SIAM における環境影響とヒト健康影響についての勧告は、FW(The substance is a candidate for further work) または LP (The substance is currently of low priority for further work) として示されている。FW は「今後も追加の調査研究作業が必要である」、LP は「現状の使用状況においては追加作業の必要はない」ことを示す。FW となる理由には、追加試験が必要とされる場合の他、曝露情報の調査、詳細なリスク評価、リスク管理などが必要と判断される場合がある。しかし、曝露情報の調査、詳細なリスク評価、リスク管理などへの具体的な対応は各国に任されており、日本では評価結果を参考に必要があれば各法や各省の取り組みのなかに取り込むことになっている。SIAM で合意された勧告についてはその根拠と共に解釈することが望まれており、評価内容と合わせて参照する必要がある。

（2）第 15 回既存化学物質タスクフォースの審議内容について

第 15 回既存化学物質タスクフォースは 2007 年 3 月 1-2 日にフランスのパリで開催された。OECD の HPV 点検プログラムでは、SIAP、SIAR および Dossier の初期評価文書を作成しているが、Dossier は欧州化学品局 (ECB: European Chemicals Bureau) から提供された IUCLID (International Uniform Chemical Information Database) ソフトウェアを用いて作成されている。 2000 年末に IUCLID が導入される以前は、Dossier は単独の文書ファイルとして作成されていた。第 15 回タスクフォースは、ファイル形式の統一化を図るために文書ファイルで作成された Dossier を、2009 年までに IUCLID の形式で作成しなおす必要があるとした。

2．第 24 回 SIAM での審議状況

（1）初期評価文書の審議結果

初期評価文書は加盟各国が初期評価文書の原案をオンライン会議用掲示板 (CDG : Committee Discussion Group) に掲載し、CDG上での事前討議（コメントの提出、コメントへの返答、コメントに応じたSIAPの修正）およびSIAMでの対面討議で審議される。第24回SIAMでの初期評価文書の審議は、CDGでの事前討議を基に修正したSIAPを用いて行われた。日本政府は 2-sec-Butyl-4,6-dinitrophenol (CAS: 88-85-7)、Ferrous sulfate heptahydrate (CAS:7782-63-0) およびN-(2-Octadecanoylamidoethyl)octadecanamide (CAS: 110-30-5・5136-44-7・5518-18-3 混合物）の 3 物質の初期評価文書を提出した。なお、N-(2-Octadecanoylamidoethyl)octadecanamideについては、日本/ICCAが原案作成を行った。また、 Ferrous sulfate heptahydrateの初期評価文書は、フィンランド/ICCAが担当した「物質カテゴリー : Iron salts and their hydrates」を構成する物質の一つとして作成され、日本政府の専門家がレビューを行った後に提出された。 会議では計39物質の初期評価文書が審議され、34物質の初期リスク評価結果および評価結果に基づく措置に関する勧告が合意された（表1）。以下の物質については、通常の審議と異なる点があったため特筆する。

1）ドイツ/ICCAが提出したFormamide (CAS:75-12-7) の初期評価文書については、がん原性および遺伝毒性についての新たな情報がピアレビューされている段階であり、最終的な初期評価文書はCDG上で審議されることになった。しかし、会議は初期リスク評価結果および評価結果に基づく措置に関する勧告は変更されるべきではないと結論した。

2）今回の会議では英国/ICCAが原案作成をしたDiethanolamine (CAS:111-42-2) の初期評価文書を審議した。本物質はすでに第2回SIAM (1994年7月) および第3回SIAM (1995年3月) で、英国政府がスポンサー国となり審議されており、第3回SIAMでは、吸入亜慢性毒性、神経毒性および生殖発生毒性などの危険性を理由にFWという勧告に合意が得られたが、将来のSIAMでの再審議が予定されていた。今回のSIAMでは、眼刺激性、反復投与毒性および生殖発生毒性についての危険性はあるものの、スポンサー国で十分なリスクマネジメントがなされていることからLPという勧告に合意が得られた。現在のHPV点検プログラムでは、スポンサー

化学生物総合管理 第 3 巻第 2 号 (2007.12) 180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日 受理日：2007 年 10 月 5 日

国から寄せられる曝露情報をもとに勧告を定めることが出来る。この物質のように他の加盟国における曝露状況が不明の場合は、曝露シナリオを各国で点検することが望ましい可能性がある旨を勧告の根拠欄に示している。

3）スイス/ICCAが提出したDiethylbenzene mixed isomers（CAS:25340-17-4）については、生分解性より示唆される環境に対する有害性の有無をCDG上で審議することになった。しかし、会議は初期リスク評価結果および評価結果に基づく措置に関する勧告は変更されるべきではないと結論した。

4）スウェーデン：eu（欧州連合のリスク評価文書を基にしたことを意味する）が担当したHexabromocyclododecane（CAS: 25637-99-4 / 3194-55-6)の初期評価文書は、第9回SIAM（1996年6月）で予備的に審議されていたが、今回のSIAMにおいて初期リスク評価結果および勧告が合意された。この物質については、フガシティーモデルによる環境分布予測およびlogKowのデータが新たに入手される予定であり、最終的な初期評価文書についてはCDG上で審議される。しかし、会議は初期リスク評価結果および評価結果に基づく措置に関する勧告は変更されるべきではないと結論した。なお、この物質にはCAS番号が2つあるが、それらは同一の物質を示す。

5）デンマーク：euが担当したニッケル関連５物質（CAS:3333-67-3、7440-02-0、7718-54-9、7786-81-4、13138-45-9）についての初期評価文書は人健康影響に関する結論にのみ合意が得られた。環境影響に関する評価については、物質ごとに勧告を定める必要があるとされ、第26回SIAM（2008年4月）において再審議されることになった。

（2）HPV 点検プログラムにおける全般的な議題

1）グローバルポータルの開発について

OECD では、加盟国や国際機関が有している既存化学物質のハザード情報などに関するデータベースを一括して検索できるグローバルポータルサイト「eChemPortal™」を構築している。グローバルポータルサイトの構築作業には日本も参加しており、化学物質総合情報システム（CHRIP：Chemical Risk Information Platform）で公開されている既存化学物質安全性点検データ（生分解性・蓄積性の情報）が組み込まれることになっている。OECD 事務局が今回の会議で紹介したグローバルポータルサイトの初版は、2007 年夏に一般に公開された。今回の会議では、英国からの質問を受け、グローバルポータルサイトそのものに参加するのではなく、Web ページにリンクを貼る形で参加する方法も可能であることが確認された。

