between the soil and water. After shaking, the water phase was separated from the soil by centrifugation at 3500 rpm for 10 min, followed by filtration through a glass filter (GF/F, ϕ = 0.7 μm, Whatman Japan K. K., Tokyo, Japan). The water phase was then extracted with 10 mL of *n*-hexane. The extract was dried over anhydrous sodium sulphate and subjected to gas chromatography–mass spectrometry (GC–MS) analysis for quantification of isoprothiolane.

Estimation of the degradation rate constant

Ten grams (dry weight) of wet soil was added to a glass tube. Ultrapure water was then added to the tube to a water depth of 1–2 cm, which was maintained during the batch test by adding additional ultrapure water as needed. The soil–water mixture was pre-incubated for 3 days at 20 °C in the dark for conditioning. After 3 days, isoprothiolane was added to the tube at a final concentration of 7.2 mg/kg-dry soil, which is the average application dose recommended for actual paddy fields (Japan Plant Protection Association, 1994). The soil was then incubated again at 20 °C in the dark. Samples were withdrawn on days 0, 5, 10, and 20 for quantification of residual isoprothiolane as follows. The samples were centrifuged at 3500 rpm for 10 min to separate the water and soil. The procedure used for the extraction of the isoprothiolane from the water phase was the same as that described in the previous section, and the extract was subjected to the GC–MS analysis. To extract the isoprothiolane from the soil, 10 mL of acetone was added to the soil and the mixture was vortexed for 20 min. After vortexing, the mixture was centrifuged at 3500 rpm for 10 min, and the supernatant was subjected to the GC–MS analysis.

Analytical methods

Isoprothiolane was quantified by GC–MS (Agilent 6890N gas chromatograph, Agilent 5973 mass spectrometry detector) equipped with a capillary column (Agilent HP-5MS, 5% diphenyl 95% dimethylsiloxane; i.d., 0.25 mm; length, 30 m). The temperature of the ion source, injector, and transfer line was 250 °C. GC–MS was performed in selected ion monitoring mode; the fragment ions of isoprothiolane were detected at *m/z* 118. The relative contents of organic compounds in the soil were measured by NC analyzer (Sumigraph NC-800, Sumika Chemical Analysis Service, Ltd., Tokyo, Japan) so that the adsorption coefficient of soil organic compounds (K_{OC}) could be calculated from K_d.

Other model inputs and parameters

The time-series hydrological input for the model was precipitation less evapotranspiration; these data were calculated from published meteorological data (Japan Meteorological Agency, Tokyo) by a method described elsewhere (Matsui et al., 2005). The model takes into account 23 hydrologic parameters. The values of 13 parameters are provided a priori or a posteriori from observation data (Matsui et al., 2005), and those of the remaining 10 parameters are adjustable. Their values are searched for during model simulation so as to give the best fit to observed water flow rates (Ministry of Land, Infrastructure and Transport of Japan) in accordance with the minimum error criterion of the Nash-Sutcliffe coefficient (Nash and Sutcliffe, 1970).

RESULTS AND DISCUSSION

Isoprothiolane adsorption and degradation in soil

The isoprothiolane concentration changes in the soils due to degradation were well described by first-order reaction kinetics (data not shown), and the degradation rate was parameterised by the first-order reaction constant (k). The degradation rate constant (k) varied greatly depending on the soil type (Figure 3). Even within the same soil group (wet Andosols), rate constants differed by a factor of 12. For soil samples within the same soil subgroup (for example, wet Andosols 2) collected from different paddy fields, the difference in rate constants became smaller. The

adsorption coefficient of the soils sampled from various paddy fields, expressed as the ratio of the amount of pesticide adsorbed per unit weight of organic carbon (K_{OC}), also varied depending on the soil type. However, in soils of the same soil subgroup, K_{OC} was roughly similar. The organic carbon contents of soils from the same soil subgroup were similar. Since soils of the paddy fields in the target catchment were mostly wet Andosols, we assumed that in the model, the isoprothiolane degradation rate could be described by a first-order reaction with the degradation rate constant determined in accordance with the soil type. K_{OC} and OC values in the model were also determined in accordance with soil type. In addition, we confirmed that the literature-reported values of the degradation rate (half-life) and the soil adsorption coefficient (Uchida, 1978; Kuwatsuka and Yamamoto, 1998; Kishimoto et al., 1999)were in the same range as our values.

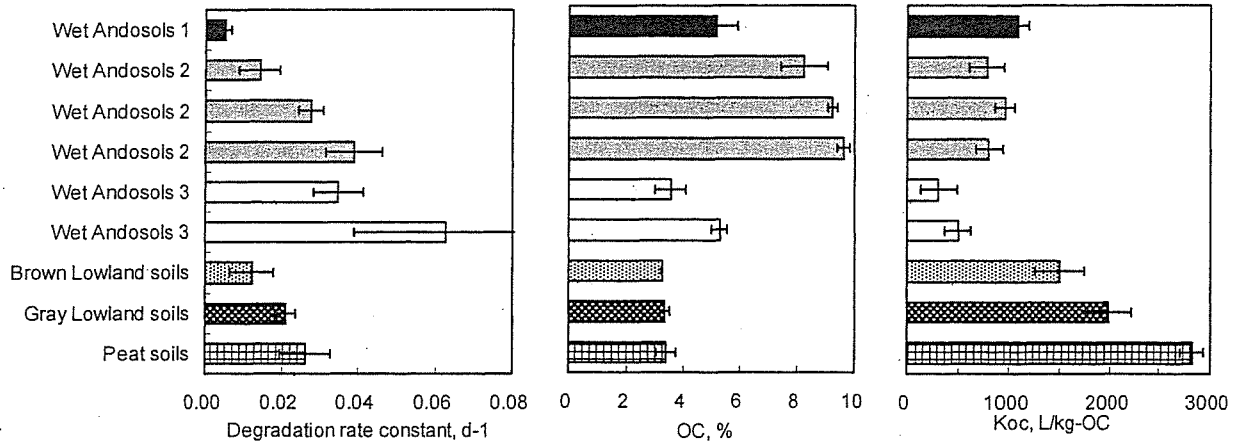

Figure 3 Soil characteristics for isoprothiolane degradation and adsorption in soils sampled from nine paddy fields. Error bars were calculated from data of three soil samples from each paddy field.

