Updated Hazard Assessment of Bisphenol A

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The Research Institute of Science
for Safety and Sustainability (RISS)

National Institute of
Advanced Industrial Science
and Technology (AIST)

Introduction

The Research Institute of Science for Safety and Sustainability (RISS), National Institute of Advanced Industrial Science and Technology (AIST) assessed the human health hazard of bisphenol A (BPA) as well as its exposure level in Japan, and published it as "Risk Assessment Document Series No.6, Bisphenol A (in Japanese)" [1] (Nakanishi et al., 2005). Many challenges still remained at that time, such as "low dose issues". However, studies from many fields were subsequently carried out, and a tremendous amount of new information on BPA with regard to human health hazard has been published. Therefore, some information gaps generated in our previous publication now exist due to the incomparable accumulation of information regarding its potential as a human health hazard. Moreover, updates on the risk assessment of BPA by the European Union (EC, 2008) and a summary of a review report regarding the human health hazard of BPA from a joint expert meeting of the Food and Agriculture Organization and World Health Organization (WHO, 2010) were published. Under these circumstances, we believed that there was a need to promptly re-examine our hazard assessment of BPA with regard to human health and so initiated an update from July of 2010. We herein have organized the latest information as of this moment, and have summarized the updated view of our institute, although there still remain many uncertainties regarding the human health hazard associated with BPA, and all arguments have not yet been settled.

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[1] English version was published in 2007. (http://www.aist-riss.jp/main/modules/product/rad.1.html)

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Abstract

We closely investigated the information on bisphenol A (BPA) regarding its potential as a human health hazard from 2005 onwards, and updated the hazard assessment of BPA. Since reproductive toxicity in the next generation was one of concerns with regard to the human health hazard induced by BPA, a two-generation reproductive study using mice under OECD GLP was carried out. However, no toxic effects on the reproductive potential of the next generation except for a slight prolongation of gestational length of F1/F2 at 300 mg/kg bw, and a no-observed-adverse-effect level (NOAEL) of 50 mg/kg bw have been noted. Regarding the general toxicity of BPA, multinucleated giant hepatocytes, centrilobular hepatocytomegaly, centrilobular hepatocyte hypertrophy and nephropathy were observed in mice. Considering that the NOAEL of centrilobular hepatocyte hypertrophy (3 mg/kg bw) found in mice was the lowest, this finding was determined as the endpoint of general toxicity upon oral administration of BPA.

The carcinogenicity of BPA by oral administration has already been determined to be negative from bioassays. With regard to skin irritation, skin sensitization, skin photo-irritability, and photo-sensitization due to BPA, it was believed that there is almost no need for concern because these were found to be negative in animal testings at a practical dose level.

Regarding the developmental neurotoxicity of BPA, a GLP-compliant rat developmental neurotoxicity study on BPA under the OECD testing guideline 426 and testing guideline 870.6300 of the U.S. EPA OPPTS was performed. However, evaluating the developmental neurotoxicity of BPA in our institute was not carried out because the validity of the testing protocol on chemical compounds with estrogenic activities has not yet been proven, although the protocol is valid to detect known developmental neurotoxicants. Moreover, the RISS determined that any influence on the brain function as well as behavior of children exposed to BPA in-utero or via breast milk cannot currently be evaluated because reports related to sexually differentiation of the brain, sexual behavior, social behavior, brain neurotransmitters, receptor expression, etc. in experimental animals caused by prenatal or neonatal exposure of BPA were all too uncertain to conclude as being adverse to humans.

Accordingly, NOAEL for the hazard assessment of BPA was determined to be 3 mg/kg bw/day, with the uncertainty factor of 25 (= species difference: 2.5 x individual difference: 10), although NOAEL and uncertainty factor were determined to be 5 mg/kg bw/day and 100, respectively, in our previous publication (Nakanishi et al., 2005). In the present assessment, the uncertainty factor related to species difference with regard to the extrapolation of animal data to human has been determined to be 2.5 because BPA

has been shown to be detoxified mainly by glucuronide-conjugation in the liver, but it is rapidly metabolized and excreted in humans in comparison to rodents.

According to the BPA exposure estimate in Japanese individuals (Miyamoto and Kotake, 2006), exposure was highest in 1 to 6 year-olds, with an estimated 95% tile value of 3.9 μg/kg bw/day (men) and 4.1 μg/kg bw/day (women). In addition, the 95% tile value of BPA intake estimated from the amount of BPA excreted in 24-hour urine in adults was 0.037 to 0.064 μg/kg bw/ day (men) and 0.043 to 0.075 μg/kg bw/day (women). Using the 95% tile value of these exposure estimates and the NOAEL (3 mg/kg bw/day) from the animal testings, the Margin of Exposure (MOE) became 730 to 770 in 1 to 6 year-olds, and 40,000 to 81,000 in adults. These values were much larger than the MOE (25) estimated to cause health hazards in humans mentioned above or the conventional and conservative MOE (100), and thus the risk of BPA with regard to human health was believed to be very small.

1. New information on the human health hazard of BPA

1.1 Epidemiology

1.1.1 Reproductive toxicity

Sugiura-Ogasawara et al. (2005) carried out a case-controlled study to examine the effect of bisphenol A (BPA) exposure on miscarriages. More specifically, the blood BPA concentration (measured by ELISA) of 45 patients with a history of 4 consecutive first-trimester miscarriages on average was compared to that of 32 healthy women with no history of live birth and infertility. As a result, while the BPA concentration was 0.77 ± 0.38 ng/mL in the control group, it was 2.59 ± 5.23 ng/mL ($p < 0.024$) in the patient group. Among the patients, 15.6% had hypothyroidism, 13.3% were positive for anti-phospholipid antibody (Ab), 22.2% were positive for anti-nuclear Ab, and the BPA concentration in patients that were positive for anti-nuclear Ab was significantly higher compared to that of patients that were negative for anti-nuclear Ab. Among all patients, 35 patients subsequently became pregnant, with 17 patients having a normal delivery and 1 patient having an ectopic pregnancy, but 17 patients (48.6%) suffered a miscarriage again. The blood BPA in patients having another miscarriage was 4.39 ± 8.08 ng/mL, and 1.22 ± 1.07 ng/mL in patients having a normal delivery. From these results, the authors of this study concluded that an evident correlation existed between BPA exposure and miscarriage. However, Bekowitz (2006) pointed out many issues on this report, commenting that a correlation between BPA and miscarriage could not be suggested with the data used, due to the following reasons; a) The blood half-life ($t_{1/2}$) of BPA is 5 hours or less, so the timing of blood collection is important and should be carried out in close timing with the assumed phenomenon (miscarriage), but this does not seem to be the case. b) The median of blood BPA concentration was approximately the same in both patient and control groups, with several high values in the patient groups pushing up the mean value. In such cases, the values should be log-transformed for the statistical analysis. c) The fertility data of the control group, such as the history of live births, infertility and miscarriage, was not revealed, rendering it unable to be used as a control for the patient group. Additionally, other factors causing miscarriage (confounding factors) were not considered. d) There is no mention of hypothyroid disease and/or the rate of positive anti-nuclear Ab in the control group. And, e) among all patients, 35 became pregnant, with half of them having normal deliveries; however, the median blood BPA concentration in these patients (0.91 ng/mL) was higher than that of patients having a miscarriage (0.71

ng/mL), and conflicted with the hypothesis that an association exists between BPA and miscarriage.

Mendiola et al. (2010) investigated the relationship between total-BPA (free and conjugates, same hereafter) concentration in spot urine and the reproductive parameters of men with reproductive potential, such as semen quality and/or testosterone. Participants in this study (n = 375) were the partners of pregnant women in the United States, with all men donating a sample of their blood, semen, and urine. Serum reproductive hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, inhibin B, estradiol, sex hormone-binding globulin (SHBG) and free androgen index (FAI) were analyzed. As a result, no significant correlation was observed between the semen parameters and urine total-BPA concentration (median: 1.7 μg/L). However, there was a significant positive correlation between the urine total-BPA concentration and SHBG as well as a significant inverse correlation between the urine total-BPA concentration and FAI and/or FAI/LH ratio. From the results above, the authors of this study determined the effects of BPA on the reproductive functions to be slight, with any clinical significance being uncertain, although there is a possibility that exposure to BPA at low concentrations is related to the decline in free testosterone index.

Meeker et al. (2010) investigated the total-BPA concentration in spot urine, semen quality and DNA damage (comet assay) in the sperm of 190 men gathered in a fertility clinic. BPA was detected in 89% of samples, with a median concentration of 1.3 (interquartile range was 0.8 to 2.5) ng/mL. The urine total-BPA concentration appears to correlate with sperm concentration and motility, although it was not statistically significant. Moreover, the urine total-BPA concentration also correlated with the decreased rate of normal morphology of the sperm. When it was modeled as continuous dependent variables, the increase in the interquartile range of the blood total-BPA concentration correlated with sperm concentration, motility, decrease in normal morphology, and increase in sperm DNA damage. From the results above, the authors determined that the urine total-BPA may be correlated with the decline in semen quality and the increase in sperm DNA damage. However, the authors themselves indicated that these correlations need to be re-evaluated by other large and appropriately designed human epidemiologic studies that measure BPA in multiple urine samples across the exposure window of interest because some inconsistencies were observed in their results between statistical and exposure assessment approaches.

Li et al. (2010) investigated the correlation between the total-BPA concentration in

spot urine and semen quality in 218 male workers occupationally exposed to BPA in a factory in China. Six parameters including semen volume, total sperm count, concentration, sperm vitality, motility and morphology were used as parameters for semen quality. The median urine BPA concentration (μg/g creatinine) of workers occupationally exposed to BPA and workers not exposed to BPA were 38.7 and 1.4, respectively, with the 25% tile to the 75% tile being 6.3 to 354.3 and 0.0 to 17.9, respectively. When confounding factors were adjusted by linear regression analysis, the increase in urine total-BPA concentration significantly correlated with the decrease in sperm concentration and total sperm count, as well as the decline in sperm vitality and/or sperm motility. However, there was no correlation observed between semen volume and/or semen motility. Moreover, based on the findings of a logistic regression analysis, men with BPA detected in their urine were found to be 3 times or more at risk of having a low sperm concentration and low sperm vitality, 4 times or more at risk of low sperm count, and 2 times or more at risk of low sperm motility, compared to men with no BPA detected in their urine. Furthermore, when men detected as having BPA concentrations in their urine were divided into tertile numbers, the increase in urine total-BPA concentration by tertile number was related to an increased risk of low quality sperm (sperm concentration, vitality, and motility). The authors determined that these results are the first epidemiologic evidence of adverse events to sperm quality caused by BPA.

Mok-Lin et al. (2010) investigated the relationship between the total-BPA concentration in spot urine of women who underwent in-vitro fertilization and the ovarian response (number of oocytes and serum estradiol level) by a prospective cohort study. The urine total-BPA concentration in 84 women who underwent in-vitro fertilization 112 times was measured, and was corrected by the specific gravity of the urine. The geometric mean of the total-BPA concentration ranged from less than 0.4 μg/L to 29.6 μg/L, with a median of 2.28 μg/L. The mean value was then normalized by age, body mass index (BMI), and concentration of FSH on the 3rd day of menstruation, using a mixed effect model and/or Poisson's regression model. The relationship of the log-transformed total-BPA concentration with the maximum serum estradiol concentration or the total number of oocytes retrieved was evaluated. As a result, BPA was detected in the majority of women undergoing in-vitro fertilization, and an inverse correlation was observed between the urine total-BPA concentration and the numbers of follicles collected per in-vitro fertilization period and/or peak serum concentration of estradiol.

1.1.2 Effects on children

Wolff et al. (2008) reported a cross-sectional study, which was carried out from 1996 to 1997, on 192 healthy 9 years old girls residing in New York City with regard to the association of BPA exposure at pubertal stage. More specifically, the associations regarding the developments of breasts and pubic hair with the concentrations of urine phytoestrogen, urine total-BPA, serum DDE, serum PCB, serum lead were statistically analyzed. However, no relationship was observed between the urine total-BPA concentration and the pubertal development indexes.

Wolff et al. (2010) reported a large scale prospective cohort study on 1,151 girls residing in New York City, Cincinnati, and North California with regard to the association of BPA exposure with pubertal stage. During the study period (2004 to 2007), the total-BPA concentration in spot urine of girls aged 6 to 8 was measured, and subsequently, the degree of development of breasts and pubic hair was investigated. The correlation between the degree of pubertal development and urine total-BPA concentration was analyzed, but no correlation was observed.

Braun et al. (2009) carried out a prospective cohort study on 249 women and their children residing in Cincinnati regarding the association of BPA exposure and early childhood behavior. The maternal total-BPA concentrations in spot urine at gestational weeks 16 and 26, and at birth were measured. The median total-BPA concentration at gestational weeks 16 and 26, and at birth was 1.8, 1.7 and 1.3 ng/mL, respectively. The childhood behavior was evaluated by using the Behavioral Assessment System for Children (BASC-2) Parent Rating Scale for preschoolers at 2 years old, in which the externalizing behavior score of 50 is believed to be average, 60 to 69 to be at risk and 70 or more to be clinically significant. Upon analyses with consideration of various confounding factors (maternal age, race, education level, marital status, annual income, depressive state, child care environment, gender and age of the child, etc.) the log-transformed maternal urine total-BPA concentration during gestational period was found to correlate with the reduced externalizing behavior score of children. Above all, a stronger correlation was observed in the maternal urine total-BPA concentration at gestational week 16 compared to the BPA concentration at gestational week 26 or at birth, and was more strongly observed in male children than female children. More specifically, the externalizing behavior scores of female children was about 8 points lower than the average control value if the maternal urine total-BPA concentration at gestational week 16 was low, but became closer to the average value as the total-BPA concentration increased.

Miodovnik et al. (2010) collected maternal spot urine samples (137 patients) who

delivered at Mount Sinai Hospital in New York City during pregnancy between 25 and 40 (average: 31.2 weeks), measured the total-BPA, and analyzed the correlation between prenatal exposure to BPA and social behavior of the child. Social behavior of the child was evaluated by the mother using a Social Responsive Scale (SRS) when the child was 7 to 9 years old. There was no correlation observed between the maternal urine total-BPA concentration and SRS scores of the child.

1.1.3 Others

Itoh et al. (2007) compared the stage of endometriosis patients (n = 166) and total-BPA concentration in spot urine in Japanese women, but no correlation between the stages of endometriosis and the urine total-BPA concentration was observed.

Considering that mammary tumorigenesis in Korean women has increased within the last 20 years, Yang et al. (2009) suspected a correlation with BPA exposure and carried out a case-controlled study. The blood BPA-conjugate concentrations of patients diagnosed with breast cancer (n = 70) at the Seoul National University Hospital, SNUH from 1994 to 1997 and control subjects who consulted SNUH over the same period under suspicion of breast cancer but did not have breast cancer (n = 82) were compared. As a result, the median of the BPA-conjugate concentration of patients was higher than that of the control group, but there was no statistical difference.

Among all study subjects registered in the U.S. National Health and Nutrition Examination Survey (NHANES) in 2003/2004 and 2005/2006, Melzer et al. (2010) analyzed the correlation between the total-BPA concentration in spot urine and cardiovascular diseases (CVD) such as heart attack, coronary heart disease, angina pectoris, as well as diabetes and serum liver enzyme activities of patients from 1,455 and 1,493 patients, respectively, aged 18 to 74 years old using a regression model. The regression model was normalized by age, gender, race, education level, household annual income, smoking, BMI, as well as waist circumference and urine creatinine. A positive correlation was observed between the high value of total-BPA in spot urine and CVD in the subjects of 2005/2006. There was also a positive correlation when the subjects of 2003/2004 and 2005/2006 were added up. A positive correlation between the urine total-BPA concentrations with diabetes was not observed in the subjects from 2005/2006, but was observed when joined with subjects from 2003/2004. Of serum concentrations of liver enzymes, there was no correlation between urine total-BPA concentrations with γ-glutamyl transferase (GGT), but a positive correlation with alkaline phosphatase (ALP) and lactate dehydrogenase

(LDH) when the subjects from 2003/2004 and 2005/2006 were joined together.

1.2 Acute toxicity

There is no new information related to acute toxicity.

1.3 Irritability and sensitization potential

New information mentioning a 30% concentration of BPA as being negative in skin sensitization, skin photo-irritation, and photo-sensitization tests in mice based on a GLP-compliant study using modified LLNA (local lymph node assay) method is available, according to EC (2008). In support of the animal data, no reports related to skin sensitization due to BPA exposure according to the recent medical checks of a total of 875 workers in 5 of 6 BPA plants within the EU have been informed. However, this information was personally provided to the European Chemical Bureau (ECB) by Plastic Europe (EC, 2008).

1.4 Repeated dose toxicity

Data from two new studies are now available. The first study was a 13-week dietary toxicity study using CD-1 mice (unpublished, cited in EC, 2008), carried out to determine the dose levels for a two-generation reproductive toxicity study. According to EC (2008), there were no effects in terms of toxic signs, deaths, or body weight changes in males by the administration of BPA except an increase in centrilobular hepatocyte hypertrophy from the lowest dose level, 500 ppm (74 mg/kg bw), and an increase in the relative liver weight from 2,000 ppm (298 mg/kg bw). In females, an increase in the relative liver weight at 500 ppm (100 mg/kg bw) and more, degeneration and necrosis of hepatocytes at 2,500 ppm (487 mg/kg bw) and more, and nephropathy at 3,500 ppm (728 mg/kg bw) were noted. NOAEL was not obtained in this study because toxic effects were observed at the lowest dose level.

A two-generation reproductive toxicity study using CD-1 mice was conducted (Tyl et al., 2008a). Neither toxic symptoms nor deaths caused by BPA administration were observed in parental generation (F0) or next generation (F1), but there was an increase in the kidney weight from the lowest dose level (0.18 ppm) in F1 males and at 300 ppm (50 mg/kg bw) in F0 males. Moreover, an increase in the incidence of centrilobular hepatocyte hypertrophy in F0/F1 of both sexes at 300 ppm and more, and increased liver weight in F0/F1 of males at the highest dose level (3,500 ppm = 600 mg/kg bw) were observed. However, centrilobular hepatocyte hypertrophy was

observed in the F0/F1 control group as well, with the incidence in males and females of 10.7%/12.7% and 1.8%/3.6%, respectively. From these findings, Tyl et al. (2008a) determined the NOAEL for the general toxicity of BPA in this study to be 30 ppm (5 mg/kg bw).

1.5 Genotoxicity

Hunt et al. (2003) reported that an increase in the defects in the alignment of the chromosomes on the first meiotic spindle due to BPA administration. In this study oocytes were retrieved from neonatal female C57BL/6 mice who were administered BPA (0, 20, 40 or 100 μg/kg bw) in corn oil by gavage for 6 to 8 days. Oocytes in the second metaphase were selected after an overnight incubation, and the alignment of the chromosomes on the first meiotic spindle was observed after immunostaining with anti-microtubule Ab. Meanwhile, Pacchierotti et al. (2008) reported that BPA (0.2 or 20 mg/kg bw) was administered to C57BL/6 mice, but there were no effects due to BPA on the number of aneuploids in the metaphase oocytes or fertilized ovum .

1.6 Carcinogenicity

Takashima et al. (2001) dietary administered BPA (10,000 ppm) to female Wistar rats from 6 weeks old to 16 weeks old, and subsequently from mating to weaning, for a total of 23 to 25 weeks. Offspring (32 to 50 rats/group) were administered N-nitrosobis (2-hydroxypropyl) amine (BHP) at 2,000 ppm in the drinking water from 3 weeks after birth, and carcinogenicity in multiple organs (lung, thyroid, tongue, liver and thymus) was investigated at 25 weeks old; however, no effects due to BPA were observed. Moreover, Ichihara et al. (2003) administered BPA at 0, 0.05, 7.5, 30 or 120 mg/kg bw to female F344 rats by gavage during both the gestational and lactational periods. Therefore, offspring were exposed to BPA transplacentally (in-utero) and lactationally. Then, 7, 12-dimethylbenz [a] anthracene (DMBA) at 50 mg/kg bw was subcutaneously injected to male offspring 10 times, every 2 weeks from 5 weeks old, and the incidence rate of prostate cancer was investigated at 65 weeks old; however, there were no effects due to BPA. Furthermore, Yoshida et al. (2004) administered BPA to pregnant Donryu rats at 0, 0.006 or 6 mg/kg bw from gestational day 2 to postnatal day (PND) 21. Subsequently, female offspring received a single injection of N-ethyl-N'–nitro–N–nitrosoguanidine (ENNG) of 20 mg/kg bw into the uterine horn, and were examined for uterine cancer and premalignant lesions 15 months later; however, no effects due to BPA were observed.