2）（定量的）構造活性相関アプリケーションツールボックスについて

（定量的）構造活性相関「（Q)SAR：(Quantitative) Structure-Activity Relationships」は、化学物質の構造と活性との間に成り立つ数量的関係を示し、構造的に類似した化学物質の毒性を予測することを目的として注目されている。OECD における(Q)SAR モデル使用の可能性については第 34 回 Joint Meeting (2002 年 11 月)において審議され、2004 年 11 月の第 37 回 Joint Meeting は、(Q)SAR アプリケーションツールボックス（ツールボックス）の開発が必要であるとした。このツールボックス開発の目的は、(Q)SAR モデルの複雑さを軽減させ、信頼できる情報を容易に入手できるようにし、(Q)SAR モデルを用いた化学物質のカテゴリー化を支援することである。現在、オランダの RIVM （Het Rijksinstituut voor Volksgezondheid en Milieu：The National Institute for Public Health and the Environment）が OECD との契約の下、ツールボックスの作成を行っている。今回の会議では、前回の会議に引き続き OECD 事務局および RIVM が機能の説明を行った。OECD 事務局は、第 25 回 SIAM でツールボックスのベータ版を配布することを伝えた。また、ツールボックスは 2008 年 3 月までに作成され、CD-ROM で無料配布される予

化学生物総合管理 第 3 巻第 2 号 (2007.12) 180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日 受理日：2007 年 10 月 5 日

定であることが確認された。

3）物質カテゴリーについてのガイダンス

OECD 事務局は第 23 回 SIAM に引き続き、EU と OECD の共同プロジェクトである「物質カテゴリーの構成と使用について」のマニュアルのガイダンス文書の修正案について報告した。このプロジェクトはもともと EU の化学物質の登録・評価・認可および制限に関する規則である REACH（Registration, Evaluation, Authorisation of Chemicals）の履行プロジェクト(RIP: REACH Implementation Projects) 3.3 の一つとして開始された。共同プロジェクトの目的は OECD の HPV 点検プログラムおよび EU の REACH で使用されているマニュアルのガイダンスを作成することである。第 23 回 SIAM 後に BIAC、カナダ、オランダからコメントが寄せられ、コメントを反映した最新版の草案が今回の会議に先立って CDG 上に掲載された。第 24 回 SIAM は、オーストラリアのコメントに従って文書中の登録者に対するリファレンスを削除することに合意した。しかし、それ以外の字句の修正などのコメントについては、限定的過ぎるとして合意されなかった。修正した文書は、承認を得るために既存化学物質タスクフォースおよび Joint Meeting に提出される。マニュアルのガイダンス文書は「Series on Testing and Assessment」のモノグラフとして 2007 年中に Web 上に公開される。

4）HPVに対するGHS適用について

GHS（Globally Harmonized System of Classification and Labelling of Chemicals）とは、世界的に統一されたルールに従って化学物質を危険有害性ごとに分類し、その情報を一目でわかるようなラベルの表示や安全データシートで提供するというものである。持続可能な開発に関する世界首脳会議は、2002年9月にヨハネスブルグで採択した行動計画において、2008年までにGHSを完全に実施することを目指して各国ができる限り早期にGHSを実施することに合意した。HPV点検プログラムではGHS適用のためのパイロットプロジェクトが設けられ、日本も有志国として参加した。有志国はSIAMで審議される物質についてのGHS分類を作成し、加盟各国がCDG上でレビューを行った。2007年7月にスイスのベルンで行われたワークショップでは、パイロットプロジェクトの成果が報告された。ワークショップには、分類および表示の調和に関するOECDのタスクフォースおよびSIAMからの代表者が出席し、GHS分類についてのガイダンスについて審議を行った。なお、今回のパイロットプロジェクトは単発のものであり、今後継続して行われる予定はないことが確認された。

5）国や地域レベルの評価プログラムとOECDのHPV点検プログラムについて

EUの化学物質の登録・評価・認可および制限に関する規則であるREACHの施行をうけ、ECは、REACHおよびHPV点検プログラムの相乗的な効果について文書をまとめた。REACHは人の健康と環境を化学物質の危険から守ることおよびEU化学産業の競争力を強化することを目的として施行され、年間1トン以上の化学物質を製造又は輸入する企業は、化学物質の情報をデータベースに登録する必要がある。OECDのHPVプログラムの文書作成は、OECD加盟各国の有志が協力し合い行っているが、REACHにおける登録文書の作成は製造者又は輸入者の義務である。登録期限は製造量・輸入量などにより異なるが、2010年11月、2013年6月、2018年6月とされる。現在考えられているREACHおよびHPV点検プログラムの相互関係は次の通りである。

- REACHの登録文書は、Technical Dossier（1トン以上の製造又は輸入の場合に提出）および化学物質安全性報告書（CSR : Chemical Safety Report ; 10トン以上の製造又は輸入の場合に提出）の2つであるが、それぞれOECDのDossierおよびSIARと類似している。
- REACHでは製造・輸入量に応じて残留性や蓄積性などの情報についても記載する必要があり、高残留性や高蓄積性の物質については、曝露シナリオを記載する必要があるなどの相違点がある。

化学生物総合管理　第 3 巻第 2 号 (2007.12)　180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日　　受理日：2007 年 10 月 5 日

- CSRはSIARにハザード評価に必要となる情報を加え、若干の修正を加えることによって作成でき、SIARはCSRから不要部分を削除し若干の修正を加えることによって作成することが可能である。
- HPV点検プログラムのDossierとREACHのTechnical Dossierは共にIUCLIDを用いて作成されるが、Technical Dossierに要求される情報の範囲は前述のようにHPV点検プログラムより広い。
- REACHの登録以前にDossierおよびSIARがSIAMで合意されている場合は、Technical Dossier およびCSR作成にその情報を利用し、DossierおよびSIARの内容については、SIAMの合意に従う。
- REACHの登録までの手順は、OECDのHPV点検プログラムの文書作成からSIAMで審議されるまでの手順に類似しており、OECDのHPV点検プログラムで用いられているカテゴリー評価や（Q）SAR評価は、REACHでも同様に用いられる。
- 新たな情報が必要な場合は、OECDのテストガイドラインを用いて試験を行う。
- HPV点検プログラムのFWの勧告とREACHでのFWの勧告の表現は類似するが、要求される範囲が異なるため、FWの求める内容は相違する。OECDのHPV点検プログラムでの勧告はREACHに登録する製造者又は輸入者がCSRを作成する上でどこに注意すべきかを示唆することになる。

既存化学物質タスクフォースは、他の国や地域レベルの評価プログラムとOECDのHPV点検プログラムについて、同様の文書を作成することが有用であるとした。その最終的な目標は、次の2点である。

① 国や地域レベルの評価プログラムを利用し、HPV点検プログラムの効率化を図る術がないか模索すること。
② 現状のHPV点検プログラムについて、変更の必要性がないか確認すること。

カナダ、日本、米国、オーストラリアは自国の既存化学物質の評価プログラムについて紹介した。日本は「官民連携既存化学物質安全性情報収集・発信プログラム」（通称：「Japan チャレンジプログラム」）について紹介した。Japan チャレンジプログラムでは、国内年間製造・輸入量が 1,000 トン以上である有機低分子化合物について、情報を収集・発信することとしており、OECD の HPV 点検プログラムや US チャレンジプログラムでの情報収集予定がない約 140 物質を優先的に行うものとしている。これまで、約８０物質についてスポンサー企業が登録されているが、今後、引き続きスポンサー企業を募集するとともに、収集された情報については web ページを通じて積極的に公表することとしている。

