In addition to these state-of-the-art updates on existing OECD methods, the following reports have been published for test systems for which no OECD guidelines currently exist:

- Photochemical genotoxicity [13].
- Single cell gel electrophoresis (Comet) assay *in vitro* and *in vivo* [14 and this issue].
- DNA adduct determination [15].
- *In vitro* micronucleus test [16,17].
- *In vivo* transgenic mutation assays [18,19].

3. The Fourth IWGT Workshop

The Fourth IWGT Workshop was recently held in San Francisco, USA as a satellite to the 2005 International Conference on Environmental Mutagens (ICEM). The majority of discussions and recommendations were in the area of strategic use of genotoxicity tests, but some new recommendations for methods were also made. The Working Group reports from this Workshop are published elsewhere in this issue.

Since OECD and ICH guidances (guidelines) constitute the two major sets of internationally harmonised genotoxicity guidelines in regulatory use, it is hoped that recommendations made by IWGT working groups are of particular help in supplementing test design and interpretation of genotoxicity test packages that are based on these guidelines. They may serve as a basis to open discussions for the revision of OECD test guidelines and the maintenance of ICH S2 guidance.

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Summary

Summary of major conclusions from the 4th IWGT, San Francisco, 9–10 September, 2005

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1. Introduction

Seven individual working groups addressed either specific aspects of the strategy for genetic toxicology testing or the design of protocols for specific assays. Comprehensive summaries of the outcome of each Working Group (WG) are given in the individual WG reports. The following outline summarises the main points that either differ from existing published recommendations (as in the case of mouse lymphoma test and *in vivo* micronucleus assay) or are key features to be considered in the development of new guidelines for an improved testing strategy.

2. Protocol design

Historically, there has been a tendency to recommend protocols that are as extensive as possible in the hopes of not failing to identify a genotoxic chemical. Based on previous experience [1–3] and the large databases that are now available for all of the assays evaluated in this workshop, it is clear that no assay, however extensive the protocol, can detect all genotoxic chemicals. This has led to the adoption of combinations of tests for genotoxicity screening (*i.e.* the use of test batteries). Further, recent data [4–6] have shown that *in vitro* assays commonly employed in regulatory screening strategies are often positive for agents considered not to present a significant genotoxic or carcinogenic risk *in vivo*. The rate of positive responses for non-carcinogens becomes exceptionally high when test batteries are employed [4]. Therefore, the WGs dealing with test methods were asked to define the basic features of the protocol that are essential for the detection of the majority of genotoxic agents. They were then encouraged to identify special cases, namely compounds or classes of compounds for which specific protocol adaptations might be needed.

2.1. *In vivo* erythrocyte micronucleus assay

Reaffirming this WG's report from the 2nd workshop [7], it was agreed that flow cytometric systems to detect

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the induction of micronucleated immature erythrocytes have advantages over manual scoring in that they give good reproducibility, are rapid and provide improved statistical power. A major new conclusion was that flow cytometric analysis of rat peripheral blood is acceptable as the sole assay endpoint for screening purposes. Previously, bone marrow analysis was required as part of the routine assay. This new conclusion allows integration of the micronucleus test with routine toxicology studies in either rats or mice, as only small (μL) quantities of peripheral blood are required. Data were presented (some still unpublished) that suggested that flow cytometry of blood reticulocytes may have the potential to allow monitoring of chromosome damage in other species. This could include dogs and non-human primates as part of routine toxicology studies, and humans in clinical trials or as part of biomonitoring studies, as long as the potential confounding effects of splenic activity are considered. At present the use of anti-CD71 fluorescent staining has been the most extensively validated, but other flow cytometric methods may be chosen as long as they meet the validation criteria previously published by this group [7]. The group also confirmed that rat peripheral blood reticulocytes can be used as the sole assay endpoint when young reticulocytes are analysed under proper assay protocol and sample size.

This WG also reviewed the assay using tissues other than those from the haematopoietic system, *e.g.* liver, colon, skin and testes. The group consensus was that the assay using young rat liver as the target organ to detect micronucleus induction was acceptable as an alternative to approaches such as hepatectomy in adult rats. Assay results from other tissues were incorporated into the database published previously [7].