Durando et al. (2007) continuously injected (0.25 μL/hr) BPA into female Wistar rats for 14 days from gestational days 8 to 23 by an osmotic pump. The offspring were lactated for 21 days and then received a single intraperitoneal injection of N-nitroso-N-methylurea (MNU) at less or equal to the carcinogenic dosage level. As a result, an increased number of hyperplastic ducts and augmented stromal nuclear density was noted in the mammary glands of the offspring at 110 days old and 180 days old. In addition, a dense stroma layer around the mammary epithelial structures, and a fibroblastic stroma which replaced the normal adipose tissue of the mammary gland were observed. From these results, it was determined that prenatal exposure of BPA at low dose increased the sensitivity of rats to chemical carcinogens such as MNU.

Moreover, Moral et al. (2008) administered BPA (25 or 250 μg/kg bw) to pregnant SD rats from gestational days 10 to 21, and found a significant increase in the number of mammary terminal end buds (TEB) in 21 days old neonatal rats in the high dosage group compared to those of the low dosage group (however, with no significant difference from the control group), and along with this, a decline in the gene expression involved in cell differentiation in the BPA groups. From these results, the authors determined that there was an increase in TEB due to BPA administration, thus suggesting that prenatal exposure of BPA affects the susceptibility of mammary gland to transformation because TEB is prone to transformation.

Jenkins et al. (2009) examined the effects of BPA exposure via breast milk on mammary carcinogenesis induced by DMBA in SD rat offspring. The rat offspring were lactationally exposed to BPA by oral administration of BPA (25 or 250 μg/kg bw) to their dam from PND 2 to PND 20, although the BPA concentration in the breast milk was not measured. Subsequently, DMBA (30 mg/kg bw) was administered to the offspring at 50 days old, and the effects on mammary carcinogenesis were investigated. As a result, the number of mammary tumor per rat induced by DMBA significantly increased in the BPA group (2.84 ± 0.31, 3.82 ± 0.43, 5.00 ± 0.88/rat for the control group, BPA 25 μg/kg bw group and BPA 250 μg/kg bw group, respectively). In the absence of DMBA treatment, lactational BPA exposure resulted in increased cell proliferation and decreased apoptosis at 50 days, but not 21 days postpartum (shortly after last BPA treatment). Furthermore, up-regulation of the progesterone (PR) receptor-A (PR-A), as well as steroid receptor coactivator 1 (SRC-1) and SRC-3, which is a transcription-coupled activation factor in nuclear receptor protein, were observed in 50 days old rats. An over expression of PR is known to be found in cases of breast cancer, so it was suggested that the sensitivity of rats to hormones

(progesterone) after sexual maturation increased due to lactational exposure to BPA, and as a result, the sensitivity towards carcinogenesis due to DMBA increased. Betancourt et al. (2010), from the same study group as Jenkins et al. (2009), examined the effects of BPA exposure via breast milk on mammary carcinogenesis induced by DMBA in rat offspring, too. The same experimental design as that of Jenkins et al. (2009) was employed except that DMBA was administered to female offspring at either 50 days old or 100 days old. There was no increase in the number of mammary cancer in the test group in which DMBA was administered at 50 days old (Dosage of BPA to dam: 25 or 250 μg/kg bw), but it increased remarkably (p = 0.022) in the test group (83.3%) in which DMBA was administered at 100 days old (Dosage of BPA to dam: 250 μg/kg bw), compared to the control group (53.6%). Moreover, the expression of the estrogen receptor (ER)-α, PR, and B-cell lymphoma 2 (Bcl-2) in the mammary gland of offspring who received DMBA at 50 days old decreased whereas they increased when received DMBA at 100 days old. The authors of this study suggested that DMBA-induced mammary cancer was enhanced due to the increased target structure of DMBA in mammary glands by in-utero exposure of BPA because of the following reasons; a) the terminal duct (TD), said to be highly susceptible to mammary carcinogenesis, increased at 100 days old but not at 50 days old when BPA (250 μg/kg bw) was exposed in-utero; and b) the epithelial structure has been reported to increase at 180 days old when BPA is continuously injected by an osmotic pump during gestation. Moreover, it was suggested by the authors that these shifts in carcinogenic susceptibility may be due to the increased expression of ER-α and sex steroid hormone controlling proteins.

As for carcinogenesis related to the estrogen-like action of BPA, Ho et al. (2006) reported that the number of prostatic intraepithelial neoplasia (PIN), which are precancerous lesions of prostate cancer, increased in SD rats as in humans when rats were subcutaneously injected with BPA for a short term along with the subsequent continuous exposure to estrogen and testosterone, thereby imitating the increased level of sex hormones in humans due to aging. More specifically, BPA (0.1 μg/pup or 0.001 μg/pup, equivalent to 100 μg/kg bw or 0.1 μg/kg bw, respectively) or estradiol benzoate (EB) (25 μg/pup equivalent to 2,500 μg/kg bw) was subcutaneously injected into male neonates at PND 1, PND 3, and PND 5, and then divided into two groups at PND 90. 17β-estradiol (E2) and testosterone (T) were continuously exposed to one group afterwards, with the other group as the control, which was sacrificed at 28 weeks old, and neoplastic changes at the prostate were histopathologically examined. As a result, in the neonatal rats that were subcutaneously injected BPA immediately

after birth and subsequently exposed to E2+T, a significant increase in the number of PIN along with a decrease in the methylation of phosphodiesterase type 4 (PDE 4) which is involved in the breakdown of cAMP and an increase in PDE 4 gene expression. The authors of the study suggested that the increased occurrence of PIN in the prostate was caused by the increased PDE 4 activity. In addition to this study, a follow-up study (Prins et al., 2010) was carried out because BPA was subcutaneously injected in the above study and was regarded as problematic from the toxicokinetic viewpoint. More specifically, BPA was orally administered or subcutaneously injected at 10 μg/kg bw to neonates at PND 3, and the occurrence rate of PIN after 16 weeks of injection of E2 and T was investigated at PND 90 along with comparing the kinetics of free-BPA and total-BPA in the blood. As a result, similarly to subcutaneous injection, an increase in PIN was observed when BPA was orally administered at a neonatal period and E2 and T were injected for 16 weeks from 90 days after birth. Furthermore, it was confirmed that the blood concentration of free-BPA in the neonates was higher by subcutaneous injection than by oral administration, and that there was a difference in the internal dosimetry of BPA depending on the route of administration, even for 3 days old neonates. Moreover, it was suggested by the authors that the findings obtained by Ho et al. (2006) and this study, that PIN increases due to BPA, is directly involved in the present health effects of BPA on humans because the blood concentration of free-BPA by subcutaneous injection is approximately the same as the internal dosimetry in humans (blood plasma of a pregnant women and her child, amniotic fluid, fetal tissue or milk) under the current BPA exposure level from the environment.

In addition, Murray et al. (2007) subcutaneously injected BPA into Wistar-Furth rats at 2.5 to 1,000 μg/kg bw continuously from embryonic day 9 until PND 1 using an osmotic pump, and investigated the sexual maturation index after birth as well as the presence of precancerous lesions in the mammary gland at PND 50 and PND 95. As a result, an increase in mammary hyperplasia was observed in all BPA groups at PND 50, and was observed in only the BPA 2.5 μg/kg bw group at PND 95. Moreover, a sieve-like structure determined to be an intraductal carcinoma insitu (CIS) was observed in the 250 and 1,000 μg/kg bw groups, and it was concluded that fetal exposure of BPA induces mammary cancer and its precancerous lesions. Furthermore, Newbold et al. (2007) subcutaneously injected BPA (0, 0.01, 0.1 or 1 mg/kg bw) into neonatal CD-1 mice from PND 1 to PND 5, and histopathologically investigated the effects on female reproductive tract (uterus, ovary, oviduct) at 18 months old. As a result, an increasing trend in cystic ovary, cystic endometrial hyperplasia (CEH),

adenomyosis, and uterine polyps and a decreasing trend in corpora lutea were observed in BPA groups. In 0.1 mg/kg bw group, in particular, the increase in cystic ovary and CEH was statistically significant. In addition, para-ovarian cysts, progressive proliferative lesions of the oviduct, Wolffian duct remnants, leiomyomas, and atypical hyperplasia of the uterus, that were not seen in the control group, were observed in the BPA groups, and it was thus concluded that long-term adverse effects in the female reproductive tracts were induced following exposure to BPA during the critical period of differentiation.

1.7 Reproductive and developmental toxicity

Tyl et al. (2008a) reported a GLP-compliant two-generation reproductive toxicity study on BPA using CD-1 mice employing OECD testing guideline 416. BPA was dietary administered to mice at 0, 0.018, 0.18, 1.8, 30, 300 or 3,500 ppm (0, 0.003, 0.03, 0.3, 5, 50 or 600 mg/kg bw). A concurrent positive control group received dietary 17β-estradiol (E2) at 0.5 ppm (0.08 mg/kg bw) developed decreased live F1 offspring number, F1 litter size and F1 fertility index, facilitation of the vaginal opening, delay of preputial separation, and increased female reproductive organ weight, along with a decreased weights of testes and testicular epithelium and increased hypoplasia of testicular seminiferous epithelium at the weaning of F1 and F2. It was thus confirmed that the effects of E2 can be detected in the study protocol used. A decline in body weight, splenic and testes weight at weaning, as well as a mild delay of preputial separation and delay of testicular descent of F1/F2 at 3,500 ppm was observed in the BPA groups. These changes were all believed to be secondary to the general toxicity of BPA in the dam. Moreover, the gestational period for F1/F2 generations was prolonged for 0.3 days in the same group, but this was presumed to not be acceptable as evidence of any toxicological significance. There were no effects due to BPA observed in the reproductive organ weight, histopathological examination, sperm parameters, ovarian primordial follicles counts, and estrous cyclicity of females in F0 and F1 mice. Moreover, there was no effect on the adult mating or fertility of F0 and F1. Effects due to BPA and/or parameters indicating a bell-shaped response curve were not observed in low dosages from 0.018 to 30 ppm. From the above, the NOEL for the reproductive and developmental toxicity of BPA was determined to be 300 ppm (ca. 50 mg/kg bw), and BPA was not considered a selective reproduction or developmental toxicant in mice.

Howdeshel et al. (2008) examined the effect of BPA on the development of male reproductive organs using Long-Evans rats. BPA (2, 20 or 200 μg/kg bw) was

administered to dam from gestational day 7 to PND 18. As a result, no effects due to BPA were found regarding the body weight, reproductive indexes of the dam (number of implantations, number of live pups at weaning, etc.) or the following parameters in male offspring; body weight and anogenital distance (AGD) on PND 2, organ weights of the seminal vesicle, testes, prostate, glans penis, and bulbocavernosus, epididymal sperm count, blood hormone level (LH, testosterone, estradiol, PR, corticosterone and total thyroxin), and histopathological figures of testes, epididymides, glans penis, prostate, seminal vesicle, Cowper's gland and bulbocavernosus, at 4 months old. As for the effects of BPA on female offspring in the above study, Ryan et al. (2010) reported that there was no effects on the body weights at birth and at weaning, reproductive parameters (number of implantations, number of live pups, etc.), anogenital distance (AGD), age and body weight at vaginal opening, cleft phallus, urethrovaginal distance (UVD), fecundity, saccharin preference, lordosis behavior, or the figure 8-maze spontaneous locomotor activity.

On the other hand, Timms et al. (2005) reported that an increased volume of the coagulating gland and urethral stricture was observed along with an increased volume and increased numbers of dorsal, lateral, and ventral prostate ducts when BPA (10 μg/kg bw) was administered by gavage to pregnant CD-1 mice (5 to 6 mice for each group) from gestational days 14 to 18. Similar effects were observed in ethinylestradiol (EE) or diethylstilbestrol (DES) at low doses (0.1 μg/kg bw). However, in contrast to the findings at low dosages, a complete hypoplasia was observed in the dorsal and lateral prostate upon the oral administration of DES at a high dosage (200 μg/kg bw). Considering that the profile of adverse effects differs with high and low dosages of DES, the authors of the study claim that the health risks regarding BPA and/or DES at a dosage lower than those to which human fetuses are exposed should be re-evaluated.

Furthermore, there was a report (Japanese Health and Labor Sciences Research Grant, Chemical Substance Risk Research Project, 2007[3]) mentioning that when BPA (0, 0.5, 5 or 50 μg/kg bw) was given to pregnant SD rats by gavage from gestational day 6 until PND 20, and the estrous cycle of the female offspring was observed until 12 months old, cases showing menoxenia were observed at 0.5 μg/kg bw in a statistically significant manner from 4 months old onwards. In addition, it was concluded by the authors that low dosages of BPA induce a late-onset of an abnormal

[3] Studies on the development of the definitive testing methods and the overall evaluation guideline for EDCs (2004 – chemistry – general – 001) 2006 General/shared research report (March, 2007)

estrous cycle. However, the number of animals examined in the study was not sufficient (22 to 31 rats/group) to determine the effect because the abnormal estrous cycle determined to be an effect in this study also showed an age-related increase in the control group. In such cases, comparisons with their historical data will normally help to determine the significance of the change, but this was not the case in this instance. Consequently, even though there were statistically significant differences, it cannot necessarily be said that the change occurred specifically due to BPA exposure.

1.8 Developmental neurotoxicity

Stump et al. (2010) carried out a GLP-compliant developmental neurotoxicity study according to OECD testing guideline 426 as well as the U.S. EPA OPPTS testing guideline 870.6300. More specifically, BPA (0, 0.15, 1.5, 75, 750 or 2,250 ppm; 0, 0.01, 0.1, 5, 50 or 150 mg/kg bw) was dietary-administered to SD rats from gestational day 0 to PND 21. Dam was examined for general signs, detailed clinical observations, body weight, food consumption, and gestational period. The offspring were evaluated using the following tests: detailed clinical observations, auditory startle, motor activity, learning and memory using Bile-maze test, brain and nervous system neuropathology and brain morphometry. As a result, no effects due to BPA were observed except a decrease in food consumption and body weight increase in dams in the BPA group of 750 ppm or more at the first week of gestation. Regarding the offspring, there were no effects in terms of general signs, righting reflex, sexual maturation period, detailed clinical observations, motor activity, auditory startle reaction, learning and memory, neuropathological figure of the brain/nervous system or morphometry of the brain except lower body weights of the offspring in the BPA group of 750 ppm or more at PND 7 to PND 14 in comparison with the control group. From the above results, NOAEL for the developmental neurotoxicity of BPA was determined to be 2,250 ppm (164 mg/kg bw during gestation: 410 mg/kg bw during lactation), which is the highest dose level employed in the study.

1.9 Developmental neurotoxicity studies using atypical test protocols

Developmental neurotoxicity of BPA has been examined using atypical test protocols by many scientists: sexually differentiation of the brain (Kubo et al., 2001; Kubo et al., 2003; Patisaul et al., 2006; Ceccarelli et al., 2007; Patisaul et al., 2007) sexual behaviors (Farabollini et al.; 2002; Della Seta et al., 2006; Fujimoto et al., 2006; Ryan and Vandenberg, 2006), fear behavior (Negishi et al., 2003 Negishi et al., 2004),

aggressive behavior (Kawai et al., 2003; Kawai et al., 2007), exploratory behavior (Farabollini et al., 1999), playing behavior (Dessi-Fulgheri et al., 2002; Porrini et al., 2005), maternal behavior (Palanza et al., 2002), pain response (Aloisi et al., 2002), hippocampal function (Carr et al., 2003), amount of neural transmission factors inside the brain (Honma et al., 2006), development of neurons (Funabashi et al., 2004; Nakamura et al., 2006; Tando et al., 2007), ligand binding to somatostatin receptors (Facciolo et al., 2002; Facciolo et al., 2005), occurrence of dopamine receptor (Suzuki et al., 2003; Mizuo et al., 2004a), and the brain amine levels (Adriani et al., 2003; Mizuo et al., 2004b; Laviola et al., 2005; Narita et al., 2006; Narita et al., 2007).

1.10 Toxicokinetics, metabolism and distribution

Völkel et al. (2002) studied the metabolism and kinetics of BPA in three healthy men and three healthy women by the oral administration of a single dose of ^{2}D-BPA (5 mg/human, 54 to 88 μg/kg bw). BPA was absorbed into the body rapidly and completely, and was excreted in the urine within 24 hours. The late blood half-life of BPA was 5.3 hours in both males and females, and the half-life by urinary excretion was 5.4 hours. The same amount of BPA was orally administered to 4 healthy men in order to determine the detailed kinetics of the blood concentration. As a result, the maximum blood concentration (C_{max}) of BPA-glucuronide conjugate was observed 80 minutes after administration, and was 840 pM (0.19 ng/mL blood plasma). The initial half-life of BPA in the blood was 89 minutes, while subsequently disappearing at a half-life of 3.4 hours. The blood concentration slightly decreased (by about 0.15 nM = 0.03 ng/mL blood plasma) when plasma samples were treated with β-glucuronidase, but the authors mentioned that free-BPA was not detected in the blood plasma. These findings suggest the absence of any enterohepatic circulation because BPA is completely excreted via the urine within 24 hours. When the dosage was reduced by 200 fold (0.27 - 0.44 μg/kg bw), 75% (women) to 85% (men) of the administered BPA was collected in the urine within 5 hours after administration, and the excretion half-life was 4 hours (Völkel et al., 2005). When ^{14}C-BPA at 100 μg/kg bw was orally administered to non-human primates, BPA rapidly disappeared at a blood half-life of approximately 10 hours in cynomolgus monkeys (Kurebayashi et al., 2002), and at 3.5 hours in the rhesus monkeys (Doerge et al., 2010a). On the other hand, the half-life of BPA in the blood plasma in rats by the oral administration of ^{14}C-BPA at 100 or 500 μg/kg bw was 18 to 22 hours (Kurebayashi et al., 2005), and thus the excretion rate of BPA from the blood in rats was about 4 times slower than in humans.

When ^{14}C-BPA was intravenously injected into cynomolgus monkeys, 79 to 86% of

the radioactivity was excreted in the urine and 2% in the feces within 7 days after injection (Kurebayashi et al., 2002). 94% of the blood radioactivity was bound to proteins, and the half-life of radioactivity in the blood plasma was 14 hours. The half-life of ^{14}C-BPA by intravenous injection was 13.5 hours in males and 14.7 hours in females, and was longer than the cases of oral administration. On the other hand, the half-life of ^{2}D-BPA of total-BPA in the serum of rhesus monkeys after intravenous injection was 3.6 hours, with almost no difference observed compared to the cases of oral administration (3.5 hours) (Doerge et al., 2010a).

In cynomolgus monkeys of both sexes, BPA, BPA-glucuronide, BPA-diglucuronide, and two unidentified metabolites were detected in the urine and blood plasma either by intravenous injection or oral administration of BPA (Kurebayashi et al., 2002). BPA-glucuronide was the major radioactivity (95 to 100% of radioactivity) in the blood plasma upon oral administration (100 μg/kg bw), and free-BPA represented 1.4% or less of the radioactivity in the blood plasma (Kurebayashi et al., 2002). Also, Doerge et al. (2010a) reported that the C_{max} in rhesus monkeys following the oral administration of BPA and the ratio of free-BPA to total-BPA in the area under the blood concentration-time curve (AUC) was 0.21% and 0.19%, respectively. On the other hand, it has been reported that after intravenous injections of BPA, 57 to 82% of the radioactivity in the blood plasma was from glucuronide conjugates and 27 to 29% was from free-BPA in cynomolgus monkeys (Kurebayashi et al., 2002). Also, Doerge et al. (2010a) reported that the ratio of free-BPA to the total-BPA at 5 minutes after the injection of BPA to be 29% for C_{max} and 14% for AUC in rhesus monkeys. These data show that BPA becomes rapidly metabolized even with parenteral routes in non-human primates. Moreover, Kurebayashi et al. (2002) suggested that the radio-activities that were detected at a maximum of 3.9% in the blood plasma by intravenous injection of BPA are attributable to BPA-sulfate and 5-hydroxy BPA.