米国は、新しい評価文書形式（SIAP より詳細だが SIAR よりは簡略されているもの）を紹介した。これは、US チャレンジプログラムで情報収集された化学物質の有害性（Hazard Characterizations）を示す文書であり、2007 年 9 月に約 100 物質についての文書が公開された。米国は紹介した文書フォーマットについてはまだ変更可能であり、将来的に OECD の HPV 点検プログラムの条件に合わせる可能性があるとした。

カナダは、環境保護法(CEPA: Canadian Environmental Protection Act)に基づいた新規および既存の化学物質の評価プログラムについて説明した。また、2006 月 12 月にカナダ政府が発表した化学物質管理計画（Chemical Management Plan)についても概要を説明した。

オーストラリアは、工業化学品（届出・審査）制度（NICNAS: National Industrial Chemicals Notification and Assessment Scheme）による既存化学物質の再評価について報告した。

会議ではプログラム間の類似点および相違点に焦点があてられた。OECD 事務局は、2007 年 10 月に予定されている第 25 回 SIAM および既存タスクフォースまでに、各プログラムについての文書をまとめることになった。

化学生物総合管理 第 3 巻第 2 号 (2007.12) 180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日 受理日：2007 年 10 月 5 日

6）試験および評価に関する統合的アプローチについて

2007 年 12 月にワシントン DC において、試験および評価に関する統合的アプローチについてのワークショップの開催が予定されている。このワークショップの目的は、様々な法規制や評価プログラムの条件を満たす総合的な評価アプローチを新たに模索することである。現在は米国が中心となって、異なる 3 つのグループの化学物質（Triadimefon「抗真菌剤」；Sulfosuccinates「食品における界面活性剤」；Ethylene glycols「HPV のカテゴリー物質」）について Dossier を作成し、事例研究を行っている。事例研究についての協議は CDG 上で行われる。ワークショップでは、現在使用されている評価方法（in vivo および in vitro による試験、(Q) SAR、Read across および カテゴリー評価）が、新規および既存化学物質や、殺虫剤および農薬などの異なる枠組みの法規制にどのように利用できるかなどを検討する。

7）SIAM 前・SIAM 後の化学物質の情報について

HPV 点検プログラムでは、CDG を通じて SIAM の開催前後に情報の公開や審議を行っている。OECD 事務局は、CDG 上での審議の質を高く保つために CDG を積極的に活用するよう勧告した。また、CDG に掲載されている第 22 回 SIAM で審議された物質カテゴリー：PFOA（CAS:335-67-1、3825-26-1）の修正文書については、既存化学物質タスクフォースおよび Joint Meeting による承認が得られた後に出版されることとなる。

8）MERAG プロジェクトについて

国際金属鉱業評議会（ICMM：International Council on Mininig and Metals）の代表者が、金属環境リスクアセスメント・ガイダンス（MERAG：Metal Environmental Risk Assessment Guidance）プロジェクトの紹介を行った。人健康影響のリスクアセスメントについても類似するプロジェクトが進行中であり、金属に関する統一的な評価や基準が提供されることが期待されている。

おわりに

OECD の HPV 点検プログラムでは、プログラム進捗の加速化を目指し、2010 年までに 1,000 物質についてデータを収集することを目標にしている。2007 年 6 月より REACH が段階的に施行されるにあたって、HPV 点検プログラムと REACH の間での相互的な作用が期待される。また、Japan チャレンジプログラムや他の国の化学物質の評価プログラムの HPV 点検プログラムへの貢献も期待される。

勧告の判定については前回の会議に引き続き、環境影響またはヒト健康影響に対する有害性が認められ、かつ曝露情報が不足している、または高曝露が予測される物質については FW と結論される傾向にあった。一方、環境影響またはヒト健康影響に対する有害性の低いもの、或いは有害性は認められるが低曝露が予測される物質(ヒト健康影響)および速やかに生分解される物質(環境影響)などは、LP と結論される傾向にあった。

参照資料

- OECD (2007a) Draft Summary Record of the Twenty-third SIDS Initial Assessment Meeting (SIAM 24) ENV/JM/EXCH/SIAM/M(2007)1
- OECD (2007b) OECD integrated HPV database. http://cs3-hq.oecd.org/scripts/hpv/
- UNEP (2007) Chemicals Screening information dataset (SIDS) for high volume chemicals. http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html
- 江馬　眞（2006）：OECD の高生産量化学物質安全性点検プログラムとその実施手順．化学

化学生物総合管理　第 3 巻第 2 号 (2007.12)　180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1　E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日　　受理日：2007 年 10 月 5 日

生物総合管理, 2-1, 83-103
・高橋美加, 松本真理子, 川原和三, 菅野誠一郎, 菅谷芳雄, 広瀬明彦, 鎌田栄一, 江馬 眞 (2006a) : OECD化学物質対策の動向（第8報）. 化学生物総合管理, 2-1, 147-162
・高橋美加, 松本真理子, 川原和三, 菅野誠一郎, 菅谷芳雄, 広瀬明彦, 鎌田栄一, 江馬 眞 (2006b) : OECD化学物質対策の動向（第9報）. 化学生物総合管理, 2-1, 163-175
・高橋美加, 松本真理子, 川原和三, 菅野誠一郎, 菅谷芳雄, 広瀬明彦, 鎌田栄一, 江馬 眞 (2006c) : OECD 化学物質対策の動向（第11報）. 国立医薬品食品衛生研究所報告, 124, 62-68
・高橋美加, 松本真理子, 川原和三, 菅野誠一郎, 菅谷芳雄, 広瀬明彦, 鎌田栄一, 江馬 眞 (2007a) : OECD化学物質対策の動向（第10報）. 化学生物総合管理, 2-2, 286-301
・高橋美加, 松本真理子, 川原和三, 菅野誠一郎, 菅谷芳雄, 広瀬明彦, 鎌田栄一, 江馬 眞 (2007b) : OECD化学物質対策の動向（第12報）. 化学生物総合管理, 3-1, 43-55
・松本真理子、高橋美加、平田睦子、広瀬明彦、鎌田栄一、長谷川隆一、江馬 眞 (2006) : OECD 高生産量化学物質点検プログラム : 第 18 回初期評価会議までの概要. 化学生物総合管理, 2-1, 104-135
・松本真理子、大井恒宏、宮地繁樹、菅谷芳雄、江馬 眞 (2007) : OECD 高生産量化学物質点検プログラム : 第 23 回初期評価会議概要、化学生物総合管理, 3-1, 56-65

化学生物総合管理 第 3 巻第 2 号 (2007.12) 180-189 頁
連絡先 : 〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日 : 2007 年 8 月 8 日 受理日 : 2007 年 10 月 5 日

