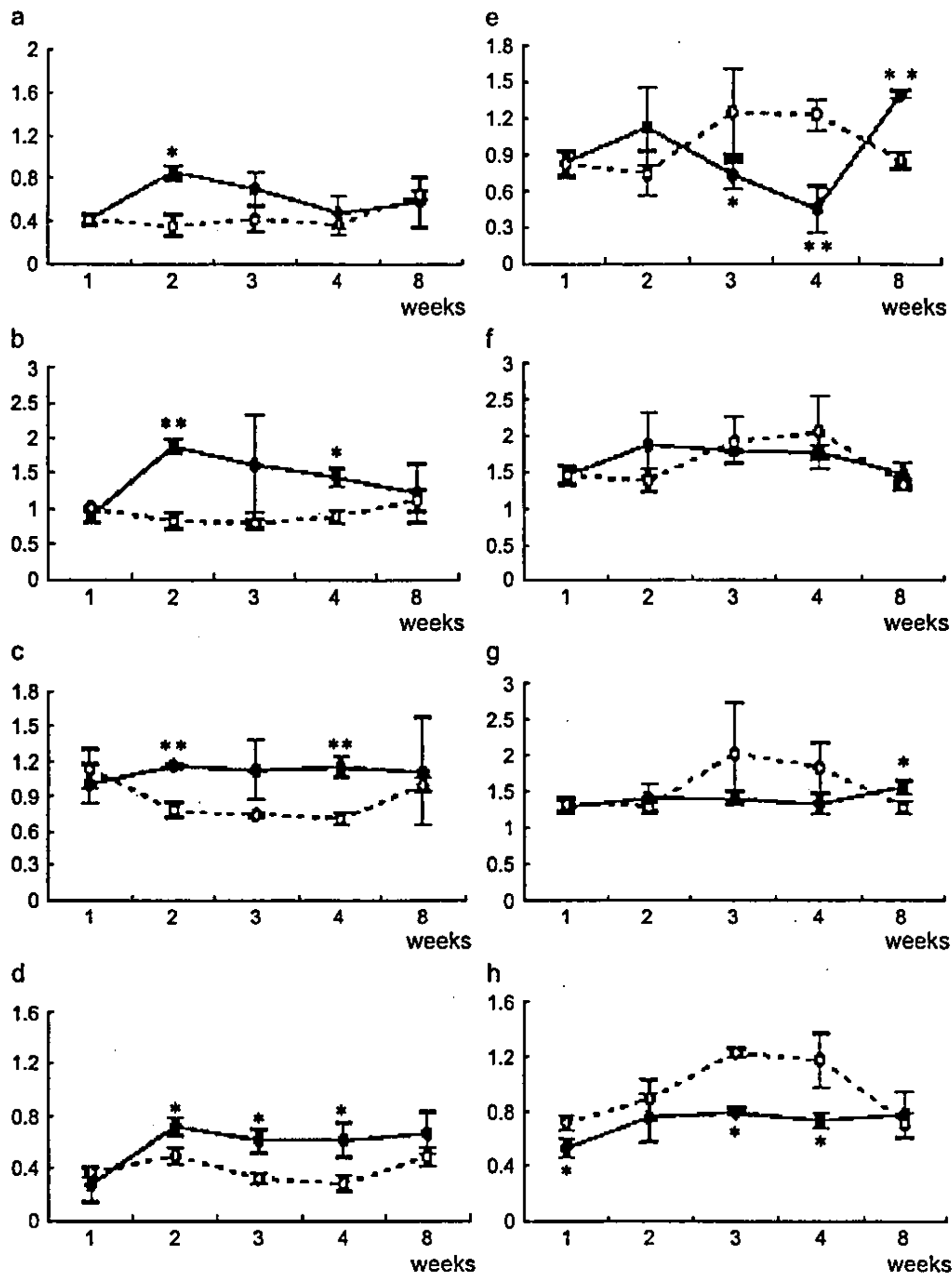



Fig. 3. Sequential changes in the expression of CD74 antigen mRNA (a and e), MHC class II alpha chain mRNA (b and f), RT1.B-1 beta chain mRNA (c and g), and RT1.D mRNA (d and h) in BN (a–d) and Wistar rats (e–h). —: Porcine serum-treated group; ----: -: saline-treated group. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the control group.

lupus erythematosus, and myasthenia gravis. Fc receptor (Nishi et al., 1981) and Ig active lambda2-like chain (Storb et al., 1988) are related to the production of immunoglobulins. In this connection, in the PS-induced hepatic fibrosis, antibody level for PS was elevated (Bhunchet et al., 1996) and immunoglobulin-related glomerulonephritis occurred (Kitamura et al., 1984), along with the development of hepatic fibrosis. Therefore, there may be a certain relation between the up-regulation of these genes and immunoglobulin production in the PS-induced hepatic fibrosis.

At 2 weeks, the expression of growth response protein (CL-6) gene was elevated in BN rats and that of metallothionein-1 gene increased in Wistar rats. On the other hand, the expression of albumin promoter D-site binding protein gene decreased at 2 weeks in both BN and Wistar rats. CL-6 is known to be induced during hepatic regeneration (Diamond et al., 1993), and metallothionein-1 functions as metal metabolism and radical scavenger (Sato, 1991). For example, when the hepatic damage is induced by carbon tetrachloride, the expression of metallothionein-1

gene was elevated in the liver (Schroeder and Cousins, 1990). This phenomenon may indicate that metallothionein-1 protects the liver from damage by free radicals. In addition, albumin promoter D-site binding protein is known as an important transcriptional factor (Lee et al., 1994) and rapidly decreased with hepatic regeneration (Mueller, 1992). Although PS-induced hepatic fibrosis showed a little histopathological hepatocyte damage, changes in the expression of these genes suggest an existence of latent or functional hepatocyte damage.

A participation of MHC class II in hepatic diseases has been reported in relation to hepatitis C (Mangia et al., 1999), primary biliary cirrhosis (Agarwal et al., 1999), and schistosomiasis mansoni-related hepatic disorder (Assaad-Khalil et al., 1993). However, it is reported in only a few cases of experimentally induced hepatic fibrosis models (Ide et al., 2003; Jezequel et al., 1987). As mentioned before, Ide et al. (2003) described an immunohistochemical finding suggesting a relationship between MHC class II and thioacetamide-induced rat hepatic fibrosis.

In conclusion, the present results suggest that MHC class II may participate in the initiation of the PS-induced hepatic fibrosis in rats. They also suggest that many genes related to immunoglobulin production and hepatocyte damage, which were up-regulated in the present study, may have a certain relation to the development of hepatic fibrosis in this model. Further study on the activation of B cells and the production of immunoglobulins is now in progress in relation to the activation of MHC class II in the PS-induced rat hepatic fibrosis model.

## References

Agarwal, K., Jones, D.E., Bassendine, M.F., 1999. Genetic susceptibility to primary biliary cirrhosis. Eur. J. Gastroenterol. Hepatol. 11, 603–606.

Akiyoshi, H., Terada, T., 1998. Mast cell, myofibroblast and nerve terminal complexes in carbon tetrachloride-induced cirrhotic rat livers. J. Hepatol. 29, 112–119.

Armbrust, T., Batusic, D., Ringe, B., Ramadori, D., 1997. Mast cells distribution in human liver disease and experimental rat liver fibrosis. Indications for mast cell participation in development of liver fibrosis. J. Hepatol. 26, 1042–1054.

Assaad-Khalil, S.H., Helmy, M.A., Zaki, A., Mikhail, M.M., el-Hai, M.A., el-Sawy, M., 1993. Some genetic, clinical and immunologic interrelations in schistosomiasis mansoni. Ann. Biol. Clin. 51, 619–626.

Baba, Y., Uetsuka, K., Nakayama, Y., Doi, K., 2004. Rat strain differences in the early stage of porcine-serum-induced hepatic fibrosis. Exp. Toxicol. Pathol. 55, 325–330.

Ballardini, G., Fallani, M., Biagini, G., Bianchi, F.B., Pisi, E., 1988. Desmin and actin in the identification of Ito cells and in monitoring their evolution to myofibroblasts in experimental liver fibrosis. Virchows Arch., B Cell Pathol. Include Mol. Pathol. 56, 45–49.

Bhunchet, E., Eishi, Y., Wake, K., 1996. Contribution of immune response to the hepatic fibrosis induced by porcine serum. Hepatology 23, 811–817.

Bhunchet, E., Fujieda, K., 1993. Capillarization and venularization of hepatic sinusoids in porcine serum-induced rat liver fibrosis: a mechanism to maintain liver blood flow. Hepatology 18, 1450–1458.

Bhunchet, E., Wake, K., 1992. Role of mesenchymal cell population in porcine serum-induced rat liver fibrosis. Hepatology 16, 1452–1473.

Diamond, R.H., Du, K., Lee, V.M., Mohn, K.L., Haber, B.A., Tewari, D.S., Taub, R., 1993. Novel delayed-early and highly insulin-induced growth response genes. Identification of HRS, a potential regular of alternative pre-mRNA splicing. J. Biol. Chem. 268, 15185–15192.