Predicting isoprothiolane concentration in river water

Agricultural records were collected for all 372 farmers engaged in paddy-rice cultivation in the river basin, including dates of rice transplanting, dates and amounts of herbicide, fungicide, and insecticide applications, irrigation practices and water level of rice-paddy pondings, and harvest time. From these, a model input data set for all of the farmers were created. Model inputs for isoprothiolane adsorption and degradation in the soil were developed from the abovementioned observational data. Uncertainty in model inputs was minimised by using these data, making it possible to test the prediction capability of the model. We compared predicted and observed time variations in the isoprothiolane concentrations in river water (Figure 4). In 2003, the concentration was predicted to peak on July 20, but regrettably there were no observed data on that day. Therefore, the ability of the model to predict peak concentration could not be confirmed. In 2004, fairly good agreement was obtained for both concentration peak height and timing between predicted and observed values, because adequate water samples had been collected at suitable times. Overall, the predicted concentrations were close to observed values. These results suggested that the model was capable of predicting pesticide concentration in river water when precise model inputs and parameter values were provided. In other words, the model realistically predicted pesticide fate without neglecting significant processes such as pesticide transport and decomposition.

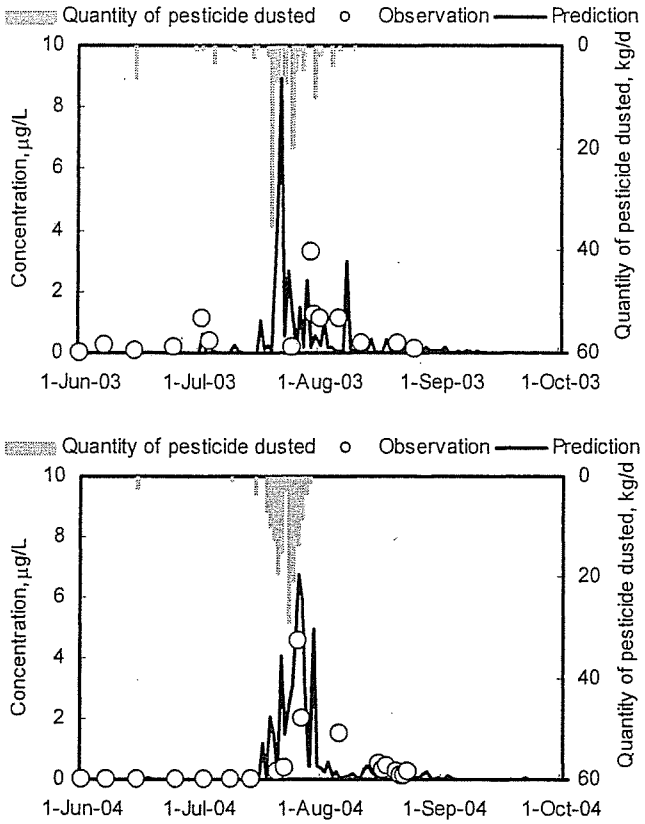

Figure 4 Comparison of observed time-series isoprothiolane concentrations with those predicted by the model.

Sensitivity analysis

A sensitivity analysis of the model was conducted to elucidate pesticide runoff phenomena.

Effects of pesticide application and precipitation date accuracy

The pesticide concentration in the runoff increased several days after pesticide application to the rice paddy (Figure 4). Therefore, the effect of the accuracy of input dates of pesticide application and irrigation was studied by model simulation. The pesticide concentrations predicted with imprecise input data, when the pesticide application dates input were either 1 week ahead or 1 week behind the actual schedule, did not yield accurate predictions (Figure 5). However, the concentration variation pattern shifted 11 days ahead when the input date was shifted ahead by 1 week, whereas it shifted behind by 1 week when the input date was shifted 1 week behind. Thus, although the date of pesticide application was the dominant factor determining the period of pesticide runoff, the shift in the runoff dates did not correspond simply to the shift in pesticide application timing.

Pesticide runoff can be caused by spill-over of rice-paddy water during or after a rainfall or by artificial drainage of rice-paddy water. To investigate the effect of the timing of rainfall, model simulations were conducted with time-series model inputs in which weather (precipitation) events were shifted by 1 week either behind or ahead. A 1-week delay or acceleration of the weather pattern changed both the peak height and time-course variation in pesticide concentration (Figure 6). However, the pattern did not shift ahead or behind by 1 week, suggesting that pesticide runoff was

not caused primarily by spill-over of rice-paddy water during or after rainfall but was probably related to artificial drainage of rice-paddy water.

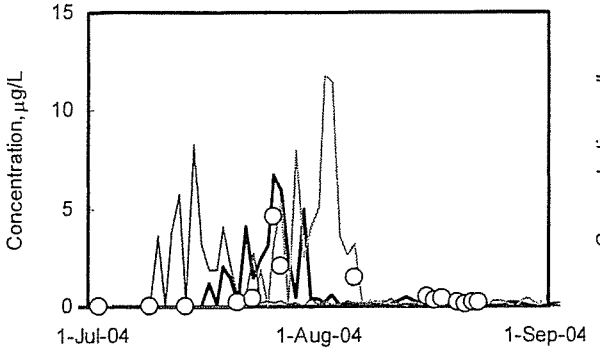

Figure 5 Effect of the accuracy of agricultural practice data on model prediction. Black line, prediction with accurate input data; blue line, prediction with agricultural schedule inputs moved forward by 1 week; red line, prediction with inputs moved back by 1 week.

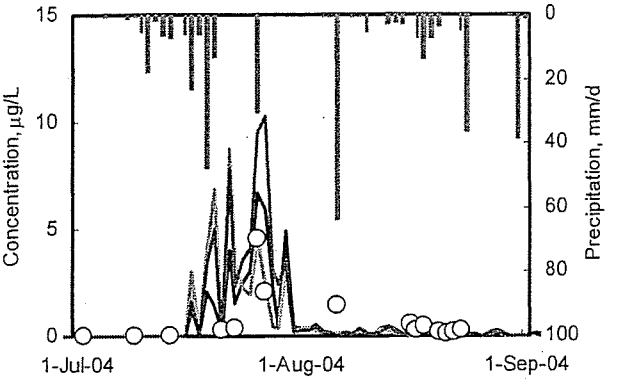

Figure 6 Effect of precipitation dates on model prediction. Green bars, actual precipitation; black line, prediction with actual precipitation data; blue line, prediction with precipitation input moved forward 1 week; red line, prediction with precipitation input moved back 1 week.