In rats, ^{14}C-BPA that was either orally administered or intravenously injected was excreted in 48 hours, with the majority (78 to 82%) via the feces and a small amount (10 to 13%) via the urine. Meanwhile, 58 to 66% or 45 to 50% of BPA was excreted into the bile within 6 hours after intravenous injection or oral administration of BPA at 0.1 mg/kg bw, and 84 to 88% of radioactivity in the bile was attributable to BPA-glucuronide. When unlabelled BPA at 100 mg/kg bw was orally administered, 41% was excreted in the bile as a BPA-glucuronide within 18 hours. Although 61% of BPA was excreted via the feces by 72 hours, conjugates were not detected. Whereas, 8% was excreted via the urine, but 82% of this was attributable to BPA-glucuronide, 4% to BPA-sulfate conjugates, and 14% to free-BPA (Kurebayashi et al., 2003).

According to a comparative study of the metabolism of BPA (Tominaga et al., 2006) using rats, cynomolgus monkeys, and chimpanzees, C_{max} and AUC of free-BPA by oral administration of BPA (10 or 100 mg/kg bw) was much lower than by subcutaneous injection in all animal species used (C_{max} and AUC by oral administration of BPA 10 mg/kg bw in rats, chimpanzees, cynomolgus monkeys were 2.1, 5.5, 11.5 μg/L and 7.2, 3.1, 42.5 μg·hr/L, respectively, and C_{max} and AUC by subcutaneous injection were 746, 703, 4,213 μg/L and 1,977, 6,000, 18,855 μg·hr/L, respectively). The bioavailability was shown to be low with oral administration. Moreover, the ratio of C_{max} of BPA-metabolites (conjugates) to that of free-BPA upon oral administration of BPA (10 mg/kg bw) was 65 times in rats (138 vs. 2.1 μg/L), 751 times in cynomolgus monkeys (8,638 vs. 11.5 μg/L), and 184 times in chimpanzees (1,013 vs. 5.5 μg/L). BPA was rapidly metabolized at the first pass in all animal species. According to Tominaga et al. (2006), the "apparent" intrinsic clearance (CL_{int}) estimated from K_m and V_{max} of enzymes involved in BPA-glucuronidation in rat liver microsomes is 139 L/hr/kg bw, and is much larger than the hepatic blood flow of rats (5.1 L/hr/kg bw), and it was suggested that the speed of glucuronidation of BPA in the liver depends on the hepatic blood flow. Moreover, even if CL_{int} of humans is assumed to be 1/10 that of rats, it is still much larger than the hepatic blood flow of humans (1.2 L/hr/kg bw), and similarly to rats, the glucuronidation speed of BPA in humans was determined to be dependent on the hepatic blood flow. In any case, the glucuronide conjugation capacity of BPA in the liver was also sufficiently high from the biochemical data of liver microsomes regardless of the animal species, and was proven to be rapidly metabolized in the liver. Furthermore, the free-BPA concentration in the blood by subcutaneous injection of BPA was significantly high compared to oral administration in all animal species examined (Tominaga et al., 2006), and the glucuronidation rate of BPA is shown to be slower upon subcutaneous injection compared to oral administration.

How BPA metabolic activities change with pregnancy is important. In an analysis using ^{2}D-BPA and the LC/MS/MS method, the half-life of serum total-BPA of 3 days old SD rats following oral administration of BPA at 100 μg/kg bw was 6.7 hours (Doerge et al., 2010b), approximately 1.5 times slower compared to that of 5 days old neonatal rhesus monkeys (4.6 hours; Doerge et al., 2010a). Furthermore, in another study (Domoradzki et al., 2004), when ^{14}C-BPA at 10 mg/kg bw was orally administered to neonatal rats, there was a peak of plasma radioactivity within 1 hour after administration, and the radioactivity declined according to the average half-life of 4 to 6 hours; however, there was no second peak, suggesting no enterohepatic circulation

of BPA in neonatal rats. Moreover, Domoradzki et al. (2003) reported that there was no difference in the distribution of radioactivity in the dam following an oral administration of ^{14}C-BPA at 10 mg/kg bw to SD rats regardless of the gestational status. Moreover, they reported that radioactivity of less than or equal to 0.1% was detected in embryo/fetus at gestational day 17, although there was no distribution specific to the embryo/fetus by 6 to 14 days of gestation. Zalko et al. (2003) reported that when BPA was subcutaneously injected into pregnant CD-1 mice, BPA was not only rapidly glucuronide-conjugated but became metabolized into diglucuronide conjugates and methoxy derivatives, etc. and 4% or more of the dosage was distributed to the fetus 24 hours after administration, being present as free-BPA, BPA-glucuronide, and BPA-diglucuronide. Moreover, Kurebayashi et al. (2005) reported that the radioactivity was detected in the bladder and small intestine of the fetus on gestational day 18 but not on gestational day 12 or 15 when ^{14}C-BPA with high specific radioactivity was orally administered to dams. However, the radioactivity in the fetal tissue was approximately 30% of that found in the blood of the dam, and it cannot be said that BPA distributed specifically to the fetal tissue.

There are several reports on the toxicokinetics of BPA in neonates. Domoradzki et al. (2004) reported that when ^{14}C-BPA at 10 mg/kg bw was orally administered, the C_{max} of free-BPA was 48.3 (male) or 10.2 μg/g plasma (female) in 4 days old neonatal SD rats, while it was 0.024 (male) or 0.063 μg/g plasma (female) in adults; when compared to adults, the C_{max} of neonates was approximately 2,000 times higher in male neonates and approximately 160 times higher in female neonates, but this difference shrank to about 10 times at 21 days old. Moreover, Doerge et al. (2010b) also reported that when BPA at 100 μg /kg bw was orally administered to female neonatal SD rats, the blood concentration of total-BPA and free-BPA in 3 days old neonates was much higher than that of adults (total-BPA: 445 nM vs. 70 nM, free-BPA: 29 nM vs. 0.4 nM), but the difference shrank in accordance with growth, and at 21 days old, the blood half-life, AUC, and C_{max} of free-BPA was of the same level as adults.

These reports show that the ability of neonatal rats to metabolize BPA to conjugates is low, but will reach the level of adults at PND 21. However, the free-BPA was merely 16% of the total-BPA upon oral administration of BPA at about 100 μg/kg bw in 3 days old neonates, suggesting that BPA is efficiently glucuronidized in neonates as well (Doerge et al., 2010b). In addition, Doerge et al. (2010a) reported that the metabolic profile of BPA (100 μg/kg bw) by intravenous injection in 70 days old rhesus monkeys had no difference compared to adults, showing that metabolism of

BPA was also effective in 70 days old neonates regardless of the dosing route. Moreover, the blood concentration of total BPA by oral administration to neonatal rhesus monkeys was high immediately after delivery, and declined with growth, but the blood concentration of free-BPA was 1% or less of the total BPA in all periods from 5 days old to 70 days old (Table 1). This shows that orally administered BPA is inactivated by Phase II metabolism in neonates as well at the same level as adults. Moreover, the reason why blood concentration of total-BPA was higher at a younger age is suggested as being because the renal functions to excrete BPA-glucuronide are immature immediately after birth (Doerge et al., 2010a).

Table 1 Serum pharmacokinetic parameters for free-BPA and total-BPA from neonatal rhesus monkeys administered a single intravenous (iv) or oral (po) dose of 100 μg/kg bw. (Doerge et al., 2010a)

Age	Parameter	iv	po
5 days old	Free-BPA		
	C_{max} (nM)		2.0 ± 2.4
	AUC (nM x hr)		5.7± 4.8
	$t_{1/2}$ (hr)		2.0 ± 1.4
	Total-BPA		
	C_{max} (nM)		690 ± 130
	AUC (nM x hr)		4,010 ± 1,610
	$t_{1/2}$ (hr)		4.6 ± 1.6
35 days old	Free-BPA		
	C_{max} (nM)		1.1 ± 0.88
	AUC (nM x hr)		3.7 ± 2.6
	$t_{1/2}$ (hr)		1.7 ± 1.1
	Total-BPA		
	C_{max} (nM)		550 ± 230
	AUC (nM x hr)		2,250 ± 1,500
	$t_{1/2}$ (hr)		4.3 ± 1.8
70 days old	Free-BPA		
	C_{max} (nM)	—	1.5 ± 0.70
	AUC (nM x hr)	190 ± 57	3.4 ± 2.8
	$t_{1/2}$ (hr)	0.63 ± 0.18	1.5 ± 1.2
	Total-BPA		
	C_{max} (nM)	—	250 ± 250
	AUC (nM x hr)	1,950 ± 880	750 ± 760
	$t_{1/2}$ (hr)	3.6 ± 2.8	2.6 ± 1.6

Taylor et al. (2008) claimed that the same reproductive toxicity of BPA can be obtained regardless of the dosing route because the blood concentration of free-BPA in neonatal CD-1 mice at 3 days old was the same level for both oral administration and subcutaneous injection. However, Prins et al. (2010) demonstrated that free-BPA

as well as total-BPA were both clearly higher upon subcutaneous injection than with oral administration in 3 days old neonatal SD rats; blood C_{max} (mean value ± SEM) of free-BPA and total-BPA by subcutaneous injection and oral administration of BPA (10 μg/kg bw) were 1.77 ± 0.63 ng/mL (subcutaneous) vs. 0.26 ± 0.04 ng/mL (oral) and 2.00 ± 1.00 ng/mL (subcutaneous) vs. 1.02 ± 0.30 ng/mL (oral), respectively.

2. The view of RISS on the health hazard of BPA

2.1 Epidemiology

Cross-sectional epidemiology studies regarding the relationships between BPA exposure and semen quality, sperm count, female sterility, endometriosis, breast cancer, and onset of cardiovascular disease (CVD), or the relationships between prenatal exposure of BPA and the behavior of the children and/or autism have been reported. However, in these studies, the exposure dose of BPA was estimated from blood concentrations or from the total-BPA (free or conjugate) concentration in the spot urine at the same time as the period in which the diseases and/or symptoms manifested or a certain period before delivery.

The drawback of these cross-sectional studies has been indicated by the European Food Safety Authority (EFSA, 2010) as well as the joint expert meetings of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) (hereafter, FAO/WHO expert committee) (WHO, 2010); the blood BPA concentration and/or BPA concentration in the spot urine does not necessarily reflect the exposure concentration of BPA because the in-vivo half-life of orally exposed BPA is short in humans. In particular, regarding an epidemiology study on cardiovascular disease (CVD) by Melzer et al. (2010), problems are indicated by EFSA (2010) such as not having exposure data before and halfway during onset, not being able to eliminate the contribution of confounding factors that have not been considered (genetic background, lifestyle, environmental factors, etc.), and the consideration on the possible concurrent diseases (e.g. diabetes with CVD or any of the three components) being unclear. Although Braun et al. (2009) reported that there was a correlation between the in-utero exposure of BPA and the decline in scores regarding the behavior of female children, the significance of declined scores is not clearly explained; increased external behavioral scores are considered to be problematic. EFSA (2010) questioned whether the behavioral change in children determined to correlate with BPA exposure had any clinical significance because they determined that scores related to child behavior are within the range of physiological variation, and indicated that it may be due to hidden confounding factors (mental status of parents, alcohol and/or drug use, attitude of the mother to children, etc.). Although Li et al. (2010) reported that the exposure concentration of BPA correlated with the decline in semen quality of factory workers, those who were determined to have been affected may have been exposed to other chemical substances, and the analysis of confounding factors was believed to be insufficient, as indicated by the FAO/WHO

Expert Meeting (WHO, 2010).

As mentioned above, most epidemiology studies had significant shortcomings in their study design, such as the possibility of confounding factors regarding the onset of studied diseases not being sufficiently eliminated, together with insufficient exposure estimates of BPA. Thus, the results of these epidemiology studies were not believed to be useful for use in human health risk assessments of BPA.

2.2 Acute toxicity

There were no new information, and no changes were made from our previous hazard assessment with regard to acute toxicity of BPA. More specifically, the acute toxicity of LD_{50} by oral administration of BPA was 4,100 mg/kg bw (male) and 3,300 mg/kg bw (female) in rats, and 5,200 mg/kg bw (male) and 4,100 mg/kg bw (female) in mice (NTP, 1982). Upon inhalation administration, there were no cases of death at 6 hours of exposure at the maximum testable concentration of 170 mg/m^3 in rats, and upon percutaneous administration, death was observed in rabbits at an exposure of 2,000 mg/kg bw or more (ECB, 2003). Therefore, similarly to previous evaluations, it can be presumed that “the acute toxicity of BPA was generally weak; with no cases of death in rats after 6 hours of exposure at the maximum testable concentration of 170 mg/m^3 by inhalation, and if worker exposure is not evaluated, then there is no need to regard it as problematic.”

2.3 Irritability and skin sensitization properties

The skin sensitization and photo-sensitization potential of BPA was of concern in the previous evaluation, but according to EC (2008), skin sensitization, skin photo-irritation or photo-sensitization for 30% concentrations of BPA in a LLNA test (GLP test) using mice were all negative. Moreover, there were no reports of skin sensitization in 875 BPA plant workers (EC, 2008). Because details are described in EC (2008), the above information was determined to be highly reliable, and here, skin irritation, skin sensitization, skin photo-irritation, and photo-sensitization of BPA are determined to be substantially negative. Furthermore, although respiratory tract irritations was detected at 50 mg/m^3 in an 13-week rat inhalation toxicity study on BPA (unpublished data, cited in EC, 2003), it was believed that there is no need to select it as a subject for risk assessment because the possibility of the consuming public being exposed to large quantities of BPA dust was close to none.

2.4 Repeated dose toxicity

At the time of the previous evaluation, a suppression of increased body weight was commonly noted in oral toxicity studies on BPA with dosing period of from 2 weeks to 2 years; suppression of body weight increase by BPA was noted upon administration of BPA at 500 mg/kg bw or more in repeated dose toxicity studies with dosing period of from 2 weeks to 44 days, at 200 mg/kg bw or more in a one-generation reproductive toxicity study, at 100 mg/kg bw or more in a 13 week-dietary toxicity study, at 74 mg/kg bw or more, which is the lowest dosage level, in a 2 year-carcinogenicity study, and 50 mg/kg bw or more in a three-generation reproductive toxicity study.

When a suppression of body weight increase was determined to be the endpoint of general toxicity, LOAEL in rats was 40 mg/kg bw, which is the lowest dosage level in the 13 week-dietary toxicity study, and was 50 mg/kg bw in the three-generation reproductive toxicity study, being roughly accordant in both tests. Therefore, in the previous evaluation, NOAEL was determined to be 5 mg/kg bw, which is the lowest dosage level in the three-generation reproductive toxicity study. With regard to the uncertainty factor involved in the dosing period, we determined not to apply this factor because the suppression of body weight increase in rats at 1,000 ppm and 2,000 ppm in the 13 week-dietary toxicity study (NTP , 1982) was -23% and -18% in males in comparison with the control group, respectively, and -11% and -12% in females, respectively, and that of 1,000 ppm and 2,000 ppm in the carcinogenicity study (NTP , 1982) was -4.5% and -8.7% in males, respectively, and -6.3% and -10.9% in females, respectively, thus indicating that no aggravation due to the prolonged administration period exists.

In addition, multinucleated giant hepatocytes were observed in a dose-dependent manner in males in the 13-week dietary toxicity study on BPA using B6C3F1 mice (NTP, 1982). In addition, centrilobular hepatocytomegaly (karyomegaly and cytomegaly), multinucleated giant hepatocytes, and renal tubular cell nuclear variability were observed in both sexes in the reproduction and fertility study using B6C3F1 mice (NTP, 1985). Multinucleated hepatocytes, along with anemic changes, thrombocytosis, degeneration or fibrosis of renal tubules, suppression of body weight increase, ovarian weight loss, and increased kidney weight were observed in another study in which BPA was orally administered to B6C3F1 mice for 13 weeks (Furukawa et al., 1994). Moreover, multinucleated giant hepatocytes were also observed in the 2-year carcinogenicity study in B6C3F1 mice (NTP, 1982). As for dogs, BPA was dietary-administrated to beagles for 90 days, and the only change obtained in this

study was an increased relative liver weight in the high dose group, and the NOAEL in this study was determined to be 74 mg/kg bw (ECB, 2003).

According to the new information, increased centrilobular hepatocyte hypertrophy, increased relative kidney weight, and increased nephropathy were observed in the 13 week-dietary toxicity study in CD-1 mice (unpublished data, cited in EC 2008), that was a dose-range finding study for the two-generation reproductive toxicity study (Tyl et al., 2008a). In the two-generation reproductive toxicity study, increased liver and kidney weights, centrilobular hepatocyte hypertrophy, and nephropathy were observed in F0/F1 mice, and increased liver and kidney weights as well as centrilobular hepatocyte hypertrophy were observed in male F0 mice. The results of these two studies strongly suggested that liver and kidneys are the target organs with regard to the general toxicity of BPA. Meanwhile, no histological changes were observed in the liver of dogs administered BPA in spite of the increased organ weight, and no effects on the liver were reported in rat toxicity studies either.

The multinucleated giant hepatocytes that were found in B6C3F1 mice in response to BPA are observed in this strain of mice in an age-related manner, and is known to increase due to exposure to some chemical substances (Haighton et al., 2002), but its pathogenic mechanism and toxicological significance in humans both remain unclear. Likewise, the pathogenic mechanism of giant hepatocyte and hepatocellular hypertrophy by BPA also remains unclear. Therefore, these findings were considered to be an endpoint for risk assessment of BPA in humans because there is no data that clearly rules it out, although the toxicological significance of these findings are not definite in humans as well as in rodents. Meanwhile, although BPA does not induce micronuclei, it has been shown in human fibroblasts that BPA binds with intracellular microtubules and stops cell division at G2 or G1 phase, (Lehman and Metzler, 2004), and these interactions with the microtubules may be the cause of multinucleated giant hepatocytes by BPA; however, the details are not clear. Moreover, effects on the liver were all strongly observed in male mice, and thus it is highly possible that this may be due to non-specific adaptive responses which are induced by the induction of drug-metabolizing enzymes, but no relevant data are available. Meanwhile, according to Nakagawa and Tayama (2000), BPA is cytotoxic to hepatocytes in-vitro, and as its mechanism, it has been clearly demonstrated that BPA partially uncouples oxidative phosphorylation in mitochondria and that the intracellular ATP level drops due to the suppression of both NAD^+ dependent- and FAD dependent-mitochondrial respiration. The decline of intracellular ATP level due to the suppression of mitochondrial functions appears to be the possible pathogenic

mechanism for the hepatocyte degeneration or necrosis due to the high dosages of BPA (487 mg/kg bw or more) because the same mechanism was seen in common in many cytotoxic chemical substances, and included in the pathological changes such as necrosis and apoptosis. Additionally, it is highly possible that nephrotoxicity due to BPA observed in B6C3F1 and CD-1 mice is caused by the same mechanism. However, these pathogenic mechanisms and the species difference with regard to both hepatotoxicity and nephrotoxicity of BPA found in mice were not necessarily clear, and, as of now, determination on which finding is related to human health hazard cannot be carried out. Therefore, the NOAEL value for each finding was estimated by the benchmark dose (BMD) method as BMDL (the lower limit of 95% confidence range of benchmark response (BMR)), and the findings showing the lowest BMDL were determined to be the endpoint of hazard assessment of BPA.

In the previous evaluation, multinucleated giant hepatocyte was believed to be the main lesion (Table 2), and the BMDL for this finding in males in the reproduction and fertility study on BPA using B6C3F1 mice (NTP, 1985) was estimated to be 0.019% in the food (equivalent to 23 mg/kg bw) by BMD method using a BMD software program (BMDS) ver. 1.3.2 of U.S. EPA, where BMR was set to 5% extra risk.