Dowton, S.B., Colten, H.R., 1988. Acute phase reactants in inflammation and infection. Semin. Hematol. 25, 84–90.

Fujiwara, K., Ogata, I., Ohta, Y., Hayashi, S., Mishiro, S., Takatsuki, K., Sato, Y., Yamada, S., Hirata, K., Oka, H., 1988. Decreased collagen accumulation by a prolylhydroxylase inhibitor in pig serum-induced fibrotic rat liver. Hepatology 8, 804–807.

Geller, D.A., Kispert, P.H., Su, G.L., Wang, S.C., Silvio, M.D., Tweardy, D.J., Billiar, T.R., Simmons, R.L., 1993. Induction of hepatocytes lipopolysaccharide binding protein in models of sepsis and the acute-phase response. Arch. Surg. 128, 22–28.

Geraci, J.P., Mariano, M.S., Jackson, K.L., 1992. Radiation hepatology of the rat: microvascular fibrosis and enhancement of liver dysfunction by diet and drugs. Radiat. Res. 129, 322–332.

Haczku, A., Chung, K.F., Sun, J., Barnes, P.J., Kay, A.B., Moqbel, R., 1995. Airway hyperresponsiveness, elevation of serum-specific IgE and activation of T cells following allergen exposure in sensitized Brown-Norway rats. Immunology 85, 598–603.

Ide, M., Yamate, J., Machida, Y., Nakanishi, M., Kuwamura, M., Kotani, T., Sawamoto, O., 2003. Emergence of different macrophage populations in hepatic fibrosis following thioacetamide-induced acute hepatocytes injury in rats. J. Comp. Pathol. 128, 41–51.

Ishida, N., Nakata, K., Takase, K., Tanaka, M., Mita, S., 1991. The protective effects of SA3443, a novel cyclic disulfide, on chronic liver injuries in rats. J. Hepatol. 13, 200–207.

Jezequel, A.M., Mancini, R., Rinaldesi, M.L., Macarri, G., Venturini, C., Orlandi, F., 1987. A morphological study of the early stages of hepatic fibrosis induced by low doses of dimethylnitrosamine in the rat. J. Hepatol. 5, 174–181.

Jonker, A.M., Dijkhuis, F.W., Hardonk, M.J., Moerkerk, P., Ten, K.J., Grond, J., 1994. Immunohistochemical study of hepatic fibrosis induced in rats by multiple galactosamine injections. Hepatology 19, 775–781.

Kageyama, R., Kitamura, N., Ohkubo, H., Nakanishi, S., 1985. Differential expression of the multiple forms of rat prekininogen mRNAs after acute inflammation. J. Biol. Chem. 260, 12060–12064.

Kitamura, K., Yasoshima, A., Iwasaki, H.O., Doi, K., Okaniwa, A., 1984. Hepatic fibrosis produced in rats by repeated intraperitoneal injections of swine serum. Jpn. J. Vet. Sci. 46, 265–271.

Lee, Y.H., Alberta, J.A., Gonzalez, F.J., Waxman, D.J., 1994. Multiple, functional DBP sites on the promoter of the cholesterol 7alpha-hydroxylase P450 gene, CYP7. Proposed role in diurnal regulation of liver gene expression. J. Biol. Chem. 269, 14681–14689.

Lukjan, H., Czokalo, M., Kiersnowska, B., Bielawiec, M., 1975. Studies of the plasma kinin-forming system in cirrhosis of the liver. Acta Hepatogastroenterol. 22, 89–92.

Mangia, A., Gentile, R., Cascavilla, I., Margaglione, M., Villani, M.R., Stella, F., Modola, G., Agostiano, V., Gaudiano, C., Andriulli, A., 1999. HLA class II favors clearance of HCV infection and progression of the chronic liver damage. J. Hepatol. 30, 984–989.

Mueller, C.R., 1992. The down-regulation of albumin transcription during regeneration is due to the loss of HNF-1 and the D-site transcription factors. DNA Cell Biol. 11, 559–566.

Nakano, M., 1986. Early morphological changes of porcine serum-induced hepatic fibrosis. Acta Pathol. Jpn. 36, 415–422.

Nishi, T., Bhan, A.K., Collins, A.B., McCluskey, R.T., 1981. Effect of circulating immune complexes on Fc and C3 receptors of Kupffer cells in vivo. Lab. Invest. 44, 442–448.

Ozeki, T., Kan, M., Iwaki, K., Ohuchi, K., 1986. Orosomucoid as the accelerator of hepatic fibrosis. Br. J. Exp. Pathol. 67, 731–736.

Peng, R.Y., Wang, D.Y., Xu, Z.H., Gao, Y.B., Yang, R.B., Liu, P., Wang, Z.P., Li, Y.P., 1994. The changes and significance of mast cells in irradiated rat liver. J. Environ. Pathol., Toxicol. Oncol. 44, 655–661.

Rioux, K.P., Sharkey, K.A., Wallace, J.L., Swain, M.G., 1996. Hepatic

mucosal mast cell hyperplasia in rats with secondary biliary cirrhosis. Hepatology 23, 888–895.

Sato, M., 1991. Dose-dependent increases in metallothionein synthesis in the lung and liver of paraquat-treatment rats. Toxicol. Appl. Pharmacol. 107, 98–105.

Schroeder, J.J., Cousins, R.J., 1990. Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocytes monolayer cultures. Proc. Natl. Acad. Sci. U. S. A. 87, 3137–3141.

Senoo, H., Wake, K., 1985. Suppression of experimental hepatic fibrosis by administration of vitamin A. Lab. Invest. 52, 182–194.

Shiga, A., Shirota, K., Ikeda, T., Nomura, Y., 1997. Morphological and immunohistochemical studies on porcine serum-induced rat liver fibrosis. J. Vet. Med. Sci. 59, 159–167.

Steinhoff, D., 1975. Effect of diethylnitrosamine on the livers of rats after high oral doses administered at intervals varying between three and twenty-four days. Acta Hepatogastroenterol. 22, 72–77.

Storb, U., Engler, P., Manz, J., Gollahon, K., Denis, K., Lo, D., Brinster, R., 1988. Expression of immunoglobulin genes in transgenic mice and transfected cells. Ann. N. Y. Acad. Sci. 546, 51–56.

Su, G.L., Rahemtulla, A., Thomas, P., Klein, R.D., Wang, S.C., Nanji, A.A., 1998. CD14 and lipopolysaccharide binding protein expression in a rat model of alcoholic liver disease. Am. J. Pathol. 152, 841–849.

Sugihara, A., Tujimura, T., Fujita, Y., Nakata, Y., Terada, N., 1999. Evaluation of role of mast cells in the development of liver fibrosis using mast cell-deficient rats and mice. J. Hepatol. 30, 859–867.

Sundewall, A.C., Lefvert, A.K., Olsson, R., 1985. Anti-acetylcholine receptor antibodies in primary biliary cirrhosis. Acta Med. Scand. 217, 519–525.

Uyama, O., Ihaku, D., Kitada, O., Miyasaka, M., Sugita, M., 1995. Role of eosinophils and cell adhesion molecules in the allergen-induced asthmatic response of rats. Res. Commun. Mol. Pathol. Pharmacol. 90, 3–15.

Xu, X., Ruan, Y.B., Wu, Z.B., 1994. Study on the role of mast cells in the development of liver fibrosis during diethylnitrosamine (DEN)-induced hepatocarcinogenesis in rats. J. Tongji Med. Univ. 14, 193–199.

Yang, M.C., Ruan, Q.G., Yang, J.J., Eckenrode, S., Wu, S., McIndoe, R.A., She, J.X., 2001. A statistical method for flagging weak spots improves normalization and ratio estimates in microarrays. Physiol. Genomics 7, 45–53.

Yang, W., Zeng, M., Fan, Z., Mao, Y., Song, Y., Jia, Y., Lu, L., Chen, C.W., Peng, Y.S., Zhu, H.Y., 2002. Prophylactic and therapeutic effect of oxymatrine on D-galactosamine-induced rat liver fibrosis. Zhonghua Gan Zang Bing Zazhi 10, 193–196.