Effects of pesticide adsorption and decomposition

Pesticide adsorption coefficient and degradation rate constant did not greatly affect pesticide concentration in the river water (Figures 7 and 8). In general, the smaller the K_{OC} value was, the larger the pesticide concentration was, but an increase in K_{OC} had a smaller effect than a decrease. An increase in the degradation rate constant by a factor of 10 decreased the pesticide concentration in river water by about 30%, but a decrease in the degradation rate constant changed the pesticide concentration by a lesser amount. These results suggest that the pesticide isoprothiolane is somewhat hydrophobic and persistent, so further enhancement of these tendencies would not affect the runoff of the pesticide. Overall, the effects of pesticide adsorption and degradation was not linear, and a parameter value change in the direction of constraining pesticide runoff likely is characterised by diminishing returns. These parameters did not significantly influence peak height of time-varying concentrations in the pesticide pollutograph (data not shown), but instead affected the low concentrations of the decreasing limb of the concentration peaks. Pesticide runoff at these low concentrations probably occurs through soil and groundwater percolation, leading to greater dependence on the values of the pesticide decomposition and adsorption parameters.

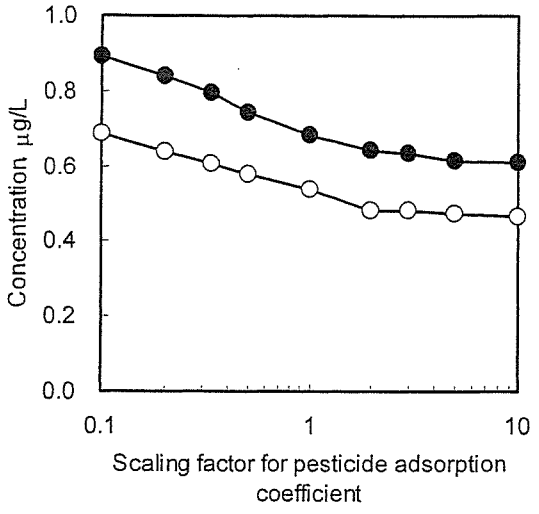

Figure 7 Effect of the pesticide adsorption coefficient (*Koc*) on average and peak concentrations in July and August.

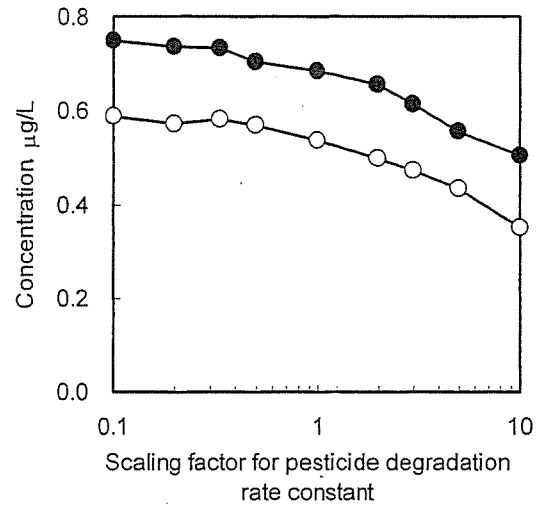

Figure 8 Effect of the pesticide degradation rate constant on average and peak concentrations in July and August.

Effect of quantity of pesticide applied and runoff rate

The quantity of pesticide applied directly affects the pesticide concentration in the river water. As expected, an explicit linear relationship was obtained between concentration and applied quantity. The total pesticide discharge to the river was also linearly proportional to the total quantity of pesticide applied to the paddy field (data not shown). However, all of the pesticide applied to the paddy fields was not discharged to the river water. The pesticide discharge rate, defined as the annual pesticide discharge in the river flow divided by the annual quantity of pesticide applied to the paddy fields in the catchment, was 28% in 2003 and 42% in 2004, indicating that more than half of the pesticide applied to the paddy fields did not reach the river. Nonetheless, the rates of adsorption and degradation of pesticide in the soil did not significantly affect the concentration in the river water. Further study is needed to elucidate the significant pesticide runoff processes.

CONCLUSIONS

Pesticide concentration in river water was successfully predicted by a diffuse pollution model provided with precise model inputs, including agricultural practices of individual farmers and experimentally derived data on pesticide adsorption and degradation rates in paddy field soils. Although rates of both pesticide adsorption and degradation differed, depending on soil type, similar values were obtained for soils belonging to the same soil subgroup. The timing of concentration increases in river water was determined mostly by agricultural practices (pesticide application and irrigation) and not greatly by weather (precipitation) patterns. These results suggest that artificial drainage of paddy water may be a significant process affecting pesticide runoff. However, the pesticide discharge rate was less than 50%, possibly because of loss from pesticide degradation. Nonetheless, the pesticide concentration in river water

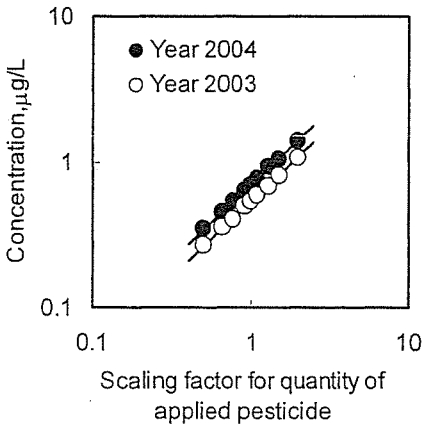

Figure 9 Effect of quantity of pesticide applied on average river water concentrations in July and August.

was not greatly affected by pesticide adsorption and degradation rates in paddy field soils.

ACKNOWLEDGEMENTS

The authors thank Ms. Nakano of Iwate Agricultural Research Center, Japan, for providing the pesticide concentration data and for her assistance in collecting copies of Cultivation Management Register. This work was supported in part by a Grant-in-aid (Hazardous Chemicals) from the Ministry of Agriculture, Forestry and Fisheries of Japan (HC-06-2114-2) and a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare (H16-Health-066).

REFERENCES

Borah D. K. and Bera M. (2003) Watershed-scale hydrologic and nonpoint-source pollution models: review of mathematical bases. *Trans. ASAE* **46**(6), 1553–1566.

Borah D. K. and Bera M. (2004) Watershed-scale hydrologic and nonpoint-source pollution models: review of applications. *Trans. ASAE* **47**(3), 789–803.