The extension of the application of a single dose level assay was proposed, but the WG decided not to suggest any alteration to the current recommendations in the OECD guideline 474 for use of a single dose level only in a limit test.

2.2. *In vivo comet assay workgroup*

The Comet assay has been considered previously by IWGT [8]. At this most recent meeting, the WG discussed aspects of study design and conduct that needed clarification, with the primary focus being on the alkaline (pH > 13) version of the assay as it is applied to *in vivo* rodent systems. With regard to the numbers of dose levels required for a valid *in vivo* test, due to the lack of sufficient test data to demonstrate that downturns in dose response do not exist for this endpoint, it was concluded that a single dose level would not be sufficient even when conducted at the limit dose of 2 g/kg. A discussion on the relative merits of different methods for processing solid tissues (*i.e.* using isolated nuclei versus isolated cells) did not result in a conclusion that one method was superior to the other. However, it was recognised that more data are needed, and it was recommended that the proposed international Comet assay validation study include investigation of both processing methods. The impact of cytotoxicity on DNA migration formation was discussed, and there was consensus agreement that measures of cytotoxicity need to be included in all studies so that the impact of cytotoxicity on interpretation of Comet assay data can be addressed. For *in vivo* studies, histopathology was recognised as the most reliable way to identify the presence of apoptosis or necrosis in solid tissues, but it was agreed that there is a need to standardise the presentation of histopathological findings, as is normally done for chronic animal toxicity studies.

Scoring of comets by manual methods and image analysis was discussed, as were the various measures of DNA migration. The WG agreed that image analysis is preferred but not required, and that the percentage of tail DNA is the measure that seems most linearly related to dose and the easiest to understand, but other measures of DNA migration are equally acceptable. There was agreement that if a measure of tail moment is used, then percentage of tail DNA and tail length data should also be presented. It was also recommended that negative control treatments should exhibit measurable DNA migration as a means for evaluating run-to-run variability across time. Such historical data can be used as part of the acceptance criteria for new studies. In addition, it was recognised that, with sufficient migration in the negative controls, substances that induce DNA cross-linking could be detected.

2.3. *Mouse lymphoma thymidine kinase gene mutation assay*

This WG has met informally on a number of occasions in addition to the formal meetings at the Washington [9] and Plymouth [10] workshops, and has recently published recommendations on assay acceptance criteria, positive controls and data evaluation [11]. The WG met again informally during the 4th IWGT workshop, and the Steering Committee decided to include its report along with the formally constituted WGs at this meeting.

The main objectives of the San Francisco workshop were to review various aspects of the 24 h treatment protocol for the mouse lymphoma assay (MLA). The WG agreed to continue their support of the International Conference on Harmonisation (ICH) recommendation that

the MLA assay should include a 24-h treatment (without S-9) in those situations where the short treatment (3–4 h) gives negative results. Recommendations were made concerning the acceptable values for the negative/solvent control (mutant frequency, cloning efficiency and suspension growth) and the criteria to define an acceptable positive control response. Consensus was also reached concerning the use of both the global evaluation factor (GEF) and appropriate statistical trend analysis to define positive and negative responses.

3. Classification of genotoxic agents and strategy for risk assessment

At the Plymouth workshop a WG discussing strategies for classification and risk assessment of genotoxic agents was first established. A number of key conclusions were reached [12] but a number of important issues were not discussed. At the present workshop, four individual WGs addressed the key issues identified previously.

3.1. Strategy for genotoxicity testing: hazard identification and risk assessment in relation to in vitro testing

The objective of this WG was to develop recommendations for interpretation of results from tests commonly included in regulatory genotoxicity test batteries, and to propose an appropriate strategy for follow-up testing when positive *in vitro* results were obtained in these assays. Firstly, it was agreed that in most cases, a chemical found negative in an initial regulatory battery of tests (*e.g.* as proposed by ICH for pharmaceuticals [13]), does not require follow-up testing. However, some examples where metabolism may not be appropriate, and where positive *in vivo* results or tumours are subsequently found would require additional testing. The topics of metabolism and of rodent carcinogens that are negative in the standard screening battery were discussed by other subgroups and are summarised in the following sections. A structurally alerting chemical might trigger additional testing, but generally only if the negative *in vitro* battery was considered likely not to be sensitive to that chemical class.