New Horizon of Mycotoxicology for Assuring Food Safety (Proceedings of ISMYCO Kagawa'03)
Edited by Takumi Yoshizawa ©2004 Japanese Association of Mycotoxicology


# T-2 toxin-induced toxicity in pregnant rats - histopathology and gene expression profiles -

Shinya SEHATA[*1, *2], Munehiro TERANISHI[*2], Takashi YAMOTO[*2], Naochika MATSUNUMA[*2] and Kunio DOI[*1]

[*1]Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo
(1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan)
[*2]Medicinal Safety Research Laboratories, Sankyo Co., Ltd.
(717 Horikoshi, Fukuroi-shi, Shizuoka 437-0065, Japan)

## Summary

T-2 toxin is a trichothecene mycotoxin produced by various species of *Fusarium* spp. Single dose or subacute dose of T-2 toxin induces damage in the lymphoid and hematopoietic tissues, resulting in lymphopenia and immunosuppression in many species. Necrosis/apoptosis is also reported in the gastrointestinal tract and liver. As there are a few reports of T-2 toxin-induced toxicity in pregnant rats, we performed a study to examine T-2 toxin-induced morphological changes in pregnant rats. Single cell necrosis was observed in the thymus, liver, intestines, placenta and fetal liver in pregnant rats treated with T-2 toxin on day 13 of gestation. To investigate gene expression profiles in the liver, placenta and fetal liver in the pregnant rats treated with T-2 toxin, Wistar rats on day 13 of gestation were orally administered with T-2 toxin at a single dosage of 2 mg/kg. Twenty-four hours after treatment, rats were sacrificed. Microarray analysis in the liver, placenta and fetal liver was performed using the Affymetrix Rat Genome U34A chip. The results in these tissues showed the same changes in lipid metabolism-related genes, apoptosis-related genes and oxidative stress-related genes. From these results, the mechanism of T-2 toxin-induced toxicity is speculated that T-2 toxin caused oxidative stress, following the impairment of lipid peroxidation and metabolism-related enzymes. These changes may cause the changes in the intracellular environments, finally resulting in the induction of apoptosis. Further study on the gene expression profiles at the earlier time point should be done to clarify the whole mechanisms of T-2 toxin-induced toxicity.

**Key words**: T-2 toxin, pregnant rats, histopathology, gene expression

## Introduction

T-2 toxin is a trichothecene mycotoxin produced by various species of *Fusarium* spp. T-2 toxin has been found to contaminate foods, animal foods and agricultural

products, and has been reported in many parts of the world[1]. Single dose or subacute dose of T-2 toxin induces damage in the lymphoid and hematopoietic tissues, resulting in lymphopenia and immunosuppression in many species. Necrosis/apoptosis is also reported in the gastrointestinal tract and liver[2-4]. Furthermore, it is said that pregnant mice treated with T-2 toxin exhibited fetal death and fetotoxicity mainly in the central nervous and skeletal systems[5]. However, mechanisms of fetotoxicity induced by T-2 toxin are still unknown, because there are only a few reports of T-2 toxin-induced toxicity in pregnant rats. Therefore, we first performed the histopathological examination in pregnant rats, placenta and fetal liver[6].

Molecular approach is necessary to investigate mechanisms of toxic damage induced by chemicals. Recent years DNA Microarray technologies have been developed that allow one to detect the expression of many genes at the same time. To examine the gene expression profiles, we performed microarray analysis in the liver, placenta, and fetal liver in pregnant rats treated with T-2 toxin[7]. We especially focused on genes related to apoptosis, metabolism, and oxidative stress as major mechanisms involved in the T-2 toxin-induced toxicity.

## Materials and Methods

***Animals*** Total 18 pregnant Wistar: Slc rats (Japan SLC Co., Ltd., Hamamatsu, Japan) on day 11 of gestation (GD11) were obtained from Japan SLC Co., Ltd., Hamamatsu, Japan. Animals were kept using an isolator caging system (Niki Shoji Co., Ltd., Tokyo, Japan) under controlled conditions (23 ± 2 ℃ with 55 ± 5 % humidity and a 14 hr light/10 hr dark cycle), and fed commercial pellets (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum.*

***Treatments*** Animals were used on GD13. For histopathological study, 6 animals were treated with a single oral dose of 2 mg/kg T-2 toxin (Sigma Chemical Co., St. Louis, MO, USA), and 3 animals were sacrificed by exsanguinations under ether anesthesia at 24 and 48 hours after treatment (HAT), respectively. For microarray study, 3 rats were treated with same way and sacrificed at 24 HAT. In addition, control animals were treated with the vehicle, and were sacrificed in the same way. T-2 toxin was dissolved in corn oil and a dosing volume was adjusted to 2.5 mL/kg.

***Pathological examinations*** After animals were sacrificed, macroscopic examination was performed. Dam's organs and fetuses were weighed. Dam's organs, placentae and fetuses were fixed in 10 % neutral-buffered formalin. Four-mm paraffin sections were stained with hematoxylin and eosin (HE) and subjected to microscopic examination. Cells with fragmented DNA were detected by the TdT mediated dUTP nick end labeling (TUNEL) method using an apoptosis detection kit (Apop Tag, Intergen, Purchase, NY, USA).

*RNA extraction and microarray analysis* Total RNA was extracted from frozen tissues (up to 0.5 g) from the liver, placenta and fetal liver using the RNeasy Mini Kit (QIAGEN Inc., CA, USA) or TRIzol (Invitrogen, CA, USA). Microarray analysis (total 18 arrays) was performed according to the Affymetrix protocol.
*Data analysis* The microarray data were analyzed using the Affymetrix software analysis and Spotfire Pro Version 4.2 program (Spotfire Inc., MA, USA). In brief, 4 % trimmed mean normalization was performed in each gene data[8]. Following this, data were compared between the treated group and control group by calculating the Pearson Correlation Value[9]. In the present study, we only analyzed known genes. Finally, we selected genes that gave a value of greater than +1.5 or less than -1.5. The mean value on each gene in the treated group was also statistically compared with that of control by Student's *t*-test or Welch's *t*-test after analysis of homogeneity of variance by F-test.

## Results

*Body weight, organ weight and mortality* In the dams of the T-2 toxin group, although not significant, the body weight showed a tendency to decrease. The weights of the thymus, spleen and liver significantly decreased at 24 HAT. In the fetuses of the T-2 toxin group, a small number of fetal deaths occurred and survival rate of fetuses decreased at 24 HAT, and a decrease in fetal weight was observed.
*Histopathological findings* Histopathological changes attributable to the T-2 toxin were observed in the lymphoid and hematopoietic tissues, gastrointestinal tracts and genital glands. In the liver, apoptosis and fatty change of hepatocytes and inflammatory cell infiltration were observed (Fig. 1A). In the uterus and placenta, hemorrhage and apoptosis of cytotrophoblasts were observed (Fig. 1B). At 48 HAT, similar changes were observed. In the fetuses of the T-2 toxin group, changes attributable to the treatment were observed in the nervous system, liver, gastrointestinal tracts, and cartilage primordium at 24 HAT. At 48 HAT, sinusoidal dilatation and apoptosis of hematopoietic cells and hepatocytes in the liver were observed (Fig. 1C). There were no histopathological changes in the dams and fetuses in the control group. TUNEL staining demonstrated that apoptosis was induced in the liver, placenta, and fetal liver by T-2 toxin treatment.
*Microarray analysis* In the present study, we especially focused on genes related to apoptosis, metabolism, and oxidative stress as major mechanisms involved in the T-2 toxin-induced toxicity. In the dam's liver, the expression of 235 genes was induced and the 382 genes were suppressed by T-2 toxin. The expression of cell cycle- and apoptosis-related genes (*p53, p21*, and *Bax-a* etc.) was increased. In addition, the expression of oxidative stress-related genes (*Thioredoxin reductase* etc.) was also increased. On the other hand, the expression of lipid metabolism-related genes and drug metabolizing

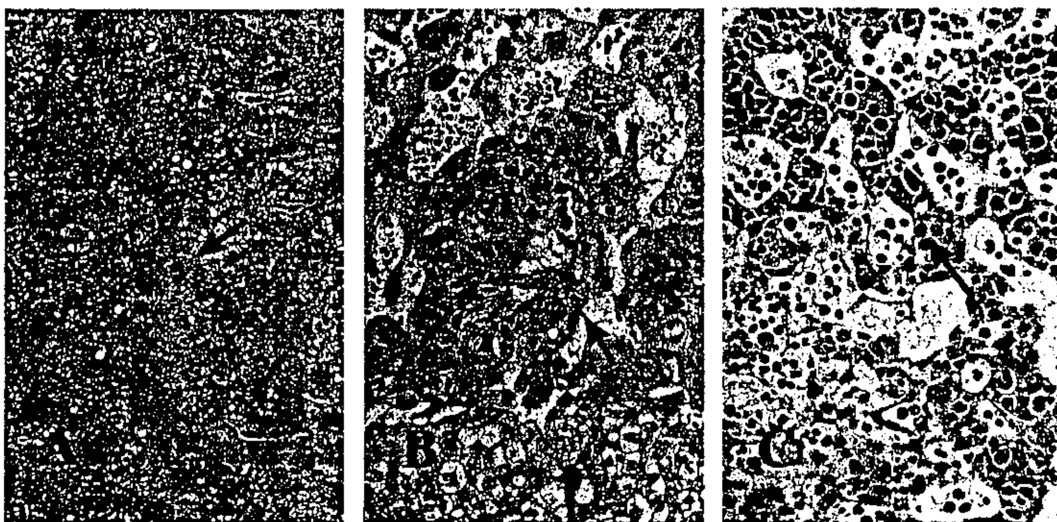


**Fig. 1. Histopathological photographs.** A) Liver from a dam treated with T-2 toxin (24 HAT). Fatty change and apoptosis of hepatocytes (arrow) are observed. B) Placenta from a dam treated with T-2 toxin (24 HAT). Apoptosis of cytotrophoblasts (arrow) is observed. C) Fetus liver treated with T-2 toxin (48 HAT). Apoptosis of hematopoietic cells and hepatocytes (arrow) is observed. HE, × 165. (modified from J. Toxicol. Pathology, **16**, 59-65, 2003)

enzymes-related genes (P450s and GSTs) was suppressed. In the placenta, the expression of 187 genes was increased and the expression of 181 genes was suppressed by T-2 toxin. Gene expression of cell cycle- and apoptosis-related genes (*c-fos, c-jun,* and *GADD45* etc.) was induced. Gene expression of *Heat shock protein 70* was strongly increased by the treatment. On the other hand, the expression of lipid metabolism-related genes and glutathione S-transferase-related genes was suppressed by T-2 toxin. In the fetal liver, the expression of 15 genes was increased and the expression of 103 genes was suppressed by T-2 toxin. The expression of *heat shock protein 70* (oxidative stress-related gene) was strongly increased. The expression of *Bax-a* was also increased. The expression of metabolism-related genes was generally not induced by T-2 toxin. On the other hand, the expression of lipid metabolism-related genes and GSTs was suppressed. Gene expression of cell cycle- and apoptosis-related genes (*cyclin D, c-myc, and 14-3-3 protein*) was suppressed.

