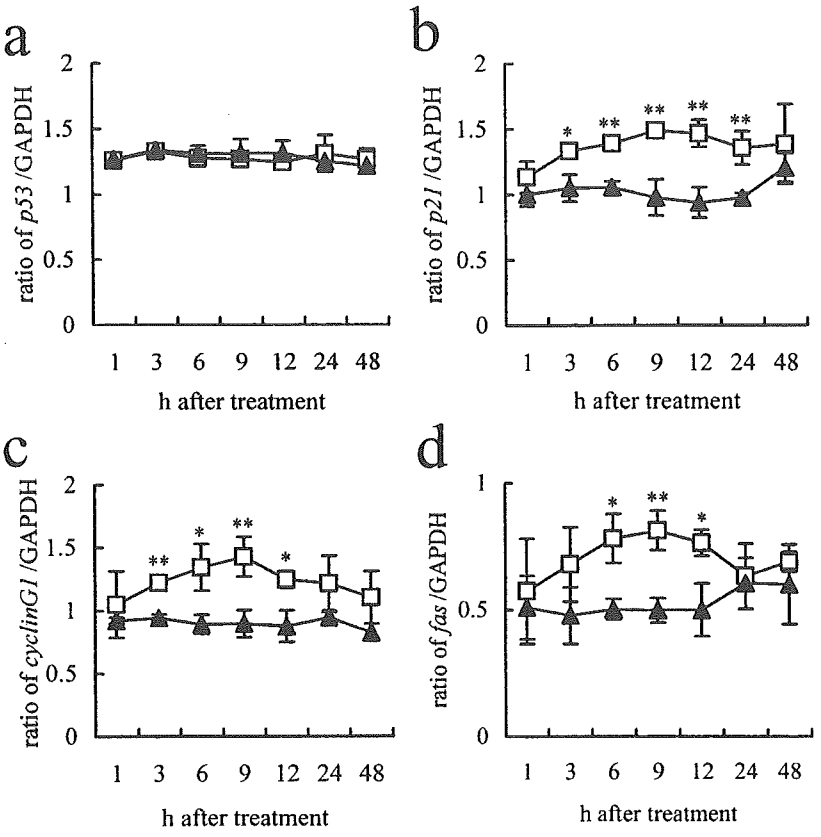

FIG. 9. Sequential changes in the expression of *p53* (a), *p21* (b), *cyclinG1* (c), and *fas* (d) mRNAs in the placenta exposed to Ara-C. Data represent the mean ± SD (n = 3). *, $P < 0.05$, **, $P < 0.01$; significantly different from controls. Open squares, Ara-C-treated group; closed triangles, control group.

growth, development, and well being of the fetus. In the present study, we demonstrated that Ara-C caused increased apoptotic cell death and impaired cell proliferation in the placental labyrinth zone. These toxic effects on trophoblastic cells would disrupt the function of the placental labyrinth zone, resulting in abnormal fetal development.

In the placenta, the expression of various genes that regulate apoptosis and proliferation, including p53, has been reported [39–42], and the death as well as growth of placental component cells is believed to be strictly controlled by these genes. In the present study, immunohistochemical examination revealed that a substantial elevation of p53 protein expression preceded Ara-C-induced changes in the apoptosis and proliferation of trophoblastic cells. In response to DNA damage, p53 protein is modified via phosphorylation or acetylation and becomes stabilized and activated. In a lot of cases, the function of p53 is mediated by those posttranscriptional mechanisms increasing the half-life and transcriptional activity of the protein [43, 44]. In our case, overexpression of *p53* mRNA was not detected in spite of the increase in the expression of p53 protein, and this suggests that p53 is regulated by such a posttranscriptional mechanism. On the other hand, the up-regulation of the p53 target genes mRNAs was observed following the increase in the expression of p53 protein, implying that p53 exerts its function by transactivation. Therefore, it is suggested that p53 and its transcriptional target genes are involved in the Ara-C-induced trophoblastic cell apoptosis and inhibition of cell proliferation in the placental labyrinth zone.

Ethylnitrosourea, a DNA alkylating teratogenic drug, was also reported to cause apoptosis and cell-cycle arrest with up-regulation of p53 protein in trophoblastic cells in the placental labyrinth zone [21]. In fetal central nervous system, targeted disruption of p53 critically suppressed apoptotic cell death induced by the administration of Ara-C [17] and ethylnitrosourea [45] to dams. The results of studies using Ara-C and ethylnitrosourea indicate that trophoblastic cells in the placental labyrinth zone are highly susceptible to genotoxic stimuli and p53 plays an important role in mediating the toxic effect of these stresses. Increased expression of p53 protein is also reported in human placenta complicated with fetal growth restriction [46]; thus, there is a possibility that stimuli such as hypoxia other than genotoxic stress cause placental disability in a p53-mediated way.

In addition to DNA damaging agents, endocrinological abnormality, inflammatory cytokines, and oxidative stress are demonstrated to induce placental apoptosis, and the distribution of the lesion is specific to the stimulus [5, 11, 12], suggesting that the pathways of increased placental apoptosis differ. Trophoblastic cells in the placental labyrinth zone actively proliferate and synthesize DNA [47], and this would have some relation to their sensitivity because Ara-C is cytotoxic to proliferating cells, especially in the DNA synthetic phase of the cell cycle [48]. However, the mechanism of the zone-dependent placental apoptosis under various pathological conditions is still obscure, and further study is needed to clarify this point.

In conclusion, the placenta is susceptible to genotoxic stress, including DNA damaging agents, which disrupt the regulation of trophoblastic cell death and proliferation. In addition, our findings suggest that p53 and its transcriptional target genes play an important role in the pathogenesis of Ara-C-induced placental toxicity. Probably, this induces placental growth inhibition and subsequent dysfunction of the placenta, which severely affects the development of the fetus, resulting in the induction of fetal growth restriction and other abnormal fetal development.

REFERENCES

1. Bauer MK, Harding JE, Bassett NS, Breier BH, Oliver MH, Gallaher BH, Evans PC, Woodall SM, Gluckman PD. Fetal growth and placental function. Mol Cell Endocrinol 1998; 140:115–120.
2. Smith SC, Baker PN, Symonds EM. Placental apoptosis in normal human pregnancy. Am J Obstet Gynecol 1997; 177:57–65.
3. Lea RG, Riley SC, Antipatis C, Hannah L, Ashworth CJ, Clark DA, Critchley HO. Cytokines and the regulation of apoptosis in reproductive tissues: a review. Am J Reprod Immunol 1999; 42:100–109.
4. Halperin R, Peller S, Rotschild M, Bukovsky I, Schneider D. Placental apoptosis in normal and abnormal pregnancies. Gynecol Obstet Invest 2000; 50:84–87.
5. Waddell BJ, Hisheh S, Dharmarajan AM, Burton PJ. Apoptosis in rat placenta is zone-dependent and stimulated by glucocorticoids. Biol Reprod 2000; 63:1913–1917.
6. Smith SC, Baker PN, Symonds EM. Increased placental apoptosis in intrauterine growth restriction. Am J Obstet Gynecol 1997; 177:1395–1401.
7. Smith SC, Baker PN. Placental apoptosis is increased in postterm pregnancies. Br J Obstet Gynaecol 1999; 106:861–862.
8. Axt R, Kordina AC, Meyberg R, Reitnauer K, Mink D, Schmidt W. Immunohistochemical evaluation of apoptosis in placentae from normal and intrauterine growth-restricted pregnancies. Clin Exp Obstet Gynecol 1999; 26:195–198.
9. Erel CT, Dane B, Calay Z, Kaleli S, Aydinli K. Apoptosis in the placenta of pregnancies complicated with IUGR. Int J Gynaecol Obstet 2001; 73:229–235.
10. Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol 2002; 186:158–166.
11. Miller MJ, Voelker CA, Olister S, Thompson JH, Zhang XJ, Rivera D, Eloby-Childress S, Liu X, Clark DA, Pierce MR. Fetal growth retardation in rats may result from apoptosis: role of peroxynitrite. Free Radic Biol Med 1996; 21:619–629.
12. Ejima K, Koji T, Tsuruta D, Nanri H, Kashimura M, Ikeda M. Induction of apoptosis in placentas of pregnant mice exposed to lipopolysaccharides: possible involvement of Fas/Fas ligand system. Biol Reprod 2000; 62:178–185.
13. Chiba K, Ishikawa H, Rahman ME, Endo A. Neonatal mouse hip joint and hindlimb anomalies induced by prenatal exposure to Ara-C. Reprod Toxicol 1996; 10:215–219.
14. Vlahovic M, Bulic-Jakus F, Juric-Lekic G, Fucic A, Maric S, Serman D. Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine. Int J Dev Biol 1999; 43:843–846.
15. Wang B, Ohyama H, Haginoya K, Odaka T, Yamada T, Hayata I. Prenatal radiation-induced limb defects mediated by Trp53-dependent apoptosis in mice. Radiat Res 2000; 154:673–679.
16. Katayama K, Ishigami N, Suzuki M, Ohtsuka R, Kiatipattanasakul W, Nakayama H, Doi K. Teratologic studies on rat perinates and offspring from dams treated with ethylnitrosourea (ENU). Exp Anim 2000; 49: 181–187.
17. D'Sa-Eipper C, Leonard JR, Putcha G, Zheng TS, Flavell RA, Rakic P, Kuida K, Roth KA. DNA damage-induced neural precursor cell apoptosis requires p53 and caspase 9 but neither Bax nor caspase 3. Development 2001; 128:137–146.
18. Katayama K, Ohtsuka R, Takai H, Nakayama H, Doi K. Expression of p53 and its transcriptional target genes mRNAs in the ethylnitrosourea-induced apoptosis and cell cycle arrest in the fetal central nervous system. Histol Histopathol 2002; 17:715–720.
19. Ueno M, Katayama K, Nakayama H, Doi K. Mechanisms of 5-azacytidine (5AzC)-induced toxicity in the rat foetal brain. Int J Exp Pathol 2002; 83:139–150.
20. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996; 10: 1054–1072.
21. Katayama K, Ueno M, Takai H, Ejiri N, Uetsuka K, Nakayama H, Doi K. Ethylnitrosourea induces apoptosis and growth arrest in the trophoblastic cells of rat placenta. Biol Reprod 2002; 67:431–435.
22. Wagner VM, Hill JS, Weaver D, Baehner RL. Congenital abnormalities in baby born to cytarabine treated mother. Lancet 1980; 2:98–99.
23. Schafer AI. Teratogenic effects of antileukemic chemotherapy. Arch Intern Med 1981; 141:514–515.
24. Volkenandt M, Buchner T, Hiddemann W, Van de Loo J. Acute leukaemia during pregnancy. Lancet 1987; 2:1521–1522.
25. Chaube S, Kreis W, Uchida K, Murphy ML. The teratogenic effect of 1-β-D-arabinofuranosylcytosine in the rat. Protection by deoxycytidine. Biochem Pharmacol 1968; 17:1213–1216.
26. Ritter EJ, Scott WJ, Wilson JG. Teratogenesis and inhibition of DNA synthesis induced in rat embryos by cytosine arabinoside. Teratology 1971; 4:7–13.
27. Yamauchi H, Katayama K, Yasoshima A, Uetsuka K, Nakayama H, Doi K. 1-β-D-Arabinofuranosylcytosine (Ara-C)-induced apoptosis in the rat fetal tissues and placenta. J Toxicol Pathol 2003; 16:223–229.
28. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119:493–501.
29. Los M, Wesselborg S, Schulze-Osthoff K. The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity 1999; 10:629–639.
30. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth Differ 1991; 2:209–214.
31. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75:817–825.
32. Okamoto K, Beach D. Cyclin G is a transcriptional target of the p53 tumor suppressor protein. Embo J 1994; 13:4816–4822.
33. Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med 1998; 188:2033–2045.
34. Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ, Reed SI. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994; 76:1013–1023.
35. Okamoto K, Li H, Jensen MR, Zhang T, Taya Y, Thorgeirsson SS, Prives C. Cyclin G recruits PP2A to dephosphorylate Mdm2. Mol Cell 2002; 9:761–771.
36. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991; 66:233–243.
37. Levin S, Bucci TJ, Cohen SM, Fix AS, Hardisty JF, LeGrand EK, Maronpot RR, Trump BF. The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists. Toxicol Pathol 1999; 27:484–490.
38. Knipp GT, Audus KL, Soares MJ. Nutrient transport across the placenta. Adv Drug Deliv Rev 1999; 38:41–58.
39. Rees WD, Hay SM, Fontanier-Razzaq NC, Antipatis C, Harries DN. Expression of the growth arrest genes (GAS and GADD) changes during organogenesis in the rat fetus. J Nutr 1999; 129:1532–1536.
40. Levy R, Nelson DM. To be, or not to be, that is the question. Apoptosis in human trophoblast. Placenta 2000; 21:1–13.
41. Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez J, Samoto T, Maruo T. Changes in proliferative potential, apoptosis and Bcl-2 protein expression in cytotrophoblasts and syncytiotrophoblast in human placenta over the course of pregnancy. Endocr J 2000; 47:317–327.
42. Ka H, Hunt JS. Temporal and spatial patterns of expression of inhibitors of apoptosis in human placentas. Am J Pathol 2003; 163:413–422.
43. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene 1999; 18:7644–7655.
44. Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003; 15: 164–171.
45. Leonard JR, D'Sa C, Klocke BJ, Roth KA. Neural precursor cell apoptosis and glial tumorigenesis following transplacental ethyl-nitrosourea exposure. Oncogene 2001; 20:8281–8286.
46. Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, Nelson DM. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. Am J Obstet Gynecol 2002; 186:1056–1061.
47. Peel S, Bulmer D. Proliferation and differentiation of trophoblast in the establishment of the rat chorio-allantoic placenta. J Anat 1977; 124:675–687.
48. Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res 1998; 72:197–233.