Table 2 Incidence of hepatocellular lesions in the reproduction and fertility study on BPA using B6C3F1 mice (NTP, 1985)

sex	BPA conc. In the food (%)	Group size (n)	Multinucleated giant hepatocyte	Centrilobular hepatocytomegaly
male	0	19	0	0
	0.25	19	13	14
	0.5	20	19	18
	1.0	11	10	10
female	0	20	0	-
	0.25	19	0	-
	0.5	20	1	-
	1.0	11	4	-

Here, the number of incidences of multinucleated giant hepatocytes in males in the reproduction and fertility study (NTP, 1985) was re-analyzed using the new BMDS ver. 2.1.2[4] that was extensively improved from the previous version (ver. 1.3.2). The BMR was set as the default (10%), so as to be the same in the analysis of hepatocellular hypertrophy described below.

Data from the high dosage group were excluded from the analysis because the compatibility with the model was not good when data from all dosages were used. The

[4] http://www.epa.gov/ncea/bmds/index.html

arithmetically averaged BMDL with regard to the incidence of multinucleated giant hepatocytes in males in the reproduction and fertility study (NTP, 1985) was 0.015% in the food (Table 3). When this value was converted to dosage by using the default value (body weight of male mouse: 0.03 kg, food consumption: 3.6 g/day) (ECB, 2003), it became 18 mg/kg bw.

Table 3 Results of BMD analysis on the multinucleated giant hepatocytes found in the mouse reproduction and fertility study (NTP, 1985)

Model	*p*- value	AIC	Scaled Residual of Interest	BMD (%)	BMDL (%)	Mean BMDL (%)
Gamma	1.0000	36	0.000	0.057	0.015	0.015
Logistic	~~0.0959~~	~~39~~	~~-0.850~~	~~0.094~~	~~0.056~~	
LogLogistic*	1.0000	36	0.000	~~0.097~~	~~0.006~~	
LogProbit*	1.0000	36	0.000	~~0.088~~	~~0.002~~	
Multistage	0.9951	36	-0.006	0.031	0.015	
Probit	~~0.0789~~	~~39~~	~~-0.883~~	~~0.084~~	~~0.053~~	
Weibull	1.0000	36	0.000	0.044	0.015	
Quantal Linear	0.8405	34	0.000	0.020	0.014	

AIC: Akaike's Information Criterion.
The figure under the cancellation line was excluded from the analysis.
* The response curve was determined to be inappropriate by visual inspection.

Regarding the centrilobular hepatocytomegaly that were seen in mice in the reproductive and fertility study (NTP, 1985), BMDL was calculated using the BMD method, too. Similar to multinucleated giant hepatocytes, data from the high dosage group was excluded from the analysis because the compatibility with the model was not good when the data from all dosages were used. When the BMDL obtained in this calculation was arithmetically averaged, feeding concentration of 0.013% (equivalent to 16 mg/kg bw) was obtained.

Regarding the centrilobular hepatocyte hypertrophy observed in the two-generation reproductive toxicity study in mice, Tyl et al. (2008a) determined the NOAEL to be 30 ppm (5 mg/kg bw) from their viewpoint as the specialists. However, NOAEL could not be easily determined ourselves due to the lack of clear dose-response relationships among the data. We therefore tried to calculate the BMDL for the centrilobular hepatocyte hypertrophy in F0/F1/F1R males and F0/F1 females, by using BMDS ver. 2.1.2 of U.S. EPA. The results from the analysis of the incidence of centrilobular hepatocyte hypertrophy in F0 males are shown in Table 4. Meanwhile, EC (2008) determined the NOAEL to be 300 ppm (50 mg/kg bw) because they regarded the hepatocellular hypertrophy by BPA as being toxicologically

insignificant because the finding was mild and not accompanied by any organ weight changes.

Table 4 Results of BMD analyses on the centrilobular hepatocyte hypertrophy in F0 males in the two-generation reproductive toxicity study (Tyl et al., 2008a).

Model	*p*-value	AIC	Scaled Residual of Interest	BMD (ppm)	BMDL (ppm)	Mean BMDL (ppm)
Gamma	0.5946	88	0	222	42	52
Logistic	0.6997	86	-1.228	128	76	
LogLogistic	0.5946	88	0	247	54	
LogProbit	0.5946	88	0	213	50	
Multistage	0.7269	86	-1.157	157	42	
Probit	0.6970	86	-1.237	122	71	
Weibull	0.5946	88	0.001	229	42	
Quantal Linear	0.6559	87	-1.321	80	37	

The BMDL value for the centrilobular hepatocyte hypertrophy in F0 males was 52 ppm from the estimate by BMDS. The BMDL value for the same finding in F1 and F1R males as well as F0 and F1 females was calculated in the same way. The summary of all analysis results are shown in Table 5.

Table 5 Summary of BMD analyses regarding the centrilobular hepatocyte hypertrophy in F0/F1/F1R mice in the two-generation reproductive toxicity study (Tyl et al., 2008a).

sex	Group	BMDL (ppm)	Mean BMDL (ppm)	Dose (mg/kg bw/day)
male	F0	52	89	15
	F1	Not fitted*		
	F1R	125		
female	F0	389	252	42
	F1	114		

* *p* value of the chi-square test was 0.1 or under in all models

Data regarding the centrilobular hepatocyte hypertrophy in F1 males did not fit to any models. The arithmetically averaged value of BMDL regarding hepatocellular hypertrophy of neonatal mice was 89 ppm in males, and 252 ppm in females. The lower value was selected, and the NOAEL was determined to be 89 ppm. When the concentration in the food (ppm) was converted to dosage, it was 15 mg/kg bw. Moreover, this value was approximately the same level as the BMDL (16 mg/kg bw) for the centrilobular hepatocytomegaly observed in the reproduction and fertility study

in B6C3F1 mice (NTP, 1985).

Since multinucleated giant hepatocytes induced by BPA tend to be aggravated by the prolongation of the dosing period, 5 was applied as the uncertainty factor for the extrapolation from short-term study to long-term study, in our previous evaluation. With regard to other findings in the liver by BPA, such as centrilobular hepatocytomegaly and centrilobular hepatocyte hypertrophy, the aggravation by the prolongation of the dosing period is not clear due to the lack of data to evaluate this. We therefore hypothesized that they may be aggravated due to long-term dosing period, similarly to multinucleated giant hepatocytes, and 5 was applied as the uncertainty for the extrapolation from short-term study to long-term study. As a result, if the three types of liver pathology above were used as endpoints, the NOAEL for the chronic toxicity study of BPA was estimated to be 3.6, 3.2, and 3 mg/kg bw, respectively. Here, centrilobular hepatocyte hypertrophy was determined as endpoint for the hepatotoxicity of BPA, considering that the NOAEL value (3 mg/kg bw) of this finding was the lowest.

Table 6 Summary of BMD analysis regarding the effects on the kidneys of F0/F1 mice in the two-generation reproductive toxicity study (Tyl et al., 2008a)

Findings	sex	Group	BMDL (ppm)	Mean BMDL (ppm)	Dose (mg/kg bw/day)
Nephropathy	male	F0	474	539	90
		F1	502		
		F1R	642		
Increase in the left kidney weight	female	F0	968	968	161
		F1	Not fitted		
		F1R	Not fitted		

With regard to the NOAEL for the nephrotoxicity of BPA, it was also difficult to glance and to determine the NOAEL because an increased kidney weight of F1 males was observed at the lowest dose level, and moreover, nephropathy was also seen in the control group in the two-generation reproductive toxicity study (Tyl et al., 2008a). Accordingly, BMD analysis was also carried out on the kidney weight and the incidence of nephropathy. As a result, values of 474, 502 and 642 ppm were obtained as the BMDL from the data involved in the nephropathy of F0, F1 and F1R males, with an arithmetic mean value of 539 ppm (Table 6). With regard to the increased weight of the left kidney in F0/F1/F1R males, only data from F0 conformed to the model, and 968 ppm was obtained as the BMDL. From the analysis above, the BMDL for nephropathy and kidney weight increase were 539 ppm and 968 ppm, respectively.

The lower value (539 ppm) was determined to be NOAEL for the nephrotoxicity of BPA in the two-generation reproductive toxicity study (Tyl et al., 2008a). When this was converted to oral dosage, it was 90 mg/kg bw (Table 6). If these findings were hypothesized to be aggravated by the dosing period and when the uncertainty factor of 5 for the extrapolation from short-term study to long-term study was applied, the NOAEL for the nephrotoxicity of BPA in the chronic oral toxicity was estimated to be 18 mg/kg bw.

From the above analysis, the NOAEL for the hepatotoxicity (centrilobular hepatocyte hypertrophy) and nephrotoxicity (nephropathy) of BPA in mice were estimated to be 3 mg/kg bw and 18 mg/kg bw, respectively.

2.5 Genotoxicity and carcinogenicity

According to the new information, there was a report mentioning that BPA increased the defects in the alignment of the chromosomes on the first meiotic spindle in oocytes of neonatal mice when the oocytes were harvested from mice administered BPA and then were incubated overnight in-vitro (Hunt et al., 2003). However, it is highly possible that the above effect is reversible in-vivo, because no actual abnormalities were observed in the mitotic figures of oocytes or fertilized ova harvested from mice who received BPA orally for 7 days (Pacchierotti et al., 2008). Therefore, it is strongly suggested that there are no in-vivo effects on oocytes and fertilized ova due to BPA.

The promoter activity of BPA on the tumor initiator has not been observed, as shown by the findings that orally administered BPA had no effects on the outbreak of carcinogenesis in multiple organs in Wistar rats by BHP (Takashima et al., 2001), prostate cancer in F344 rats by DMBA (Ichihara et al., 2003), or uterine cancer in Donryu rats by ENNG (Yoshida et al., 2004).

There was a report mentioning that the number of mammary terminal end buds (TEB) increased in SD rats that were exposed to BPA in-utero (Moral et al., 2008). However, no causal relationship with BPA exposure was evident because no significant difference was observed with the control group in this study. In addition, there was a report mentioning that BPA accelerated/reinforced the mammary carcinogenesis due to DMBA in SD rats that were exposed to BPA via breast milk (Jenkins et al., 2009). However, the direct causal correlation of this finding with BPA was not definite because the BPA concentration in the breast milk was not measured. Betancourt et al. (2010), from the same study group, reported that BPA enhanced the mammary carcinogenesis at 12 months old in female offspring who were exposed to

BPA in-utero (250 μg/kg bw of dam), and then received DMBA of 30 mg/kg bw by gavage at PND 100. However, there were no enhancements of the mammary carcinogenesis by DMBA when administered at PND 50. The authors of the study suggested that the expression of ER-α as well as the sex steroid hormone controlling proteins in the fetus may have been enhanced by gestational exposure to BPA. However, the mechanism for the different response generated by the different timing for the DMBA dosing (PND 50 vs. PND 100) was not evident. It therefore believed that the study results could not be immediately applied to humans due to the clear difference in the kinetics of BPA between rodents and primates, and the lack of carcinogenicity in experimental animals.

In addition, there are reports mentioning that subcutaneously injected BPA promoted the action of tumor initiator in rats (Durando et al., 2007) and developed the precancerous lesions in the reproductive organs in rats and mice (Ho et al., 2006; Murray et al., 2007; Newbold et al., 2007). However, it was determined that these study results cannot be used in the hazard assessment of BPA in humans due to the parenteral dosing routes, because there is a clear difference in the kinetics of BPA between oral and parenteral routes.

Following a weight-of-evidence approach, it has been concluded that BPA is not likely to be carcinogenic to humans (Haighton, 2002). This was due to the fact that; a) BPA did not cause gene mutations or chromosomal aberrations in bacteria/fungi/mammalian cells in standard in-vitro genetic tests, b) BPA was negative in in-vivo chromosomal aberration tests, and c) BPA was negative in all of the bone-marrow micronucleus tests in mice, dominant lethal tests in rats, and carcinogenicity study in rats and mice. None of the new information supported overturning this conclusion.

2.6 Reproductive and developmental toxicity

In the previous evaluation, there were no adverse effects due to BPA administration on reproductive potential of parents and the next generation in a single generation reproductive or two-generation reproductive toxicity studies (Ema et al., 2001, ECB, 2003). A decline in litter size was observed at 50 mg/kg bw in the three-generation reproductive toxicity study (Tyl et al., 2002), but it was believed to be a secondary effect due to the general toxicity in the parent animals, and the NOAEL of the reproductive and developmental toxicity of BPA in the three-generation reproductive toxicity study in rats was determined to be 50 mg/kg bw. In a reproduction and fertility study using B6C3F1 mice (NTP, 1985), NOAEL could not be

obtained because there was a decline in the fertility at 600 mg/kg bw or more, and a decline in epididymal weight in the F1 generation in the lowest dose level of 300 mg/kg bw. With regard to the decline in epididymal weight, it was the only effect observed in the reproductive organ due to BPA administration, but its toxicological significance was not evident. From the above, we determined the NOAEL for the reproductive toxicity in rats to be 50 mg/kg bw, in the previous evaluation.

The GLP-compliant two-generation reproductive toxicity study in mice under OECD testing guidelines (Tyl et al., 2008a) is one of the important information here. 17β-estradiol (E2) was used as a positive control. The validity of the test protocol to E2 was confirmed separately (Tyl et al., 2008b; 2008c). As a result, no direct effects due to BPA on the reproductive potential of the offspring other than a slight prolongation of the gestational period in F1/F2 at the highest dose level (600 mg/kg bw) were observed. Moreover, it was confirmed that there are no parameters showing toxic effects due to BPA and/or bell-shaped dose-response curves at low doses of 0.003 to 5 mg/kg bw. Therefore, the NOAEL for the reproductive and developmental toxicity of BPA was determined to be 300 ppm (50 mg/kg bw), and BPA was determined not to be a reproductive and developmental toxicants in mice (Tyl et al., 2008a). In addition, it has been reported that there were no effects on the reproductive potential of the dam or reproductive functions of female and male offspring when low dose BPA (maximum of 200 μg/kg bw) was orally administered to rats during either the gestational or lactational period (Howdwshel et al., 2008; Ryan et al., 2010).

In the two-generation reproductive toxicity study by Tyl et al. (2008a), various experts participated in all aspects of the study, from designing the study protocol to the statistical analysis of the data. Particularly, the study was designed to detect the presence of the bell-shaped dose-response curve at low doses. EC (2008) has valued this study design as the gold standard, considering that this test conforms to GLP and internationally approved testing guidelines, and thus the study was most reliable as a test for evaluating the reproductive and developmental toxicity of BPA. Regarding the reproductive toxicity of BPA, 50 mg/kg bw was believed to be the appropriate NOAEL value because the value in this study coincides with that obtained from previous studies in rats and mice. Moreover, it was believed that there was no bell-shaped responsiveness at low doses. Meanwhile, EFSA (2006) decided that the additional uncertainty factor 5, which was adopted in their hazard assessment in 2002 due to the lack of sufficient information, is now unnecessary because the database regarding reproductive/developmental toxicity has been reinforced.

2.7 Developmental neurotoxicity

A GLP-compliant developmental neurotoxicity study on BPA under the OECD testing guideline 426 as well as the testing guideline 870.6300 of the U.S. EPA OPPTS (Stump et al., 2010) was carried out. As a result, the NOAEL of developmental neurotoxicity was determined to be 2,250 ppm (164 mg/kg bw during gestational period; 410 mg/kg bw during lactational period), which was the highest dose level used in the study. However, EFSA (2010) determined that there was a problem in the study protocol for evaluating the effects on learning and memory using the Bile-maze test by Stump et al. (2010), and determined that the effects due to BPA on learning and memory cannot be evaluated. The reason for this was because only 3 minutes were given to complete the tasks in the Bile-maze test, so by limiting the time, data that could have been obtained if a longer time had been spent may have been censored out.

The purpose of the developmental neurotoxicity study is to detect the effects on behavior and learning/memory functions or motility regulatory functions by test articles exposed in-utero or neonatal periods. Therefore, a test article was administered to dams from gestational day 6 to PND 10, and as a result in-utero exposure to the fetus and lactational exposure to offspring were carried out. Locomotion, behavior, reflex, and learning/memory of the offspring were observed and evaluated in detail for approximately two months after birth, and a detailed histopathological investigation was carried out on the nerve tissues of the whole body along with measurement of brain weight at PND 11 and at the end of the study (U.S. EPA Health Effects Test Guidelines, OPPTS 870.6300; OECD Guidelines for the Testing of Chemicals 426). These developmental neurotoxicity tests can detect about 5 to 20% of changes compared to a control group, and have been confirmed to be effective in the detection of known human developmental neurotoxicants (ethanol, lead, methylmercury, PCBs, DDT, etc) (Makris et al., 2009). Meanwhile, estradiol has the potential to stimulate the induction of neurite in organ cultures of the preoptic area, hypothalamus, and cerebral cortex in-vitro, and moreover, it is known that estradiol influences apoptosis of neurons at the site known as the sexual dimorphic nucleus. In addition, it is also known that estrogen receptors (ER) are widely distributed in the brain in the developmental stage and there is a difference between sexes in terms of ER distribution at specific sites (McCarthy, 2008). Sex hormones required for brain development are locally *de novo*-synthesized, and their synthesis is highly controlled, simultaneously with the inactivation of circulating hormones by binding with α-fetoprotein (McCarthy, 2008). However, chemical substances with estrogen-like

activity are not controlled in the same way as endogenous estrogen because most of the chemicals have low affinity with α-fetoprotein. Thus, the possibility of chemical substances with estrogen activity influencing structure and functions of the brain during development, such as in-utero exposure of BPA, is a concern.

In a neuropathological test by Stump et al. (2010), there were no abnormalities of the brain/nervous system of offspring due to the administration of BPA to their dam. From this result, it was strongly suggested that BPA is, at least, not a conventional developmental neurotoxicant. At the same time, this data presents a question with regards to the validity of the current study protocol to detect toxic effects under the assumption of developmental neurotoxicity due to estrogen-like substances. Thus, in order to evaluate the developmental neurotoxicity of BPA, it is necessary to confirm that presumed developmental neurotoxicity due to estrogen activity is effectively detected by the current study protocol. Therefore, the RISS will withhold its evaluation of BPA regarding developmental neurotoxicity until the validity of the study protocol is confirmed.

In addition, various types of developmental neurotoxicity of BPA under the atypical test protocol have been reported. However, the test protocol used was not well-established as a toxicity tests, and the toxicological significance of the data from these atypical protocol was not evident. Moreover, the effects on central nervous systems at various sites are observed in the offspring exposed to BPA in-utero, but in many cases, the recommendation of ILSI (Holson et al., 2008) regarding data analysis to avoid the litter effect was not followed. Meanwhile, the FAO/WHO Expert Meeting (WHO, 2010) determined that the findings have a shortcoming due to the inappropriate experimental method, and that the uncertainty is too large to extrapolate any effects observed in laboratory animals to humans. It was thus believed to be too hasty to regard these test data immediately as adverse effects of BPA in humans.

As an supporting evidence for the effects of BPA on the sexual differentiation of the brain and/or sexual behavior, there is a report (Ehrhardt et al., 1989) mentioning the loss of parenting interest as an abnormality in the sexual differentiation of women's brain due to the in-utero exposure to diethylstilbestrol (DES) (Ehrhardt et al., 1989). However, this has not been reproduced in the subsequent large-scale studies (Lish et al., 1991). Newbold (1993) claims that the published abnormal behaviors found in women exposed to DES in-utero are not definite because they were observed in studies lacking sufficient number of cases, some without appropriate control subjects, and moreover, included cases of DES exposure that were not of in-utero exposure. Moreover, she warns that caution is required when extrapolating findings obtained

from animal tests to humans, because the factors related to social behaviors and their sex differences in experimental animals are not only diverse and complicated but different causes are involved depending on the animal species. In fact, Wallen (2005), who studied the hormonal influences on the sexual dimorphic behavior in non-human primates, stated that the developmental stage of brain and sexually differentiated behavior at birth differs in monkeys and rodents, and also the role of sex hormones in monkeys with regard to the sexual differentiation of the brain is different from that of rodents. Moreover, McCarthy (2008) presented an issue with a working hypothesis that endocrine–disrupting action will effect sexually differentiation of the brain, by quoting two papers negatively (Funabashi et al., 2004 and Fujimoto et al., 2006) where the effects of BPA on the development of corticotropin-releasing hormone-immunoreactive neurons in rat brain and on the “depression-like behaviors” in rats, respectively, were examined under the above working hypothesis. That issue is based on the following reasons; a) it was originally not understood whether or not there is a difference by sex in the above phenomenon, and b) many other causes that effect sexually differentiation of the brain were conceivable. In addition, development of the brain becomes temporarily exuberant at a certain point during the perinatal period, an event known as the brain growth spurt, but the fact that this period widely varies by animal species (Dobbing and Sands, 1979) suggests the difficulty in extrapolating animal test data to humans with regard to the effects on the development of the brain function. As stated above, it is believed that one should be deliberate when applying the data obtained in rodents regarding the impacts on sexually differentiation of the brain by BPA presuming estrogen-like actions to humans.