In comparison with the results of 3 tissues (liver, placenta, and fetal liver), changes in lipid metabolism-, apoptosis- and cell cycle-, and oxidative stress-related genes were detected in all tissues. Namely, decreased expression of lipid metabolism-related genes, increased expression of apoptosis- and cell cycle-related genes, and increased expression of oxidative stress-related genes were detected (Fig. 2).

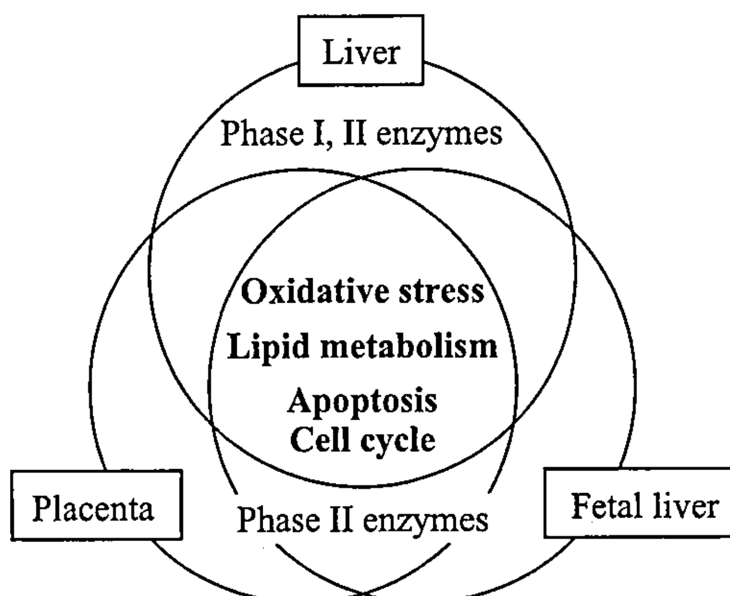



**Fig. 2. Summary of microarray analysis in the dam liver, placenta and fetal liver.**

## Discussion

Pregnant Wistar rats on GD13 were treated with a single oral dose of 2 mg/kg T-2 toxin, and were sacrificed at 24 and 48 HAT, and subjected to histopathological examination. It is reported that T-2 toxin induces apoptosis in the lymphoid tissues and intestine[2-4]. In our study, apoptotic changes were observed in the lymphoid tissues including the thymus and spleen, intestine, and fetal brain[6]. It has been considered that fetotoxicity of T-2 toxin is secondary to maternal toxicity. T-2 toxin easily passes the placenta and distributes to fetal tissues[10], and the changes observed in fetuses were essentially the same as those observed in dams in the present study. Judging from those findings, fetotoxicity is not secondary to maternal toxicity but a direct toxicity to fetuses. Therefore, T-2 toxin-induced changes may be caused by the same mechanisms in these tissues.

From the results of microarray analysis, the expression of apoptosis/cell cycle-related genes, lipid metabolism-related genes, drug metabolizing enzyme genes and oxidative stress-related genes was detected in the liver, placenta and fetal liver. T-2 toxin enhances lipid peroxidation in the liver[11]. Oxidative stress causes lipid peroxidation and induces mitochondrial dysfunction. This mitochondrial dysfunction causes fatty acid b-oxidation and induces fatty liver[12]. In our study, fatty liver was observed in the liver, suggesting that the fatty change in the liver may be related with oxidative stress caused by T-2 toxin.

Increased apoptosis in the liver, placenta and fetal liver was observed histopathologically in the present study, and microarray analysis also showed the

increased expression of apoptosis-related genes. At the same time, the expression of cell cycle accelerating genes was also induced by T-2 toxin. Furthermore, the expression of oxidative stress-related genes and repressor of apoptosis was induced in the placenta and fetal liver. This suggests that T-2 toxin induced not only apoptosis but also cell repair and proliferation at 24 HAT.

In our study, the expression of drug-metabolizing enzyme genes was suppressed or showed the tendency of suppression by T-2 toxin treatment in the liver, placenta and fetal liver. This result was consistent with those of reported study[13]. It is reported that lipid peroxidation caused by oxidative stress might affect the P450 content in the liver[14]. Therefore, oxidative stress might be an effective factor of P450 suppression.

In conclusion, histopathological study showed that T-2 toxin may induce the apoptotic changes of the same nature in the liver, placenta, and fetal tissues. Microarray analysis in the liver, placenta and fetal liver showed the same changes in lipid metabolism-related genes, drug metabolizing enzyme genes, apoptosis-related genes and oxidative stress-related genes. From these results, the mechanism of T-2 toxin-induced toxicity is speculated that T-2 toxin causes oxidative stress, following the impairment of lipid peroxidation and metabolism-related enzymes. These changes may cause the changes in the intracellular environments, finally resulting in the induction of apoptosis. Further study on the gene expression profiles at the earlier time point should be done to clarify the whole mechanisms of T-2 toxin-induced toxicity.

## References

1 ) WHO (World Health Organization): Selected mycotoxins; ochratoxins, trichothecenes, ergot, Environmental Health Criteria, **105** (1990), WHO, Geneva

2 ) Pang, V.F., Lorenzana, R.M., Beasley, V.R., Buck, W.B., Haschek, W.M.: Fundam. Appl. Toxicol., **8**, 298-309 (1987)

3 ) Bratich, P.M., Buck, W.B., Haschek, W.M.: Arch. Toxicol., **64**, 251-253 (1990)

4 ) Sugamata, M., Hattori, T., Ihara, T., Okumura, H., Yoshino, N., Ueno, Y.: J. Toxicol. Sci., **23** (Suppl. II), 148-154 (1998)

5 ) Ishigami, N., Shinozuka, J., Katayama, K., Nakayama, H., Doi, K.: Exp. Toxic. Pathol., **52**, 493-501 (2001)

6 ) Sehata, S., Teranishi, M., Atsumi, F., Uetsuka, K., Nakayama, H., Doi, K.: J. Toxicol. Pathol., **16**, 59-65 (2003)

7 ) Sehata, S., Kiyosawa, N., Sakuma, K., Ito, K., Yamoto, T., Teranishi, M., Uetsuka, K., Nakayama, H., Doi, K.: Exp. Toxic. Pathol. (in press)

8 ) Yang, M.C.K., Ruan, Q.G., Yang, J.J., Eckenrode, S., Wu, S., McIndoe, R.A., She, J.X.: Physiol. Genomics, **7**, 45-53 (2001)

9 ) Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M.,. Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S.: Science, **286**, 531-537 (1999)

10) Lafarge-Frayssinet, C., Chakor, K., Lafont, P., Frayssinet, C.: J. Environ. Pathol. Toxicol. Oncol., **10**, 64-68 (1990)
11) Chang, I.-M., Mar, W.-C.: Toxicol. Lett., **40**, 275-280 (1988)
12) Jaeschke, H., Gores, G.J., Cederbaum, A., Hinson, J.A., Pessayre, D., Lemasters, J.J.: Toxicol. Sci., **65**, 166-176 (2002)
13) Guerre, P., Eeckhoutte, C., Burgat, V., Galtier, P.: Food. Addit. Contam., **17**, 1019-1026 (2000)
14) Serbinova, E.A., Kadiiska, M.B., Bakalova, R.A., Koynova, G.M., Stoyanovsky, D.A., Karakashev, P.C., Stoytchev, Ts.S., Wolinsky, I., Kagan, V.E.: Toxicol. Lett., **47**, 119-123 (1989)

Exp Toxic Pathol 2004; 55: 467–480
http://www.elsevier.de/etp

[1] Exploratory Toxicology and DMPK Research Laboratory, Tanabe Seiyaku Co. Ltd., Saitama, Japan
[2] Department of Veterinary Medical Sciences, Clinical Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[3] Safety Research Laboratory, Tanabe Seiyaku Co. Ltd., Osaka, Japan
[4] Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

# Hepatic changes in the acute phase of streptozotocin (SZ)-induced diabetes in mice

EISUKE KUME[1], HISAKO FUJIMURA[1], NAOAKI MATSUKI[2], MASAHITO ITO[1], CHINAMI ARUGA[1], WATARU TORIUMI[1], KAZUYUKI KITAMURA[3], and KUNIO DOI[4]