The WG was able to agree and define the circumstances in which the pattern and magnitude of positive results *in vitro* are such that there is very low or no concern, and no further testing is needed (*e.g.* non-reproducible or marginal responses). Consideration of historical control data is important in this context.

The criteria for determining when follow-up testing is needed include factors such as evidence of reproducibility, level of cytotoxicity at which increased DNA damage or mutation frequency is observed, relationship of results to the historical control range of values, and total weight of evidence across assays.

When follow-up testing is needed, it should be based on the knowledge about the mode of action that is available, gleaned from the published literature and previous experimental observations. Initial findings and available information on the biochemical and pharmacological nature of the agent should allow conclusions as to whether the responses are consistent (or not) with certain molecular mechanisms. Follow-up tests should be chosen so as to be sensitive to the endpoints known to be capable of inducing the initial observed response, and non-standard tests may be more appropriate than standard tests in this regard.

The WG recognised that genotoxic events might arise from processes other than direct reactivity with DNA, that these mechanisms may often have a non-linear, or threshold, dose–response relationship. Such dose–response relationships are often also associated with indications of an overload of the biochemical processes within the test organism, and hence may not linearly relate to what is observed at lower concentrations. In cases in which a non-linear or threshold response can be demonstrated, it may be possible to determine an exposure level below which there is negligible concern for humans.

3.2. Strategy for genotoxicity testing: metabolic considerations

This WG considered the role of metabolism in producing *in vitro* genotoxicity results that may not be predictive of rodent carcinogenicity, or relevant for the evaluation of human risk. The basic question is whether a human metabolite(s) of interest is (are) represented in the assays used for genotoxicity and carcinogenicity testing. Alternative (and more "competent", *i.e.* capable of generating the metabolite of interest) metabolic activation or test systems may need to be evaluated. Since the default species for carcinogenicity testing are rats and mice, the impact of human metabolism relative to these species needs to be assessed in most cases. Also, appropriate action triggers, based on the extent of human exposures (*i.e.* "major" or unique), consideration of structural knowledge of the metabolite (*e.g.* evidence of reactivity), and evidence of genotoxicity obtained with conventional metabolic activation systems (*e.g.* induced liver S9), need to be defined. The WG emphasised the

need to consider these points in relation to the timing of human ADME studies in the case of pharmaceutical development. They therefore proposed both proactive and retroactive strategies to assess metabolite genotoxic potential, including use of an alternative/optimised *in vitro* metabolic activation system or direct testing of metabolites, and study of both point mutations and chromosomal aberrations.

The WG also identified specific areas where there is insufficient understanding, experience or scientific basis to achieve full consensus. The definition of a quantitative human metabolite exposure as a trigger for safety assessment requires broader discussions and debate (*e.g.* on the significance of absolute or relative metabolite abundance) to reach consensus. The WG expressed the desire to consider further an absolute exposure definition in order to better support risk assessment, analogous to the threshold of toxicological concern (TTC) concept. This absolute exposure definition could be associated with a re-definition of the highest suitable test concentrations for *in vitro* assays. Justification for such re-definition could be supported by the capability limitations for most biochemical/metabolic processes (K_ms) within tissues and cells, the overload of which can generate results of questionable meaning. A universal recommendation for the timing of human ADME studies in the case of pharmaceutical development was not agreed, and neither was how to use structural knowledge and/or physicochemical properties (*e.g. in silico* systems, literature, or expert analysis) of metabolites to assess safety *in lieu* of genotoxicity testing. Lastly, the group was unable to define discrete triggers for direct metabolite testing vs. use of an alternative activation system. This was largely driven by the inability to define a universal metabolite exposure level that was considered "sufficient" to characterise potential genotoxic hazards when generated by an alternative activation system.

3.3. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards and identification of in vivo-only positive compounds in the bone marrow micronucleus test

This WG reviewed the growing body of (published and unpublished) evidence that compound-related disturbances in the physiology of rodents used for bone marrow micronucleus tests can result in positive responses not relevant to human exposures. These disturbances include significant and sustained increases or decreases in core body temperature, increases in erythropoiesis in the bone marrow (*e.g.* following prior toxicity to erythroblasts or by direct stimulation of division in these cells), and inhibition of protein synthesis. The potential for a test compound to operate through any one of these modes of action should be considered when interpreting the results of *in vivo* micronucleus studies.