2.8 Toxicokinetics

As of the previous evaluation, information regarding the kinetics and metabolism of BPA was limited to the studies in mainly rats and mice. More specifically, orally administered BPA is rapidly absorbed from the gastrointestinal tract, metabolized in the liver (first-pass effect), and rapidly excreted from the blood in rats (ECB, 2003). The major metabolite in rats is glucuronide conjugates, but 5-hydroxy BPA and BPA-sulfate conjugates are formed at high BPA concentrations in-vitro (Elsby et al., 2001). BPA is excreted mainly in feces in rodents, and it has been shown to enterohepatically circulate (Upmeier et al., 2000), but most is excreted by 72 hours after administration. Lactational excretion of BPA was also observed, but the quantity was small (Snyder et al., 2000). Moreover, a difference by sex was observed in the

kinetics of BPA in rats; the blood concentration and the quantity of urinary excretion of BPA for both oral and parenteral administrations in females were larger than that of males. Moreover, a strain difference was also observed; F344 rats were found to have a greater quantity of absorption and urinary extraction of BPA compared to SD rats (ECB, 2003).

When BPA was orally administered to healthy humans (men and women) at 5 mg (54 to 88 μg/kg bw), BPA was completely absorbed from the gastrointestinal tract and rapidly excreted from the blood with the initial half-life of 1.5 hours and the late half-life of 3.4 hours. Moreover, when plasma samples were treated with β-glucuronidase, the blood concentration slightly declined, but the authors determined that free-BPA was not detected in the blood plasma (Völkel et al., 2002). Furthermore, when BPA was orally administered to cynomolgus monkeys of both sexes at 0.1 mg/kg bw, it was rapidly excreted at a half-life of approximately 10 hours (Kurebayashi et al., 2002).

From 2005 onwards, papers on the toxicokinetics of BPA in chimpanzees and monkeys, on the effects of gestation on the metabolism of BPA, and on the potential of neonatal rats to metabolize BPA have been published. As a result, it has become evident that the glucuronide-conjugation of orally administered BPA is rapid in both rodents and primates. More specifically, according to a comparative study of BPA metabolism using rats, cynomolgus monkeys and chimpanzees (Tominaga et al., 2006), the ratio of the C_{max} of BPA-metabolites (conjugate) to free-BPA upon oral administration of BPA (10 mg/kg bw) was 65 times in rats, 751 times in cynomolgus monkeys, and 184 times in chimpanzees; BPA is rapidly metabolized at the first-pass and became detoxified in all animal species. This has been supported by the fact that the apparent intrinsic clearance (CL_{int}) is much larger than the hepatic blood flow in rats and humans (Tominaga et al., 2006).

Orally administered BPA is rapidly metabolized to glucuronide-conjugates in the liver in both primates and rodents. However, the excretion of BPA from the body is delayed in rodents due to the enterohepatic circulation. On the other hand, BPA are excreted from the body in primates including humans faster than in rodents because BPA-glucuronides are not excreted into the bile in primates. The blood half-life of BPA was reportedly about 5 hours in humans (Völkel et al., 2002) and 18 to 22 hours in rats (Kurebayashi et al., 2005), and the excretion of BPA from the blood in humans is about 4 times faster than in rodents. When the BPA-glucuronidation ability of various recombinant UDP-glucuronosyltransferase (UGT) isozymes in humans is compared in terms of the K_{cat}/K_m value, 1A9 isozymes that are expressed in the gastrointestinal tract and liver are the strongest (Doerge et al., 2010a), indicating a large contribution

of UGT in the organs other than the liver to the detoxification of BPA in humans. In addition, it has been reported that sulfate conjugates were 20% or less in rhesus monkeys and 5% or less in rats (Doerge et al., 2010a), suggesting the large contribution of the sulfation route in humans with regard to detoxication of BPA. All these findings support metabolic detoxication of BPA in humans stronger than that of rodents.

The issue is how this metabolism of BPA changes due to pregnancy, or how the metabolic activity changes in fetus or neonates. It has been reported that in rats, the metabolism of orally administered BPA is not affected by pregnancy, and that there is no distribution specific to the embryo/fetus until gestational days 6 to 14. However, 0.1% or lower of the radioactivity from BPA was detected in rat fetus at gestational day 17 (Domoradzki et al., 2003). Similarly, Kurebayashi et al. (2005) reported that radioactivity from BPA was not detected in rat fetus at gestational day 12 or 15 when BPA was orally administered to their dams, but radioactivity was detected in the bladder and small intestine of the fetus at gestational day 18. These reports suggest that free-BPA or BPA-metabolites are incorporated into the fetus via the placenta from the blood of dams in the late gestational period with the critical period of around gestational day 17. The radioactivity detected in rat fetuses appears to be attributable to free-BPA produced by β-glucuronidase in the placenta where β-glucuronidase has presumably become dominant in comparison with placental UGT (Aitio, 1974). That is because BPA-glucuronide cannot pass the placental blood barrier due to its high water solubility, but free BPA, which is presumably produced by β-glucuronidase in the placenta, can pass. However, it should be emphasized that UGT activity of 22 nmoles/min/g tissue has been demonstrated in rat fetal liver of gestational days 18 to 19, which is about 20 times lower of the UGT activity in adult liver (Aitio, 1974). It is highly possible that UGT activity in fetus liver participates in the detoxification of BPA, too.

Recently, metabolic profile of BPA in neonatal rhesus monkeys have become evident; the blood concentration of free-BPA in neonates of all ages from 5 days old to 70 days old was the same level as that of adults while the blood concentration of total-BPA including free and conjugates was higher in younger neonates, and decreased to adult levels with growth, upon oral administration of BPA at 100 μg/kg bw. This shows that neonatal rhesus monkeys effectively metabolize BPA even 5 days after the birth (Doerge et al., 2010a). The reason for the high blood concentration of total-BPA in 5 days old neonates was determined to be attributable to the immature function of kidneys to excrete BPA-conjugates at this age (Doerge et al., 2010a).

On the other hand, Domoradzki et al. (2004) orally administered BPA to neonatal rats at 1 and 10 mg/kg bw, and showed that the blood level of BPA-glucuronide in the neonatal rats immediately after birth was evidently lower than that of adults. Subsequently, Doerge et al. (2010b) also showed that the blood concentration of total-BPA and free-BPA in 3 days old neonatal rats that were orally administered BPA at 100 μg/kg bw was much higher compared to adults (total-BPA: 445 nM vs. 70 nM, free-BPA: 29 nM vs. 0.4 nM). However, even in 3 days old neonates, the free-BPA was merely 16% of the total-BPA, and thus it was clarified that BPA is also efficiently glucuronidized in neonates.

There is no information regarding BPA metabolism in human fetuses or neonates, but there are several reports that become reference. More specifically, hepatic UGT in humans decreased in the late-1st trimester (from week 14 of pregnancy onwards) (Collier et al., 2002; 2009), and when it was tracked by UGT1A4 isozyme activity, neonates also had low hepatic UGT activity for about 3 months after birth (Miyagi and Collier, 2007). However, isozymes of UGT involved in BPA-glucuronidation in humans is a matter of controversy; Edginton and Ritter (2009) assume it to be UGT2B7, and Doerge et al. (2010a) to be UGT1A9. UGT2B7 is nearly the same level as adults in a one year old child, but it is considered to be 5% of adults in neonates, and 30% of adults in 3 months old (Edginton et al., 2006). Conversely, β-glucuronidase activity, which produces free-BPA by degrading BPA glucuronides, is the same level in 4 months old as adults, but expresses a higher activity in the fetal liver than in the adult liver (Miyagi and Collier, 2007). Thus, regarding UGT2B7 in humans, it was suggested that the glucuronidation ability of BPA from gestational week 14 onwards, until 3 months old is lower than that of adults. Accordingly, an estimation of the plasma concentration of free-BPA in human neonates is 11 times higher than that of adults by the PBPK model analysis under such a hypothesis, considering the low UGT activity in neonates, the histology between neonates and adults, and physiological differences (Edginton and Ritter, 2009). However, the metabolic profile of BPA in neonatal rhesus monkeys (Doerge et al., 2010a) clearly shows that, contrary to expectations, orally administered BPA is rapidly detoxified to glucuronides as well as to sulfate-conjugates as in adults, and that the blood free-BPA concentration is almost of a negligible level. Thus, as in the analysis by Doerge et al. (2010a), it is highly conceivable that in addition to the fact that UGT is expressed not only in the liver but also in the digestive tract, the contribution of the sulfate conjugation is not so small. Particularly, glutathione S-transferase (GST) activity in the liver and kidneys in human fetuses is higher than in adults (Mukhtar et al., 1981) in contrast to rats (Mukhtar and Bresnick,

1976). Thus, there is a possibility that the presumed low glucuronide conjugation capacity for BPA is supplemented by the sulfate conjugation by GST. This possibility has been examined in detail in EFSA (2008) and concluded that BPA can be detoxified by sulfation in fetuses and neonates because; a) acetaminophen, which has the simple phenolic structure without steric hindrance of the OH groups like BPA, is transformed to sulfates in human fetuses and neonates, b) BPA-sulfate is generated in cultured human hepatocytes, and c) BPA-sulfate is detected in humans as urine metabolite. In fact, in both neonatal and adult rhesus monkeys, BPA is transformed to glucuronide-conjugates together with sulfate conjugates, and 20% or less of all metabolites are products of the sulfate conjugation route (Doerge et al., 2010a).

As mentioned above, the excretion rate of BPA in humans is about 4 times faster than rodents, and moreover, the blood concentration of free-BPA was negligible when BPA of about 50 to 88 μg/kg bw were orally administered to humans. Also, it is highly conceivable that human neonates and fetuses have sufficiently high metabolic potential to detoxify BPA at concentrations that exposed via the environment because human neonates presumably have the same potential to metabolize BPA as in neonatal rhesus monkeys, and also the sulfate conjugation route appears to participate in the biotransformation of BPA in human fetuses.

Ginsberg and Rice (2009) emphasized the role of β-glucuronidase in the placenta, and strictly criticized the view of EFSA (2008), which determined that BPA is rapidly detoxified to glucuronide-conjugates in humans. However, they neglected the fact that the placenta of humans and rats has the large quantity of UGT (Litterst et al., 1975; Collier et al., 2002), together with β-glucuronidase, that a large quantity of GST is present in the human fetus and neonates, and that UGT is available in rat fetal liver (Aitio, 1974) . Moreover, their criticism contradicts the metabolism profile of BPA in neonatal rhesus monkeys, in which the blood free-BPA concentration is almost of a negligible level (Doerge et al., 2010a). Meanwhile, Taylor et al. (2008) reported that the blood free-BPA concentration in neonatal CD-1 mice at PND 3 was the same regardless of the dosing route; oral or subcutaneous. However, they measured only t-buty ether-extractable free-BPA, and thus the toxicokinetics of total BPA including metabolites is not evident, as indicated by EFSA (2008). In contrast to the results by Taylor et al. (2008), clear difference in blood concentration of free-BPA in neonatal rats between subcutaneous injection and oral administration has been shown (Doerge et al. 2010b; Prins et al., 2010), in which BPA contamination from the experimental environment was carefully avoided.

3. Overall evaluation of the health hazard of BPA

The reproductive toxicity in the next generation was of concern as one hazard of BPA on human health, and a GLP compliant two-generation reproductive toxicity study on BPA under the current testing guidelines was carried out. Regarding the reproductive toxicity of BPA to the next generation, no toxic effects were observed other than a slight prolongation of the gestational period of F1/F2 at 300 mg/kg bw, and consequently, NOAEL of 50 mg/kg bw was obtained. Furthermore, we determined not to apply any additional uncertainty factor to the conventional uncertainty factor (100 = species difference 10 x individual variability 10) with regard to the uncertainty of the low dose effects of BPA, because a clearly negative result was obtained in the well designed two-generation reproductive toxicity study on BPA.

It has been reported that BPA accelerated/reinforced the mammary carcinogenesis due to DMBA in SD rats that were lactationally exposed to BPA (Jenkins et al., 2009; Betancourt et al., 2010). However, this finding was not believed to be a hazardous risk to humans because of the following reasons; a) the oral carcinogenicity of BPA has already been determined to be negative from several bioassays, b) the mechanism for the different susceptibility of neonatal rats to tumor initiator, that was generated by the different timing for the DMBA dosing (PND 50 vs. PND 100), was not evident, and c) there is a clear difference in the potential for the biotransformation of BPA between rodents and primates, and thus the above findings appear to be limited to rodents.

Skin irritation, skin sensitization, photo-irritability, and photo-sensitization due to BPA was negative in animal testings at a practical exposure level, so it was believed that there is almost no need for concern.

Regarding the developmental neurotoxicity of BPA, a GLP-compliant developmental neurotoxicity study under the OECD testing guidelines 426 and testing guidelines 870.6300 of the U.S. EPA OPPTS has been carried out (Stump et al., 2010). However, it was not evident if the current study protocol, that is designed originally to detect developmental neurotoxicity of known neurotoxicants, was effective for chemical substances having estrogenic activity. Thus, the RISS withholds the evaluation of BPA regarding developmental neurotoxicity until the validity of the study protocol for estrogenic substances is confirmed.

In addition, the effects on the sexually differentiation of the brain, sexual behavior, various social behaviors, brain neurotransmitters as well as its receptor expressions, by prenatal or neonatal exposure of BPA either in humans or experimental animals have been reported. However, significant methodological defects in the

epidemiological studies (e.g. exposure concentration of BPA is not evident, considerations for the confounding factors are not sufficient, etc.) and insufficient scientific basis for the working hypothesis in animal studies (e.g. the presumed effects of endocrine–disrupting action on the sexually differentiation of the brain are not confirmed, extrapolation of animal data to humans with regard to brain function needs further investigation, etc.) have been found. Therefore, the RISS decided to withhold the assessment of the effects of BPA exposed in-utero or via breast milk on the brain functions or behaviors of children at present.

Regarding the NOAEL of oral general toxicity of BPA, we determined that centrilobular hepatocyte hypertrophy with NOAEL of 3 mg/kg bw/day as the endpoint. This NOAEL value was roughly the same level as the value from the previous evaluation (5 mg/kg bw/day for the multinucleated giant hepatocytes).

The RISS decided to set the uncertainty factor related to toxicokinetics as 1, and subsequently to have the uncertainty factor for species difference as 2.5, although it is the conventional way to have the uncertainty factor for species difference of 10 (uncertainty factor for toxicokinetics: 4, uncertainty factor for toxicodynamics: 2.5) (IPCS, 1999). The reason for this is as mentioned below.

BPA enterohepatically circulates in rodents, delaying excretion from the body, whereas there is no enterohepatic circulation in primates including humans, and the rate of excretion from the blood is faster in primates than in rodents. Also, presuming from the blood concentration half-life of BPA in humans and laboratory animals, the metabolic activity in adult humans to detoxify BPA to conjugates is at least 4 times higher compared to rodents and is believed to be the same or better in comparison with monkeys. Moreover, the C_{max} of free-BPA in adult humans when BPA at 54 to 88 μg/kg bw was orally administered was roughly 0.15 nM, that is substantially negligible (Völkel et al., 2002). C_{max} and AUC of free-BPA by the oral administration of BPA at 100 μg/kg bw in adult rats were 0.39 nM and 2.6 nM·hr, respectively (Table 9, Doerge et al., 2010a; 2010b). Thus, it is highly conceivable that C_{max} and AUC of free-BPA by oral administration of BPA at about 100 μg/kg bw in humans is either comparable to or at least 2 times lower than that of rats. Regarding the metabolic potential of neonates for BPA, C_{max} and AUC of free BPA of 3 days old rats and 5 days old rhesus monkeys were 29 nM vs. 2 nM and 56 nM·hr vs. 5.7 nM·hr, respectively. Thus, the free-BPA concentration in the blood and AUC of human neonates are hypothesized to be about 10 times lower than in rat neonates. Furthermore, it has been clearly shown that hepatocellular damage and/or hormonal activity due to BPA was caused by not conjugated BPA, but free-BPA. Thus, it was believed legitimate to consider the

uncertainty factor for the toxicokinetics involved in species differences to be 1 regardless of adults or neonates.

Meanwhile, the FAO/WHO Expert Meeting (WHO, 2010) suggests from the toxicokinetic profile of BPA that the uncertainty factor of 4 for toxicokinetics is unnecessary for adults. Moreover, EFSA (2010) also stresses that the uncertainty factor of 10 involved in species differences in the risk assessment of BPA is substantially conservative. On the other hand, EC (2008) determined that the approach of making the uncertainty factors involved in toxicokinetics and toxicodynamics smaller cannot be taken, considering that species differences are not only due to differences in metabolic rate, and from the fact that causes of the toxic effects in liver are not limited to free-BPA.

Here, we determined the NOAEL to be 3 mg/kg bw/day, and uncertainty factor to be 25, although we previously determined them to be 5 mg/kg bw/day and 100, respectively, in the detailed risk assessment document published in 2005. Meanwhile, it is believed that there is no place for the glucuronidation ability of BPA to be effected by genetic polymorphisms of UGT in humans, because various UGT isozymes such as 1A1 or 1A3 in addition to 1A9 are expressed (Doerge et al., 2010a).

According to the BPA exposure estimate in Japanese people (Miyamoto and Kotake, 2006), the highest BPA exposure was found in 1 to 6 years old children, with a 95% tile value of estimated figures of 3.9 μg/kg bw/day (men) and 4.1 μg/kg bw/day (women). Moreover, the 95% tile value for the intake amount of BPA estimated from the 24-hour urine BPA concentration in adults was 0.037 to 0.064 μg/kg bw/day (men) and 0.043 to 0.075 μg/kg bw/day (women). When the 95% tile value of these exposure estimates and the NOAEL (3 mg/kg bw/day) from the animal tests are used, the Margin of Exposure (MOE) was 730 to 770 in 1 to 6 years old children, and was 40,000 to 81,000 in adults. These values were much larger than the MOE (25) that was presumed to cause health effects in humans or the conventional and conservative MOE (100) mentioned above, and thus the risk of BPA with regard to human health was believed to be very small.

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Appendix 1. BMD analysis of hepatocellular lesions regarding the mouse reproduction and fertility study

An analysis of the binary data regarding multinucleated giant hepatocytes and centrilobular hepatocytomegaly in male mice in the reproduction and fertility study (NTP, 1985) was carried out using Benchmark Dose Software (BMDS) Version 2.1.2, developed by U.S. EPA.

Eight models of Gamma, Logistic, Log-Logistic, Log-Probit, Multistage, Probit, Weibull, Quantal Linear were used for the analysis. All parameters for the BMD analysis were set to default, including the benchmark response (BMR) of 10% extra risk.

The compatibility of the models was determined from *p* values for the chi-square test of 0.1 or more and the absolute value for Scaled Residual of Interest of fewer than 2 as well as the visual inspection of the estimated dose-response curve. Also, Akaike's Information Criterion, AIC, was used to compare the model's goodness of fit (the smaller, the better the fit).