Dubus I. G., Brown C. D. and Beulke S. 2003. Sources of uncertainty in pesticide fate modeling. *Sci. Total Environ.* **317**, 53–72.

Gilliom R. J., Barbash J. E., Kolpin D. W. and Larson S. J. (1999) Testing water quality for pesticide pollution. *Environ. Sci. Technol.* **33**(7), 164A–169A.

Iwate Agricultural Research Center (1997) *Soil Map of Iwate Prefecture, No.6.*

Japan Plant Protection Association (1994) *Pesticide Handbook.* Tokyo (in Japanese).

Kishimoto M., Momonoi K., Komatsu T. and Kameya T. (1999) Behavior of isoprothiolane in a paddy field. *Annual Conference Abstract Book of Japan Society on Water Environment.* pp.508 (in Japanese).

Kuwatsuka, S. and Yamamoto, H. (1998) *Soils and Pesticides—Fates of Pesticides in Environment.* Japan Plant Protection Association (in Japanese).

Matsui Y., Itoshiro S., Buma M., Hosogoe K., Yuasa A., Shinoda S., Matsushita T. and Inoue T. (2002) Predicting pesticide concentrations in river water by hydrologically calibrated basin-scale runoff model. *Water Sci. & Tech.* **45**(9), 141–148.

Matsui Y., Inoue T., Matsushita T., Yamada T., Yamamoto M. and Sumigama Y. (2005) Effect of uncertainties of agricultural working schedule and Monte-Carlo evaluation of the model input in basin-scale runoff model analysis of herbicides. *Water Sci. & Tech.* **51**(3-4), 329–337.

Matsui Y., Narita K., Inoue T. and Matsushita T. (2006) Screening level analysis for monitoring pesticide in river water using a hydrological diffuse pollution model with limited input data. *Water Sci. & Tech.* **53**(10), 173–181.

Nash J. E. and Sutcliffe J. V. 1970. River flow forecasting through conceptual models. *J. Hydrol.* **10**, 282–290.

Organisation for Economic Co-operation and Development [OECD] (2000) *Adsorption–Desorption. Using a Batch Equilibrium Method.* OECD Guideline for Testing of Chemicals 106. http://www.oecd.org/findDocument/0,2350,en_2649_34377_1_1_1_1_1,00.html

Ministry of Agriculture, Forestry and Fisheries of Japan. (2005) http://www.maff.go.jp/nouyaku/aca-info.htm (in Japanese).

Ministry of Land, Infrastructure and Transport of Japan. (2005) Water Information System. http://www1.river.go.jp/ (in Japanese).

Uchida M. (1978) Fuji-one and soils. *Pesticide* **25**(3), 50–55 (in Japanese).

Application of "Water Safety Plan" to drinking water quality management in Japan

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Introduction

"Water Safety Plan (WSP)", a highlight in the third edition of WHO Guidelines for Drinking-water Quality [WHO, 2004], is a very important tool to achieve health-based targets. WSP requires the systematic management of drinking water quality from a source to consumer taps. In this paper, Japan's approaches to WSP incorporation in drinking water quality management are described.

Japan approaches to WSP incorporation in drinking water quality management

Activities for the introduction and application of WSP to the drinking water quality management in Japan include a research study on WSP application in municipal water supplies and the development of guidelines on WSP application. Key steps in developing a WSP are shown in Figure 1 for reference.

Research study on WSP application in municipal water supplies

A research study, with its objective of exemplifying the way of WSP introduction to municipal water supplies in Japan and funded by a grant of MHLW, started in FY2004. Five water supplies, such as Tokyo, Yokohama, Osaka, Osaka (bulk water supply) and Kobe, are going to reformulate their drinking water quality management programs applying the concept of WSP. The study originally started on a trial basis, but it actually contributes to streamlining and upgrading their current programs of drinking water quality management. Tokyo Metropolitan Water Supply and Osaka Municipal Water Supply are going to obtain or have obtained ISO 9001 certifications regarding to their water treatment and/or distribution systems along with WSP application.

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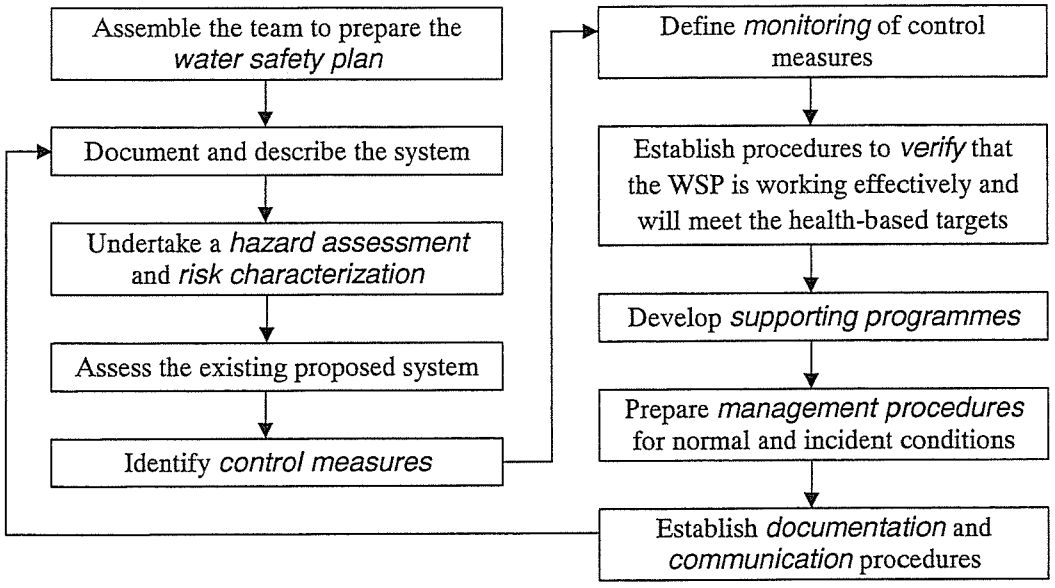

Figure 1 Overview of the key steps in developing a Water Safety Plan (WSP)

Development of guidelines on WSP application

A committee for the development of guidelines on WSP introduction was organized in Japan Water Works Association (JWWA) under the financial support of MHLW in FY2005. A few trials of WSP application to small drinking water supplies are being implemented. There exist more than eight thousand small water supplies, with a population served of 101-5,000 persons, in Japan at present. Their water quality management is rather poor compared with municipal water supplies, and its improvement is of vital importance for preventing waterborne disease outbreaks caused by drinking water contamination. Most of them lack manpower, technology and financial resources. Therefore, small water suppliers will become the main audience of the guidelines on WSP application. Their WSP should be simple, user-friendly and easy to improve. Draft guidelines will be prepared until March 2007.