Not all compounds that are positive (or more readily detected) in an *in vivo* micronucleus test, yet give negative or marginal results for *in vitro* genotoxicity, operate through the kinds of physiological disturbances described above. Reasons may be due to metabolic differences, the influence of gut flora, higher exposures *in vivo* compared to *in vitro*, and effects on pharmacology, in particular folate depletion. A number of receptor kinases fall into the category of pharmacologically mediated activity. Amongst the compounds reviewed by the WG, many interfere with cell cycle kinetics and this can result in either aneugenicity or chromosome breakage. It is possible that some of these compounds could be detected *in vitro* if a specific test were chosen as part of the test battery, but the "correct" choice may not always be obvious when testing a compound of unknown genotoxicity. The WG considered that a sufficient number of "unique" *in vivo* positive compounds exist that it may not be appropriate, at this time, to eliminate the *in vivo* micronucleus test from those batteries in which it is an integral part. However, as the relevant compounds act *via* a number of specific mechanisms, *e.g.* compounds metabolised by CYP2E1, folate inhibitors, kinase inhibitors, *etc.*, it may be possible to consider additional modified *in vitro* testing when such compounds are negative in conventional *in vitro* assays.

3.4. Follow-up testing of rodent carcinogens not positive in the standard genotoxicity testing battery

This WG focussed on when it would be appropriate to conduct additional genotoxicity studies, as well as what types of studies, if the initial standard battery of tests is negative, but tumour formation is observed in the rodent carcinogenicity assessment. Standard genetic toxicology tests can help to determine the mode of action for carcinogenesis (genotoxic vs. non-genotoxic) but there are limitations. The entire toxicological profile of a compound (*e.g.* structure activity relationships, the nature of the tumour finding, metabolic profiles) needs to be considered before conducting any additional testing. If the need for further genotoxicity testing is identified, test models for investigating genotoxicity in the tumour target organ(s) are highly recommended. Transgenic mutation assays, the Comet assay, or DNA binding studies are considered appropriate for this purpose. However, it should be remembered that only those assays that directly assess the induction of mutations, in particular

allowing the repeat dosing protocol as previously recommended [14,15], can provide definitive information as to the ability of the compound to induce mutation. A positive finding in any genotoxicity study indicates a genotoxic potential for the compound, but not necessarily that genotoxicity, or more specifically DNA reactivity, is the principal mode of carcinogenic action. It requires a thorough weight of evidence assessment of all the available and relevant information, including effect and/or extent of exposure at the target sites, in order to decide whether tumourigenesis is mediated *via* a genotoxic mode of action.

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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. © 2007 Wiley Periodicals, Inc. 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.,

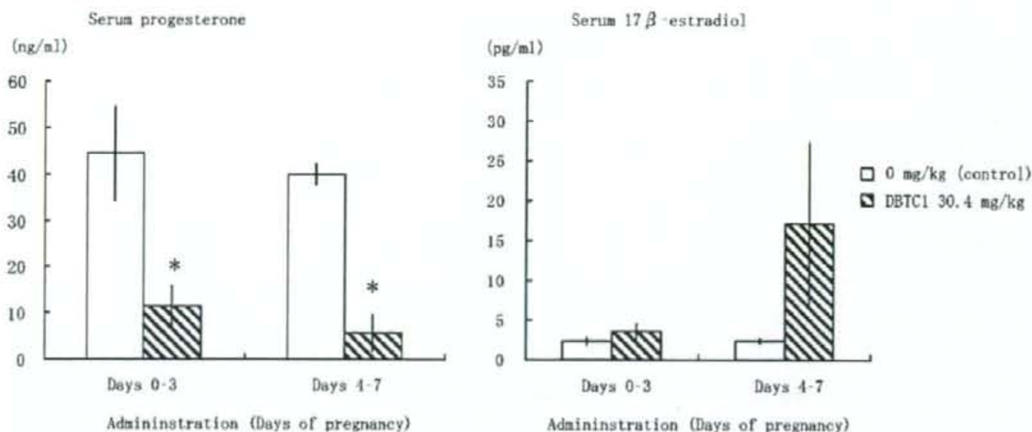

Fig. 1. Serum progesterone and 17β-estradiol levels in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Blood samples were collected on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± SEM of seven or eight mice. *Significantly different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

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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.