Table 1 Incidence of multinucleated giant hepatocyte (NTP, 1985)

Dose (%)	Observed (n)	Size (n)
0	0	19
0.25	13	19
0.5	19	20
1.0	10	11

Table 2 Results of BMD analysis on multinucleated giant hepatocyte (all dose levels included)

Model	AIC	P-value	Specified Effect	Risk Type	BMD (%)	BMDL (%)	Scaled Residual of Interest	Error log
Gamma	44	0.1069	0.1	Extra risk	0.024	0.018	0	
Logistic	56	~~0.0000~~	0.1	Extra risk	~~0.075~~	~~0.051~~	-1.608	
LogLogistic	44	0.4204	0.1	Extra risk	0.040	0.005	0	
LogProbit	44	0.3927	0.1	Extra risk	0.027	0.000	0	
Multistage	44	0.1069	0.1	Extra risk	0.024	0.018	0	
Probit	60	~~0.0000~~	0.1	Extra risk	~~0.073~~	~~0.054~~	-1.979	
Weibull	44	0.1069	0.1	Extra risk	0.024	0.018	0	
Quantal-Linear	44	0.1069	0.1	Extra risk	0.024	0.018	0	
				Mean	0.027	0.013		

* Row 1: Gamma Error

Table 3 Results of BMD analysis on multinucleated giant hepatocyte (the highest dose level excluded)

Model	AIC	P-value	Specified Effect	Risk Type	BMD (%)	BMDL (%)	Scaled Residual of Interest	Error Log
Gamma	36	1.000	0.1	Extra risk	0.057	0.015	0	
Logistic	39	~~0.096~~	0.1	Extra risk	~~0.094~~	~~0.056~~	-0.85	
LogLogistic**	36	1.000	0.1	Extra risk	~~0.097~~	~~0.006~~	0	*
LogProbit**	36	1.000	0.1	Extra risk	~~0.088~~	~~0.002~~	0	
Multistage	36	0.995	0.1	Extra risk	0.031	0.015	-0.006	
Probit	39	~~0.079~~	0.1	Extra risk	~~0.084~~	~~0.053~~	-0.883	
Weibull	36	1.000	0.1	Extra risk	0.044	0.015	0	
Quantal-Linear	34	0.841	0.1	Extra risk	0.020	0.014	0	
				Mean	0.038	0.015		

* Row 3: LogLogistic Error: Warning: Likelihood for the fitted model larger than the Likelihood for the full model.

** Dose response curve did not fit the data according to the visual inspection.

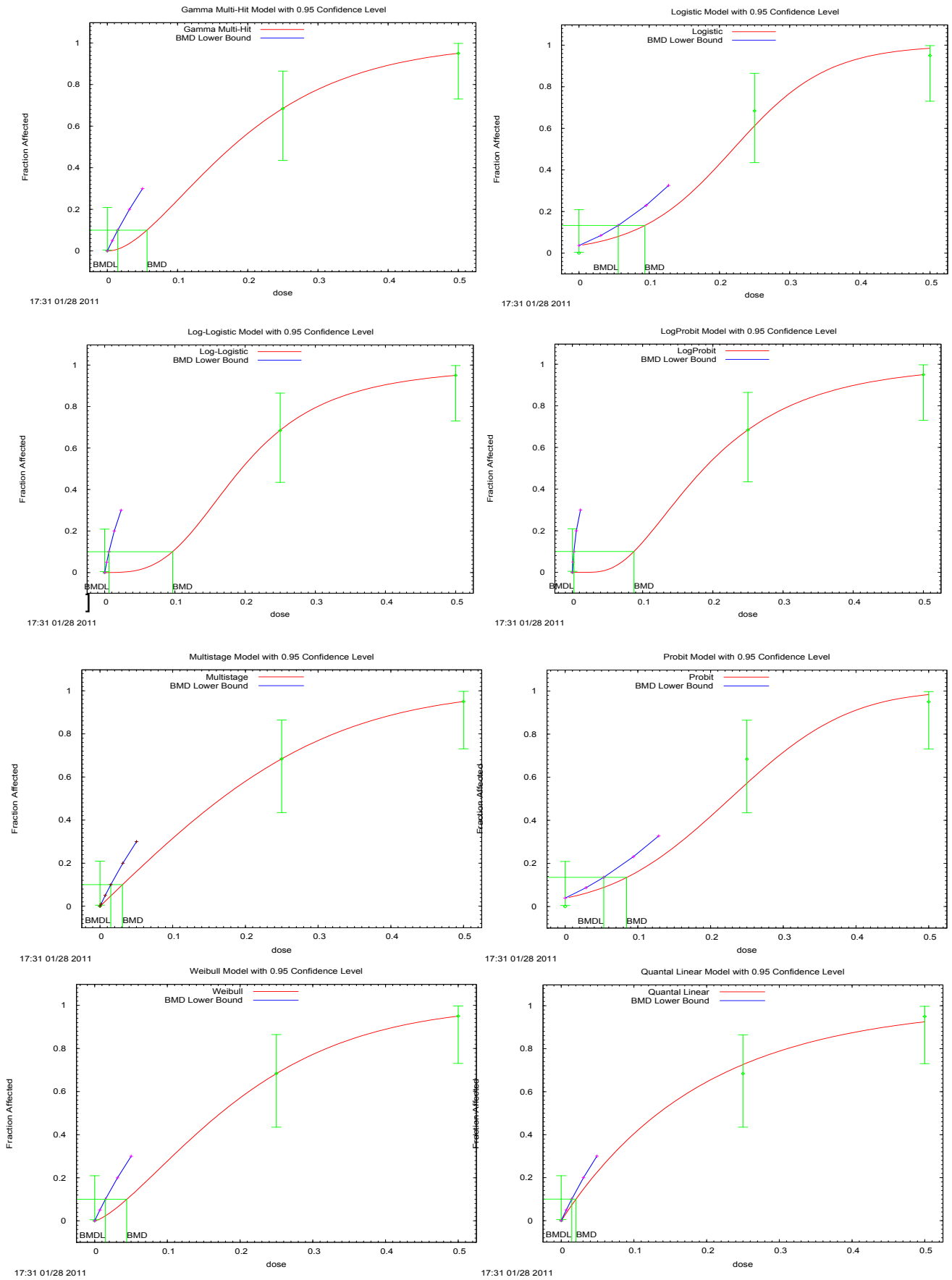

Fig.1 Dose response curves for the multinucleated giant hepatocyte
(the highest dose level excluded)

Table 4　Incidence of centrilobular hepatocytomegaly (NTP, 1985)

Dose (%)	Observed (n)	Size (n)
0	0	19
0.25	14	19
0.5	18	20
1.0	10	11

Table 5　Results of BMD analysis on centrilobular hepatocytomegaly (all dose level included)

Model	AIC	P-value	Specified Effect	Risk Type	BMD (%)	BMDL (%)	Scaled Residual of Interest	Error log
Gamma	46	0.1624	0.1	Extra risk	0.025	0.018	0	
Logistic	60	~~0~~	0.1	Extra risk	~~0.073~~	~~0.051~~	~~-1.808~~	
LogLogistic	46	0.8512	0.1	Extra risk	0.014	0.004	0	
LogProbit**	46	0.8308	0.1	Extra risk	~~0.009~~		0	
Multistage	46	0.1624	0.1	Extra risk	0.025	0.018	0	
Probit	64	~~0~~	0.1	Extra risk	~~0.073~~	~~0.055~~	~~-2.112~~	
Weibull	46	0.1624	0.1	Extra risk	0.025	0.018	0	
Quantal-Linear	46	0.1624	0.1	Extra risk	0.025	0.018	0	
				Mean	0.023	0.016		

* Row 1: Gamma Error

** Dose response curve did not fit the data according to the visual inspection.

Table 6　Results of BMD analysis on centrilobular hepatocytomegaly (The highest dose level excluded)

Model	AIC	P-value	Specified Effect	Risk Type	BMD (%)	BMDL (%)	Scaled Residual of Interest	Error Log
Gamma	37	0.936	0.1	Extra risk	0.021	0.015	0	
Logistic	45	~~0.0166~~	0.1	Extra risk	~~0.080~~	~~0.051~~	~~-1.197~~	
LogLogistic	39	1	0.1	Extra risk	0.037	0.004	0	
LogProbit*	39	1	0.1	Extra risk	~~0.032~~		0	
Multistage	37	0.936	0.1	Extra risk	0.021	0.015	0	
Probit	64	~~0~~	0.1	Extra risk	~~0.073~~	~~0.055~~	~~-2.112~~	
Weibull	37	0.936	0.1	Extra risk	0.021	0.015	0	
Quantal-Linear	37	0.936	0.1	Extra risk	0.021	0.015	0	
				Mean	0.024	0.013		

* Dose response curve did not fit the data according to the visual inspection.

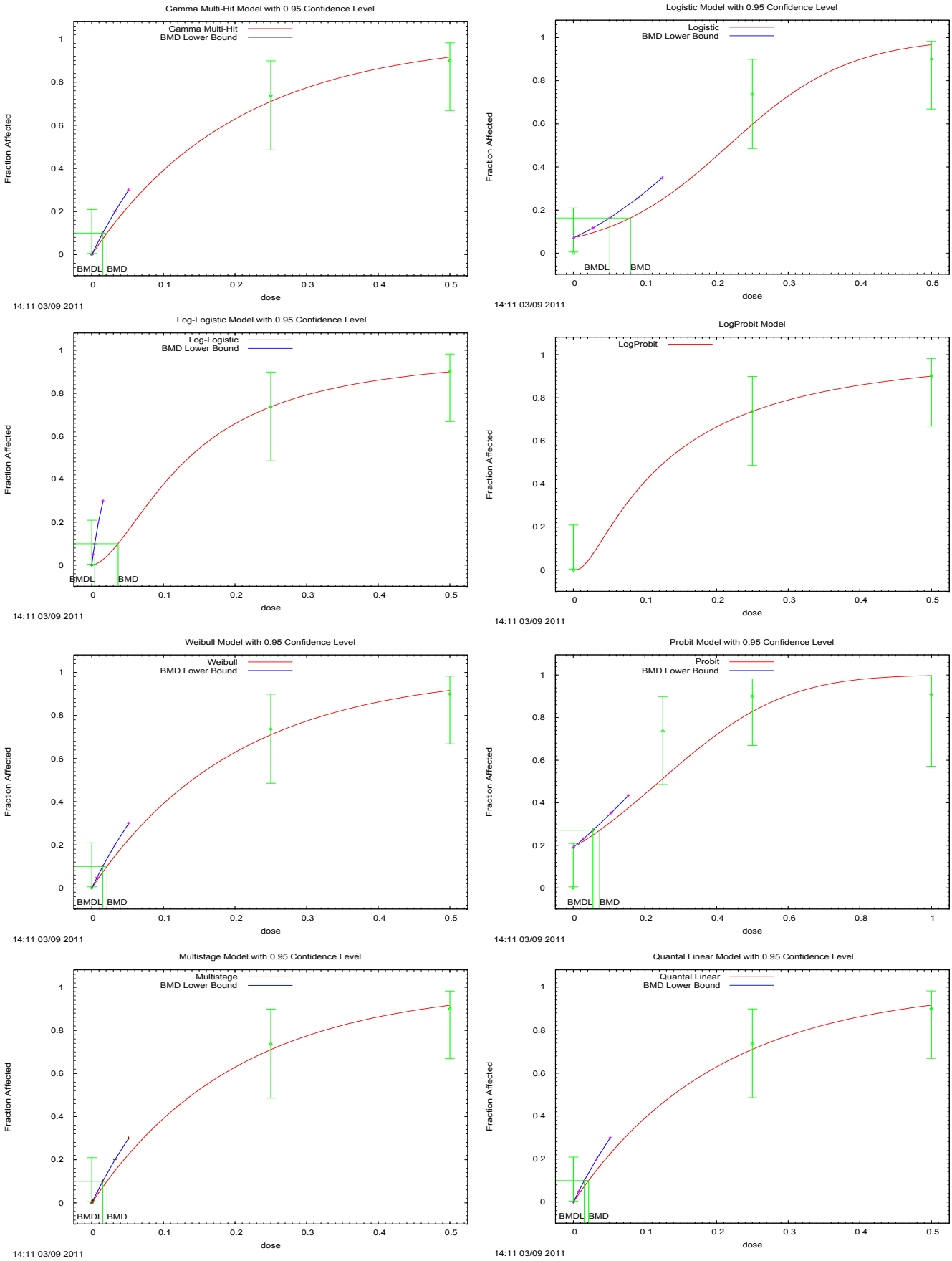

Fig.2 Dose response curves for the centrilobular hepatocytomegaly (the highest dose level excluded)

Appendix 2. BMD analysis of lesions in the liver and kidney regarding the mouse two-generation reproduction toxicity study

An analysis of the binary data regarding centrilobular hepatocyte hypertrophy in male F0/F1/F1R and female F0/F1 mice, and nephropathy in male F0/F1/F1R in the two generation reproduction toxicity study (Tyl et al., 2008a) was carried out using Benchmark Dose Software (BMDS) Version 2.1.2, developed by U.S. EPA.

Eight models of Gamma, Logistic, Log-Logistic, Log-Probit, Multistage, Probit, Weibull, Quantal Linear were used for the analysis of binary data (incidence of nephropathy). All parameters for the BMD analysis were set to default, including the benchmark response (BMR) of 10% extra risk. With regard to the continuous data (kidney weight), 7 models of Linear, Polynomial, Power and Exponential-2 ~ -5 were used. All parameters for the BMD analysis were set to default and the BMR was set to be one standard deviation (1 SD).

The compatibility of the models was determined from *p* values for the chi-square test of 0.1 or more and the absolute value for Scaled Residual of Interest of fewer than 2 as well as the visual inspection of the estimated dose-response curve. Also, Akaike's Information Criterion, AIC, was used to compare the model's goodness of fit (the smaller, the better the fit).

Table 1 Incidence of centrilobular hepatocyte hypertrophy in male F0 mice
(Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	6	56
0.018	1	10
0.18	2	10
1.8	2	10
30	0	10
300	4	10
3500	10	10

Table 2 Results of BMD analysis on centrilobular hepatocyte hypertrophy
in male F0 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	88	0.5946	0.1	Extra risk	222	42	0
Logistic	86	0.6997	0.1	Extra risk	128	76	-1.228
LogLogistic	88	0.5946	0.1	Extra risk	247	54	0
LogProbit	88	0.5946	0.1	Extra risk	213	50	0
Multistage	86	0.7269	0.1	Extra risk	157	42	-1.157
Probit	86	0.697	0.1	Extra risk	122	71	-1.237
Weibull	88	0.5946	0.1	Extra risk	229	42	0.001
Quantal-Linear	87	0.6559	0.1	Extra risk	80	37	-1.321
				Mean	175	52	

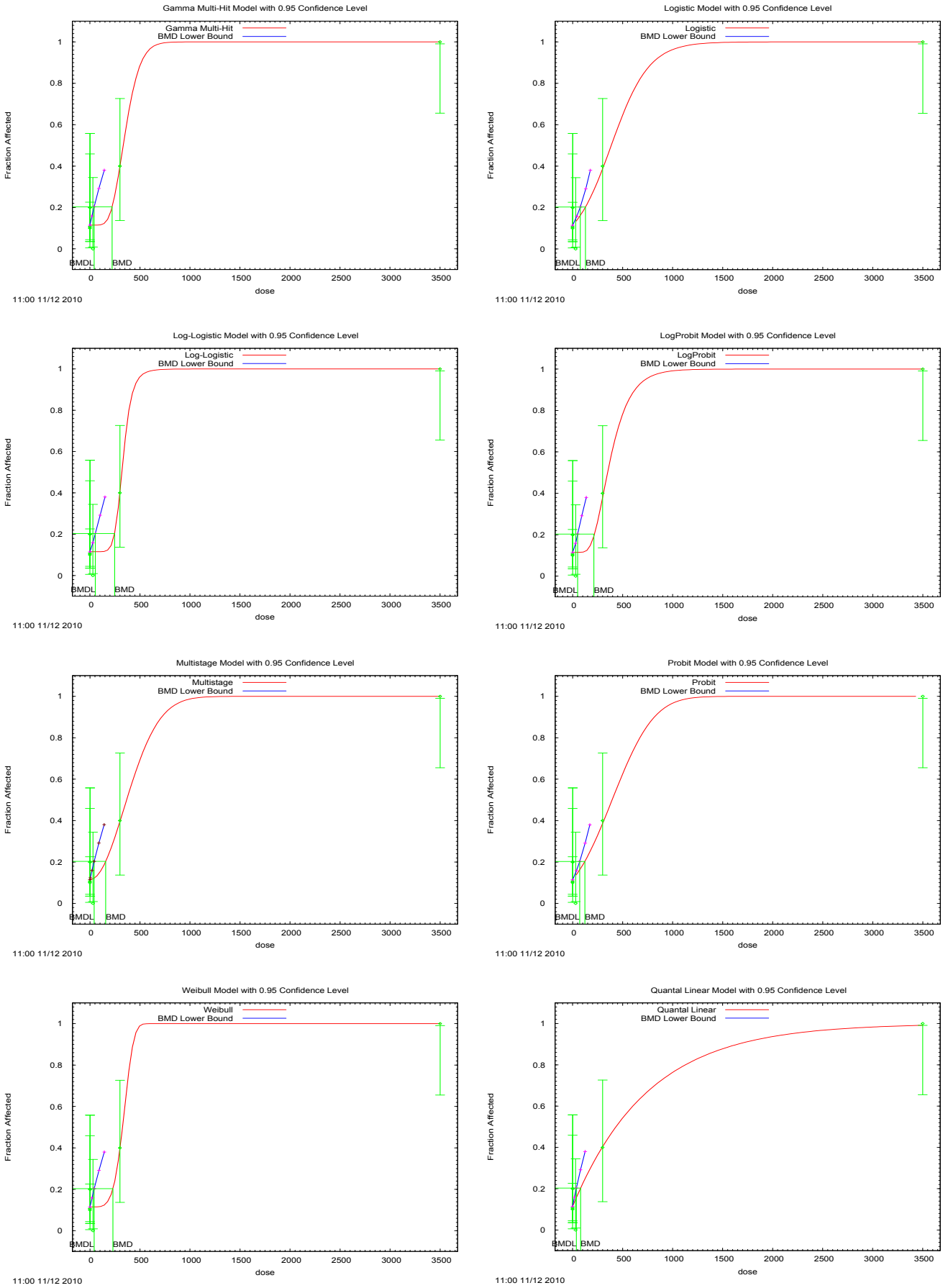

Fig.1 Dose response curves for the centrilobular hepatocyte hypertrophy in male F0 mice

Table 3　Incidence of centrilobular hepatocyte hypertrophy in male F1 mice
(Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	7	55
0.018	0	10
0.18	0	10
1.8	4	10
30	2	10
300	1	10
3500	6	10

Table 4 Results of BMD analysis on centrilobular hepatocyte hypertrophy
in male F1 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	112	~~0.0002~~	0.1	Extra risk	2.50E+13		~~3.555~~
Logistic	100	~~0.0761~~	0.1	Extra risk	942	613	-0.482
LogLogistic	102	~~0.0452~~	0.1	Extra risk	2422	158	0
LogProbit	104	~~0.0265~~	0.1	Extra risk	27	0.0901371	-0.048
Multistage	100	~~0.0819~~	0.1	Extra risk	1294	260	-0.348
Probit	100	~~0.0755~~	0.1	Extra risk	879	578	-0.5
Weibull	102	~~0.0452~~	0.1	Extra risk	2522	261	0
Quantal-Linear	100	~~0.069~~	0.1	Extra risk	506	249	-0.678

No fitted model obtained.