WSP dissemination to developing countries through international cooperation

NIPH has been serving as the coordinator of Operation and Maintenance Network (OMN) Group since several years ago. OMN is a NGO established in 1990 under WHO for the purpose of improving the operation and maintenance of water supply and sanitation facilities especially in developing countries through the exchange of experiences, knowledge and information. The main activities of OMN include training tool development, convening workshops/seminars and information exchange through the web. Recently, OMN is contributing to the improvement of drinking water quality management through WSP dissemination to developing countries in Asia

collaborating with WHO, JICWELS (Japan International Corporation of Welfare Services) and other institutions as written below.

- JICWELS workshop on WSP and other topics, Hue, Vietnam, June 2005; Collaboration with OMN and WHO/WPRO
- JICWELS seminar on water supply management (WSP and other topics), Tokyo, January 2006; Collaboration with ONM, WHO/WPRO and NZ
- WHO-OMN workshop for PPWSA (Phnom Penh Water Supply Authority) on water supply management (WSP and other topics), Phnom Penh, Cambodia, October 2006

Conclusions

There is no doubt that WSP is essential for ensuring drinking water safety. Hazard identification seems a key component of WSP. Catchment management should be paid more attention to by drinking water suppliers in order to achieve drinking water safety. The way of WSP application may vary according to the situations of individual water supply.

Reference

World Health Organization: Guidelines for Drinking-water Quality, 3rd Edition, Vol.1: Recommendations, 2004

Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

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ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. Environ Toxicol 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

INTRODUCTION

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during the melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment. The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation of butyltin in oysters (Waldock and Thain, 1983), mud crabs (Evans and Laughlin, 1984), marine mussels (Laughlin et al., 1986), Chinook salmon (Short and Thrower, 1986), and dolphin, tuna, and shark (Kannan et al., 1996) has been reported. These findings indicate that butyltins accumulate in the

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food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotins possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimatized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ-irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at 22°C ± 3°C, with a relative humidity of 50% ± 20%, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 μmol/kg bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g)[a]	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g)[a]				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain[b]	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g)[a]				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at −80°C for later assay of steroid hormones. Serum progesterone and 17β-estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β-estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β-estradiol were below 6.4% and 8.9%, respectively. The intra- and interassay coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β-estradiol were analyzed by Student's *t*-test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female[a,b]	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%)[a,b]	9.7	29.7[c]	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter[a,d]	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter[a,d]	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%)[d,e]	1.5	3.3	1.1	0
No. of post-implantation loss per litter[a,d]	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%)[d,f]	10.1	14.1	41.3*	32.2
No. of live fetuses per litter[a,d]	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g)[a]				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg)[a]	125 ± 56	116 ± 15	120 ± 17	119 ± 16

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

RESULTS

Administration of DBTCl on Days 0–3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0–3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0–4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0–4, days 4–8, and days 8–18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0–4 at 7.6 and 30.4 mg/kg bw/day and on days 8–18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0–3 of pregnancy are shown in Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g)[a]	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g)[a]				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	−0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain[b]	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g)[a]				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day, and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17β-Estradiol Levels

The serum progesterone and 17β-estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17β-estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17β-estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of

TABLE IV. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	1	2	1
No. of pregnant females	11	11	10	11
No. of implantations per female[a,b]	12.6 ± 4.4	13.2 ± 4.6	7.5 ± 5.7*	11.1 ± 5.4
Pre-implantation loss per female (%)[a,b]	8.9	8.9	24.7	18.3[c]
No. of pregnant females surviving until scheduled sacrifice	11	11	9	10
No. of litters totally resorbed	0	2	8*	10*
No. of corpora lutea per litter[a,d]	13.8 ± 2.1	14.5 ± 2.3	10.6 ± 5.2	13.9 ± 2.8
No. of implantations per litter[a,d]	13.7 ± 2.1	14.4 ± 2.2	9.4 ± 5.1	12.7 ± 4.1
Pre-implantation loss per litter (%)[d,e]	0.6	0.6	10.7	10.2
No. of postimplantation loss per litter[a,d]	0.6 ± 1.0	7.2 ± 6.1*	8.7 ± 4.8*	12.7 ± 4.1*
Post-implantation loss per litter (%)[d,f]	4.3	48.3*	94.4*	100*
No. of live fetuses per litter[a,d]	13.1 ± 2.0	7.2 ± 5.6*	0.8 ± 2.3*	0
Sex ratio of live fetuses (male/female)	82/62	50/29	4/3	
Body weight of live fetuses (g)[a]				
Male	1.45 ± 0.10	1.23 ± 0.10*	1.27	
Female	1.39 ± 0.10	1.18 ± 0.14*	1.18	
External examinations of fetuses				
No. of fetuses (litters) examined	144 (11)	79 (9)	7 (1)	
No. of fetuses (litters) with anomalies	0	2 (2)	0	
Omphalocele	0	1	0	
Exencephaly and open eyelids	0	1	0	
Placental weight (mg)[a]	102 ± 10	99 ± 12	114	

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

pregnancy following maternal exposure during early pregnancy in mice. The most striking finding in the present study is pregnancy failure, decrease in the pregnancy rate, and litters totally resorbed, in females given DBTCl during early pregnancy.

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of pregnancy, recovery by the end of the study was not found in females given DBTCl at 7.6 mg/kg bw/day and higher after the administration on days 4–7 of pregnancy. Following the administration on days 4–7 of pregnancy, a significantly lower adjusted weight gain was also noted in females given DBTCl at 15.2 mg/kg/day and higher. These findings indicate that more severe adverse effects on body weight gain were induced by DBTCl on days 4–7 of pregnancy than that on days 0–3 of pregnancy. More severe effects of DBTCl on body weight gain following the administration on days 4–7 may be attributable to the significant decrease in the number of live fetuses.