表 1　第 24 回 SIAM で審議された化学物質と合意結果

CAS	物質名・物質カテゴリー名	担当国	勧告	
			ヒト健康	環境
75-12-7	Formamide	DE/ICCA	LP	LP
88-85-7	2-sec-butyl-4,6-dinitrophenol	JP	LP	FW
100-97-0	Methenamine	DE/eu	FW	LP
カテゴリー (5 CAS)	Phosphates	US/ICCA	LP	LP
111-42-2	Diethanolamine	UK/ICCA	LP	LP
872-50-4	1-methyl-2-pyrrolidone	US/ICCA	LP	LP
1461-22-9	Tributyltin chloride	US/ICCA	FW	FW
7646-78-8	Tin tetrachloride	US/ICCA	LP	LP
1461-25-2	Tetrabutyltin	US/ICCA	FW	FW
3590-84-9	Tetraoctyltin	US/ICCA	LP	FW
(110-30-5, 5136-44-7, 5518-18-3 混合物)	N-(2-octadecanoylamidoethyl)octadecan amide	JP/ICCA	LP	LP
2487-90-3	Trimethoxysilane	US/ICCA	LP	LP
7759-02-6	Strontium sulfate	KO	LP	LP
25340-17-4	Diethylbenzene mixed isomers	CH/ICCA	LP	LP
3194-55-6・25637-99-4 *1	Hexabromocyclododecane	SE/eu	FW	FW
カテゴリー (10 CAS)	Iron salts and their hydrates	FI+(JP)*2 /ICCA	LP	LP
カテゴリー (4 CAS)	Ammonia	US/ICCA	LP	LP
3333-67-3 (12122155, 12607704) 7440-02-0 7718-54-9 7786-81-4 13138-45-9	Nickel carbonate (2:3 basic nickel carbonate 1:2 basic nickel carbonate) Nickel (metal) Nickel chloride Nickel sulfate Nickel dinitrate	DK/eu	FW	-
51000-52-3	Neodecanoic acid ethenyl ester	UK/ICCA	LP	FW

FW = The substance is a candidate for further work. (追加の調査研究作業が必要)

化学生物総合管理　第 3 巻第 2 号 (2007.12)　180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1　E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日　　受理日：2007 年 10 月 5 日

LP = The substance is currently of low priority for further work.（現状では追加作業の必要なし）
ICCA は国際化学工業協会協議会による原案提出を示す。
eu は欧州連合でのリスク評価文書を基にしたことを意味する。
略号は、CH：スイス、DE：ドイツ、FI：フィンランド、JP：日本、KO：韓国、SE：スウェーデン、UK：英国、US：米国である。
*1: CAS 番号（3194-55-6、25637-99-4）は同一の物質を示す。
*2: 物質カテゴリー「Iron salts and their hydrates Ferrous」を構成する 10 物質のうち、sulfate heptahydrate（CAS:7782-63-0）は、日本およびフィンランドが担当国となり文書を提出した。

化学生物総合管理 第 3 巻第 2 号 (2007.12) 180-189 頁
連絡先：〒158-8501 東京都世田谷区上用賀 1-18-1 E-mail: ema@nihs.go.jp
受付日：2007 年 8 月 8 日 受理日：2007 年 10 月 5 日

Available online at www.sciencedirect.com
ScienceDirect
Reproductive Toxicology 23 (2007) 12–19
Reproductive Toxicology
www.elsevier.com/locate/reprotox

Developmental toxicity of dibutyltin dichloride in cynomolgus monkeys

Makoto Ema[a,*], Katsuhiro Fukunishi[b], Mariko Matsumoto[a], Akihiko Hirose[a], Eiichi Kamata[a], Toshio Ihara[b]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Shin Nippon Biomedical Laboratories Ltd., Kagoshima, Japan*

Received 20 June 2006; received in revised form 15 August 2006; accepted 6 September 2006
Available online 12 September 2006

Abstract

Dibutyltin dichloride (DBTCl) has been shown to be teratogenic in rats. The present study was conducted to determine the teratogenic potential of DBTCl given to pregnant monkeys during the entire period of organogenesis. Cynomolgus monkeys were dosed once daily by nasogastric intubation with DBTCl at 0, 2.5 or 3.8 mg/kg on days 20–50 of pregnancy, the whole period of organogenesis. The pregnancy outcome was determined on day 100 of pregnancy. In both DBTCl-treated groups, a significant increase in the incidence of pregnant females with soft stool and/or diarrhea, and with yellowish stool was observed. Maternal body weight gain at 3.8 mg/kg and food consumption at 2.5 and 3.8 mg/kg were decreased during the administration period. The survival rate of fetuses at terminal cesarean sectioning was decreased in the DBTCl-treated groups and significantly decreased at 2.5 mg/kg. There were no changes in the developmental parameters of surviving fetuses, including fetal body weight, crown-rump length, tail length, sex ratio, anogenital distance and placental weight, in the DBTCl-treated groups. No external, internal or skeletal malformations were found in the fetuses in any group. Although internal and skeletal variations were found, no difference in the incidence of fetal variation was noted between the control and DBTCl-treated groups. No effect on skeletal ossification was observed in fetuses in the DBTCl-treated groups. The data demonstrate that DBTCl is embryolethal but not teratogenic in cynomolgus monkeys.

Keywords: Dibutyltin; Organotin; Teratogenicity; Embryolethality; Monkey

1. Introduction

Organotin compounds are widely used in agriculture and industry. The most important non-pesticidal route of entry of organotin compounds into the environment is through the leaching of organotin-stabilized polyvinyl chloride (PVC) by water [1], and its use in antifouling agents, resulting in the entry of organotin into the aquatic environment [2]. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for PVC plastics to prevent degradation of the polymer during melting and the forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers [3,4]. The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms [5,6] and marine products [7] has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment [8,9]. Organotin compounds are introduced into foods by the use of pesticides and antifoulants and via the migration of tin from PVC materials [4].

We previously demonstrated that tributyltin chloride (TBTCl) during early pregnancy caused early embryonic loss [10–12], and TBTCl on days 10–12 and on days 13–15, but not on days 7–9 of pregnancy, produced fetal malformations in rats [13]. The predominant malformation induced by TBTCl was cleft palate [13,14]. It has been reported that TBT is metabolized to DBT and MBT, and DBT was metabolized to monobutyltin (MBT) [15–17]. DBT is also reported to have toxic effects on reproduction and development in rats [18]. The oral administration of dibutyltin dichloride (DBTCl) during early pregnancy caused early embryonic loss in rats [19–21]. The oral administration of DBTCl to rats throughout the period of organogenesis resulted in a significant increase in the incidence of fetuses with malformations [22], and rat embryos were highly susceptible to the

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

0890-6238/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2006.09.003

teratogenic effects of DBTCl when it was administered on day 7 and 8 of pregnancy [23]. Dibutyltin diacetate (DBTA) [24–28], dibutyltin maleate, dibutyltin oxide, and dibutyltin dilaurate [26] were teratogenic in rats when administered orally. Developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from that of tetrabutyltin (TeBT), TBT and MBT in its mode of action because the period of susceptibility to teratogenicity and the types of malformations induced by DBT are different from those induced by TeBT, TBT and MBT [29,30]. DBTCl had dysmorphogenic effects in rat embryos in a whole embryo culture system [31,32]. DBT was detected in rat maternal blood at 100 ng/g and embryos at 720 ng/g at 24 h after gavage of DBTA at 22 mg/kg on day 8 of pregnancy [27]. The dysmorphogenic concentrations of DBTCl in cultured embryos were within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT. These findings suggest that DBT itself is a causative agent in DBT teratogenesis, which may be due to direct interference with embryos.