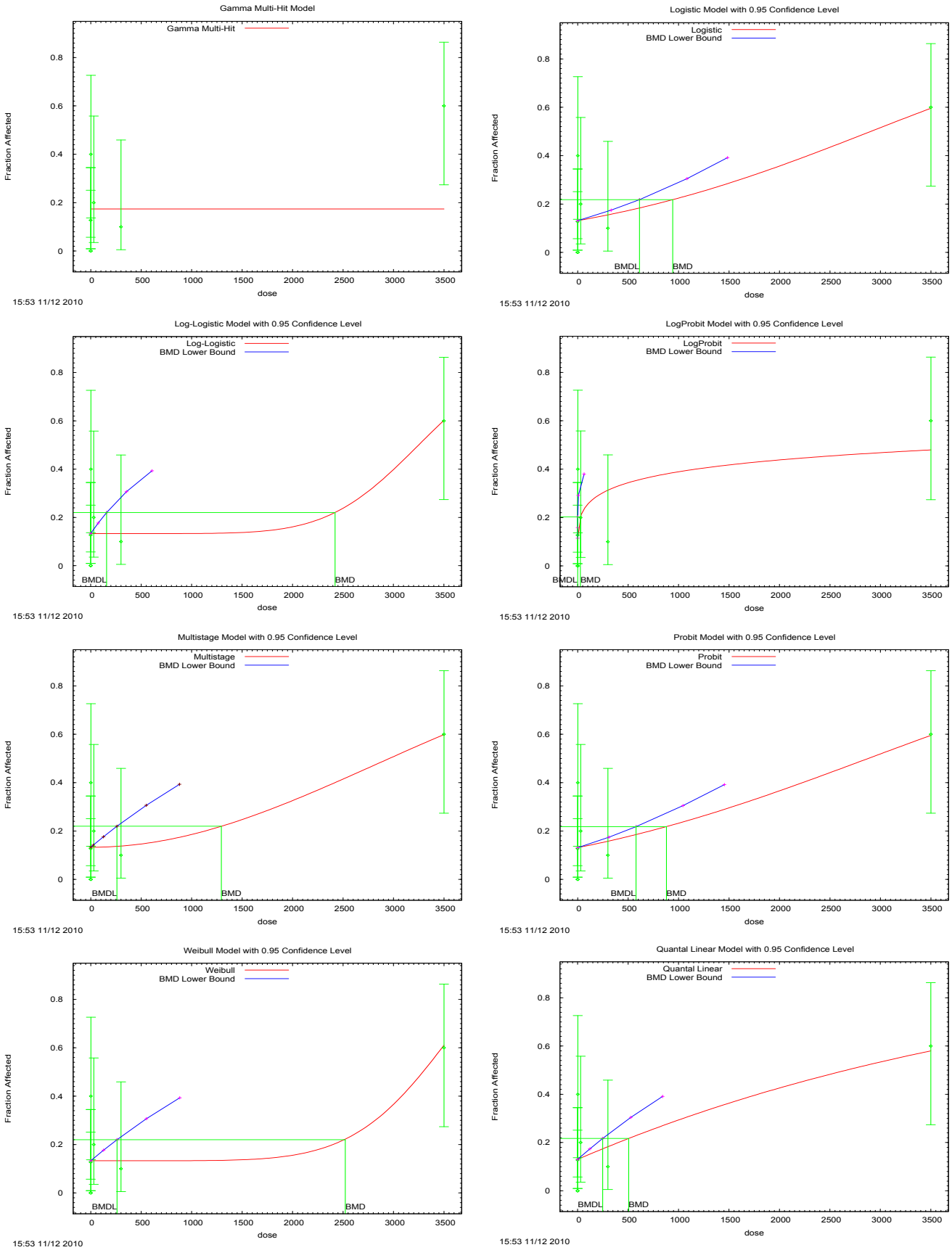

Fig.2　Dose response curves for centrilobular hepatocyte hypertrophy in male F1 mice

Table 5　Incidence of centrilobular hepatocyte hypertrophy in male F1R mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	4	50
0.018	1	10
0.18	3	10
1.8	2	10
30	2	10
300	5	10
3500	7	10

Table 6　Results of BMD analysis on centrilobular hepatocyte hypertrophy in male F1R mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	104	0.1445	0.1	Extra risk	278	147	1.928
Logistic	106	~~0.0686~~	0.1	Extra risk	~~695~~	~~450~~	~~2.413~~
LogLogistic	102	0.2865	0.1	Extra risk	101	35	0.335
LogProbit*	101	0.5935	0.1	Extra risk	~~0~~	~~0~~	1.198
Multistage	104	0.1445	0.1	Extra risk	278	147	1.928
Probit	106	~~0.0717~~	0.1	Extra risk	~~656~~	~~442~~	~~2.392~~
Weibul	104	0.1445	0.1	Extra risk	278	147	1.928
Quantal-Linear	104	0.1445	0.1	Extra risk	278	147	1.928
Mean					243	125	

* Dose response curve did not fit the data according to the visual inspection

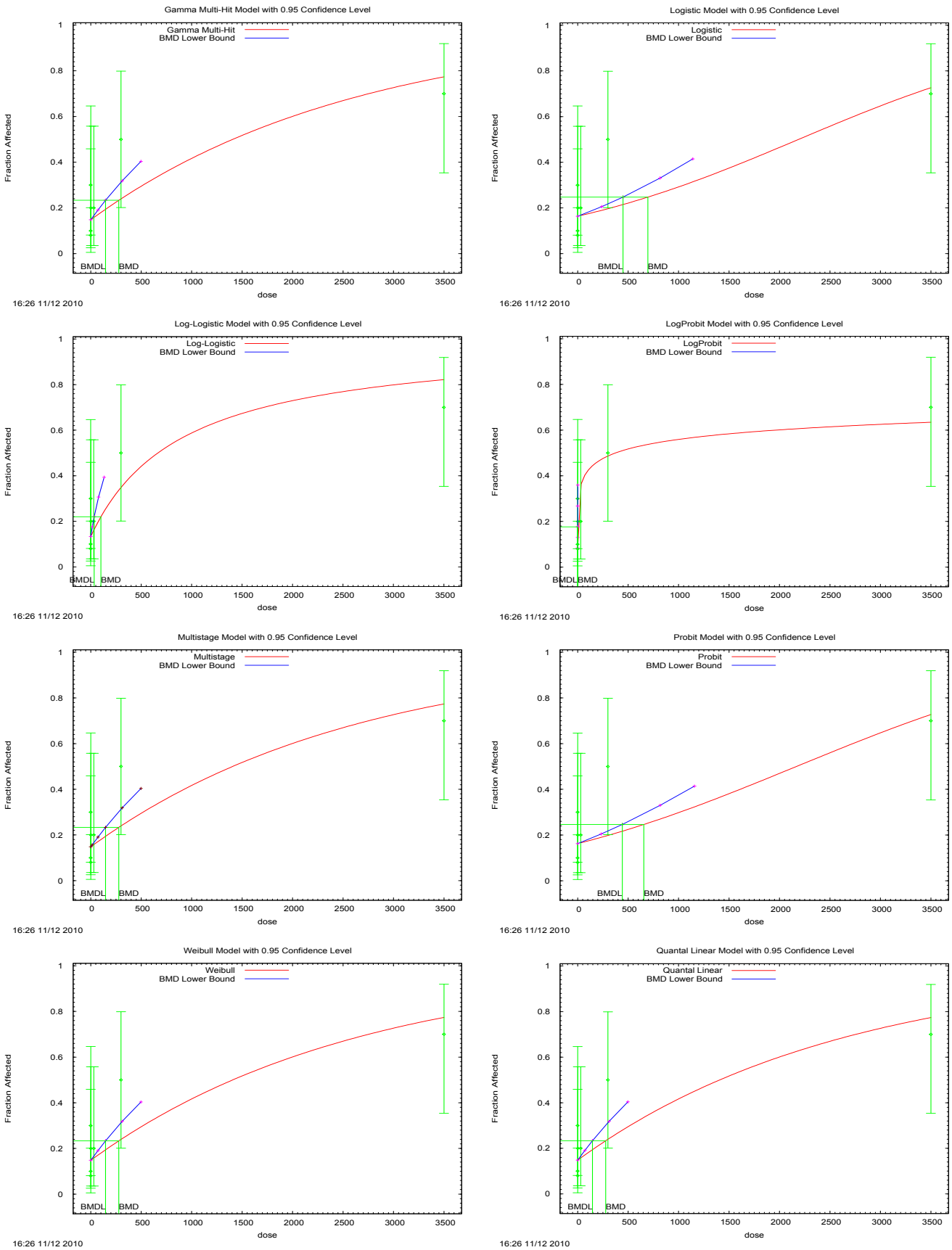

Fig.3 Dose response curves for the centrilobular hepatocyte hypertrophy in male F1R mice

Table 7　Incidence of centrilobular hepatocyte hypertrophy in female F0 mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	1	56
0.018	0	10
0.18	0	10
1.8	0	10
30	0	10
300	1	10
3500	6	10

Table 8　Results of BMD analysis on centrilobular hepatocyte hypertrophy in female F0 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	37	0.9347	0.1	Extra risk	420	221	0.211
Logistic	37	0.7047	0.1	Extra risk	1566	1094	1.5
LogLogistic	37	0.9394	0.1	Extra risk	381	124	0.145
LogProbit	37	0.9462	0.1	Extra risk	347	63	0.069
Multistage	35	0.9755	0.1	Extra risk	403	221	0.168
Probit	37	0.7515	0.1	Extra risk	1348	947	1.401
Weibull	37	0.9349	0.1	Extra risk	415	221	0.196
Quantal-Linear	35	0.9755	0.1	Extra risk	403	221	0.168
Mean					660	389	

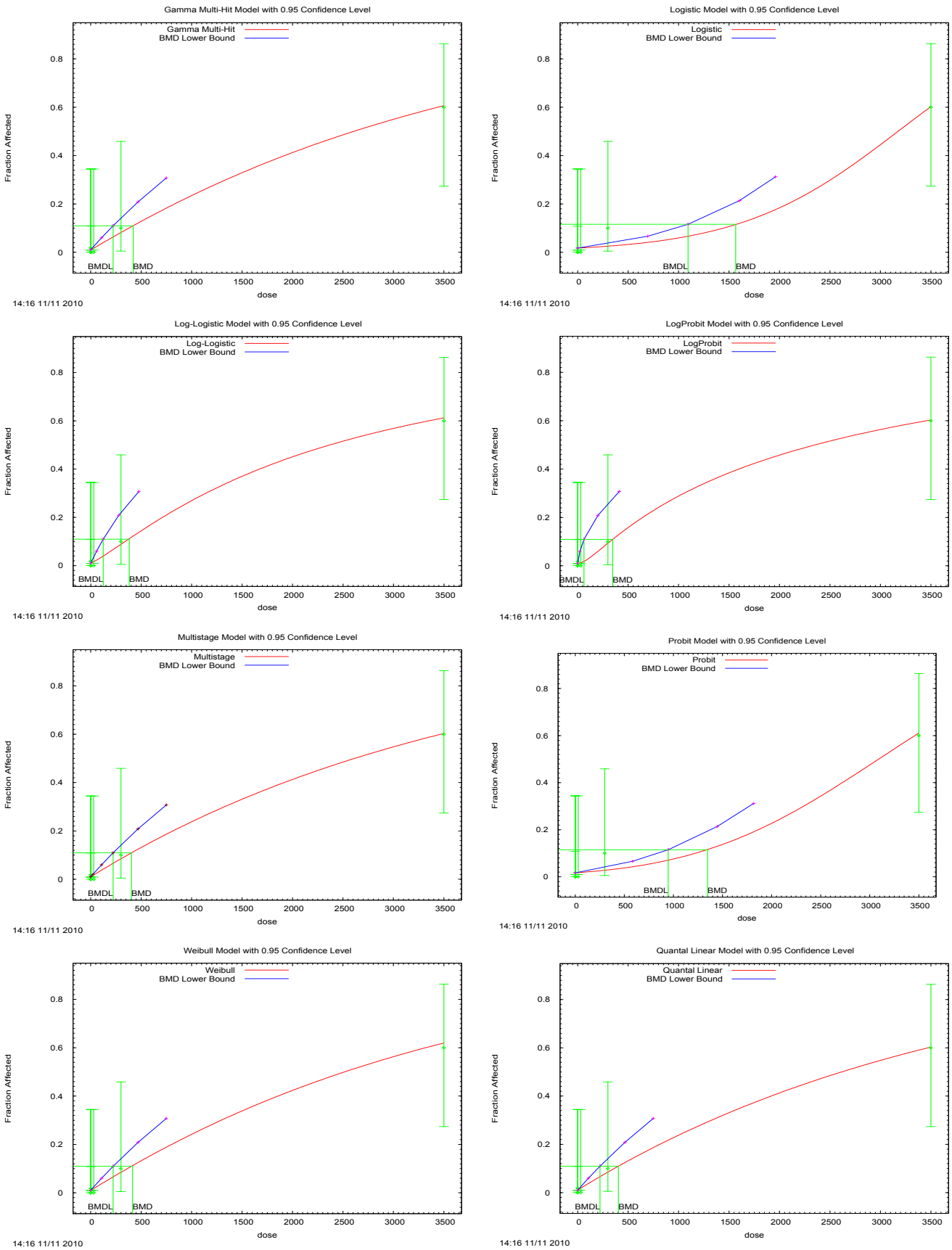

Fig.4 Dose response curves for the centrilobular hepatocyte hypertrophy in female F0 mice

Table 9　Incidence of centrilobular hepatocyte hypertrophy in female F1 mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	2	55
0.018	0	10
0.18	0	10
1.8	0	10
30	0	10
300	3	11
3500	7	10

Table 10　Results of BMD analysis on centrilobular hepatocyte hypertrophy in female F1 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	50	0.6263	0.1	Extra risk	258	150	1.323
Logistic	55	~~0.058 1~~	0.1	Extra risk	~~1101~~	~~756~~	~~2.96~~
LogLogistic	49	0.841 3	0.1	Extra risk	141	60	-0.681
LogProbit	51	0.7708	0.1	Extra risk	131	23	-0.63
Multistage	50	0.626 3	0.1	Extra risk	258	150	1.323
Probit	55	~~0.0739~~	0.1	Extra risk	~~962~~	~~679~~	~~2.866~~
Weibull	50	0.6263	0.1	Extra risk	258	150	1.323
Quantal-Linear	50	0.6263	0.1	Extra risk	258	150	1.323
				Mean	217	114	

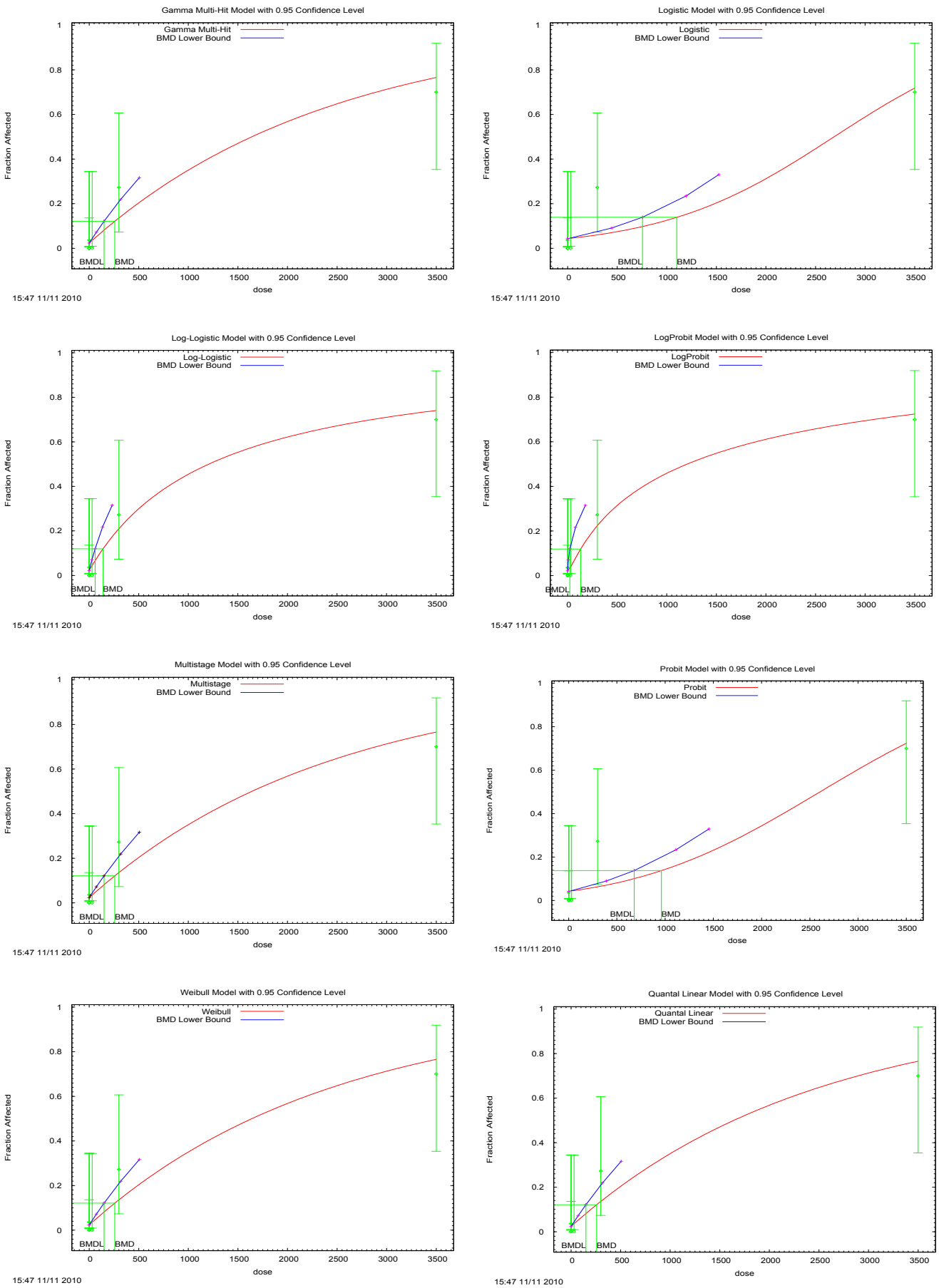

Fig.5 Dose response curves for centrilobular hepatocyte hypertrophy in female F1 mice

Table 11 Incidence of nephropathy in male F0 mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	12	56
0.018	0	10
0.18	3	10
1.8	2	10
30	2	10
300	1	10
3500	4	10

Table 12 Results of BMD analysis on nephropathy in male F0 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Logistic	120	0.542	0.1	Extra risk	1591	784	-0.802
LogLogistic	122	0.419	0.1	Extra risk	2885	359	0
LogProbit	122	0.419	0.1	Extra risk	2483	8	0
Multistage	120	0.5606	0.1	Extra risk	2076	477	0.009
Probit	120	0.5411	0.1	Extra risk	1556	750	-0.806
Weibull	122	0.419	0.1	Extra risk	2924	478	0
Quantal-Linear	120	0.534	0.1	Extra risk	1343	461	-0.837
				Mean	2123	474	