With 10 figures and 5 tables

Received: August 19, 2003; Revised: March 3, 2004; Accepted: April 15, 2004

**Address for correspondence:** EISUKE KUME, Exploratory Toxicology and DMPK Research Laboratory, Tanabe Seiyaku Co. Ltd., 2-2-50, Kawagishi, Toda, Saitama 335, Japan;
Phone: +81-48-433-8122, Fax: +81-48-433-8171, E-mail: e-kume@tanabe.co.jp

**Key words:** Streptozotocin; diabetes, acute phase; hepatic change; mouse.

## Summary

We have reported the streptozotocin (SZ)-induced hepatic lesions in the subacute phase (4 to 12 weeks after the treatment), which are characterized by appearance of oncocytic hepatocytes, cytomegalic hepatocytes and bile duct hyperplasia. In this study, we focused on the acute phase (6 to 48 hours after the treatment) of the SZ-induced hepatic lesions of mice to clarify the onset of the hepatic alterations, especially before the induction of hyperglycemia. Livers were taken from 8-week-old Crj:CD-1 (ICR) male mice at 6, 12, 24, 36 and 48 hours after the 200 mg/kg b.w. of SZ-injection. SZ-induced hyperglycemia was noted at 36 and 48 hours after the treatment, but the hepatic changes including lipid peroxidation, mitochondrial swelling, peroxisome proliferation and inhibition of hepatocyte proliferation occurred before the elevation of the serum glucose levels. The present findings indicate the direct effects of SZ on hepatocytes rather than the secondary effects of diabetes, and certain correlations between the hepatocytic changes in the acute phase and those in the subacute one. In addition, ulcer and submucosal edema of the gallbladder were observed at 36 or 48 hours after the SZ-treatment, which can be a novel finding in SZ-treated animal.

## Introduction

Although streptozotocin (SZ), an extract from *Streptomyces achromogenes*, was originally developed as an antibiotic and/or antitumor agent (VARVA et al. 1959–60; WHITE 1963), it has been attracting a great attention as a useful tool for the induction of diabetes mellitus and its complications especially in laboratory rodents (SIBAY et al. 1971; STEFFES and MAUER 1984; KUME et al. 1992) because of its toxic action on islet β cells. It is also known that SZ has acute toxic effects on many organs (SCHEIN et al. 1974; WEISS 1982) as well as carcinogenic ones in pancreas, liver and kidney (RAKIETEN et al. 1968; BERMAN et al. 1973; LEVINE et al. 1980; OKAWA and DOI 1983). We have reported the details of SZ-induced hepatic lesions in the subacute phase (4 to 12 weeks after the treatment), which are characterized by appearance of oncocytic hepatocytes, cytomegalic hepatocytes and bile duct hyperplasia (KUME et al. 1994a; KUME et al. 1994b; DOI et al. 1997). The purpose of this study is to clarify the biochemical and pathological characteristics of SZ-induced hepatic alterations in mice in the acute phase (6

**Table 1.** Organ weights in male mice treated intrapenitoneally with streptozotocin.

| | Untreated | | Vehicle | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| Absolute weight | | | | | | | | | | | | |
| Body Weight (g) | 6 | 36.5 ± 0.7 | 6 | 37.4 ± 2.9 | 6 | 36.7 ± 0.6 | 6 | 33.8 ± 1.2 | 6 | 36.2 ± 2.2 | 6 | 38.2 ± 1.1 |
| Liver (g) | 6 | 2.04 ± 0.10 | 6 | 1.95 ± 0.16 | 6 | 1.68 ± 0.26 | 6 | 1.94 ± 0.15 | 6 | 1.84 ± 0.10 | 6 | 2.24 ± 0.20 |
| Kidneys (mg) | 6 | 543 ± 35 | 6 | 580 ± 64 | 6 | 585 ± 36 | 6 | 501 ± 72 | 6 | 529 ± 65 | 6 | 598 ± 50 |
| Heart (mg) | 6 | 190 ± 18 | 6 | 187 ± 27 | 6 | 195 ± 14 | 6 | 162 ± 9 | 6 | 184 ± 13 | 6 | 199 ± 18 |
| Lungs (mg) | 6 | 188 ± 7 | 6 | 193 ± 11 | 6 | 195 ± 10 | 6 | 181 ± 10 | 6 | 185 ± 14 | 6 | 195 ± 14 |
| Spleen (mg) | 6 | 123 ± 12 | 6 | 126 ± 22 | 6 | 116 ± 24 | 6 | 99 ± 22 | 6 | 113 ± 12 | 6 | 115 ± 29 |
| Relative weight (ratio to body weight) | | | | | | | | | | | | |
| Liver (g%) | 6 | 5.58 ± 0.22 | 6 | 5.21 ± 0.44 | 6 | 4.58 ± 0.69 | 6 | 5.75 ± 0.39 | 6 | 5.08 ± 0.24 | 6 | 5.86 ± 0.48 |
| Kidneys (mg%) | 6 | 1491 ± 116 | 6 | 1548 ± 77 | 6 | 1597 ± 125 | 6 | 1488 ± 252 | 6 | 1461 ± 159 | 6 | 1567 ± 150 |
| Heart (mg%) | 6 | 521 ± 50 | 6 | 502 ± 80 | 6 | 531 ± 33 | 6 | 479 ± 32 | 6 | 509 ± 44 | 6 | 523 ± 50 |
| Lungs (mg%) | 6 | 517 ± 30 | 6 | 517 ± 30 | 6 | 533 ± 29 | 6 | 537 ± 42 | 6 | 511 ± 29 | 6 | 512 ± 44 |
| Spleen (mg%) | 6 | 338 ± 37 | 6 | 337 ± 58 | 6 | 317 ± 61 | 6 | 292 ± 63 | 6 | 311 ± 27 | 6 | 299 ± 68 |

| | SZ 200 mg/kg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| Absolute weight | | | | | | | | | | |
| Body Weight (g) | 6 | 36.4 ± 1.4 | 6 | 38.0 ± 1.4 | 6 | 33.1 ± 0.9 | 6 | 36.1 ± 1.8 | 6 | 35.6 ± 3.6 |
| Liver (g) | 6 | 2.25 ± 0.24 *a | 6 | 2.22 ± 0.10 **a | 6 | 1.94 ± 0.11 | 6 | 1.88 ± 0.15 | 6 | 1.89 ± 0.33 *a |
| Kidneys (mg) | 6 | 564 ± 70 | 6 | 496 ± 45 **a | 6 | 456 ± 43 | 6 | 510 ± 38 | 6 | 548 ± 38 |
| Heart (mg) | 6 | 186 ± 16 | 6 | 177 ± 17 | 6 | 149 ± 14 | 6 | 167 ± 12 *a | 6 | 179 ± 16 |
| Lungs (mg) | 6 | 193 ± 14 | 6 | 178 ± 10 *a | 6 | 174 ± 14 | 6 | 179 ± 20 | 6 | 179 ± 24 |
| Spleen (mg) | 6 | 114 ± 37 | 6 | 93 ± 13 | 6 | 86 ± 7 | 6 | 93 ± 15 *a | 6 | 97 ± 26 |
| Relative weight (ratio to body weight) | | | | | | | | | | |
| Liver (g%) | 6 | 6.19 ± 0.65 | 6 | 5.84 ± 0.38 **a | 6 | 5.86 ± 0.28 | 6 | 5.21 ± 0.30 | 6 | 5.28 ± 0.56 |
| Kidneys (mg%) | 6 | 1555 ± 237 *a | 6 | 1312 ± 158 **a | 6 | 1380 ± 138 | 6 | 1414 ± 117 | 6 | 1548 ± 130 |
| Heart (mg%) | 6 | 511 ± 52 | 6 | 466 ± 45 *a | 6 | 451 ± 36 | 6 | 463 ± 31 | 6 | 506 ± 45 |
| Lungs (mg%) | 6 | 532 ± 46 | 6 | 469 ± 26 **a | 6 | 528 ± 49 | 6 | 497 ± 44 | 6 | 506 ± 55 |
| Spleen (mg%) | 6 | 311 ± 91 | 6 | 244 ± 33 *a | 6 | 262 ± 26 | 6 | 258 ± 36 *a | 6 | 271 ± 58 |

*a: Significantly different from the vehicle group ($P < 0.05$) by Student's t-test.
**a: Significantly different from the vehicle group ($P < 0.01$) by Student's t-test.