As described above, the teratogenic effects of organotin compounds, including DBT, were extensively investigated in rodents [18]. No reports on the assessment of the teratogenicity of DBT in any other species are available. It appears that conclusive evidence in support of the teratogenicity of DBT is still lacking, because the teratogenicity of DBT only has been reported in a single animal species. Studies in non-rodents would be of great value in estimating the teratogenicity of DBT in humans. The present study was conducted to determine the teratogenic potential of DBTCl given to pregnant cynomolgus monkeys during the entire period of organogenesis.

2. Materials and methods

2.1. Animals

Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were obtained from Guangxi Primate Center of China (Guangxi, China) through Guangdong Scientific Instruments and Materials Import/Export Co. (Guangzhou, China). The monkeys were quarantined for 4 weeks, and confirmed to be free from tuberculosis, *Salmonella* and *Shigera*. The animals were maintained in an air-conditioned room at 23.0–29.0 °C, with a relative humidity of 45–58%, under a controlled 12/12 light/dark cycle, with a ventilation rate of 15 air changes/hour, and were housed individually, except during the mating period. The monkeys were fed 108 g/day of diet (Teklad global 25% protein primate diet; Harlan Sprague-Dawley Inc., Madison, USA) and tap water ad libitum from automatic lixit devices. Healthy male and female monkeys were selected for use. Only females showing 25–32 days menstrual cycles were used in the experiment. Each female monkey was paired with a male of proven fertility for three consecutive days between days 11–15 of the menstrual cycle. The visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the

Table 1
Maternal findings in monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of pregnant females	12	12	10
Number of females showing toxicological signs			
Death	0	0	0
Soft stool/diarrhea	1	12*	10*
Yellowish stool	0	8*	8*
Vomiting	0	3	3
Initial body weight	3.53 ± 0.59	3.49 ± 0.43	3.79 ± 0.36
Body weight gain during pregnancy (g)[a]			
Days 0–20	76 ± 114	42 ± 160	73 ± 142
Days 20–51	57 ± 237	−242 ± 423	−556 ± 526*
Days 51–100	710 ± 162	755 ± 174	848 ± 263
Food consumption during pregnancy (g/day)[a]			
Days 20–21	99 ± 18	93 ± 23	76 ± 33
Days 23–24	91 ± 27	71 ± 31	55 ± 31*
Days 27–28	77 ± 28	47 ± 19*	37 ± 34*
Days 30–31	63 ± 32	33 ± 15*	22 ± 10*
Days 34–35	88 ± 25	53 ± 42	23 ± 17*
Days 37–38	86 ± 28	53 ± 42*	25 ± 24*
Days 41–42	87 ± 27	59 ± 59	36 ± 29*
Days 44–45	95 ± 22	62 ± 40	41 ± 31*
Days 48–49	98 ± 18	70 ± 48	59 ± 44
Days 51–52	94 ± 20	97 ± 24	71 ± 39
Days 55–56	102 ± 12	107 ± 2	100 ± 20
Days 58–59	106 ± 7	108 ± 0	104 ± 10
Days 62–63	106 ± 7	108 ± 0	106 ± 5
Days 80–81	108 ± 0	108 ± 0	108 ± 0
Days 90–91	106 ± 7	108 ± 0	108 ± 0
Days 99–100	108 ± 0	108 ± 0	108 ± 0

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.

median day of the mating period was regarded as day 0 of pregnancy. Pregnancy was confirmed on day 18 or 19 of pregnancy by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St. Louis, USA). Pregnant females, weighing 2.51–4.50 kg on day 0 of pregnancy, were allocated randomly to three groups, each of 10–12 monkeys, and housed individually. Animal experiments were performed at Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) during 2004–2005 in compliance with the Guideline for Animal Experimentation (1987) [33], and in accordance with the Law Concerning the Protection and Control of Animals (1973) [34] and the Standards Relating to the Care and Management of Experimental Animals (1980) [35]. This study has been approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

2.2. Dosing

The monkeys were dosed once daily with DBTCl (lot no. GG01, 98% pure, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) at 0, 2.5 or 3.8 mg/kg by nasogastric intubation on days 20–50 of pregnancy, i.e., the entire period of organogenesis [36]. Dosing was terminated in the dams in which embryonic/fetal loss occurred. The dosage levels were determined from the results of previous studies in rats, in which DBTCl administered by gavage at 7.6 or 15.2 mg/kg on days 0–3 and days 4–7 of pregnancy caused significant increases in pre- and/or post-implantation embryonic loss in rats [19–21], and in which DBTCl by gavage at 5, 7.5 or 10.0 mg/kg throughout the period of organogenesis resulted in a significant increase in the incidence of fetuses with malformations [22]. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dose volume was adjusted to 0.5 ml/kg of the most recent body weight. The control monkeys received olive oil only.

2.3. Observations

The pregnant monkeys were observed for clinical signs of toxicity twice a day during the administration period and once a day during the non-administration period. The body weight was recorded on days 0, 20, 27, 34, 41, 51, 60, 70, 80, 90 and 100 of pregnancy. The food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62, 80 and 90 of pregnancy. Embryonic/fetal heartbeat and growth were monitored using ultrasound under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride on days 25, 30, 35, 40, 50, 60, 70, 80, 90 and 99 of pregnancy. In the dams in which embryonic/fetal cardiac arrest was confirmed by ultrasound, necropsy was performed under anesthesia induced by intraperitoneal injection of pentobarbital Na (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). The uterus, including the embryo/fetus and placenta and ovaries, was removed from the maternal body and stored in 10% neutral buffered formalin. Dead or aborted embryos/fetuses were morphologically examined.

Terminal cesarean sectioning was performed on day 100 of pregnancy, under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), and contraction was induced with atropine (0.01 mg/kg, Tanabe Seiyaku Co. Ltd., Osaka, Japan). The fetus and placenta were removed from the dams. The placenta was weighed and stored in 10% neutral buffered formalin. Dams that underwent cesarean sectioning were not necropsied.

Fetal viability was recorded, and the fetuses were anesthetized by intraperitoneal injection of pentobarbital Na and euthanized by submersion in saline for 30–40 min at room temperature. Fetuses were sexed and examined for external anomalies after confirmation of the arrested heart-beat. Fetal and placental weights were recorded. The head width, tail length, crown-rump length, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid and diameters of the primary and secondary placentae were measured. After the completion of external examinations, fetuses were examined for internal anomalies. The peritoneal cavity was opened and the organs were grossly examined. The brain, thymus, heart, lung, spleen, liver, kidneys, adrenal glands and testes/uterus and ovaries were weighed and stored in 10% neutral buffered formalin. The eyeballs, stomach, small and large intestine, head skin and auricles were stored in 10% neutral buffered formalin. Fetal carcasses were fixed in alcohol, stained with alizarin red S [37] and examined for skeletal anomalies. The number of ossification centers of the vertebral column, and lengths of the ossified parts of the humerus, radius, ulna, femur, tibia and fibula were recorded. Histopathological evaluations were performed on single