The earlier administration period, days 0–3 of pregnancy, corresponds to the period before implantation, and the later administration period, days 4–7 of pregnancy, corresponds to the period when implantation is in progress and the period shortly after implantation in mice (Rugh, 1968). We expected that DBTCl insult on days 0–3 of pregnancy might result in preimplantation loss of embryos; i.e., the absence or decrease of implantation sites, and DBTCl insult on days 4–7 of pregnancy might result in postimplantation loss of embryos; i.e.,

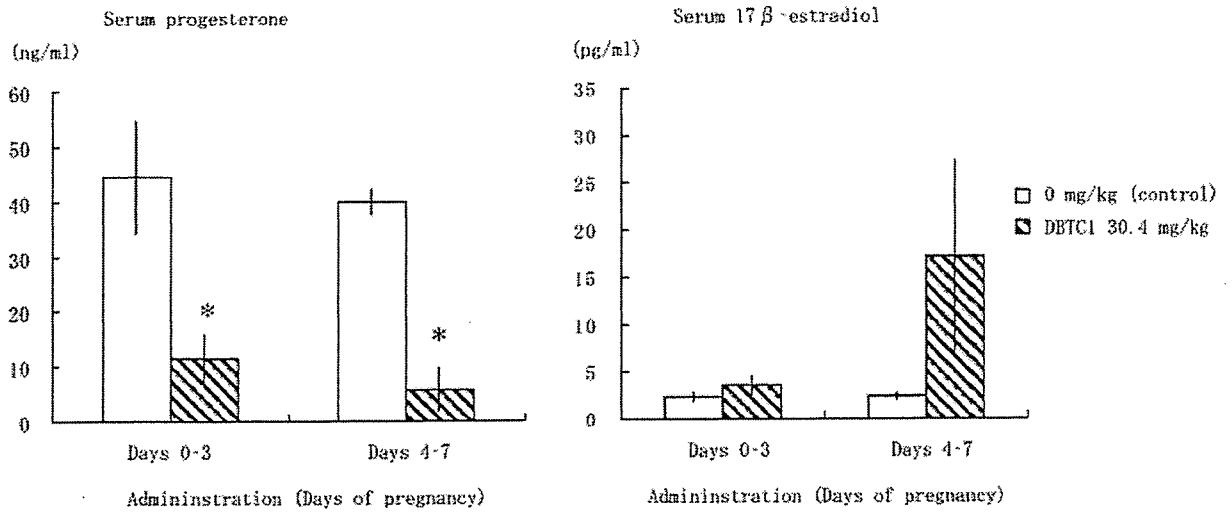

Fig. 1. Serum progesterone and 17β-estradiol levels in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Blood samples were collected on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± SEM of seven or eight mice. *Significantly different from the control group, $P < 0.05$.

the resorption of implantation sites. The most striking adverse effects of DBTCl on reproduction and development were a decrease in pregnancy rate, complete implantation failure, when DBTCl was given to mice on days 0–3 of pregnancy. The findings of an increased incidence of preimplantation embryonic loss in successfully mated females, and an increased incidence of postimplantation embryonic loss and low fetal weight in pregnant females survived until scheduled sacrifice after the administration of DBTCl on days 0–3 of pregnancy may suggest that DBTCl adversely affects preimplantation embryos and also the later survival and growth of embryos/fetuses when administered during the preimplantation period. On the other hand, the predominant adverse effects of DBTCl on reproduction and development were postimplantation loss, complete litter loss, when DBTCl was given to mice on days 4–7 of pregnancy. The findings of an increase in the incidence of postimplantation embryonic loss and a decrease in the fetal weight after administration of DBTCl on days 4–7 of pregnancy may suggest that DBTCl has effects on the later survival and growth of embryos/fetuses when administered during the peri-implantation period. Considered collectively, these findings indicate that the manifestation of adverse effects of DBTCl on reproduction and development varies with the stages of pregnancy at the time of maternal exposure.

The corpora lutea are essential up to the end of pregnancy in mice (Deansely, 1966). The embryo transport process in mice is triggered by progesterone and requires progesterone activity for its maintenance (Kendle and Lee, 1980). In mice, 24 h of progesterone priming is not only adequate for implantation, but this priming has a long-term effect on implantation (Huet-Hudson and Dey, 1990). In our previous studies in rats, increases in the incidences of early embryonic loss were observed after the administration of DBTCl during early pregnancy (Ema and Harazono, 2000ab). The suppression of uterine decidualization and reduced levels of serum progesterone were found in female rats given DBTCl on days 0–3 or days 4–7 of pseudopregnancy (Harazono and Ema, 2003), and lowered reproductive parameters in female rats given DBTCl were recovered by the administration of progesterone (Ema et al., 2003). Based on these findings, we hypothesized that the decline in serum progesterone levels in pregnant animals was a primary mechanism for the implantation failure due to DBTCl in rats. In the present study in mice, a decline in serum progesterone levels was detected after the administration of DBTCl during early pregnancy. These findings are in good agreement with previous findings that DBTCl induced early embryonic loss and decreased serum progesterone levels in pregnant rats. There is a similarity in the effects of DBTCl on progesterone levels in early pregnancy in rats and mice, and these suggest that the decline in the serum progesterone levels is also the factor responsible for the DBTCl-induced pregnancy failure in mice. Early pregnancy failure was also caused by systemic activation of the CD-40 immune costimulatory pathway in mice (Erlebacher et al., 2004). They noted that pregnancy failure resulted from impaired progesterone synthesis by the corpus luteum of the ovary, an endocrine defect in turn associated with ovarian resistance to the gonadotropic effects of prolactin and that pregnancy failure also required the proinflammatory cytokine TNF-a and correlated with the luteal induction of the prolactin receptor signaling inhibitors suppressor of cytokine signaling 1

(Socs1) and Socs3. Our results of the present study may support their argument. To further evaluate the adverse effects of DBTCl during early pregnancy, determination of the gene expression profile in the uterus of mice and rats is currently in progress.

In conclusion, DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice, and the present data suggest that the decline in progesterone is the responsible factor for the early pregnancy failure in mice.

REFERENCES

Deansely R. 1966. The endocrinology of pregnancy and foetal life. In: Parks AS, editor. Marshall's Physiology of Reproduction, Vol. 3. Boston: Little Brown. pp 891–1063.

Ema M, Harazono A. 2000a. Adverse effects of dibutyltin dichloride on initiation and maintenance of rat pregnancy. Reprod Toxicol 14:451–456.