**Table 2.** Hematology in male mice treated intrapenitoneally with streptozotocin.

| | Untreated | | Vehicle | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| RBC ($10^4/\mu l$) | 6 | 852 ± 35 | 6 | 835 ± 40 | 6 | 837 ± 20 | 6 | 820 ± 64 | 6 | 848 ± 44 | 6 | 801 ± 65 |
| Hb (g/dL) | 6 | 13.9 ± 0.6 | 6 | 13.3 ± 0.5 | 6 | 13.8 ± 0.7 | 6 | 13.2 ± 0.8 | 6 | 13.4 ± 0.6 | 6 | 12.8 ± 1.0 |
| Ht (%) | 6 | 43.2 ± 2.1 | 6 | 42.2 ± 1.8 | 6 | 42.1 ± 2.1 | 6 | 41.3 ± 2.8 | 6 | 41.8 ± 1.8 | 6 | 39.7 ± 2.9 |
| Ret (%) | 6 | 2.3 ± 0.3 | 6 | 2.8 ± 0.4 | 6 | 2.3 ± 0.6 | 6 | 3.1 ± 0.3 | 6 | 2.8 ± 0.3 | 6 | 3.7 ± 1.1 |
| WBC ($10^2/\mu l$) | 6 | 41 ± 25 | 6 | 34 ± 17 | 6 | 29 ± 16 | 6 | 42 ± 8 | 6 | 16 ± 7 | 6 | 31 ± 14 |
| Bas (%) | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 |
| Eos (%) | 6 | 1.0 ± 0.9 | 6 | 0.5 ± 1.2 | 6 | 0.7 ± 0.8 | 6 | 0.5 ± 0.8 | 6 | 0.3 ± 0.8 | 6 | 0.0 ± 0.0 |
| Neu (%) | 6 | 7.8 ± 7.2 | 6 | 20.2 ± 5.3 | 6 | 17.5 ± 6.9 | 6 | 10.7 ± 8.8 | 6 | 16.0 ± 9.4 | 6 | 21.7 ± 24.9 |
| Lym (%) | 6 | 91.2 ± 7.1 | 6 | 78.5 ± 5.0 | 6 | 81.5 ± 7.1 | 6 | 88.3 ± 8.5 | 6 | 82.8 ± 9.6 | 6 | 78.2 ± 25.3 |
| Mon (%) | 6 | 0.0 ± 0.0 | 6 | 0.8 ± 1.0 | 6 | 0.3 ± 0.5 | 6 | 0.5 ± 0.8 | 6 | 0.8 ± 1.0 | 6 | 0.2 ± 0.4 |
| | | | SZ 200 mg/kg | | | | | | | | | |
| | | | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | | | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| RBC ($10^4/\mu l$) | | | 6 | 890 ± 42 *a | 6 | 893 ± 16 **a | 6 | 828 ± 41 | 6 | 864 ± 22 | 6 | 809 ± 46 |
| Hb (g/dL) | | | 6 | 14.5 ± 0.6 **a | 6 | 14.6 ± 0.4 *a | 6 | 13.5 ± 0.5 | 6 | 13.9 ± 0.5 | 6 | 13.4 ± 0.7 |
| Ht (%) | | | 6 | 44.6 ± 1.8 *a | 6 | 44.7 ± 0.8 *b | 6 | 41.7 ± 1.3 | 6 | 43.9 ± 1.3 *a | 6 | 41.4 ± 2.1 |
| Ret (%) | | | 6 | 3.0 ± 0.3 | 6 | 2.4 ± 0.3 | 6 | 2.5 ± 0.5 | 6 | 1.8 ± 0.7 **a | 6 | 1.4 ± 0.2 **b |
| WBC ($10^2/\mu l$) | | | 6 | 24 ± 7 | 6 | 21 ± 10 | 6 | 27 ± 6 **a | 6 | 15 ± 7 | 6 | 14 ± 4 *b |
| Bas (%) | | | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 |
| Eos (%) | | | 6 | 0.2 ± 0.4 | 6 | 0.3 ± 0.5 | 6 | 0.2 ± 0.4 | 6 | 0.3 ± 0.5 | 6 | 0.7 ± 1.2 |
| Neu (%) | | | 6 | 42.5 ± 20.7 *b | 6 | 53.5 ± 21.4 **b | 6 | 21.0 ± 10.5 | 6 | 34.0 ± 20.1 | 6 | 33.2 ± 20.4 |
| Lym (%) | | | 6 | 57.3 ± 21.0 | 6 | 46.2 ± 21.5 **b | 6 | 78.5 ± 10.3 | 6 | 65.2 ± 20.9 | 6 | 65.7 ± 20.3 |
| Mon (%) | | | 6 | 0.0 ± 0.0 | 6 | 0.0 ± 0.0 | 6 | 0.3 ± 0.5 | 6 | 0.5 ± 0.8 | 6 | 0.5 ± 0.8 |

RBC: erythrocyte count, Hb: hemoglobin, Ht: hematocrit, Ret: reticulocyte ratio, WBC: leukocyte count, Bas: basophil, Eos: eosinophil, Neu: neutrophil, Lym: lymphocyte
*a: Significantly different from the vehicle group ($P < 0.05$) by Student's t-test.
**a: Significantly different from the vehicle group ($P < 0.01$) by Student's t-test.
*b: Significantly different from the vehicle group ($P < 0.05$) by Aspin-Welch's t-test.
**b :Significantly different from the vehicle group ($P < 0.01$) by Aspin-Welch's t-test.

**Table 3.** Blood chemistry in male mice treated intrapenitoneally with streptozotocin.

| | Untreated | | Vehicle | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| AST (IU/l) | 6 | 34 ± 4 | 6 | 51 ± 13 | 6 | 39 ± 7 | 6 | 40 ± 15 | 6 | 30 ± 4 | 6 | 46 ± 12 |
| ALT (IU/l) | 6 | 20 ± 4 | 6 | 25 ± 5 | 6 | 18 ± 4 | 6 | 27 ± 5 | 6 | 14 ± 2 | 6 | 26 ± 9 |
| GLU (mg/dl) | 6 | 237 ± 13 | 6 | 271 ± 14 | 6 | 250 ± 16 | 6 | 269 ± 45 | 6 | 266 ± 10 | 6 | 229 ± 22 |
| TCHO (mg/dl) | 6 | 146 ± 19 | 6 | 135 ± 17 | 6 | 134 ± 19 | 6 | 141 ± 11 | 6 | 140 ± 25 | 6 | 139 ± 34 |
| TG (mg/dl) | 6 | 104 ± 31 | 6 | 111 ± 36 | 6 | 62 ± 19 | 6 | 110 ± 35 | 6 | 73 ± 44 | 6 | 95 ± 40 |
| PL (mg/dl) | 6 | 291 ± 26 | 6 | 267 ± 36 | 6 | 245 ± 28 | 6 | 276 ± 14 | 6 | 259 ± 31 | 6 | 285 ± 57 |
| Insulin (pg/mL) | 6 | 3287 ± 1863 | 6 | 3227 ± 3321 | 6 | 1677 ± 1085 | 6 | 4558 ± 3647 | 6 | 3573 ± 1789 | 6 | 3734 ± 3997 |

| | SZ 200 mg/kg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6 h | | 12 h | | 24 h | | 36 h | | 48 h | |
| Item (Unit) | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. | N | Mean ± S.D. |
| AST (IU/l) | 6 | 92 ± 49 | 6 | 58 ± 6 **a | 6 | 54 ± 12 | 6 | 36 ± 8 | 6 | 40 ± 4 |
| ALT (IU/l) | 6 | 49 ± 14 **b | 6 | 46 ± 10 **b | 6 | 50 ± 16 *b | 6 | 33 ± 15 *b | 6 | 33 ± 8 |
| GLU (mg/dl) | 6 | 224 ± 56 | 6 | 153 ± 34 **a | 6 | 333 ± 93 | 6 | 539 ± 164 **b | 6 | 594 ± 137 **b |
| TCHO (mg/dl) | 6 | 110 ± 21 *a | 6 | 84 ± 15 **a | 6 | 97 ± 26 **a | 6 | 118 ± 23 | 6 | 129 ± 9 |
| TG (mg/dl) | 6 | 153 ± 83 | 6 | 163 ± 42 **a | 6 | 80 ± 28 | 6 | 65 ± 51 | 6 | 103 ± 58 |
| PL (mg/dl) | 6 | 146 ± 16 **a | 6 | 139 ± 24 **a | 6 | 193 ± 41 **b | 6 | 233 ± 38 | 6 | 270 ± 25 |
| Insulin (pg/ml) | 6 | 9573 ± 2883 **a | 6 | 9145 ± 1969 **a | 6 | 4154 ± 1214 | 6 | 2454 ± 2544 | 6 | 2707 ± 4920 |

AST: aspartate aminotransferase, ALT: alanin aminotransferase, GLU: glucose, TCHO: total choresterol, TG: triglyceride, PL: phospholipid
*a: Significantly different from the vehicle group ($P < 0.05$) by Student's t-test.
**a: Significantly different from the vehicle group ($P < 0.01$) by Student's t-test.
*b: Significantly different from the vehicle group ($P < 0.05$) by Aspin-Welch's t-test.
**b: Significantly different from the vehicle group ($P < 0.01$) by Aspin-Welch's t-test.