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Effects of prenatal hydroxyurea-treatment on mouse offspring

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Abstract

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, induces morphological anomalies in the central nervous system, craniofacial tissues and limb buds in animals, and neonatal respiratory distress in humans. The neonates and offspring of pregnant mice treated with 400 or 800 mg/kg of HU on day 13 of gestation were examined at 0 day and 10 weeks after birth to find a clue for clarifying the relationship between HU-induced apoptosis in the fetal tissues and teratogenicity. The offspring from dams treated with HU were retarded in growth compared with controls. But there was no significant difference in the body weight gain between the 400 and 800 mg/kg groups. In the teratologic changes, microencephaly, hydrocephalus and curved coccygeal vertebrae were observed in the offspring, and the incidence of these teratologic changes was similar but their degree was more severe in the 800 mg/kg group than in the 400 mg/kg group. Based on the above-mentioned previous and present studies of ours, we suggest that HU-induced apoptosis in fetal tissues may play an important role in the development of anomalies in the corresponding tissues of offspring.

Keywords: Anomalies; Hydroxyurea; Apoptosis; Mouse

Introduction

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, is now used for the treatment of various types of cancer, human immunodeficiency virus infection and sickle cell anemia. On the other hand, HU is also known as a teratogen. Although there are many reports of HU-related teratogenic effects in many laboratory animal species (Aliverti et al., 1980; Barr and Beaudoin, 1981; Desesso et al., 1994; Murphy and Chaube, 1964; Philips et al., 1967; Scott et al., 1971; Theisen et al., 1973; Wilson et al., 1975), there are few reports in mice (Roll and Baer, 1969). HU induces anomalies in the central nervous system (CNS), craniofacial tissues, skull and limbs (Butcher et al., 1973; Barr and Beaudoin, 1981). In addition, growth retardation was observed in offspring from dams administered with HU in the late stage of pregnancy (Butcher et al., 1973). We reported that HU administration to pregnant mice on day 13 of gestation induced a marked apoptosis in neuroepithelial cells in the CNS and mesenchymal cells in the lungs as well as in craniofacial tissues and limb buds. Therefore, we suggested that such excess cell death in the fetal tissues

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may have a certain relation to the later occurrence of morphological or functional anomalies reported in these tissues following prenatal HU-administration (Woo et al., 2003).

This study was undertaken to examine the relationship between HU-induced apoptosis in fetal tissues (Woo et al., 2003) and the anomalies in mouse offspring for up to 10 weeks after birth. There are no reports, which follow up anomalies for up to 10 weeks after birth in mice. The protocol of the present study was approved by the Animal Use and Care Committee of Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Materials and methods

Animals

Thirty 8-week-old pregnant mice of Crj:CD-1 (ICR) strain at day 8 of pregnancy were obtained from Charles River Japan Co., Yokohama, Japan. They were kept in an animal room under controlled conditions (temperature, 23±2°C; relative humidity, 55±5%) using an isolator caging system (Niki Shoji, Co., Tokyo) and were fed commercial pellets (MF, Oriental Yeast Co., Tokyo) and water ad libitum.

Chemical

HU (Sigma, St. Louis, MO) was dissolved in distilled water (DW) immediately before the treatment, and the concentration was adjusted to 60 or 120 mg/ml.

Treatments

Thirty pregnant mice were divided randomly into two groups: 0 day (0D)-group and 10 weeks (10W)-group. Ten pregnant mice of each group were injected intraperitoneally (i.p.) with 400 mg/kg b.w. (five mice) or 800 mg/kg b.w. of HU (5 mice) on day 13 of gestation (date vaginal plug found = day 0), and five pregnant mice of each group were injected i.p. with DW alone and served as controls.

In the 0D-group, the neonates from five treated dams, together with those from five control dams, were sacrificed under ether anesthesia at birth (0 day). The neonates were weighed and examined for external malformations. Half of the neonates in each litter were fixed in 10% neutral-buffered formalin to observe visceral abnormalities (Barrow and Taylor, 1969). Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE) for histological examinations. The remaining half of the neonates were fixed in alcohol, and then double-stained with alcian blue and alizarin red S to examine skeletal malformation (Inouye, 1976).

In the 10W-group, five treated dams, together with five control dams, were delivered naturally. Dams and offspring were separated at 4 weeks of age and male and female offspring were also separated at the same time. The body weights of offspring were recorded at 4, 6, 8, and 10 weeks of age. All offspring were sacrificed by heart puncture under ether anesthesia at 10 weeks after birth. The brain, lungs, heart, liver, kidneys, spleen, ovaries, testes, uterus, epididymides, stomach, and intestines were collected and weighed. All of the collected organs were fixed in 10% neutral-buffered formalin. Paraffin sections (4 μm) were stained with HE for histologic examinations.

Table 1. Reproductive data of the 0D-group

Dose	Control	400 mg/kg	800 mg/kg
Total number of litters	65	61	72
Average litter size	13	12.2	14.4
Dead neonates	0	0	0

Table 2. Reproductive data of the 10W-group

	Control	400 mg/kg	800 mg/kg
Total number of litters	53	62	50
Average litter size	10.6	12.4	10
Dead offspring (%)	0	0	0
Stunted offspring (%)	0	0	0
Anomaly (%)	0	8.1	20

Anomaly(%): curved tail and hydrocephalus.

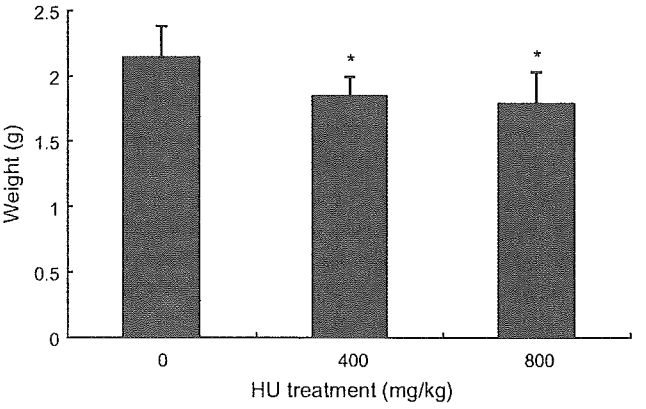

Fig 1. Body weights of the 0D-group in both sexes. *, $p < 0.05$: Significantly different from controls.

Statistical analysis

The weights of body and organs and the ratios of organs to body weight were presented as mean ± standard deviation (SD) and statistical analysis was carried out by Student's *t*-test or Welch's *t*-test.

Results

There were no significant differences in the reproduction data between the control and HU-treated groups (Tables 1 and 2). In addition, dead offspring were not observed throughout the experimental period.

The body weight of HU-treated animals was significantly lower than those of controls in both the 0D- (Fig. 1) and 10W-groups (Fig. 2). However, there were no significant differences in the body weight gain between the 400 and 800 mg/kg groups (Fig. 2). The ratio of organ to body weight and the weights of brain and lungs of HU-treated animals were significantly lower than those of controls in the 10W-group (Table 3).

As to the teratologic changes, in addition to micrencephaly, hydrocephalus and curved coccygeal vertebrae were observed in the HU-treated animals of the 10W-group (Table 4, and Figs. 3 and 4). The incidences of these changes were similar but their degree was more severe in the 800 mg/kg group than in the 400 mg/kg group.