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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, non-pregnancy 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.

Table 1
Maternal findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of rats	24	24	24	24
No. of pregnant rats	24	24	24	24
Initial body weight	256 ± 13	256 ± 13	256 ± 13	256 ± 13
No. of females showing clinical sign of toxicity				
Death	0	0	0	4
Alopecia	2	2	3	2
Bradypnea	0	0	0	2
Decreased locomotor activity	0	0	1	11**
Mydriasis	0	0	12**	24**
Prone position	0	0	0	3
Salivation	0	0	2	2
Soil of perigenital	0	0	1	4
Tremor	0	0	0	2
Body weight gain during pregnancy (g)[a]				
Days 0–6	40 ± 8	39 ± 8	40 ± 8	39 ± 8
Days 6–15	50 ± 7	49 ± 9	37 ± 11**	23 ± 10**
Days 15–20	77 ± 9	77 ± 9	71 ± 10	47 ± 16**
Days 0–20	167 ± 17	165 ± 21	148 ± 24**	109 ± 21**
Adjusted weight gain[b]	88 ± 15	87 ± 19	77 ± 15	49 ± 17**
Food consumption during pregnancy (g/day)[a]				
Days 0–6	23 ± 2	23 ± 2	23 ± 2	23 ± 2
Days 6–15	26 ± 2	26 ± 2	24 ± 3	20 ± 3**
Days 15–20	28 ± 2	28 ± 3	26 ± 2	22 ± 3**
Days 0–20	25 ± 2	26 ± 2	24 ± 2	21 ± 2**
Weight of gravid uterus (g)[a]	79 ± 10	78 ± 11	72 ± 15	59 ± 10**

[a] Values are given as the mean ± S.D.
[b] Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.
** Significantly different from the control ($p < 0.01$).

3. Results

Table 1 shows the maternal findings in rats given DTG on days 6–19 of pregnancy. At 40 mg/kg bw/day, death was found on day 8 of pregnancy in two females and on days 7 and 19 of pregnancy in one female each. Statistically significant increases in the incidence of mydriasis occurred at 20 and 40 mg/kg bw/day, and in decreased locomotor activity at 40 mg/kg bw/day. Additional findings that appeared to be treatment related, but not statistically significant were decreased locomotor activity at 20 mg/kg bw/day, salivation and soil of the perigenital area at 20 and 40 mg/kg bw/day, and bradypnea, prone position and tremors at 40 mg/kg bw/day. These signs were observed consistently throughout the dosing period and relatively higher incidences of these signs were noted during the early administration period. Maternal body weight gain was significantly decreased on days 6–15 and 0–20 of pregnancy at 20 mg/kg bw/day, and on days 6–15, 15–20 and 0–20 of pregnancy at 40 mg/kg bw/day. Adjusted weight gain, the net weight gain of maternal rats during pregnancy, and the weight of the gravid uterus were also significantly reduced at 40 mg/kg bw/day. At this dose, food consumption was significantly lowered on days 6–15, 15–20 and 0–20 of pregnancy.

Table 2 presents the reproductive findings in rats given DTG on days 6–19 of pregnancy. No dam with total litter loss was observed in any group. No effects of DTG were found on the numbers of corpora lutea and implantations, or the incidence of preimplantation loss. At 40 mg/kg bw/day, a significantly increased incidence of postimplantation loss, a decreased number of live fetuses and lowered weights of male and female fetuses and placentae were noted. The sex ratio of live fetuses was significantly reduced in the DTG-treated groups.

The summarized results of external and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in the control group. One fetus with cleft palate was found at 10 mg/kg bw/day. Fetuses with external malformations were found in 13 out of the 328 fetuses (three out of the 24 litters) at 20 mg/kg bw/day and 33 out of the 251 fetuses (11 out of the 20 litters) at 40 mg/kg bw/day, and significantly increased incidence of the total number of fetuses with external malformations was noted at 40 mg/kg bw/day. Incidences of fetuses with brachydactyly and with short tail were increased at 20 and 40 mg/kg bw/day, and significantly increased incidences were found at 40 mg/kg bw/day. As for internal malformations, one fetus each with microphthalmia in the control and 20 mg/kg bw/day groups, one fetus with dilatation of the lateral ventricles in the control group and one fetus with undescended testes in the 40 mg/kg bw/day were observed. Variations in the internal organs were observed in 11–19 fetuses in all groups. However, no significant differences in the incidences of