Table 2
Reproductive and developmental findings in monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of pregnant females	12	12	10
Number of females with embryonic/fetal loss	1	8*	4
Number of females with live fetuses until terminal cesarean section	11	4*	6
Number of live fetuses at terminal cesarean section	11	4*	6
Sex ratio of live fetuses (male/female)	6/5	1/3	3/3
Body weight of live fetuses (g)			
Male	133 ± 13	125	112 ± 24
Female	118 ± 12	108 ± 20	118 ± 13
Anogenital distance (cm)[a]			
Male	2.0 ± 0.2	1.9	1.7 ± 0.4
Female	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
Crown-rump length (cm)[a]			
Males	12.8 ± 0.6	12.4	12.4 ± 0.7
Female	12.6 ± 0.4	12.3 ± 0.5	12.6 ± 0.1
Tail length (cm)[a]			
Male	11.8 ± 1.2	11.8	11.4 ± 0.7
Female	11.9 ± 0.8	11.7 ± 1.7	12.4 ± 0.6
Placental weight (g)[a]	42.4 ± 7.2	38.9 ± 6.2	37.5 ± 9.1
Number of a single placenta	1	1	3

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.

placentas and accessory spleens after fixation, paraffin embedding, sectioning and staining with hematoxylin and eosin.

2.4. Analysis of plasma steroids hormone levels

Blood samples were collected from the femoral vein on day 51 of pregnancy, 24 h after the last administration of DBTCl. The plasma was separated and stored at −80 °C for the later assay of steroid hormones. Plasma progesterone and 17β-estradiol were measured by Teizo Medical Co. Ltd. (Kawasaki, Japan) using liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of plasma progesterone and 17β-estradiol were 10.0 pg/ml and 0.25 pg/ml, respectively. The intra- and inter-assay coefficients of variation for 17β-estradiol were below 6.4 and 8.9%, respectively. The intra- and inter-assay coefficients of variation for progesterone were below 9.0 and 7.9%, respectively.

2.5. Data analysis

The data was analyzed by MUSCOT statistical analysis software (Yukums Co. Ltd., Tokyo, Japan) using the dam or fetus as the experimental unit [38]. Data were analyzed using Bartlett's test [39] for the homogeneity of variance. When the variance was homogeneous, Dunnett's test [40] was performed to compare the mean value in the control group with that in each DBTCl group. When the variance was heterogeneous, the data were rank-converted and a Dunnett-type test [41] was performed to compare the mean value in the control group with that in each DBTCl group. The incidences of maternal and embryonic/fetal deaths and anomalous fetuses were analyzed by Fisher's exact test. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 presents maternal findings in monkeys given DBTCl on days 20–50 of pregnancy. No maternal death occurred in any group. In both DBTCl-treated groups, a significant increase in the incidence of females with soft stool and/or diarrhea, and with yellowish stool was observed. Soft stool and/or diarrhea were observed in one of the 12 females in the control group and in all females of the DBTCl-treated groups. In both groups treated with DBTCl, yellowish stool was noted in eight females and vomiting was observed in three females. Body weight gain on days 0–20, during the pre-administration period, did not significantly differ among the groups. Body weight gain on days 20–50, during the administration period, was lower in the DBTCl-treated groups, and significantly decreased at 3.8 mg/kg. No significant decrease in body weight gain on days 51–100, during the post-administration period, was found in the DBTCl-treated groups. Food consumption during the administration period was significantly reduced at 2.5 mg/kg and higher. Relatively marked decreases in the body weight gain and food consumption were observed in dams showing abortion or embryonic/fetal death.

The reproductive and developmental findings in monkeys given DBTCl on days 20–50 of pregnancy are shown in Table 2. The incidence of females with embryonic/fetal loss was increased in the DBTCl-treated groups, and a significant difference was noted at 2.5 mg/kg. Embryonic/fetal loss was observed in one of the 12 females in the control group, eight of the 12 females in the 2.5 mg/kg group and four of the 10 females in the 3.8 mg/kg group. Abortion occurred on day 30 of pregnancy in the control group, and on day 35, 44, 46, 49 or 60 of pregnancy at 2.5 mg/kg. Embryonic/fetal death was found on day 35, 40 or 64 of pregnancy at 2.5 mg/kg, and on days 38, 40 or 50 (two embryos) of pregnancy at 3.8 mg/kg. External examinations was performed in five of the eight embryonic/fetal losses at 2.5 mg/kg and four of the four embryonic/fetal losses at 3.8 mg/kg, and no anomalies were detected. Eleven, four and six females in the control, 2.5 and 3.8 mg/kg groups, respectively,

Table 3
Morphological findings in fetuses of monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of fetuses examined	11	4	6
External examination			
Number of fetuses with malformations	0	0	0
Internal examination			
Number of fetuses with malformations	0	0	0
Number of fetuses with variations	0	0	1
Accessory spleen	0	0	1
Skeletal examination			
Number of fetuses with malformations	0	0	0
Number of fetuses with variations	0	1	1
Short supernumerary rib	0	1	1
Degree of ossification[a]			
Number of ossified centers of vertebral column	53.6 ± 0.8	53.0 ± 1.2	54.2 ± 1.0
Skeletal length (mm)[a]			
Humerus	23.6 ± 0.8	23.3 ± 1.3	23.6 ± 1.2
Radius	23.0 ± 1.0	22.3 ± 1.6	23.1 ± 1.7
Ulna	24.6 ± 1.0	23.9 ± 1.5	24.3 ± 2.2
Femur	22.3 ± 1.2	21.8 ± 1.3	22.7 ± 1.6
Tibia	21.5 ± 1.3	20.5 ± 1.7	21.7 ± 1.4
Fibula	19.8 ± 1.0	19.0 ± 1.8	19.9 ± 1.6

[a] Values are given as the mean ± S.D.

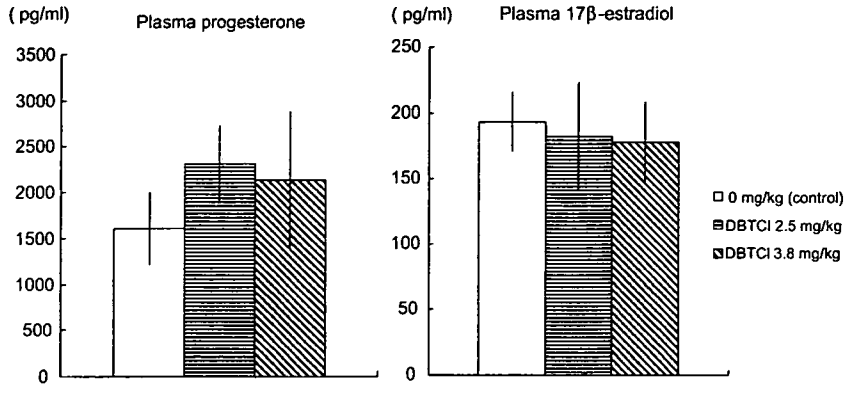

Fig. 1. Plasma progesterone and 17β-estradiol levels in pregnant monkeys given DBTCl on days 20–50 of pregnancy. Blood samples were collected on day 51 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± S.E.M. of 5–10 monkeys.

had live fetuses at terminal cesarean sectioning. There were no significant differences between the control and DBTCl-treated groups in parameters of fetal growth, such as body weight, crown-rump length and tail length. No significant differences in the head width, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid and diameters of the primary and secondary placentae were also noted between the control and DBTCl-treated groups (data not shown). No significant differences between the control and DBTCl-treated groups were found in the sex ratio of live fetuses, anogenital distance or placental weight. A single placenta was observed in one dam in the control group, one dam in the 2.5 mg/kg group and three dams in the 3.8 mg/kg group.