In the histologic examination of the 10W-group, the thickness of the cerebral cortex was reduced in HU-treated animals when compared with that in the controls (Figs. 5 and 6). In such a cerebral cortex, a decrease in the number of neural cells was observed

Table 3. The ratio of the organ to body weight in the 10W-group

	Male		
	Control	HU (400 mg/kg)	HU (800 mg/kg)
Brain	1.36±0.099	1.27±0.081**	1.21±0.14**
Lung	0.82±0.073	0.64±0.069**	0.70±0.066**
Heart	0.47±0.044	0.52±0.061	0.50±0.069
Liver	6.44±0.44	6.23±0.60	6.18±0.64
Right kidney	1.01±0.10	0.97±0.13	0.95±0.10
Left kidney	1.06±0.11	1.02±0.14	0.99±0.10**
Spleen	0.34±0.036	0.30±0.049**	0.26±0.046**
Stomach	2.29±0.81	1.89±0.59	1.95±0.51
Intestine	10.45±0.94	9.84±0.94	9.58±0.78
Testis	0.75±0.080	0.66±0.11**	0.64±0.079**
Epididymis	0.42±0.092	0.25±0.11**	0.38±0.083**
	Female		
	Control	400 mg/kg	800 mg/kg
Brain	1.73±0.15	1.61±0.14**	1.58±0.21*
Lung	0.92±0.15	0.90±0.096	0.84±0.14*
Heart	0.54±0.043	0.51±0.043	0.49±0.075
Liver	5.95±0.56	6.42±0.45	6.17±0.54
Right kidney	0.75±0.067	0.73±0.10	0.65±0.078**
Left kidney	0.78±0.066	0.79±0.097	0.68±0.075**
Spleen	0.49±0.11	0.48±0.032	0.42±0.083
Stomach	2.63±0.99	2.24±0.78	2.21±0.69
Intestine	12.04±1.50	11.07±1.42*	11.07±1.46*
Ovaries	0.16±0.017	0.18±0.0057	0.12±0.024**
Uterus	0.73±0.17	0.77±0.18	0.53±0.14**

*$p<0.05$, **$p<0.01$(Welch's *t*-test): significantly different from controls.

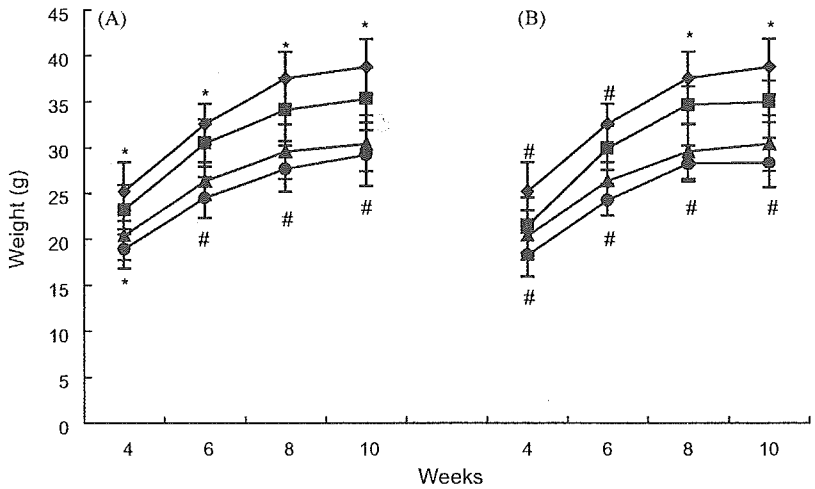

Fig 2. Changes in body weights of the 10W-group after treatment with 400 mg/kg (A) and 800 mg/kg of HU (B). ◆–◆: Male control, ■–■: Male, ▲–▲: Female control, ●–●: Female. *, $p<0.05$ (Student's *t*-test): Significantly different from controls. ♯, $p<0.05$ (Welch's *t*-test): Significantly different from controls.

Table 4. Abnormalities in the 10W-group

	Control ($n=53$)	400 mg/kg ($n=62$)	800 mg/kg ($n=50$)
Curved tail	0	5	9
Micrencephaly	0	30	25
Hydrocephalus	0	0	1

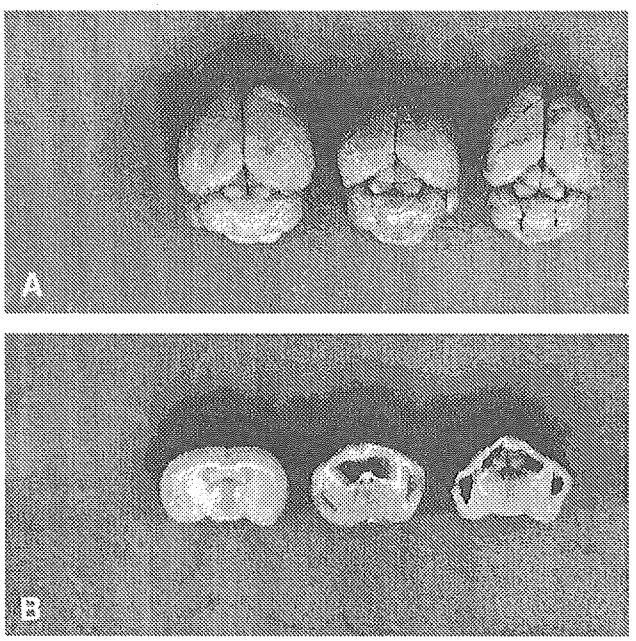

Fig. 3. Gross and cut appearance of the brain in the 10W-group: (A) Left: control, middle: 400 mg/kg, and right: 800 mg/kg. (B) Left: control, and middle and right: hydrocephalus. Brain size of HU-treated animals is reduced compared with controls.

(Fig. 7). In other organs, there were no histologic changes observed.

Discussion

The CNS is the system, which starts to develop first and finishes its differentiation last among all systems in fetuses. In addition, during the entire period of the development, the CNS is one of the organs easily affected by various toxic compounds. In our previous studies, the fetuses obtained from pregnant mice administrated with HU on day 13 of gestation developed a marked apoptosis in neuroepithelial cells in the CNS, suggesting that such excess cell death in the fetal brain might have a certain relation to the later occurrence of morphological or functional abnormalities in the CNS (Woo et al., 2003). In the present study, significant differences in the brain weight were found between the control and HU-treated groups. This corresponded well to the decrease in the thickness of the cerebral cortex in HU-treated animals. In addition, hydrocephalus was observed in one offspring of the 800 mg/kg group at 10 weeks of age.

The weights of other organs such as lungs and kidneys were also lower in the HU-treated group than in the control group. This may be related to an increase in the number of apoptotic cells in these organs of fetuses reported in our previous paper (Woo et al., 2003), and excess cell death by apoptosis in the fetal organs may bring about a lack of cell populations required for later normal histogenesis and organogenesis, resulting in anomalies in these tissues.

It is said that the calcification of the caudal part occurred in the late stage of the development of vertebrae (Rugh, 1991), and, in the present study,

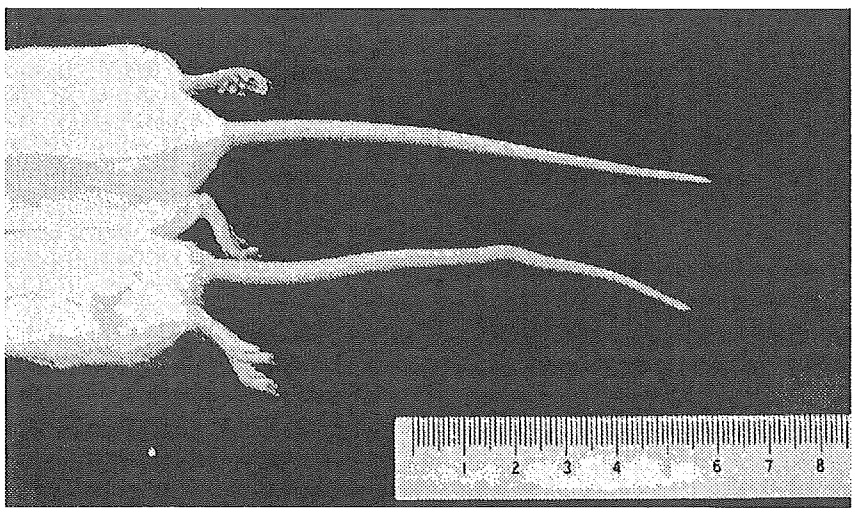

Fig. 4. Curved coccygeal vertebrae in HU-treated animals in the 10W-group.

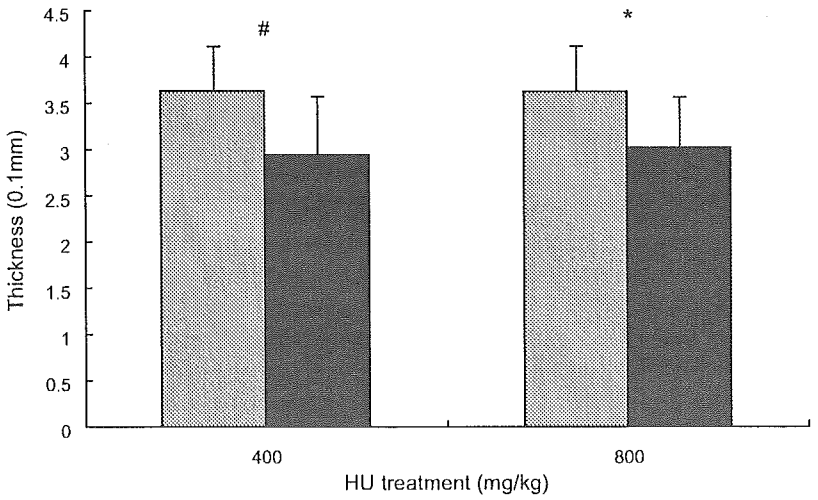

Fig. 5. The thickness of the cerebral cortex in the 0D-group. *, $p<0.05$ (Student's t-test): Significantly different from controls. ♯, $p<0.05$ (Welch's t-test): Significantly different from controls.

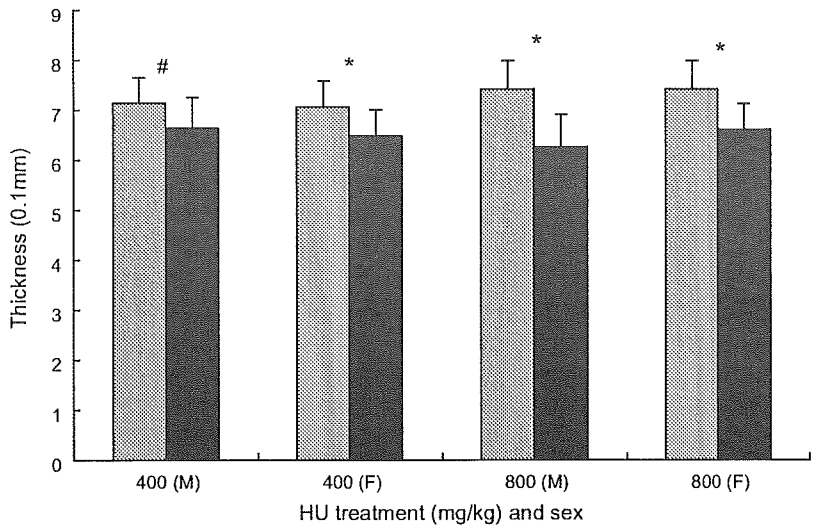

Fig. 6. The thickness of the cerebral cortex in the 10W-group. *, $p<0.05$ (Student's t-test): Significantly different from controls. ♯, $p<0.05$ (Welch's t-test): Significantly different from controls.

offspring bearing curved coccygeal vertebrae were detected in HU-treated animals in the 10W-group as mentioned above. On the other hand, there were no severe facial abnormalities in the present study. With respect to this point, some studies have reported that the injection time is very important (Aliverti et al., 1980; Roba et al., 1970). When pregnant mice were exposed to various X-ray during the fetal development, the majority of anomalies occurred following the irradiation on days 8, 9, and 10 when major organogenesis occurs. By day 13 of gestation, the organs had almost formed and congenital anomalies were not easily producible (Rugh, 1964).