Table 2
Reproductive findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of litters	24	24	24	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter[a]	15.7 ± 2.1	14.8 ± 1.6	14.9 ± 1.9	15.3 ± 1.5
No. of implantations per litter[a]	15.3 ± 1.9	14.7 ± 1.8	14.2 ± 2.7	15.2 ± 1.4
% Preimplantation loss per litter[b]	2.4	0.9	5.6	0.9
% Postimplantation loss per litter[c]	3.5	3.4	4.8	16.4**
No. of live fetuses per litter[a]	14.8 ± 1.9	14.2 ± 2.1	13.7 ± 2.9	12.6 ± 1.9**
Sex ratio of live fetuses (male/female)	0.56	0.49*	0.46*	0.46*
Body weight of live fetuses (g)[a]				
Male	3.64 ± 0.17	3.72 ± 0.18	3.59 ± 0.24	3.19 ± 0.31**
Female	3.42 ± 0.16	3.53 ± 0.25	3.41 ± 0.18	3.03 ± 0.26**
Placental weight (g)[a]	0.47 ± 0.04	0.47 ± 0.03	0.50 ± 0.16	0.40 ± 0.04**

[a] Values are given as the mean ± S.D.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c] (No. of resorptions and dead fetuses/no. implantations) × 100.
* Significantly different from the control ($p<0.05$).
** Significantly different from the control ($p<0.01$).

fetuses with internal malformations and variations were detected between the control and DTG-treated groups.

The summarized results of skeletal examinations in the fetuses of rats given DTG on days 6–19 of pregnancy are presented in Table 4. Fetuses with skeletal malformations were found in one out of the 184 fetuses (one out of the 24 litters) in the control group, one out of the 176 fetuses (one out of the 24 litters) at 10 mg/kg bw/day, 13 out of the 170 fetuses (six out of the 24 litters) at 20 mg/kg bw/day, and 26 out of the 130 fetuses (12 out of the 20 litters) at 40 mg/kg bw/day. Significantly higher incidences of the total number of fetuses with skeletal malformations were observed at 20 and 40 mg/kg bw/day. Incidences of fetuses with absence, fusion or malposition of the caudal vertebrae and with absence or fusion of phalanges were higher at 20 and 40 mg/kg bw/day, and significantly increased incidences of fetuses with these malformations and fetuses with the absence or fusion of metacarpals were found at 40 mg/kg bw/day. Although skeletal variations in the vertebral column, ribs and sternebrae were observed in all groups, no significant differences in the incidences of fetuses with skeletal variations were detected between the control and DTG-treated groups. A significantly delayed ossification, as evidenced by the numbers of sacral and caudal vertebrae, sternebrae, and metatarsi, was also noted at 40 mg/kg bw/day.

4. Discussion

In order to obtain further information on the reproductive and developmental toxicity of DTG, the present study was conducted in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16]. DTG was given to pregnant rats during the time of implantation to the term of pregnancy to

Table 3
External and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
External examination				
Total no. of fetuses (litters) examined	354 (24)	341 (24)	328 (24)	251 (20)
Total no. of fetuses (litters) with malformations	0	1	13 (3)	33 (11)**
Cleft palate	0	1	0	0
Brachydactyly	0	0	8 (3)	31 (11)**
Short tail	0	0	7 (2)	10 (7)**
Internal examination				
Total no. of fetuses (litters) examined	170 (24)	165 (24)	158 (24)	121 (20)
Total no. of fetuses (litters) with malformations	1	0	1	1
Microphthalmia	1	0	1	0
Dilatation of lateral ventricles	1	0	0	0
Undescended testes	0	0	0	1
Total no. of fetuses (litters) with variations	16 (10)	11 (9)	13 (7)	19 (12)
Thymic remnants in neck	13 (10)	8 (7)	12 (7)	17 (11)
Dilated renal pelvis	2 (2)	2 (2)	0	0
Left umbilical artery	1	1	1	2 (2)

** Significantly different from the control ($p<0.01$).