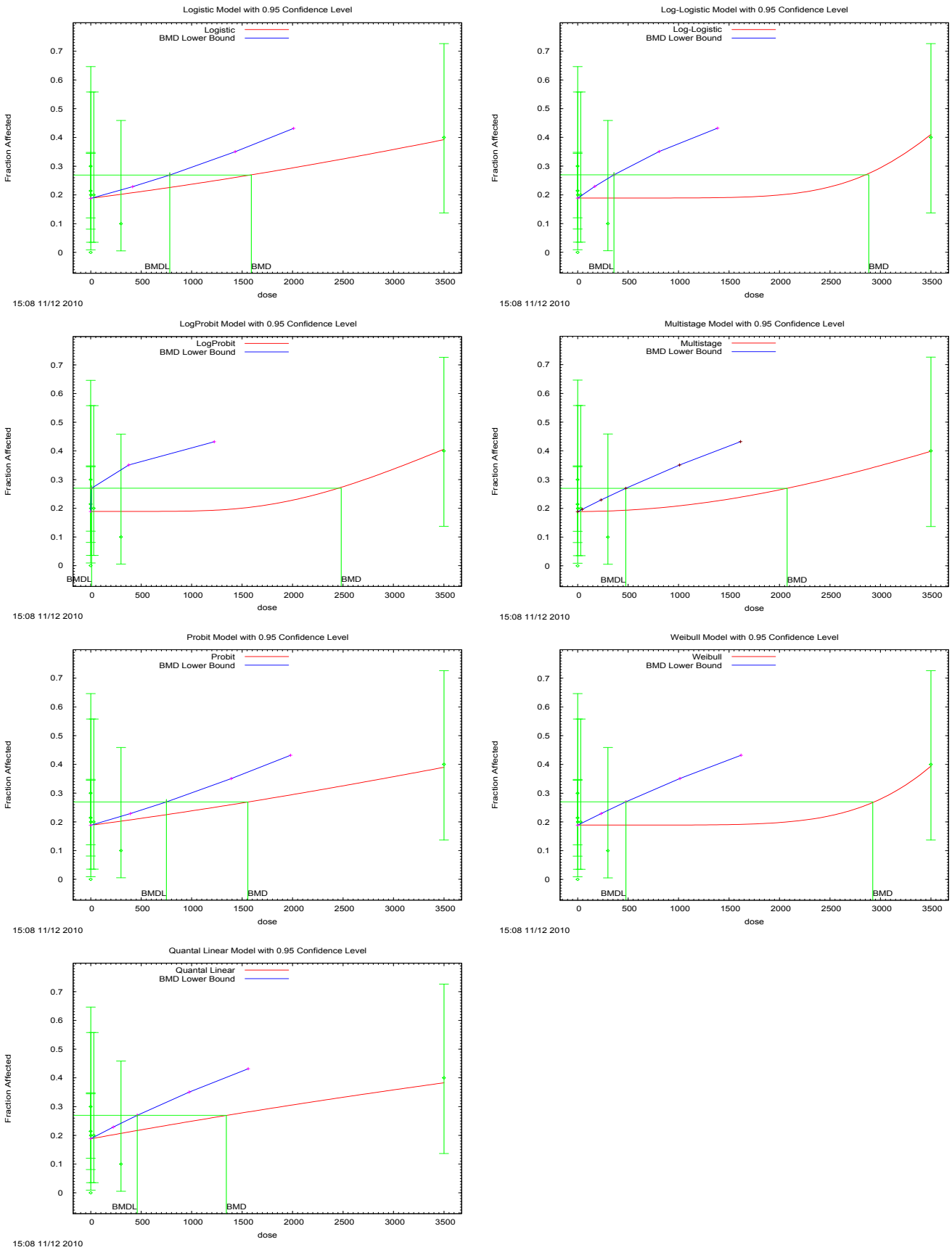

Fig. 6 Dose response curves for nephropathy in male F0 mice

Table 13　Incidence of nephropathy in male F1 mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	6	55
0.018	2	10
0.18	0	10
1.8	1	10
30	2	10
300	0	10
3500	4	10

Table 14 Results of BMD analysis on nephropathy in male F1 mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	90	0.3684	0.1	Extra risk	2527	469	0
Logistic	88	0.4854	0.1	Extra risk	1488	904	-1.16
LogLogistic	90	0.3684	0.1	Extra risk	2796	364	0
LogProbit	90	0.3684	0.1	Extra risk	2312	81	0
Multistage	88	0.5046	0.1	Extra risk	1807	465	-1.097
Probit	88	0.4835	0.1	Extra risk	1413	836	-1.169
Weibull	90	0.3684	0.1	Extra risk	2852	469	0
Quantal-Linear	88	0.4688	0.1	Extra risk	1046	430	-1.232
				Mean	2030	502	

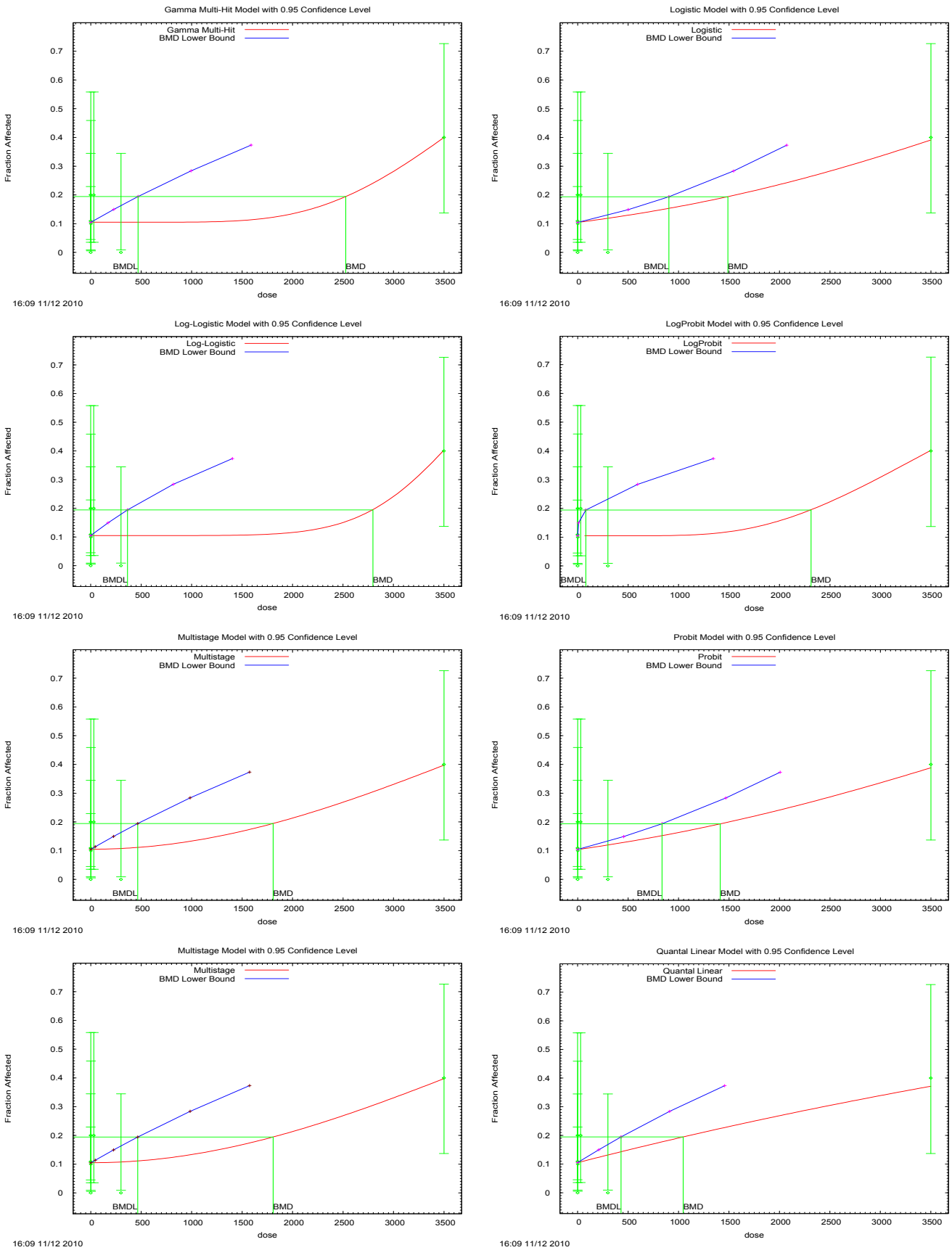

Fig. 7 Dose response curves for nephropathy in male F1 mice

Table 15　Incidence of nephropathy in male F1R mice (Tyl et al., 2008a)

Dose (ppm)	Observed (n)	Size (n)
0	8	50
0.018	1	10
0.18	0	10
1.8	0	10
30	2	10
300	0	10
3500	3	10

Table 16　Results of BMD analysis on nephropathy in male F1R mice

Model	AIC	P-value	Specified Effect	Risk Type	BMD (ppm)	BMDL (ppm)	Scaled Residual of Interest
Gamma	88	0.2128	0.1	Extra risk	2847	621	0
Logistic	86	0.3116	0.1	Extra risk	2040	1063	0.067
LogLogistic	88	0.2128	0.1	Extra risk	3049	511	0
LogProbit	88	0.2128	0.1	Extra risk	2700	128	0
Multistage	86	0.3223	0.1	Extra risk	2334	617	0.015
Probit	86	0.3111	0.1	Extra risk	1991	992	0.077
Weibull	88	0.2128	0.1	Extra risk	3082	621	0
Quantal-Linear	86	0.3077	0.1	Extra risk	1770	585	-1.203
				Mean	2477	642	

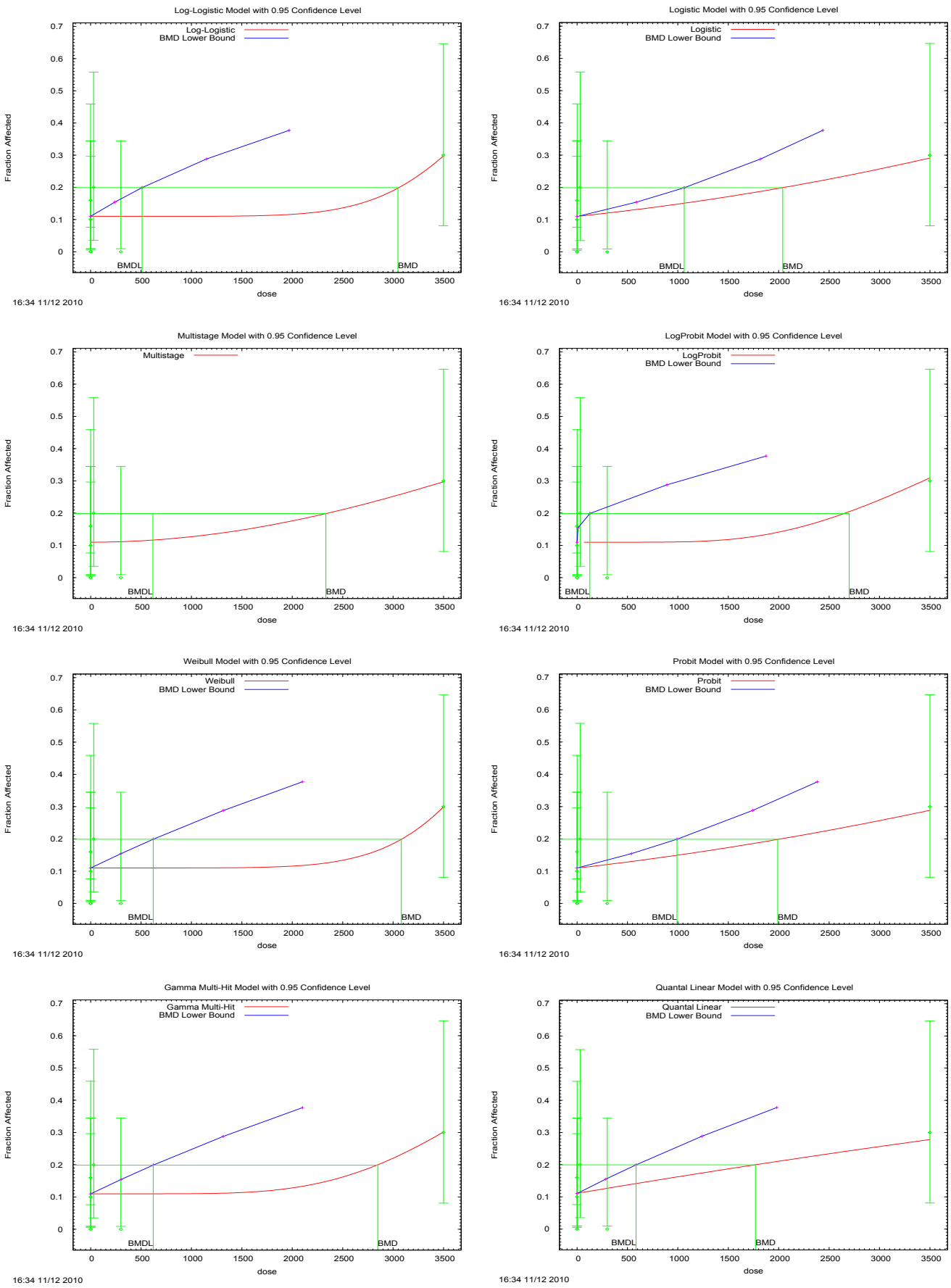

Fig. 8 Dose response curves for nephropathy in male F1R mice

Table 17　Kidney weight of male F0 mice (Tyl et al., 2008a)

Dose (ppm)	Observed Mean	Observed SEM	Size (n)
0	0.380	0.0055	50
0.018	0.380	0.0103	10
0.18	0.374	0.0086	10
1.8	0.388	0.0080	10
30	0.404	0.0137	10
300	0.414	0.0085	10
3500	0.459	0.0110	10

Table 18　Results of BMD analysis on kidney weight of male F0 mice

Model	BMD (ppm)	BMDL (ppm)	p-value Test 1	p-value Test 2	p-value Test 3	p-value Test 4-7a	AIC	Scaled Residual of Interest
Linear	1715	1330	<.0001	0.166	0.166	0.140	-641	1.920
Polynomial*	~~327~~	~~197~~	<.0001	0.166	0.166	0.495	-644	-0.171
Power	1715	1330	<.0001	0.166	0.166	0.140	-641	1.920
Exponential2	1803	1429	< 0.0001	0.166	0.166	0.131	-641	1.954
Exponential3	1803	1429	< 0.0001	0.166	0.166	0.131	-641	1.954
Exponential4	318	145	< 0.0001	0.166	0.166	0.527	-645	-0.264
Exponential5	318	145	< 0.0001	0.166	0.166	0.527	-645	-0.264
Mean	1279	968						

* Dose response curve did not fit the data according to the visual inspection.

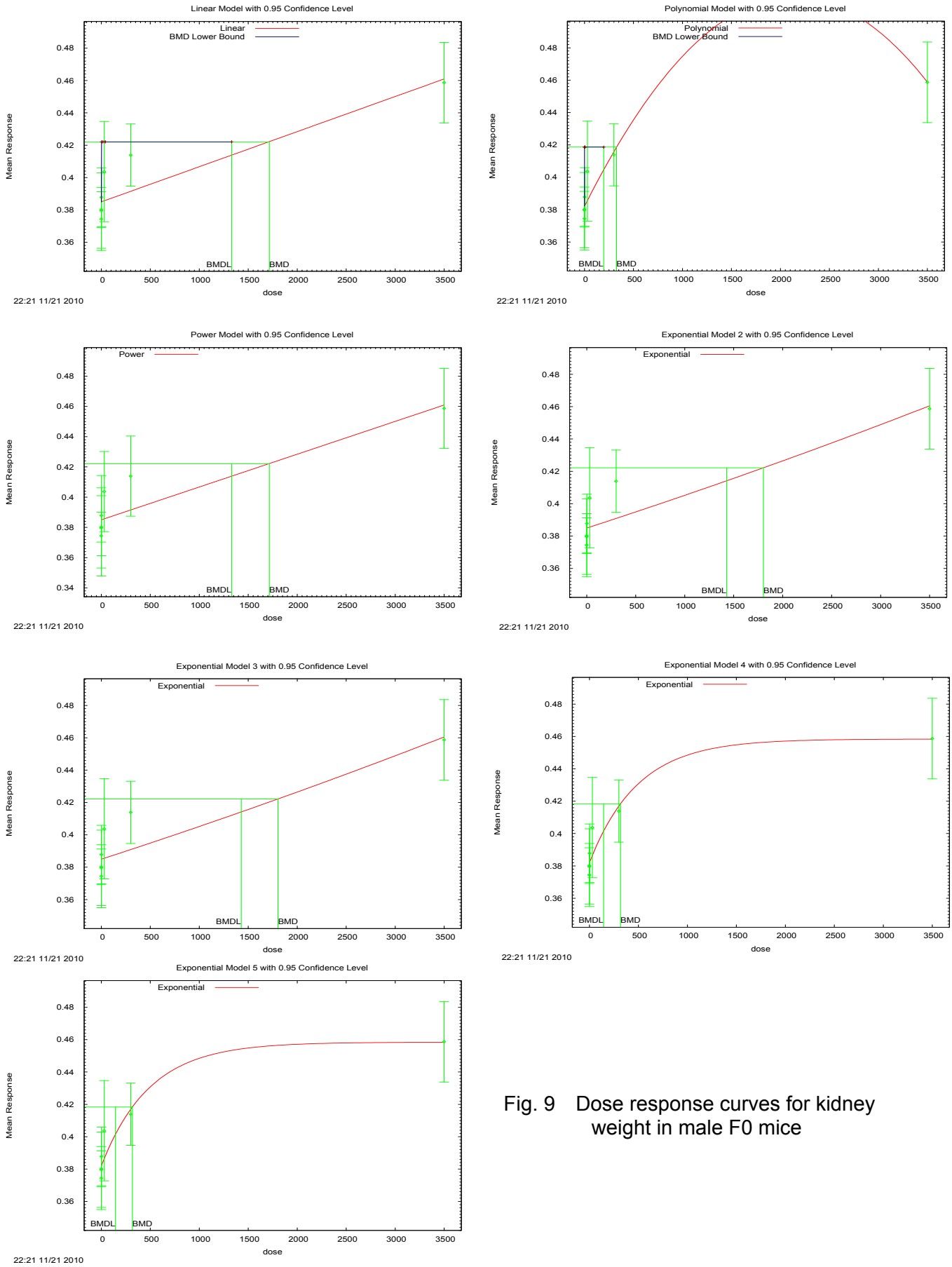

Fig. 9 Dose response curves for kidney weight in male F0 mice

Table 19　Kidney weight of male F1 mice (Tyl et al., 2008a)

Dose (ppm)	Observed Mean	Observed SEM	Size (n)
0	0.361	0.0527	55
0.018	0.393	0.0405	10
0.18	0.375	0.0262	10
1.8	0.385	0.0196	10
30	0.404	0.0332	10
300	0.393	0.0335	10
3500	0.425	0.0326	10

Table 20　Results of BMD analysis on kidney weight of male F1 mice

Model	BMD (ppm)	BMDL (ppm)	p-value Test 1	p-value Test 2	p-value Test 3	p-value Test 4-7a	AIC	Scaled Residual of Interest
Linear	~~2979~~	~~2015~~	<.0001	0.000908	0.000908	~~0.02148~~	-594	-0.103
Polynomial*	~~614~~	~~275~~	<.0001	0.000908	0.000908	~~0.02088~~	-594	-0.233
Power	~~2979~~	~~2015~~	<.0001	0.000908	0.000908	~~0.02148~~	-594	-0.103
Exponential2	~~3019~~	~~2100~~	< 0.0001	0.000908	0.000908	~~0.02109~~	-594	-0.092
Exponential3	~~3019~~	~~2100~~	< 0.0001	0.000908	0.000908	~~0.02109~~	-594	-0.092
Exponential4	~~818~~	~~4~~	< 0.0001	0.000908	0.000908	~~0.02254~~	-594	-0.391
Exponential5	~~818~~	~~5~~	< 0.0001	0.000908	0.000908	~~0.02254~~	-594	-0.391

No fitted model obtained.

Table 21　Kidney weight of male F1R mice (Tyl et al., 2008a)

Dose (ppm)	Observed Mean	Observed SEM	Size (n)
0	0.367	0.0601	50
0.018	0.376	0.0338	10
0.18	0.395	0.0332	10
1.8	0.396	0.0253	10
30	0.397	0.0386	10
300	0.417	0.0313	10
3500	0.426	0.0449	10

Table 22　Results of BMD analysis on kidney weight of male F1R mice

Model	BMD (ppm)	BMDL (ppm)	p-value Test 1	p-value Test 2	p-value Test 3	p-value Test 4-7a	AIC	Scaled Residual of Interest
Linear	~~3635~~	~~2300~~	<.0001	0.00131	0.00131	~~0.04854~~	-545	-0.182
Polynomial*	~~357~~	~~206~~	<.0001	0.00131	0.00131	0.2017	-549	-0.115
Power	~~3635~~	~~2300~~	<.0001	0.00131	0.00131	~~0.04854~~	-545	-0.182
Exponential2	~~3652~~	~~2381~~	< 0.0001	0.00131	0.00131	~~0.04745~~	-545	-0.164
Exponential3	~~3652~~	~~2381~~	< 0.0001	0.00131	0.00131	~~0.04745~~	-545	-0.164
Exponential4	NC	0	< 0.0001	0.00131	0.00131	0.3111	-550	0.000
Exponential5	NC	0	< 0.0001	0.00131	0.00131	0.3111	-550	0.000

* Dose response curve did not fit the data according to the visual inspection.
NC: not computed.
No fitted model obtained.