Based on our previous and present studies, we suggest that massive cell death by apoptosis following HU-administration may play an important role in the induction of postnatal abnormalities in corresponding organs. However, to clarify the role of apoptosis in the later development of teratogenesis, further investigations should be undertaken.

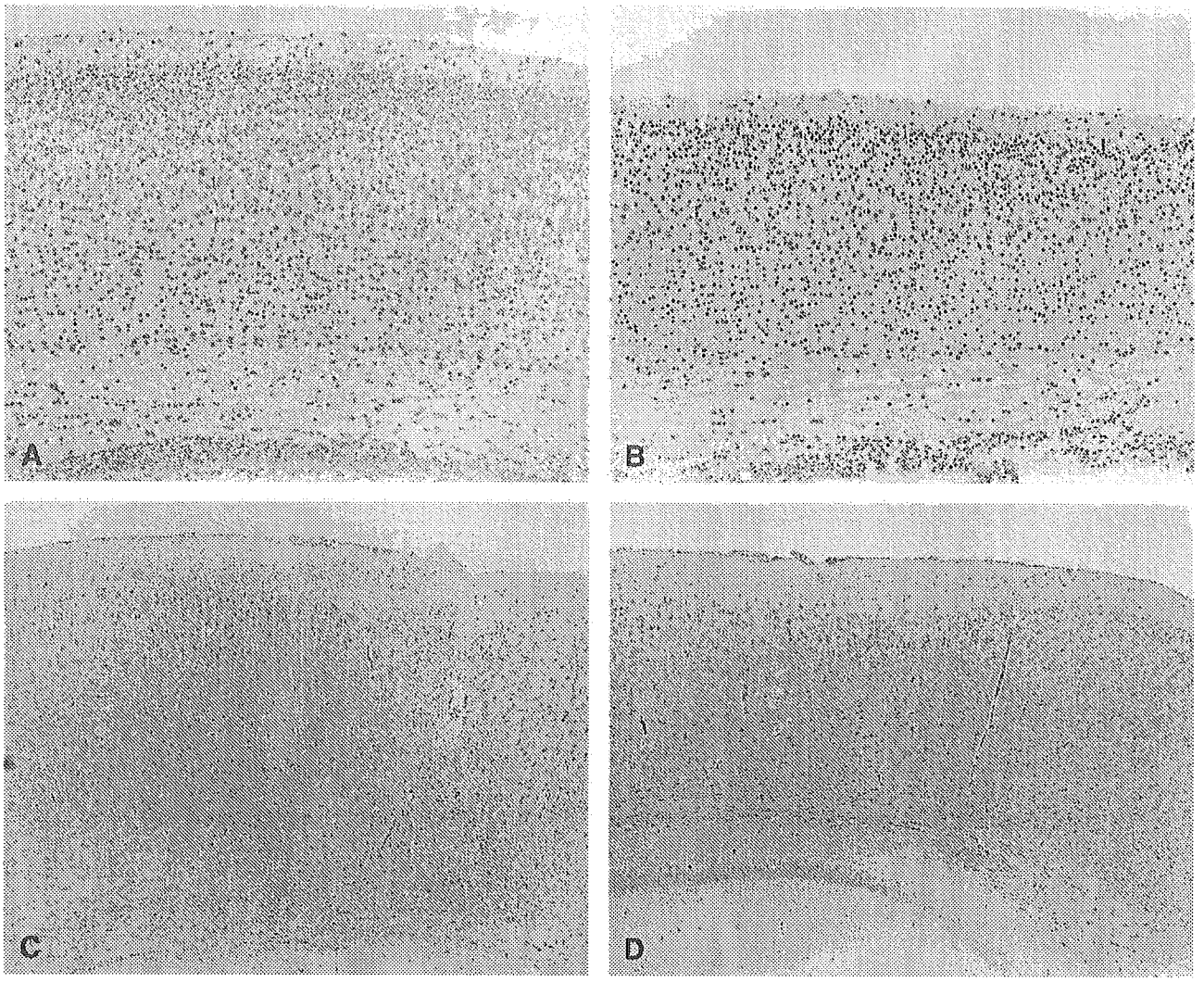

Fig. 7. Histologic appearances of the cerebrum of a control (A) and HU- treated animal (B) in the 0D-group and a control (C) and HU-treated animal (D) in the 10W-group. A, B: HE, ×210; C, D: HE, ×87.

References

Aliverti V, Bonauomi L, Giavini E. Hydroxyurea as a reference standard in teratological screening: comparison of the embryotoxic and teratogenic effects following single intraperitoneal or repeated oral administrations to pregnant rats. Arch Toxicol Suppl 1980;4:239–47.

Barr Jr M, Beaudoin AR. An exploration of the role of hydroxyurea injection time in fetal growth and teratogenesis in rats. Teratology 1981;24:163–7.

Barrow MV, Taylor WJ. A rapid method for detecting malformations in rat fetuses. J Morphol 1969;127:291–306.

Butcher RE, Scott WJ, Kazhaier KEJ. Postnatal effects in rats of prenatal treatment with hydroxyurea. Teratology 1973; 7:161–5.

Desesso JM, Scialli AR, Goeringer GC. D-Mannitol, a specific hydroxyl free radical scavenger, reduces the developmental toxicity of hydroxyurea in rabbits. Teratology 1994; 49:248–59.

Inouye M. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. Cong Anom 1976;16:171–3.

Murphy ML, Chaube S. Preliminary survey of hydroxyurea (NSC-32065) as a teratogen. Cancer Chemother Rep 1964;40:1–7.

Philips FS, Sternberg SS, Schwartz HS, et al. Hydroxyurea. I. Acute cell death in proliferating tissues in rats. Cancer Res 1967;27:61–74.

Roba J, Lambelin G, Buu-Hoi NP. Teratological studies of p-butoxyphenyl-acethydroxamic acid (CP 1044 J3) in rats and rabbits. Arzneim ForschDrug Res 1970;20:565–9.

Roll R, Baer F. Untersuchungen über die teratogene Wirkung von Hydroxyharnstoff während der frühen und embryonalen Entwicklung der Maus. Arch Toxicol 1969; 25:150–68.

Rugh R. Why radiology? Radiology 1964;82:917–20.

Rugh R. Its reproduction and development: the mouse, 1st ed. Oxford: Oxford University; 1991. p. 208–295.

Scott WJ, Ritter EJ, Wilson JG. DNA synthesis inhibition and cell death associated with hydroxyurea teratogenesis in rat embryos. Dev Biol 1971;26:306–15.

Theisen CT, Frandkin R, Wilson JG. Teratogenicity of hydroxyurea in rhesus monkeys. Teratology 1973;7: A29.

Wilson JG, Scott WJ, Ritter EJ, et al. Comparative distribution and embryotoxicity of hydroxyurea in pregnant rats and rhesus monkeys. Teratology 1975;11:169–78.

Woo GH, Karayama K, Jung JY, et al. Hydroxyurea (HU)-induced apoptosis in the mouse fetal tissues. Histol Histopathol 2003;18:387–92.

Exp Toxic Pathol 2004; 55: 357–366
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Gene expression profiles in pregnant rats treated with T-2 toxin

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With 3 figures and 5 tables

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Key words: T-2 toxin; pregnant rats; liver; placenta; fetal liver; microarray.

Summary

Pregnant rats on day 13 of gestation were treated orally with T-2 toxin at a single dose of 2 mg/kg and sacrificed at 24 hours after treatment. Histopathologically, apoptosis was increased in the liver, placenta and fetal liver. Microarray analysis was performed to examine the gene expression in the liver, placenta, and fetal liver. The results of microarray analysis showed that the changes in the expression of apoptosis genes, metabolic enzymes and oxidative stress-related genes were detected in these tissues. Suppression of phase I and II enzymes-related genes expression in the liver, and suppression of phase II enzymes-related genes expression in the placenta and fetal liver were observed. Semiquantitive RT-PCR analysis also showed the same results as those of microarray analysis. From the results of microarray analysis and histopathological examination, T-2 toxin seems to induce oxidative stress in these tissues, following the changes in metabolism-related genes expression. These changes may alter the intracellular environments resulting in the induction of apoptosis. Further studies on the gene expression profiles at the earlier time point are necessary to clarify the detailed mechanisms of T-2 toxin-induced toxicity in pregnant rats.

Introduction

T-2 toxin is a trichothecene mycotoxin produced by various species of *Fusarium* spp. *Fusarium* spp. may occur in cereals including corn, oats, rice, and wheat. T-2 toxin has been found to contaminate foods, animal foods and agricultural products, and has been reported in many parts of the world (IARC 1993; WHO 1990). 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 including chickens, mice, rats, rabbits and pigs. Necrosis/apoptosis is also reported in the gastrointestinal tract and liver (BRATICH et al. 1990; HAYES et al. 1982; HOERR et al. 1981; KOSURI et al. 1971; MARASAS et al. 1969; PANG et al. 1987). 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 in addition to maternal toxicity (ISHIGAMI et al. 2001; ROUSSEAUX et al. 1987; STANFORD et al. 1975). In rats, it is reported that T-2 toxin passes the placenta and distributes to fetal tissues, resulting in an impairment of the immune system (LAFARGE-FRAYSSINET et al. 1990). We have shown that 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 (SEHATA et al. 2003). However, mechanisms of fetotoxicity induced by T-2 toxin are still unknown.

Recent years DNA microarray technologies have been developed that allow one to detect the expression of many genes at the same time. The application of this technology to the field of toxicology, i.e. toxicogenomics, has been demonstrated. The expression of genes was reported in rats administered with hepatotoxicants, phenobarbital and carbon tetrachloride (BULERA et al. 2001; WARING et al. 2001). There is only one report of microarray analysis in mycotoxin-induced toxicity in animals (LUEHE et al. 2003). They reported that ochratoxin A-

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specific transcriptional changes were detected for genes involved in DNA damage response and apoptosis, response to oxidative stress, and inflammatory reactions. The purpose of the present study was to examine the gene expression profiles in the liver, placenta, and fetal liver in pregnant rats treated with T-2 toxin. We especially focused on genes related to apoptosis, metabolism, and oxidative stress as major mechanisms involved in the T-2 toxin-induced toxicity. The protocol of this study was approved by the Animal Care and Use Committee of Graduated School of Agricultural and Life Sciences, The University of Tokyo.

Materials and methods

Animals: Six 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 °C 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*.

Treatment: According to the results from dose-finding study (data not shown) and reported study (SEHATA et al. 2003), animals were used on GD13. Three animals were treated with a single oral dose of 2 mg/kg T-2 toxin (Sigma Chemical Co., St. Louis, MO, USA), and animals were sacrificed by exsanguination under ether anesthesia at 24 hours after treatment (HAT). T-2 toxin was dissolved in corn oil and a dosing volume was adjusted to 2.5 mL/kg. In addition, 3 control animals were treated with the vehicle, and were sacrificed in the same way at 24 HAT.