**Table 4.** Liver biochemistry in male mice treated intraperitoneally with streptozotocin.

| Item (Unit) | Vehicle 6 h N | Vehicle 6 h Mean ± S.D. | SZ 200 mg/kg 6 h N | 6 h Mean ± S.D. | 12 h N | 12 h Mean ± S.D. | 24 h N | 24 h Mean ± S.D. | 36 h N | 36 h Mean ± S.D. | 48 h N | 48 h Mean ± S.D. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TBARS (nmol/g liver) | 3 | 17.92 ± 1.10 | 3 | 41.89 ± 1.83 **a | 3 | 24.69 ± 1.92 **a | 3 | 26.63 ± 4.95 *a | 3 | 18.16 ± 2.62 | 3 | 15.98 ± 2.62 |
| PE-OOH (nmol/g liver) | 3 | 349.2 ± 20.9 | 3 | 474.3 ± 19.2 **a | 3 | 438.4 ± 10.0 **a | 3 | 508.7 ± 35.0 **a | 3 | 499.0 ± 46.5 **a | 3 | 476.1 ± 29.9 **a |
| PC-OOH (nmol/g liver) | 3 | 248.0 ± 14.2 | 3 | 321.5 ± 25.4 *a | 3 | 280.1 ± 17.4 | 3 | 286.9 ± 32.1 | 3 | 274.6 ± 15.7 | 3 | 298.8 ± 22.9 *a |

*a: Significantly different from the vehicle group ($P < 0.05$) by Student's t-test.
**a: Significantly different from the vehicle group ($P < 0.01$) by Student's t-test.
TBARS: thiobarbituric acid-reactive substances, PE-OOH: phosphatidilethanolamine hydroperoxide, PC-OOH: phosphatidilcholine hydroperoxide.

to 48 hours after the treatment) prior to the development of SZ-induced hyperglycemia. We should take such background information into consideration when we use SZ-induced diabetic models.

## Materials and methods

The study was approved by the Ethical Committee at Tanabe Seiyaku Co., Ltd. and all efforts were made to minimize animal suffering.

**Animals and treatments:** Sixty-six 8-week-old Crj:CD-1(ICR) male mice (Charles River Japan Inc., Kanagawa, Japan) were used. The animals were housed in polycarbonate cages (6 animals/cage) in a barrier system animal room under controlled conditions (temperature: 23 ± 2 °C, humidity: 55 ± 5%, 12 hour light/dark cycle) and fed CRF-1 pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum* throughout the experimental period. Thirty of them were injected intraperitoneally with 200 mg/kg b.w. of SZ (Sigma, St. Louis, MO, U.S.A.) at around 9:00 a.m. SZ was dissolved in 0.05 M citrate buffer solution (pH 4.5) just before used. Six mice each were killed by exsanguination from *aorta abdominalis* under ether anesthesia at 6, 12, 24, 36 and 48 hours after SZ-injections. Other thirty mice which were given vehicle alone and killed in the same way served as vehicle controls. In addition, the remaining six mice were used as untreated controls and killed just before dosing. All animals were observed for clinical signs during the study and their body weights were measured at each sacrifice. They were subjected to hematology, blood chemistry, necropsy including organ weight measurement, histopathology and electron microscopy. Items for the organ weight mesurement were liver, kidney, heart, lung and spleen, and their weights relative to the body weight were calculated.

To estimate the lipid peroxidation, an additional experiment was conducted. Fifteen 8-week-old Crj:CD-1 (ICR) male mice were treated with 200 mg/kg b.w. of SZ, and three mice each were killed at 6, 12, 24, 36 and 48 hours after the treatment. Three mice, which were given vehicle alone and killed at 6 hours after the treatment, served as vehicle controls. These animals were subjected to liver biochemistry.

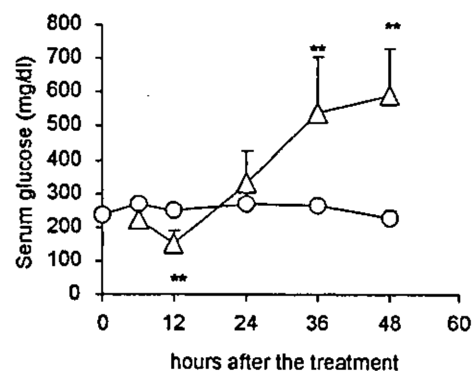



**Fig. 1.** Changes in serum glucose level. O: vehicle control group, Δ: SZ-treated group. Mean ± S.D. **: $p < 0.01$, significantly different from the vehicle control group.

**Hematology:** The following parameters were measured on blood samples obtained from all animals at each sacrifice using automatic blood cell counters M-2000 and R-3000 (Sysmex, Hyogo, Japan); erythrocyte count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean

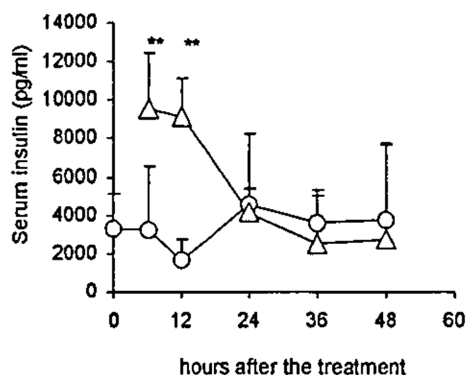



**Fig. 2.** Changes in serum insulin level. O: vehicle control group, Δ: SZ-treated group. Mean ± S.D. **: $p < 0.01$, significantly different from the vehicle control group.

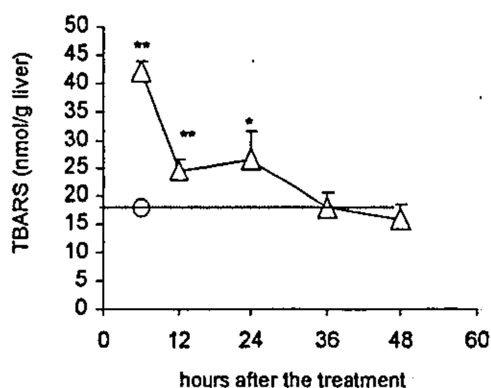


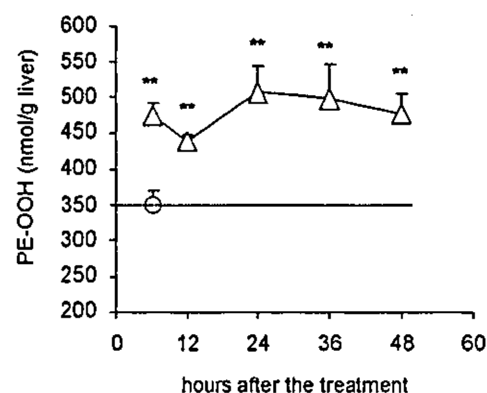

**Fig. 3.** Changes in TBA reactive substance (TBARS) in the liver of SZ-treated mice. O: vehicle control group, Δ: SZ-treated group. Mean ± S.D. *: $p < 0.05$, **: $p < 0.01$, Significantly different from the vehicle control group.

corpuscular hemoglobin concentration (MCHC), reticulocyte ratio (Ret), platelet count (PLT) and leukocyte count (WBC). Differential leukocyte count was determined on blood smears under a light microscope.

Blood chemistry: The following parameters were measured on serum samples obtained from all animals at each sacrifice using an automatic analyzer 7150 (Hitachi, Tokyo, Japan); aspartate aminotransferase (AST), alanin aminotransferase (ALT), lactose dehydrogenase (LDH), alkaline phosphatase (ALP), creatine kinase (CK), glucose (GLU), total cholesterol (TCHO), triglyceride (TG), phospholipid (PL), total protein (TP), albumin (ALB), urea nitrogen (UN), creatinine (CRE), Ca, Pi, Na, K and Cl. Serum insulin was measured by an immunoassay kit (Morinaga institute of Biological Science, Yokohama, Japan).

**Liver biochemistry:** Small pieces of the liver obtained from all animals at each necropsy in the additional experiment were quickly frozen in liquid nitrogen and used for measurement of peroxidized phospholipids (phosphatidylethanolamine hydroperoxide (PE-OOH) and phosphatidylcholine hydroperoxide (PC-OOH)) and thiobarbituric acid-reactive substances (TBARS). High performance liquid chromatography analysis was carried out for determination of peroxidized phospholipids in accordance with the method of MATSUKI et al. (1991). Free TBARS was determined by thiobarbituric acid method of SCHMEDES and HØLMER (1989)

**Light microscopy:** Liver and pancreas from all animals at each necropsy were fixed in 10% neutral buffered formalin. Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE). The liver sections from all animals at each necropsy were also subjected to immunohistochemical staining for proliferationg cell nuclear antigen (PCNA) using monoclonal mouse antigen Clone PC10 obtained from DAKO (Carpinteria, CA, U.S.A.). All of strongly positive nuclei of hepatocytes for PCNA (S-phase) in the sections were counted. Aside from this, all nuclei of hepatocytes were counted in a certain area (0.55 mm$^2$), and the whole area of each liver section was measured by using morphometry software WinROOF (Mitani corp., Tokyo, Japan), and all nuclei number was estimated. PCNA index (positive nuclei number/all nuclei number) was calculated and expressed as percent.