Ema M, Harazono A. 2000b. Developmental and reproductive toxicity of tributyltin and its metabolite, dibutyltin, in rats. Congenit Anom (Kyoto) 40:108–120.

Ema M, Hirose A. 2006. Reproductive and developmental toxicity of organotin compounds. In: Golub MS, editor. Metals, Fertility, and Reproductive Toxicity. New York: CRC Press (Taylor & Francis Group). pp 23–64.

Ema M, Itami T, Kawasaki H. 1991. Teratogenicity of di-*n*-butyltin dichloride in rats. Toxicol Lett 58:347–356.

Ema M, Itami T, Kawasaki H. 1992. Susceptible period for the teratogenicity of di-*n*-butyltin dichloride in rats. Toxicology 73: 81–92.

Ema M, Kurosaka R, Amano H, Ogasawa Y. 1995. Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats. J Appl Toxicol 15:297–302.

Ema M, Kurosaka R, Amano H, Ogawa Y. 1996. Comparative developmental toxicity of di-, tri-, and tetrabutyltin compounds after administration during late organogenesis in rats. J Appl Toxicol 16:71–76.

Ema M, Harazono A, Hirose A, Kamata E. 2003. Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats. Toxicol Lett 143:233–238.

Erlebacher A, Zhang D, Parlow AF, Glimcher LH. 2004. Ovarian insufficiency and early pregnancy loss induced by activation of the innate immune system. J Clin Invest 114:39–48.

Evans DW, Laughlin RB Jr. 1984. Accumulation of bis(tributyltin)oxide by the mud crab, *Rhithropanopeus harrisii*. Chemosphere 13:213–219.

Fish RH, Kimmel EC, Casida JE. 1976. Bioorganotin chemistry: Reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenese enzyme system. J Organomet Chem 118:41–54.

Harazono A, Ema M. 2003. Suppression of decidual cell response induced by dibutyltin dichloride in pseudopregnant rats: As a cause of early embryonic loss. Reprod Toxicol 17:393–399.

Harazono A, Ema M, Ogawa Y. 1996. Pre-implantation embryonic loss induced by tributyltin chloride in rats. Toxicol Lett 89:185–190.

Harazono A, Ema M, Kawashima K. 1998a. Evaluation of malnutrition as a cause of tributyltin-induced pregnancy failure in rats. Bull Environ Contam Toxicol 61:224–230.

Harazono A, Ema M, Ogawa Y. 1998b. Evaluation of early embryonic loss induced by tributyltin chloride in rats: Phase- and dose dependent antifertility effects. Arch Environ Contam Toxicol 34:94–99.

Huet-Hudson YM, Dey SK. 1990. Requirement for progesterone priming and its long-term effects on implantation in the mouse. Proc Soc Exp Biol Med 193:259–263.

Iwai H, Wada O, Arakawa Y. 1981. Determination of tri-, di-, and monobutyltin and inorganic tin in biological materials and some aspects of their metabolism in rats. J Anal Toxicol 5: 300–306.

Kannan K, Corsolini S, Focardi S, Tanabe S, Tatsukawa R. 1996. Accumulation pattern of butyltin compounds in dolphin, tuna, and shark collected from Italian coastal waters. Arch Environ Contam Toxicol 31:19–23.

Kendle KE, Lee B. 1980. Investigation of the influence of progesterone on mouse embryo transport by using antiprogestational steroids. J Reprod Fertil 58:253–258.

Kimmel EC, Fish RH, Casida JE. 1977. Bioorganotin chemistry. Metabolism of organotin compounds in microsomal momooxygenese system and in mammals. J Agric Food Chem 25:1–9.

Lau MM. 1991. Tributyltin antifoulings: A threat to the Hong Kong marine environment. Arch Environ Contam Toxicol 20: 299–304.

Laughlin RB Jr, French W, Guard HE. 1986. Accumulation of bis(tributyltin) oxide by the marine mussel *Mytilus edulis*. Environ Sci Technol 20:884–890.

Maguire RJ. 1991. Aquatic environmental aspects of nonpesticidal organotin compounds. Water Pollut Res J Can 26:243–260.

Ministry of the Environment. 1973. Law for the Humane Treatment and Management of Animals, Law No. 105, October 1, 1973, revised June 15, 2005. Japan: Ministry of the Environment.

Piver WT. 1973. Organotin compounds: Industrial applications and biological investigation. Environ Health Perspect 4:61–79.

Prime Minister's Office. 1980. Standards Relating to the Care and Management, etc. of Experimental Animals, Notification No. 6, March 27, 1980 of the Prime Minister's Office. Japan: Prime Minister's Office.

Quevauviller P, Bruchet A, Donard OFX. 1991. Leaching of organotin compounds from poly (vinyl chloride) (PVC) materials. Appl Organomet Chem 5:125–129.

Rugh R. 1968. The Mouse: Its Reproduction and Development. Minneapolis: Burgess Publishing. 85p.

Salewski E. 1964. Färbemethode zum makroskopischen nachweis von implantationssetellen am uterus der ratte. Naunyn-Schm Arch Exp Pathol Pharmakol 247:367–372.

Sasaki K, Ishizaka T, Suzuki T, Saito Y. 1988. Determination of tri-*n*-butyltin and di-*n*-butylin compounds in fish by gas chromatography with flame photometric detection. J Assoc Off Anal Chem 71:360–366.

Seligman PF, Valkirs AO, Stang PM, Lee RF. 1988. Evidence for rapid degradation of tributyltin in a marina. Mar Pollut Bull 19:531–534.

Short JW, Thrower FP. 1986. Accumulation of butyltins in muscle tissue of Chinook salmon reared in sea pens treated with tri-*n*-butyltin. Mar Pollut Bull 17:542–545.

Stewart C, de Mora SJ. 1990. A review of the degradation of tri (*n*-butyl) tin in the marine environment. Environ Technol 11:565–570.

Suzuki T, Matsuda R, Saito Y. 1992. Molecular species of tri-*n*-butyltin compounds in marine products. J Agric Food Chem 40:1437–1443.

Waldock MJ, Thain JE. 1983. Shell thickening in Crassostea gigas: Organotin antifouling or sediment induced? Mar Pollut Bull 14:411–415.

WHO. 1980. Environmental Health Criteria 15 Tin and Organotin Compounds: A Preliminary Review. Geneva: World Health Organization.