Histopathological examination and immunohistochemical staining: After animals were sacrificed, macroscopic examination was performed. Dam's organs (liver and thymus) and fetuses were weighed. Dam's organs, and 3 placentae and fetuses from each dam were fixed in 10% neutral-buffered formalin to confirm the changes induced by the T-2 toxin. Four-μm 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). In brief, multiple fragmented DNA-3′-OH ends on the section were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methyl green. Morphometry was performed in the liver, placenta (3 placentae/dam) and fetal liver (3 fetuses/dam) under light microscope (×400). The number of positive cells/1000 cells was represented as the mean ± SD. Statistical analysis was carried out by Student's *t*-test or Welch's *t*-test after analysis of homogeneity of variance by F-test.

RNA extraction and microarray analysis: Total RNA was extracted from frozen tissues (up to 0.5 g) using the RNeasy Mini Kit (QIAGEN Inc., CA, USA) for the fetal liver or TRIzol reagent (Invitrogen, CA, USA) for the liver and placenta. The RNA from placenta and fetal liver from each dam were pooled to generate a single sample, respectively. Microarray analysis (total 18 arrays) was performed according to the Affymetrix protocol. Briefly, cDNA was prepared from 5 μg of total RNA using the SuperScript Choice System for cDNA Synthesis (Invitrogen, CA, USA), with the exception that primer used for the reverse transcription reaction was T7-(dT)24 primer (Primer sequence: 5′-GGCCAGTGAATTGTAATACGACTCACTAT AGGGAGGCGG-$(dT)_{24}$-3′, Amersham Bioscience, Tokyo, Japan). Following this, biotin-labeled cRNA was synthesized from the cDNA using the Enzo High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, NY, USA). After 20 μg biotin-labeled cRNA was fragmentated, hybridization solution was prepared using GeneChip® Eukaryotic Hybridization Control Kit (Affymetrix), and was hybridized to the Affymetrix Rat Genome U34A oligonucleotide array for 45 °C, 16 hours in GeneChip® Hybridization Oven 640 (Affymetrix). The chips were washed and stained using the Fluidics Station (Affymetrix Inc.) and scanned with GeneArray® Scanner (Hewlett-Packard, Palo Alto, CA, USA).

Data analysis: The microarray imaging data were analyzed using the Microarray Suite ver. 5.0 (Affymetrix) and Spotfire Pro Version 4.2 program (Spotfire Inc., MA, USA). In brief, total array normalization (global normalization after trimming the top 2% and bottom 2% of the data) was performed in each experimental data (YANG et al. 2001). Following this, data were compared between the treated group and control group by calculating the prediction value in neighborhood analysis (designed as *Distinction value* in this study, given by (Average A-Average B/(Standard deviation A+ Standard deviation B, Group A and B, GOLUB et al. 1999). In the present study, we only analyzed known genes. Therefore, unknown genes, such as EST genes, were removed from the data. In addition, probes containing at least one Absence Call in the data were also removed. Finally, we selected genes that gave a distinction value of greater than +1.5 or less than −1.5. The mean value of each gene in the treated group was compared with that of control group by Student's *t*-test or Welch's *t*-test after analysis of homogeneity of variance by F-test. A significance level of $p < 0.01$ or $p < 0.05$ was considered acceptable.

Semiquantitive reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was prepared as described above. The first strand cDNA was synthesized using oligo $(dT)_{12-18}$ primer and the Superscript II RNase H^- Reverse transcriptase (Invitrogen, CA, USA). PCR was performed using oligonucleotide primers sets corresponding to the cDNA sequences (table 1; CYP1A1, CYP2B1/2, CYP2E1, CYP3A1, CYP4A1, GSTA1, GSTA2, GST-P, p53, p21, GADD45, c-fos, c-jun, Bax, hemeoxygenase-1, thioredoxin reductase, heat shock protein 70, and GAPDH) (ALBARENQUE et al. 2001; GAN et al. 2002; KATAYAMA et al. 2002; LIU et al. 2001; LUCERI et al. 2002; MORRIS and DAVILA 1996; NAKAHARA et al. 2002; UENO et al. 2002;

VANHAECKE et al. 2000). As we focused on metabolism, apoptosis, and oxidative stress as major mechanisms involved in T-2 toxin-induced toxicity, we selected the above-mentioned genes. Briefly, 100 μL reaction mixture containing 10 μL 10 × PCR buffer (100 mM Tris-HCl buffer, 500 mM KCl, and 15 mM $MgCl_2$; Takara, Shiga, Japan), 10 μL dNTP (Takara), 50 pmol sense and antisense primer, and 1 μL cDNA sample. PCR reaction (× each cycle numbers (table 1); denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min) was performed using Takara PCR Thermal cycler SP (Takara). The PCR products were electrophoresed in 2% Agarose S (Nippon Gene, Japan)/1 × TBE buffer (89 mM Tris aminomethane, 89 mM Boric acid, 10 mM EDTA). The gels were stained with ethidium bromide (Invitrogen). Fluorescent band was visualized using a UV-CCD video system ($EpiLight_{UVFA1100}$; AISIN COSMOS, Tokyo, Japan) and was analyzed using an image-analysis software, Quantity One (pdi, NY, USA) on a computer. Relative band intensity against GAPDH band was represented as the mean ± standard deviation (SD) of 3 samples. Statistical analysis for the mean values was carried out by F-test followed with Student's *t*-test or Welch's *t*-test.

Results

Histopathological examination

Dam's liver weight was significantly decreased by T-2 toxin. Although thymus weight and fetal weight were not significantly changed, the weights showed a tendency to be decreased (data not shown). At 24 HAT, apoptosis of hepatocytes, cell infiltration and fatty change were observed in the liver from pregnant rats treated with T-2 toxin (fig. 1A). In the placenta, hemorrhage and apoptosis

Table 1. Primer sequences and cycle numbers.

Gene		Sequence	Cycle number			Annealing temperature
			Liver	Placenta	Fetal liver	
CYP1A1	Sense	CTGGTTCTGGATACCCAGCTG	33	31	33	60 °C
	Antisense	CCTAGGGTTGGTTACCAGG				
CYP2B1/2	Sense	GAGTTCTTCTCTGGGTTCCTG	25	28	30	56 °C
	Antisense	ACTGTGGGTCATGGAGAGCTG				
CYP2E1	Sense	CTCCTCGTCATATCCATCTG	20	32	31	56 °C
	Antisense	GCAGCCAATCAGAAATGTGG				
CYP3A1	Sense	ATCCGATATGGAGATCAC	21	35	29	54 °C
	Antisense	GAAGAAGTCCTTGTCTGC				
CYP4A1	Sense	GGTGACAAAGAACTACAGC	24	32	30	54 °C
	Antisense	AGAGGAGTCTTGACCTGCCAG				
GST A1(Ya)	Sense	ATGAGAAGTTTATACAAAGTCC	24	30	35	54 °C
	Antisense	GATCTAAAATGCCTTCGGTG				
GST A2(Yc)	Sense	GATTGACATGTATTCAGAGGGT	23	28	29	56 °C
	Antisense	TTTGCATCCATGGCTGGCTT				
GST-P	Sense	TGCCACCGTACACCATTGTGT	26	23	23	60 °C
	Antisense	CAGCAGGTCCAGCAAGTTGTA				
p53	Sense	ATATGAGCATCGAGCTCCCTCT	34	34	30	58 °C
	Antisense	CACAACTGCACAGGGCATGT				
p21	Sense	AAGTATGCCGTCGTCTGTTCG	29	27	29	58 °C
	Antisense	GGCACTTCAGGGCTTTCTCTT				
GADD45	Sense	GATCGAAAGGATGGACACGG	28	27	28	58 °C
	Antisense	CCCACTGATCCATGTAGCGA				
c-fos	Sense	CATGGACGCTGAAGAGCTACA	34	30	32	58 °C
	Antisense	ACTGACTGAGCTGGTGCATTAC				
c-jun	Sense	CCTACGGCTACAGTAACCCTAA	30	27	27	58 °C
	Antisense	CAGCGTATTCTGGCTATGCAGTT				
Bax	Sense	TTCATCCAGGATCGAGCAGAG	28	26	25	58 °C
	Antisense	TGAGGACTCCAGCCACAAAGAT				
Heme oxygenase-1	Sense	AGCATGCCCCAGGATTTG	30	30	30	58 °C
	Antisense	CTAGCTGGATGTTGAGCAGGA				
Thioredoxin reductase	Sense	GGGAAATTCATTGGTCCTCA	27	26	25	58 °C
	Antisense	CACAGCAGCCATACTCCAAA				
Heat shock protein 70	Sense	GAGTCCTACGCCTTCAATATGAAG	35	31	35	58 °C
	Antisense	CATCAAGAGTCTGTCTCTAGCCAA				
GAPDH	Sense	GCTTCACCACCTTCTTGATGTC	23	23	23	58 °C
	Antisense	GAGTATGTCGTGGAGTCTACTG				

of cytotrophoblasts were observed (fig. 1B). In the fetuses, increased apoptosis of hematopoietic cells was observed in the liver (fig. 1C). These observed changes were the same as those of our previous report (Sehata et al. 2003). TUNEL staining and morphometry examination demonstrated that apoptosis was significantly induced in the liver, placenta, and fetal liver by T-2 toxin treatment (figs. 1D, E, F and fig. 2).

Microarray analysis

The selected results of microarray analysis in the liver, placenta, and fetal liver from pregnant rats treated with T-2 toxin are shown in tables 2, 3 and 4. Tables show the selected genes that we mainly focused on (apoptosis, metabolizing enzyme genes, and oxidative stress-related genes). In the liver, the expression of 235 genes was induced and the 382 genes were suppressed by T-2 toxin. The expression of p53, p21, cyclin G, cyclin D3, Bax-α, BOD-L and extracellular signal-related kinase (ERK3) (cell cycle- and apoptosis-related genes) was increased. In addition, the expression of Thioredoxin reductase and heme oxygenase (oxidative stress-related genes) was also increased. On the other hand, the expression of lipid metabolism-related genes and drug metabolizing enzymes-related genes was suppressed. Gene expression of DNA polymerase alpha was also suppressed. The expression of liver catalase, thioredoxin and CuZnSOD genes was decreased (table 2).