Table 3 shows the morphological changes in fetuses of monkeys given DBTCl on days 20–50 of pregnancy. No external, internal or skeletal malformations were found in fetuses in any group. Although internal and skeletal examinations revealed one fetus with an accessory spleen at 3.8 mg/kg, and one fetus with a short supernumerary rib at both 2.5 and 3.8 mg/kg, no difference in the incidence of fetuses with variation was noted between the control and DBTCl-treated groups. There were no differences between the control and DBTCl-treated groups in the number of ossified centers of the vertebral column or length of the humerus, radius, ulna, femur, tibia or fibula.

Although a significant decrease in the absolute weight of the brain and lung, and increase in the relative weight of the spleen were observed in male fetuses at 3.8 mg/kg, no significant difference in the relative weight of the brain and lung or in absolute weight of the spleen was detected between the control and DBTCl-treated groups. There were no differences in absolute and relative weights of the fetal thymus, heart, lung, liver, kidneys, adrenal glands or testes/uterus and ovaries between the control and DBTCl-treated groups (data not shown). Histopathological examinations revealed no abnormalities in single placenta and accessory spleen, and the histological structures of single placenta and accessory spleen were similar to those of normal placenta and spleen.

Plasma progesterone and 17β-estradiol levels are shown in Fig. 1. Although higher levels of plasma progesterone were observed in the DBTCl-treated groups, no statistically significant difference was noted between the control and DBTCl-treated groups. There were no significant differences in the plasma 17β-estradiol levels between the control and DBTCl-treated groups.

4. Discussion

In previous studies, the teratogenic effects of DBT were investigated in rats. The teratogenicity of DBT should be studied using other animal species to gain a better understanding of the developmental toxicity of butyltins. Non-human primates appear to provide an especially appropriate model for teratogenicity testing because of their high ranking on the evolutionary scale [42]. The close phylogenetic relatedness of old world monkeys to humans appears to render them most desirable as models in teratology studies [43]. The similarities in placentation and embryonic development indicate considerable value in the use of monkeys for investigating the developmental toxicity of chemicals [44]. In the present study, we determined the developmental toxicity, particularly the teratogenicity, of DBTCl in monkeys after administration over the entire period of organogenesis.

The doses of DBTCl set in the present study were expected to induce maternal toxicity, such as decreases in maternal body weight gain and food consumption, and were given to monkeys during organogenesis to characterize the effects of DBTCl on embryonic/fetal development. Toxicological sign, as evidenced by the significant increase in the incidence of pregnant females showing soft stool/diarrhea and yellowish stool, was found at 2.5 and 3.8 mg/kg. A significant decrease in the maternal body weight gain accompanied by significantly reduced food consumption was noted at 3.8 mg/kg. A significant decrease in food consumption was also found at 2.5 mg/kg. These maternal findings indicate that more severe adverse effects on pregnant females were noted at 3.8 mg/kg and DBTCl exerts maternal toxicity at 2.5 mg/kg and higher when administered during the entire period of organogenesis in monkeys.

Embryonic/fetal loss was observed in one dam in the control group and eight dams in the 2.5 mg/kg group and four dams in the 3.8 mg/kg group. The increased incidence of pregnant females with embryonic/fetal loss was observed at 2.5 and 3.8 mg/kg, and a significantly increased incidence of these females was found

at 2.5 mg/kg. Embryonic/fetal loss occurred on days 35–64 of pregnancy at 2.5 mg/kg, and on days 38–50 of pregnancy at 3.8 mg/kg. The embryonic mortality during organogenesis in cynomolgus monkeys of 2.4–18.2% has been reported [45]. Binkerd et al. [46] also noted that post-implantation embryonic loss was 5.4% in vehicle control pregnancies in developmental toxicity studies. Average abortion rate in cynomolgus monkeys was 26.1% in control data from 24 teratogenicity studies, and most of the abortions (66.7%) occurred during organogenesis [47]. In the background control data from 1994 to 2004 of the laboratory that performed this study, the post-implantation embryonic loss was 8.8% (29 of the 330 pregnancies). Because the incidence of embryonic/fetal loss in the DBTCl-treated groups was greater than in the historical control values, it was considered to be due to the administration of DBTCl. The data indicate that DBTCl at 2.5 mg/kg was sufficient to induce embryonic/fetal loss and the latter half of organogenesis was more susceptible for DBTCl-induced embryonic loss in cynomolgus monkeys.

We previously reported that DBTCl during early pregnancy caused pre- and post-implantation embryonic loss in pregnant rats [19,20] and that DBTCl suppressed uterine decidualization and reduced the levels of serum progesterone in pseudopregnant rats at doses that induced implantation failure [48]. We also showed that the suppression of uterine decidualization was reversed by administration of progesterone in pseudopregnant rats [48], and that progesterone protected against DBTCl-induced implantation failure [21]. Based on these findings, we hypothesized that the decline in serum progesterone levels was a primary factor for the implantation failure due to DBTCl in rats. However, no significant changes in plasma progesterone levels were noted in monkeys after the administration of DBTCl during organogenesis. The peripheral serum progesterone levels during the first 8 days of pseudopregnancy were essentially similar to those found in pregnant rats, and the serum progesterone levels rose steadily to a peak on day 4 and remained at a plateau of approximately 70 ng/ml until day 8 of pseudopregnancy [49]. In cynomolgus monkeys, plasma progesterone levels had distinct two peaks, one about 15 days postbreeding and another at about days 23–25, the progesterone decline which followed the second peak reached minimal levels (1–2 ng/ml) by about day 45 of pregnancy, and progesterone levels increased gradually throughout the rest of pregnancy with average levels of approximately 4 ng/ml [50]. In our previous study [48], rat blood samples were obtained on day 4 or 9 of pseudopregnancy. At these stages, progesterone levels could be steadily rising or remained at a plateau in pseudopregnant rats. In the present study, blood samples were collected from pregnant monkeys that were carrying their offspring and had not suffered from miscarriage on day 51 of pregnancy. At this stage, progesterone levels could be remained at a nadir in pregnant cynomolgus monkeys. The discrepancy in the effect of DBTCl on serum progesterone levels between rats and monkeys may be explained by the differences in the status and stage of pregnancy. Further studies are required to characterize more precisely the relationship between embryonic loss and maternal progesterone levels in monkeys given DBTCl.