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Prenatal developmental toxicity study of the basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Pregnant rats were given 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 10, 20 or 40 mg/kg bw/day on days 6–19 of pregnancy and the pregnancy outcome was determined on day 20 of pregnancy. At 40 mg/kg bw/day, deaths were observed in four out of 24 females. The incidences of females showing mydriasis at 20 and 40 mg/kg bw/day and showing decreased locomotor activity at 40 mg/kg bw/day were significantly increased. Alopecia, bradypnea, prone position and tremor were also observed at 40 mg/kg bw/day. The maternal body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were significantly reduced. A significantly decreased weight of the gravid uterus, increased incidence of postimplantation loss, decreased number of live fetuses, and lowered weights of fetuses and placentae were found at 40 mg/kg bw/day. The incidences of the total number of fetuses with external malformations at 40 mg/kg bw/day and with skeletal malformations at 20 and 40 mg/kg bw/day were significantly increased. Significantly higher incidences of fetuses with brachydactyly and short tail and defects of caudal vertebrae, phalanges and metacarpals were observed at 40 mg/kg bw/day. Delayed ossification was also noted at 40 mg/kg bw/day. The data indicate that DTG is teratogenic at maternal toxic doses and the NOAELs of DTG for maternal and developmental toxicity are 10 mg/kg bw/day in rats.

Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Prenatal developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

1,3-Di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the USA [1] and used as a basic rubber accelerator [2]. DTG is known to be a selective ligand receptor for the sigma site in the mammalian central nervous system [3]. Many findings have suggested that the sigma site plays a role in movement and posture through its association with brainstem and forebrain motor control circuits [4]. DTG has been reported to cause hypothermia after intraperitoneal injection in mice [5] and subcutaneous or intracerebroventricle injection in rats [6,7]. Intraperitoneal injection of DTG reduced the pain behavior in the acute phase, but increased pain behavior in the tonic phase in the formalin test in mice [8], and produced significant, but short-lived, increases in the withdrawal latencies in mice [5]. In rats, DTG also caused circling behavior after unilateral intranigral injection [4], decreased locomotor activity after intraperitoneal injection [9,10], increased bladder capacity after intravenous injection in the anaesthetized condition [11], and no change in immobility time in the forced swimming test after intraperitoneal injection [12].

It is generally assumed that the biological effects produced by chemicals should be studied in laboratory animals to investigate possible influences in human health, and the results of animal tests on chemical toxicity are relevant to humans [13]. Toxicological studies on DTG have given little information on acute animal toxicity [14]: intraperitoneal LD50 was 25 mg/kg bw in mice; the oral LD50 was 500 mg/kg bw in rats; the lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. We recently investigated the reproductive and developmental toxicity of DTG, according to the OECD guideline 421 reproduc-

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tion/developmental toxicity screening test in rats given DTG by gavage at 0, 8, 20 or 50 mg/kg bw/day [15], to obtain the preliminary information on the reproductive and developmental effects of DTG, because the testing for reproductive and developmental toxicity has become an important part of the overall toxicology. Males were given DTG for a total of 49 days beginning 14 days before mating, and females were given DTG for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. In this screening study, deaths in both sexes at 50 mg/kg bw/day, lowered body weight gain and food consumption in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day, and neurobehavioral changes such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation in both sexes at 20 and 50 mg/kg bw/day were found. Although no effects of DTG were detected on the estrous cyclicity, precoital interval, copulation, fertility and gestation indexes, numbers of corpora lutea and implantations, and gestation length, significant decreases in the number, body weight and viability of offspring and a significant increase in the incidence of fetuses with external malformations were noted at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were frequently observed at the highest dose. The total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg, and the teratogenic effect of DTG was strongly suggested. However, this screening test does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. Only external examination in the newborn rats was performed, and no internal or skeletal examinations were carried out in this screening test. The prenatal developmental toxicity study was therefore conducted to accurately evaluate the developmental toxicity, including the teratogenicity of DTG in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16] and in accordance with the principles for Good Laboratory Practice [17], "Law for the Humane Treatment and Management of Animals" [Law No. 105, October 1, 1973, revised June 15, 2005] and "Standards Relating to the Care and Management, etc. of Experimental Animals" [Notification No. 6, March 27, 1980 of the Prime Minister's Office].

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for five days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water ad libitum, and they were maintained in an air-conditioned room at 22 ± 3 °C, with a relative humidity of 50 ± 20%, a 12-h light/dark cycle, and ventilation of 10–15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm in the vaginal smear and/or vaginal plug were detected was considered to be day 0 of pregnancy. The copulated females were distributed into four groups to equalize the female body weights among groups. The copulated females were housed individually.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform, and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot no. 34K21) used in this study was 99.5% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before and after the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 10, 20 or 40 mg/kg bw on days 6 through 19 of pregnancy. The dosage levels were determined based on the results of our reproduction/developmental toxicity screening test [15], in which deaths at 50 mg/kg bw/day and neurobehavioral changes and lowered body weight gain and food consumption at 20 and 50 mg/kg bw/day in females, and decreases in the number, body weight and viability of offspring and increased incidence of fetuses with malformations at 50 mg/kg bw/day were found. DTG was suspended in 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The volume of each dose was adjusted to 5 ml/kg body weight based on daily body weight. The control rats were given only 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and each formulation was analyzed for concentration of DTG and the results revealed 90.3–99.5% of the intended concentration.

2.3. Observations

All females were observed daily during the pre-administration period and on the day of sacrifice, and twice a day (before and after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 3 and 6–20 of pregnancy. Food consumption was recorded on days 0, 3, 6, 9, 12, 15, 18 and 20 of pregnancy. The pregnant rats were euthanized by exsanguination under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the uterus was removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were counted. The live fetuses were removed from the uterus and sexed, weighed and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected, fixed in alcohol, stained with alizarin red S and alician blue [18] and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to free-hand razor-blade sectioning [19], and the thoracic areas were subjected to microdissecting [20] to reveal internal abnormalities.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, weight of the gravid uterus, food consumption, numbers of corpora lutea, implantations and live fetuses, fetal weight and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalences, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. The incidences of pre- and postimplantation embryonic loss and fetuses with malformations and variations and sex ratio of live fetuses were analyzed using Wilcoxon's rank sum test. The rates of pregnancy, nonpregnancy and females showing clinical signs of toxicity were analyzed with Fisher's exact test. The 0.05 level of probability was used as the criterion for significance.