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 c-fos, c-jun, gas-5, GADD45, and PCNA (cell cycle- and apoptosis-related

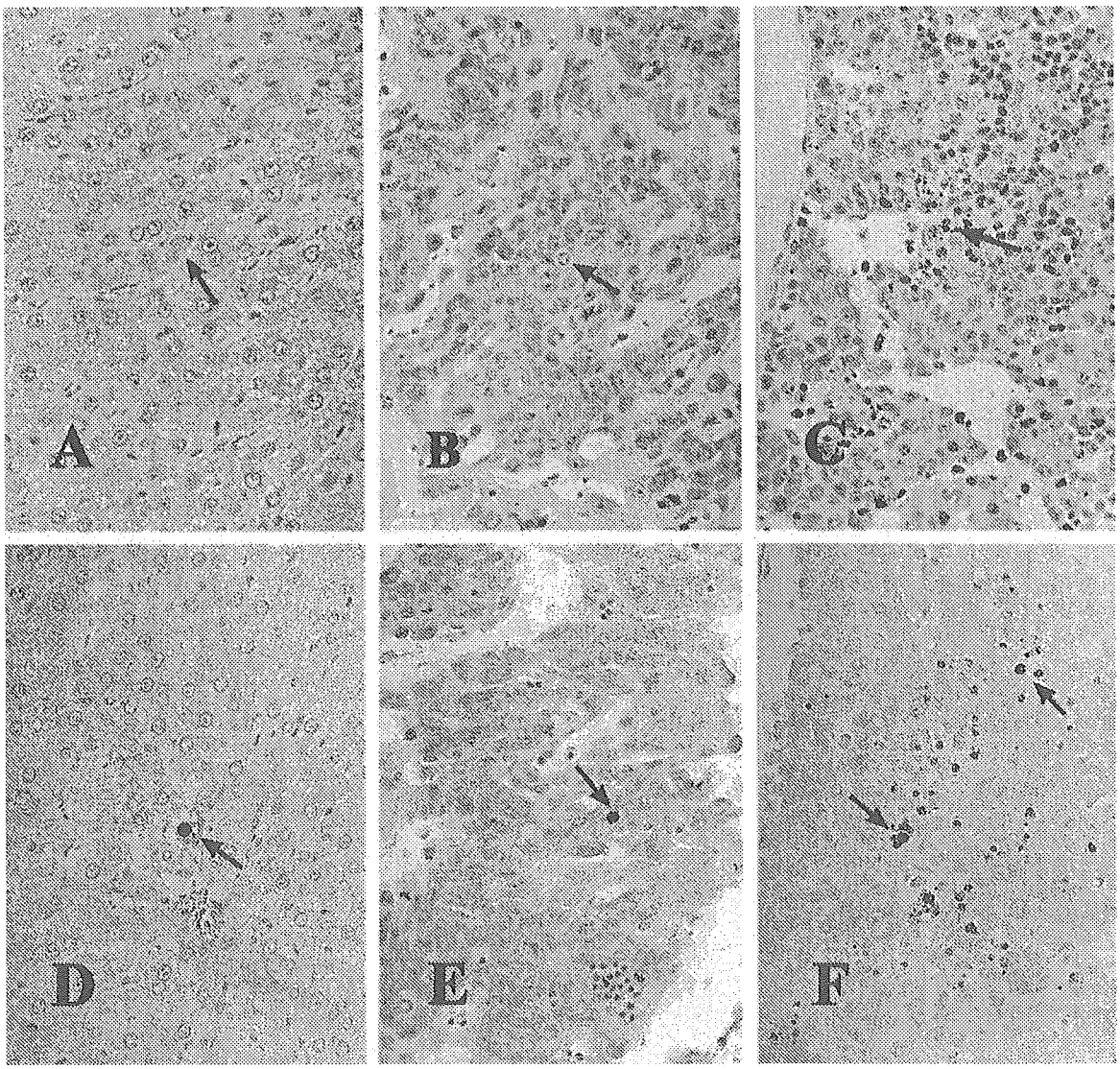

Fig. 1. Histopathological photographs of the liver, placenta and fetal liver from dams treated with T-2 toxin. Apoptotic cells (arrows) are detected in the liver (**A** and **D**), placenta (**B** and **E**) and fetal liver (**C** and **F**). HE staining, ×200 (A, B and C). TUNEL staining, ×200 (D, E and F).

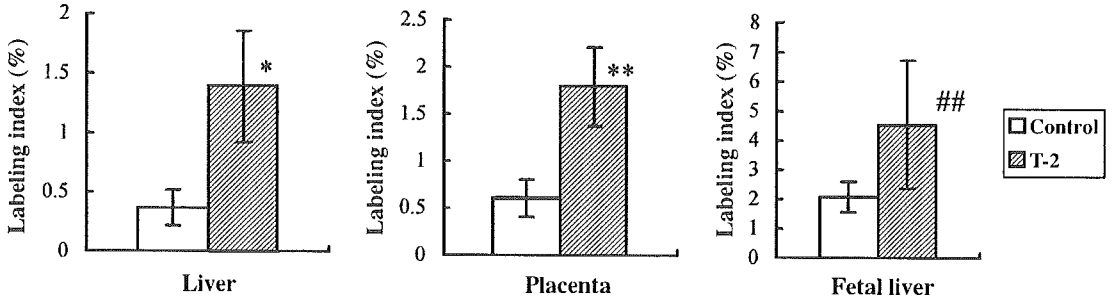

Fig. 2. TUNEL labeling index in the liver, placenta and fetal liver. Apoptosis was significantly induced by T-2 toxin in these tissues. Each value represents mean ± SD of 3 (liver) or 3/dam (placenta and fetal liver). * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test), ## $p < 0.01$ (Welch's *t*-test): significantly difference from the control group.

Table 2. Gene expression changes in the dam liver treated with T-2 toxin.

Genes	Fold change	Accession No.
Apoptosis and cell cycle-related genes		
cyclin G	9.29##	X70871
p21 protein (cip1)	13.08**	L41275
extracellular signal-related kinase (ERK3)	3.29**	M64301
cyclin D3	1.66**	D16309
Bax-alpha	2.87#	U59184
nuclear oncoprotein p53	3.38**	X13058
14-3-3 protein mRNA for MSF S1 subunit	2.53**	D30740
Bcl-2 related ovarian death gene product BOD-L	1.89*	AF065433
gas-5 growth arrest homolog non-translated mRNA sequence	4.74#	U77829
DNA polymerase alpha	–8.79**	M15114
Lipid metabolism-related genes		
phospholipase A-2-activating protein (palp)	1.69**	U17901
PS-PLA1	8.92#	D88666
3-hydroxy-3-methylglutaryl coenzyme A reductase	5.37#	M29249
fatty acid translocase/CD36	–10.19**	AF072411
peroxisomal enoyl-CoA- hydrotase-3-hydroxyacyl-CoA bifunctional enzyme	–34.63**	K03249
isovaleryl-CoA dehydrogenase (IVD)	–4.58**	J05031
type I 5 alpha-reductase	–5.47##	S81448
very-long-chain acyl-CoA synthetase	–4.62**	D85100
Drug metabolizing enzyme genes		
glutathione S-transferase Yc1 subunit	–3.59**	S72505
CYP 3A1	–6.62**	X64401
cytochrome P-450 ISF/BNF-G	–18.96##	M26127
cytochrome P450IIE1	–3.64**	M20131
liver glutathione S-transferase Ya subunit	–3.97**	K00136
cytochrome P-452	–1.78*	X07259
cytochrome P-450b (phenobarbital-inducible)	–7.23*	M11251
Stress-related genes		
thioredoxin reductase	4.14**	U63923
heme oxygenase	30.38#	J02722
liver catalase	–6.33**	M11670
thioredoxin	–1.37**	U73525
Cu, Zn superoxide dismutase	–2.04*	M25157

Significant difference from control; *, $p < 0.05$; **, $p < 0.01$ (Student's t-test); #, $p < 0.05$; ##, $p < 0.01$ (Welch's t-test).

genes) were induced. Gene expression of Nedd2/Ich-1 (Caspase-2) was also increased. 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. The expression of prolactin-like protein, spongiotrophoblast specific protein, cdk4 and DNA polymerase alpha was also suppressed (table 3).

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 was strongly increased. The expression of Bax-α (apoptosis-related gene) 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 glutathione S-transferases was suppressed. Gene expression of cyclin D, c-myc, cell cycle protein p55CDC and 14-3-3 protein was suppressed (table 4).

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.

Semiquantitative RT-PCR

We performed semiquantitative RT-PCR on selected genes that we speculated the genes involved in the T-2 toxin-induced toxicity. table 5 and figure 3 show the results of RT-PCR analysis of drug metabolizing enzyme-related genes, apoptosis-related genes and oxidative stress-related genes. The gene expression of P450 isoenzymes and GSTs was suppressed by T-2 toxin in the liver. It showed the tendency of suppression in the placenta and fetal liver. Increased expression of apoptosis-related genes, p21 and c-jun, and p53 and Bax genes showed the tendency of suppression in the liver. In the placenta, increased expression of c-jun and GADD45 was detected, but there was no clear change in the fetal liver. Regarding the oxidative stress-related genes, the expression of heme oxygenase-1 in the liver, heat shock protein 70 in the placenta, and heme oxygenase-1 and heat shock protein 70 in the fetal liver was increased by