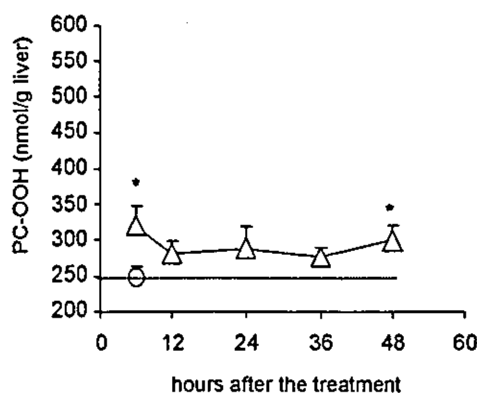




**Fig. 4.** Changes in phospholipid hydroperoxide levels in the liver of SZ-treated mice. PE-OOH: phosphatidylethanolamine hydroperoxide, PC-OOH: phosphatidylcholine hydroperoxide. O: vehicle control group, Δ: SZ-treated group. Mean ± S.D. *: $p < 0.05$, **: $p < 0.01$, Significantly different from the vehicle control group.

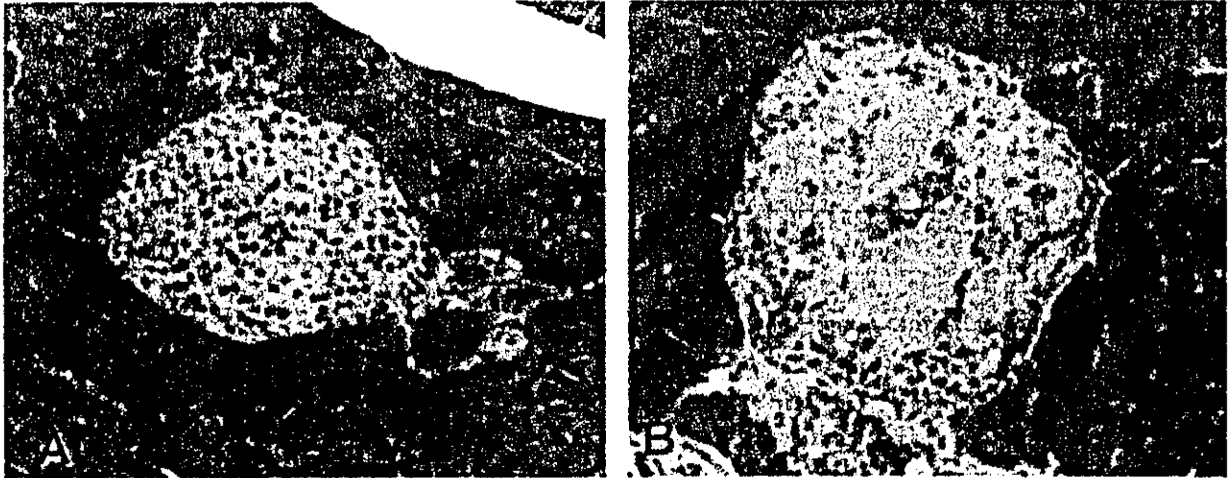



Fig. 5. Microphotographs of pancreatic islets in mice treated with vehicle (A) or streptozotocin (B) at 24 hours after the treatment. Severe islet cell necrosis is observed from 6 to 24 hours after the SZ-treatment (B). HE, ×200.

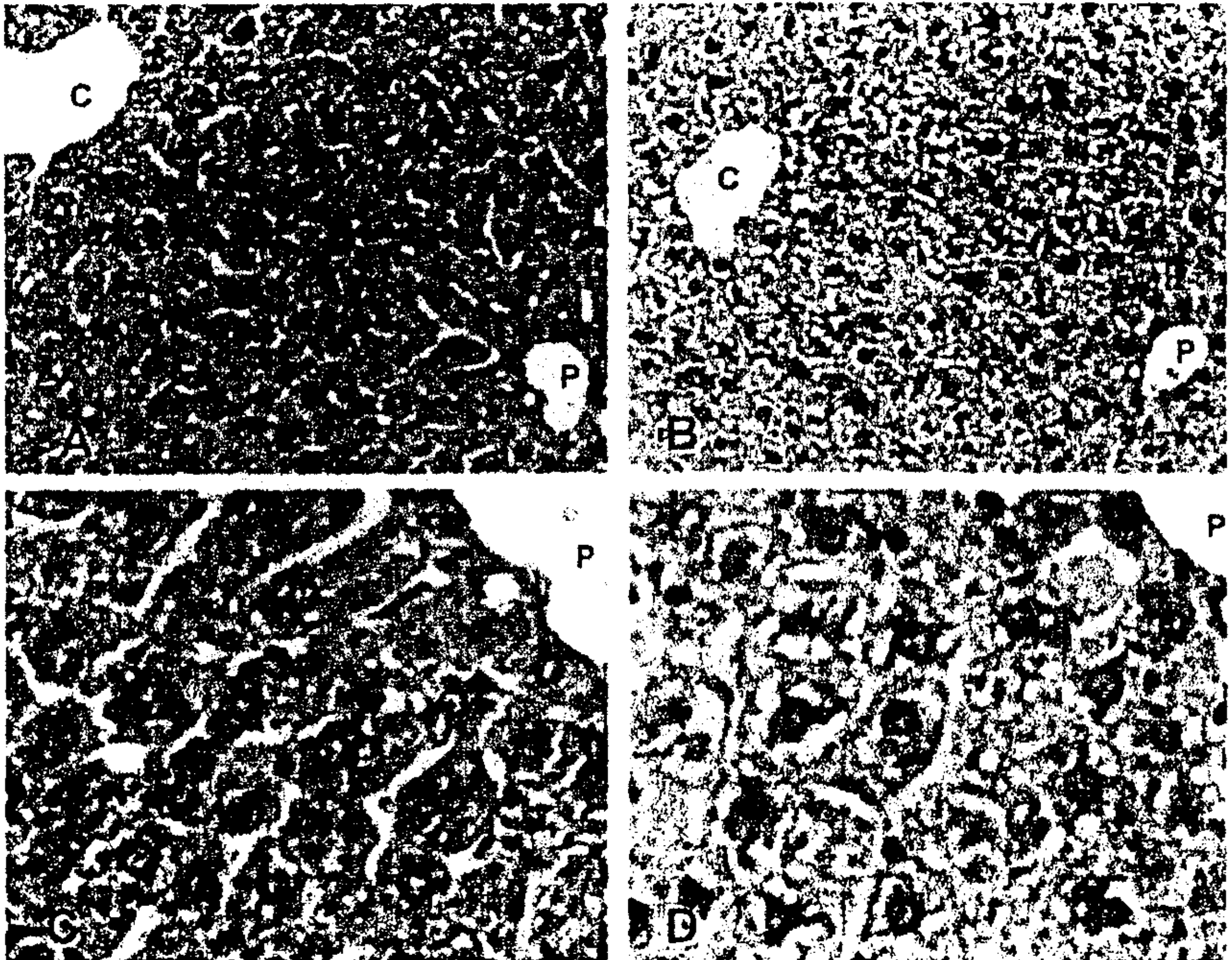



Fig. 6. Microphotographs of livers in mice treated with vehicle (A, C) or streptozotocin (B, D) at 6 hours after the treatment. Acidophilic granules are obscure in the hepatocytes of streptozotocin-treated mouse and the cytoplasm looks somewhat pale and homogeneous (B, D). C: central vein. P: portal vein. HE, ×200 (A, B) or ×600 (C, D).

Table 5. Histopathology in male mice treated intrapenitoneally with streptozotocin.

| | Untreated | | | | Vehicle | | | | SZ 200 mg/kg | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | | | 6 h | | | | 6 h | | | |
| | – | + | ++ | +++ | – | + | ++ | +++ | – | + | ++ | +++ |
| Liver (hepatocytes) | | | | | | | | | | | | |
| Decrease, glycogen area | 6 | 0 | 0 | 0 | 5 | 1 | 0 | 0 | 6 | 0 | 0 | 0 |
| Increase, glycogen area | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 2 | 4 | 0 | 0 |
| Decrease, cytoplasmic acidophilic granule | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 6 | 0 | 0 |
| Degeneration, fatty | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 |
| Gallbladder | | | | | | | | | | | | |
| (Not Examined) | (0) | | | | (0) | | | | (3) | | | |
| Edema | 6 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 3 | 0 | 0 | 0 |
| Ulcer | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |
| Pancreas | | | | | | | | | | | | |
| Necrosis, islet cell | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| Atrophy, islet | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 |

– : Normal + : Slight ++ : Moderate +++ : Severe
No noteworthy changes were observed in the other organs.

**Electron microscopy:** Small pieces of the liver from all animals at each necropsy were fixed with 2.5% glutaraldehyde and 2.0% formaldehyde, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections from two mice per a group at each necropsy were doubly stained with uranyl acetate and lead citrate and observed under a JEOL-1210 electron microscope (JEOL Co. LTD., Tokyo, Japan).

**Statistical analysis:** The data were analyzed for homogeneity of variance by F test. Statistical significance of the data was determined by Student's t-test for homogeneous data or by Aspin-Welch's t-test for heterogeneous data.

## Results

Clinical signs and body weight: No apparent changes were observed in clinical signs and body weight.

**Organ weight (table 1):** A significant increase in the liver weight and decreases in the kidney and lung weights were observed at 6 and 12 hours after the treatment in the SZ-treated group. The kidney, heart, lung and spleen weights relative to the body weights in the SZ-treated group were significantly lower than those in the vehicle control group.