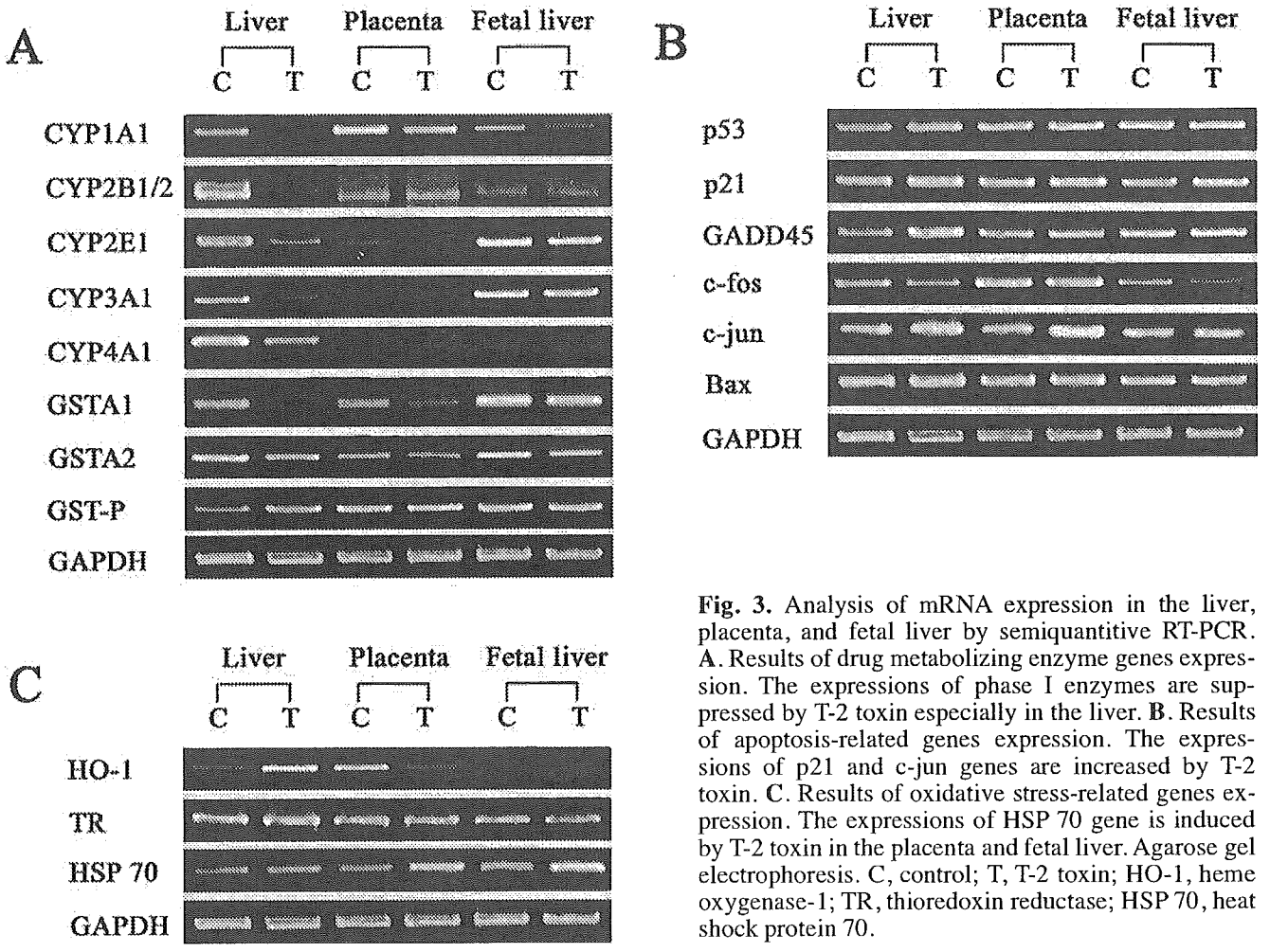

Fig. 3. Analysis of mRNA expression in the liver, placenta, and fetal liver by semiquantitive RT-PCR. **A**. Results of drug metabolizing enzyme genes expression. The expressions of phase I enzymes are suppressed by T-2 toxin especially in the liver. **B**. Results of apoptosis-related genes expression. The expressions of p21 and c-jun genes are increased by T-2 toxin. **C**. Results of oxidative stress-related genes expression. The expressions of HSP 70 gene is induced by T-2 toxin in the placenta and fetal liver. Agarose gel electrophoresis. C, control; T, T-2 toxin; HO-1, heme oxygenase-1; TR, thioredoxin reductase; HSP 70, heat shock protein 70.

Table 3. Genes expression changes in the placenta treated with T-2 toxin.

Genes	Fold change	Accession No.
Apoptosis and cell cycle-related genes		
c-fos	2.35**	X06769
c-jun oncogene mRNA for transcription factor AP-1	2.52**	X17163
gas-5 growth arrest homolog non-translated mRNA sequence	1.82**	U77829
GADD45	2.04**	L32591
Nedd2/Ich-1	1.44*	U77933
proliferating cell nuclear antigen (PCNA/cyclin)	1.85*	M24604
prolactin-like protein B	–2.24**	M31155
spongiotrophoblast specific protein	–6.11**	AB009890
cyclin-dependent kinase 4 (cdk4)	–1.49#	L11007
DNA polymerase alpha	–2.62**	M15114
Lipid metabolism-related genes		
phospholipase A-2-activating protein (palp)	1.53*	U17901
UCP2	2.04*	AB010743
thioesterase II	1.32*	Y00311
cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase	–2.61**	X52625
acyl-coA oxidase	–1.58##	J02752
squalene epoxidase	–2.81**	D37920
hepatic squalene synthetase	–1.52**	M95591
stearoyl-CoA desaturase 2	–4.11**	AF036761
Drug metabolizing enzyme genes		
glutathione S-transferase (GST) Y(b) subunit	–1.63*	X04229
glutathione S-transferase P subunit	–1.63*	X02904
Stress-related genes		
hsp70.2 (heat shock protein 70)	9.67**	Z75029
heat shock protein (Hsp27)	1.31**	M86389
thioredoxin reductase	1.45**	U63923

Significant difference from control; *, $p < 0.05$; **, $p < 0.01$ (Student's t-test); #, $p < 0.05$; ##, $p < 0.01$ (Welch's t-test).

Table 4. Gene expression changes in the fetal liver treated with T-2 toxin.

Genes	Fold change	Accession No.
Apoptosis and cell cycle-related genes		
Bax-alpha	1.74**	U59184
cyclin D1	–1.67**	X75207
c-myc oncogene and flanking regions	–1.73#	Y00396
DNA polymerase alpha	–2.56**	M15114
cell cycle protein p55CDC	–2.09**	AF052695
14-3-3 protein beta subtype	–1.51#	S55223
Lipid metabolism-related genes		
squalene epoxidase	–3.89#	D37920
cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase	–4.41**	X52625
farnesyl diphosphate synthase	–3.88**	M89945
2,3-oxidosqualene:lanosterol cyclase	–2.47#	D45252
fatty acid transporter	–4.11**	AB005743
Drug metabolizing enzyme genes		
glutathione S-transferase Yc2 subunit	–2.99#	S72506
glutathione S-transferase Yc1 subunit	–2.88*	S72505
Stress-related genes		
hsp70.2 (heat shock protein 70)	1039.12#	Z75029

Significant difference from control; *, $p < 0.05$; **, $p < 0.01$ (Student's t-test); #, $p < 0.05$ (Welch's t-test).

Table 5. Analysis of mRNA expression by semiquantitive RT-PCR.

Gene	Organs		
	Liver T-2 toxin	Placenta T-2 toxin	Fetal liver T-2 toxin
Drug-metabolizing enzyme genes			
CYP1A1	0.390 ± 0.639[a)]	0.706 ± 0.519	0.425 ± 0.374*
CYP2B1/2	0.107 ± 0.108**	1.105 ± 0.397	1.833 ± 1.318
CYP2E1	0.262 ± 0.130**	0.550 ± 0.492	0.937 ± 0.318
CYP3A1	0.281 ± 0.086**	1.828 ± 0.632	0.637 ± 0.499
CYP4A1	0.384 ± 0.203**	0.923 ± 0.166	0.646 ± 0.168*
GSTA1	0.324 ± 0.203*	0.516 ± 0.299*	0.682 ± 0.457
GATA2	0.529 ± 0.205#	0.687 ± 0.601	0.693 ± 0.467
GST-P	1.764 ± 1.071	0.873 ± 0.252	0.786 ± 0.480
Apoptosis-related genes			
p53	1.178 ± 0.297	1.215 ± 0.082	0.861 ± 0.232
p21	1.609 ± 0.382*	0.975 ± 0.196	1.061 ± 0.186
GADD45	1.469 ± 0.566	1.081 ± 0.043*	1.162 ± 0.212
c-fos	0.591 ± 0.226*	0.940 ± 0.067	0.540 ± 0.268
c-jun	1.809 ± 0.526*	1.271 ± 0.176*	0.915 ± 0.170
Bax	1.158 ± 0.156	0.928 ± 0.095	0.792 ± 0.150
Oxidative stress-related genes			
Heme oxygenase-1	5.290 ± 2.909*	0.353 ± 0.139	0.005 ± 0.004#
Thioredoxin reductase	1.198 ± 0.182	0.961 ± 0.025	0.937 ± 0.272
Heat shock protein 70	0.905 ± 0.303	3.291 ± 0.270**	2.093 ± 0.692*

Note.[a)], Relative band density. Mean ± SD.
Significant difference from control: *, $p < 0.05$; **, $p < 0.01$ (Student's t-test); #, $p < 0.05$ (Welch's t-test).

T-2 toxin. These results corresponded to the results from microarray analysis, although some genes showed opposite results from those of microarray analysis.

Discussion

It is reported that T-2 toxin induces apoptosis in the lymphoid tissues and intestine (BRATICH et al. 1990; HAYES et al. 1982; HOERR et al. 1981; KOSURI et al. 1971; MARASAS et al. 1969; PANG et al. 1987). In the present study, the induction of apoptosis in the liver, placenta and fetal liver from pregnant rats treated with T-2 toxin was also observed. Apoptotic changes were also observed in the lymphoid tissues including the thymus and spleen, intestine, and fetal brain (SEHATA et al. 2003). Therefore, T-2 toxin may induce the changes of the same nature in the liver, placenta, and fetal tissues. To examine the mechanism of T-2 toxin-induced toxicity in pregnant rats, Wistar rats on GD13 were treated orally with a single dose of T-2 toxin (2 mg/kg), and were sacrificed at 24 HAT, and Microarray analysis was performed in the liver, placenta and fetal liver.

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 (CHANG et al. 1988). Oxidative stress causes lipid peroxidation and induces mitochondrial dysfunction. This mitochondrial dysfunction causes fatty acid β-oxidation and induces fatty liver (JAESCHKE et al. 2002). In the present 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, p53, p21, Bax-α and BOD-L. At the same time, the expression of cell cycle accelerating genes, cyclin G and cyclin D3, was also induced by T-2 toxin. Furthermore, the expression of heat shock protein (HSP) 70 and HSP 27, oxidative stress-related genes and repressor of apoptosis (GARRIDO et al. 2001), 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.

It is reported that the gene expression of phase I and II enzymes was decreased by T-2 toxin (GALTIER et al. 1989; GUERRE et al. 2000). One of the causes is considered that T-2 toxin inhibits protein and DNA synthesis (ROSENTEIN and LAFARGE-FRAYSSINET 1983; THOMPSON and Wannemacher 1990). In the present study, the expression of P450 and GSTs genes in the liver was suppressed

by T-2 toxin. However, the expression of DNA synthesis-related genes was induced by T-2 toxin, suggesting that there are other mechanisms of P450 and GST suppression. As described above, T-2 toxin enhanced lipid peroxidation indicated by increased diene formation (CHANG et al. 1988). In addition, lipid peroxidation caused by oxidative stress might affect the P450 content in the liver (SERBINOVA et al. 1989). Therefore, oxidative stress might be an effective factor of P450 suppression. In the placenta and fetal liver, GST genes were suppressed by T-2 toxin in microarray analysis, and the results of RT-PCR also showed the tendency of suppression in these genes. In general, CYP3A1 expression was detected in the rat placenta and fetal liver through pregnancy (EJIRI et al. 2001; EJIRI et al. 2003). It is reported that CYP2B1, 2B2, 2E1 and 4A1 mRNAs were detected in the fetal liver, but other P450s were not done (BORLAKOGLU et al. 1993; OMIECINSKI et al. 1990). In the present study, although we detected all P450 genes examined, the expression differed by each gene. Moreover, these genes in the placenta and fetal liver showed the tendency of suppression by T-2 toxin treatment. The results obtained from RT-PCR were consistent with the results from the microarray analysis.

In comparison with the results from RT-PCR analysis on dam's liver and fetus, the expression levels of some genes showed opposite direction. The reason for these results is unclear, but it may suggest that there are differences in reaction to T-2 toxin and/or in responding genes to T-2 toxin between dam and fetus.

In the present study, rats on day 13 of gestation were treated orally with T-2 toxin and were sacrificed at 24 hours after treatment. 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

ALBARENQUE SM, SUZUKI K, SHINOZUKA J, et al.: Kinetics of apoptosis-related genes mRNA expression in the dorsal skin of hypotrichotic WBN/ILA-*Ht* rats after topical application of T-2 toxin. Exp Toxic Pathol 2001; **52**: 553–556.

BORLAKOGLU JT, SCOTT A, HENDERSON CJ, et al.: Expression of P450 isoenzymes during rat liver organogenesis. Int J Biochem 1993; **25**: 1659–1668.

BRATICH PM, BUCK WB, HASCHEK WM: Prevention of T-2 toxin-induced morphological effects in the rat by highly activated charcoal. Arch Toxicol 1990; **64**: 251–253.

BULERA SJ, EDDY SM, FERGUSON E, et al.: RNA expression in the early characterization of hepatotoxicants in Wistar rats by high-density DNA microarrays. Hepatology 2001; **33**: 1239–1258.

CHANG I-M and MAR W-C: Effect of T-2 toxin on lipid peroxidation in rats: elevation of conjugated diene formation. Toxicol Lett 1988; **40**: 275–280.

EJIRI N, KATAYAMA K, NAKAYAMA H, et al.: Expression of cytochrome P450 (CYP) isozymes in rat placenta through pregnancy. Exp Toxic Pathol 2001: **53**: 387–391.

EJIRI N, KATAYAMA K, Doi K: Induction of CYP3A1 by dexamethasone and pregnenolone-16_-carbonitrile in pregnant rat and fetal livers and placenta. Exp Toxic Pathol 2003: **54**: 273–279.

GALTIER P, PAULIN F, EECKHOUTTE C, et al.: Comparative effects of T-2 toxin and diacetoxyscirpenol on drug metabolizing enzymes in rat tissues. Food Chem Toxicol 1989; **27**: 215–220.

GAN L, LIU Q, XU H-B, et al.: Effects of selenium overexposure on glutathione peroxidase and thioredoxin reductase gene expressions and activities. Biol Trace Elem Res 2002; **89**: 165–175.

GARRIDO C, GURBUXANI S, RAVAGNAN L, et al.: Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun.2001; **286**: 433–442.

GOLUB TR, SLONIM DK, TAMAYO P, et al.: Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; **286**: 531–537.

GUERRE P, EECKHOUTTE C, BURGAT V, et al.: The effects of T-2 toxin exposure on liver drug metabolizing enzymes in rabbit. Food Addit Contam 2000; **17**: 1019–1026.

HAYES MA and SCHIFFER FB: Comparative toxicity of dietary T-2 toxin in rats and mice. J Appl Toxicol 1982; **2**: 207–211.

HOERR FJ, CARLTON WW, YAGEN B: Mycotoxicosis caused by a single dose of T-2 toxin or diacetoxyscirpenol in broiler chickens. Vet Pathol 1981; **18**: 652–664.

IARC (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER): (1993). Toxins derived from Fusarium sporotrichioides: T-2 toxin. In IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 56, pp. 467–488. IARC (WHO Secretariat), Lyon.

ISHIGAMI N, SHINOZUKA J, KATAYAMA K, et al.: Apoptosis in mouse fetuses from dams exposed to T-2 toxin at different days of gestation. Exp Toxic Pathol 2001; **52**: 493–501.

JAESCHKE H, GORES GJ, CEDERBAUM A, et al.: Mechanisms of hepatotoxicity. Toxicol Sci 2002; **65**: 166–176.

KATAYAMA K, OHTSUKA R, TAKAI H, et al.: Expression of p53 and its transcriptional target genes mRNAs in the ethylnitrosourea-induced apoptosis and cell cycle arrest in the fetal central nervous system. Histol Histopathol 2002; **17**: 715–720.

KOSURI NR, SMALLEY EB, NICHOLS RE: Toxicologic studies of *Fusarium tricinctum* (corda) Synder et Hansen from moldy corn. Am J Vet Res 1971; **32**: 1843–1850.

LAFARGE-FRAYSSINET C, CHAKOR K, LAFONT P, et al.: Transplacentral transfer of T2-toxin: pathological effect. J Environ Pathol Toxicol Oncol 1990; **10**: 64–68.

LIU H, SONG D, LEE SS: Role of heme oxygenase-carbon monoxide pathway in pathogenesis of cirrhotic cardiomyopathy in the rat. Am J Physiol Gastrointest Liver Physiol 2001; **280**: G68–G74.

LUCERI C, CADERNI G, SANNA A, et al.: Red wine and black tea polyphenols modulate the expression of cycloxygenase-2, inducible nitric oxide synthase and glutathione-related enzymes in azoxymethane-induced F344 rat colon tumors. J Nutr 2002; **132**: 1376–1379.

LUEHE A, HILDEBRAND H, BACH U, et al.: A new approach to study ochratoxin A (OTA) induced nephrotoxicity: expression profiling in vivo and in vitro employing cDNA-microarrays. Toxicol Sci 2003; **73**: 315–328.

MARASAS WFO, BAMBURG JR, SMALLEY EB, et al.: Toxic effects on trout, rats and mice of T-2 toxin produced by the fungus *Fusarium tricinctum* (corda) Synder et Hansen. Toxicol Appl Pharmacol 1969; **15**: 471–482.

MORRIS DL and DAVILA JC: Analysis of rat cytochrome P450 isoenzyme expression using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Biochem Pharmacol 1996; **52**: 781–792.

NAKAHARA T, HIRANO M, UCHIYAMA H, et al.: Chronic alcohol feeding and its influence on c-fos and heat shock protein-70 gene expression in different brain regions of male and female rat. Metabolism 2002; **51**: 1562–1568.

OMIECINSKI CJ, HASSETT C, COSTA P: Developmental expression and *in situ* localization of the phenobarbital-inducible rat hepatic mRNAs for cytochromes CYP2B1, CYP2B2, CYP2C6, and CYP3A1. Mol Pharmacol 19990; **38**: 462–470.

PANG VF, LORENZANA RM, BEASLEY VR, et al.: Experimental T-2 toxicosis in swine: III. Morphologic changes following intravascular administration of T-2 toxin. Fundam Appl Toxicol 1987; **8**: 298–309.

ROSENTEIN Y and LAFARGE-FRAYSSINET C: Inhibitory effect of Fusarium T2-toxin on lymphoid DNA and protein synthesis. Toxicol Appl Pharmacol 1983; **70**: 283–288.

ROUSSEAUX CG AND SCHIEFER HB: Maternal toxicity, embryolethality and abnormal fetal development in CD-1 mice following one oral dose of T-2 toxin. J Appl Toxicol 1987; **7**: 281–288.

SEHATA S, TERANSHIM, ATSUMI F, et al.: T-2 toxin-induced morphological changes in pregnant rats. J Toxicol Pathol 2003; **16**: 59–65.

SERBINOVA EA, KAHIISKA MB, BAKALOVA RA, et al.: Lipid peroxidation activation and cytochrome P-450 decrease in rat liver endoplasmic reticulum under oxidative stress. Toxicol Lett 1989; **47**: 119–123.

STANFORD GK, HOOD RD, HAYES AW: Effect of prenatal administration of T-2 toxin to mice. Res Comm Chem Pathol Pharmacol 1975; **10**: 743–746.

THOMPSON WL and WANNEMACHER RW JR: In vivo effects of T-2 mycotoxin on synthesis of proteins and DNA in rat tissues. Toxicol Appl Pharmacol 1990: **105**: 483–491.

UENO M, KATAYAMA K, NAKAYAMA H, et al.: Mechanisms of 5-azacytidine (5AzC)-induced toxicity in the rat foetal brain. Int J Exp Path 2002; **83**: 139–150.

VANHASECKE T, LINDORS KO, OINONEN T, et al.: Effect of long-term ethanol exposure on the acinar distribution of hepatic glutathione S-transferase. Drug Metab Disposit 2000; **28**: 1470–1474.

WARINGJF, JOLLY RA, CIURLIONIS R, et al.: Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. Toxicol Appl Pharmacol 2001; **175**: 28–42.

WHO (WORD HEALTH ORGANIZATION): Selected mycotoxins: ochratoxins, trichothecenes, ergot. Environmental Health Criteria 1990; **105**, WHO, Geneva.

YANG MCK, RUAN QG, YANG JJ, et al.: A statistical method for flagging weak spots improves normalization and ratio estimates in microarrays. Physiol Genomics 2001; **7**: 45–53.

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Full Paper

Nephrin and Podocin Expression Around the Onset of Puromycin Aminonucleoside Nephrosis

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Abstract. Decreased expression levels of the glomerular slit membrane proteins, nephrin and podocin, have been reported after the onset of puromycin aminonucleoside (PA) nephrosis. We examined nephrin and podocin expressions prior to the onset of proteinuria of PA nephrosis to elucidate the proteinuria induction mechanism of PA. PA nephrosis was induced by a subcutaneous single injection of 120 mg kg^{-1} PA. The mRNA levels of nephrin and podocin in whole kidney total RNA were quantified by the TaqMan real time PCR quantification system. The localization and levels of nephrin and podocin molecules were analyzed by immunofluorescence and Western blotting, respectively. Albuminuria and proteinuria were significant on days 3 and 4 in PA nephrosis rats. The protein levels of nephrin and podocin decreased significantly at day 3. The protein localization of nephrin and podocin changed at day 2 and day 1, respectively. The mRNA level of nephrin increased at day 2 and subsequently decreased at day 4. The podocin mRNA level did not change significantly. In conclusions, the protein level of nephrin and podocin decreased at the onset of albuminuria in the PA nephrosis. However, the first change induced by PA was the change of podocin localization from a linear pattern to a dot-like one prior to the onset of albuminuria.

Keywords: nephrin, podocin, nephrosis, puromycin aminonucleoside

Introduction

Recent studies have suggested that proteinuria is a predictive independent risk factor for the progression of renal disease (1). Therefore, the mechanism of proteinuria should be elucidated to prevent the progression of renal disease. Puromycin aminonucleoside (PA) was identified as an inducer of proteinuria in rats. PA nephrosis has been used as an experimental model of human nephrotic syndrome with minimal change (2). However, the detailed mechanism of proteinuria induction by PA has yet to be determined.

With respect to genes related to nephrosis, NPHS1 and NPHS2 were identified as mutated genes in the human congenital nephrotic syndrome of the Finnish type (3) and in patients with autosomal recessive steroid-resistant nephrotic syndrome (4), respectively. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis in humans (5). Gene targeting studies also revealed that the CD2AP gene (6) and the Nephl gene (7) were responsible for nephrosis in mice. The respective gene product NPHS1, termed nephrin, as well as the Nephl molecule, is a transmembrane glycoprotein of the slit diaphragm in the glomeruli, belonging to the immunoglobulin-like molecules (8). The respective gene product NPHS2, named podocin, is an integral protein of the slit diaphragm belonging to the stomatin family, and it has been shown to interact with nephrin (9) and Nephl (10), as well as CD2AP (11).

The disappearance of the nephrin (12, 13) and podocin (14, 15) proteins in PA nephrosis has been

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