Decreases in the absolute weights of the brain and lung, and an increase in the relative weight of the spleen, which were observed in male fetuses at 3.8 mg/kg, were not thought to de due to the toxic effects of DBTCl on fetal development, because these changes were not found in female fetuses and differences were not detected in the relative weight of the brain and lung or the absolute weight of the spleen in male fetuses. Any adverse effects on the parameters of fetal growth were also not detected in the surviving fetuses of dams given DBTCl. These findings indicate that DBTCl is not toxic to fetal growth at up to 3.8 mg/kg when administered over the entire period of organogenesis. Placental examinations revealed single placenta in all groups. In the background control data of the laboratory that performed the present study, the incidence of single placenta over a period of 10 years was 0–66.7% (mean = 13.0%, 26 of the 213 pregnancies). Histopathological examinations of single placenta revealed no changes, and the histological structure of single placenta was similar to that of normal placenta. These findings indicate that the single placenta observed in the present study was of no toxicological significance.

In the morphological examinations of the fetuses of exposed dams, a few fetuses with morphological changes were found in the DBTCl-treated groups. An accessory spleen was observed in one fetus at 3.8 mg/kg, and a short supernumerary rib was found in one fetus at both 2.5 and 3.8 mg/kg. In the background control data of the laboratory that performed the present study, the accessory spleen over the last 10 years was not observed. Leemans et al. [51] noted that the exact frequency of accessory spleen is not known, but is estimated to be between 10 and 30% in humans, and the immunohistological structure of the accessory spleen was similar to that of the normal spleens. In the present study, histopathological examinations of the accessory spleen revealed no changes, and the histological structure of accessory spleen was similar to that of the normal spleen. The accessory spleen observed in the present study contained only a minute amount of accessory tissue, and it was not considered to be a malformation. Short supernumerary rib is classified as skeletal variation [52], and the incidence of this change in the historical control data of the laboratory that performed the present study was 13.3% (31 of the 240 fetuses). DBTCl caused no skeletal retardation, as evidenced by no significant changes in the number of ossified centers of the vertebral column or the length of the humerus, radius, ulna, femur, tibia or fibula. Chahoud et al. [53] noted that variations are unlikely to adversely affect survival or health, and might result from a delay in growth or morphogenesis; the fetuses otherwise following a normal pattern of development. Furthermore, morphological examinations of aborted or dead embryos/fetuses in the DBTCl-treated groups revealed no anomalies. Considered collectively, these findings suggest that the morphological changes observed in the fetuses in the present study do not indicate a teratogenic response, and that DBTCl possesses no teratogenic potential in cynomolgus monkeys.

In conclusion, the administration of DBTCl to pregnant cynomolgus monkeys throughout organogenesis had an adverse effect on embryonic/fetal survival, but had no adverse effects on fetal morphological development, even at a maternal toxic

dose level. The data from the present study indicate that DBTCl shows embryonic/fetal lethality in monkeys.

Acknowledgement

This study was supported, in part, by a grant from the Ministry of Health, Welfare and Labor, Japan.

References

[1] Quevauviller P, Bruchet A, Donard OFX. Leaching of organotin compounds from poly (vinyl chloride) (PVC) materials. Appl Organomet Chem 1991;5:125–9.
[2] Maguire RJ. Aquatic environmental aspects of non-pesticidal organotin compounds. Water Poll Res J Canada 1991;26:243–360.
[3] Piver WT. Organotin compounds: industrial applications and biological investigation. Environ Health Perspect 1973;4:61–79.
[4] WHO. Environmental health criteria 15. Tin and organotin compounds: a preliminary review. Geneva: World Health Organization; 1980.
[5] Sasaki K, Ishizaka T, Suzuki T, Saito Y. Determination of tri-*n*-butyltin and di-*n*-butyltin compounds in fish by gas chromatography with flame photometric detection. J Assoc Off Anal Chem 1988;71:360–6.
[6] Lau MM. Tributyltin antifoulings: a threat to the Hong Kong marine environment. Arch Environ Contam Toxicol 1991;20:299–304.
[7] Suzuki T, Matsuda R, Saito Y. Molecular species of tri-*n*-butyltin compounds in marine products. J Agric Food Chem 1992;40:1437–43.
[8] Seligman PF, Valkirs AO, Stang PM, Lee RF. Evidence for rapid degradation of tributyltin in a marina. Marine Pollut Bull 1988;19:531–4.
[9] Stewart C, de Mora SJ. A review of the degradation of tri (*n*-butyl) tin in the marine environment. Environ Technol 1990;11:565–70.
[10] Harazono A, Ema M, Ogawa Y. Pre-implantation embryonic loss induced by tributyltin chloride in rats. Toxicol Lett 1996;89:185–90.
[11] Harazono A, Ema M, Ogawa Y. Evaluation of early embryonic loss induced by tributyltin chloride in rats: phase- and dose-dependent antifertility effects. Arch Environ Contam Toxicol 1998;34:94–9.
[12] Harazono A, Ema M, Kawashima K. Evaluation of malnutrition as a cause of tributyltin-induced pregnancy failure in rats. Bull Environ Contam Toxicol 1998;61:224–30.
[13] Ema M, Kurosaka R, Amano H, Ogawa Y. Further evaluation of the developmental toxicity of tributyltin chloride in rats. Toxicology 1995;96:195–201.
[14] Ema M, Harazono A, Miyawaki E, Ogawa Y. Effect of the day of administration on the developmental toxicity of tributyltin chloride in rats. Arch Environ Contam Toxicol 1997;33:90–6.
[15] Fish RH, Kimmel EC, Casida JE. Bioorgrganotin chemistry: reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenese enzyme system. J Organomet Chem 1976;118:41–54.
[16] Kimmel EC, Fish RH, Casida JE. Bioorganotin chemistry. Metabolism of organotin compounds in microsomal momooxygenese system and in mammals. J Agric Food Chem 1977;25:1–9.
[17] Iwai H, Wada O, Arakawa Y. Determination of tri-, di-, and monobutyltin and inorganic tin in biological materials and some aspects of their metabolism in rats. J Anal Toxicol 1981;5:300–6.
[18] Ema M, Hirose A. Reproductive and developmental toxicity of organotin compounds. In: Golub MS, editor. Metals, fertility, and reproductive toxicity. New York: CRC Press (Taylor & Francis Group); 2006. p. 23–64.
[19] Ema M, Harazono A. Adverse effects of dibutyltin dichloride on initiation and maintenance of rat pregnancy. Reprod Toxicol 2000;14:451–6.
[20] Ema M, Harazono A. Developmental and reproductive toxicity of tributyltin and its metabolite, dibutyltin, in rats. Congenit Anom (Kyoto) 2000;40:108–20.
[21] Ema M, Harazono A, Hirose A, Kamata E. Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats. Toxicol Lett 2003;143:233–8.
[22] Ema M, Itami T, Kawasaki H. Teratogenicity of di-*n*-butyltin dichloride in rats. Toxicol Lett 1991;58:347–56.
[23] Ema M, Itami T, Kawasaki H. Susceptible period for the teratogenicity of di-*n*-butyltin dichloride in rats. Toxicology 1992;73:81–92.
[24] Noda T, Yamano T, Shimizu M, Saitoh M, Nakamura T, Yamada A, et al. Comparative teratogenicity of di-*n*-butyltin diacetate with *n*-butyltin trichloride in rats. Arch Environ Contam Toxicol 1992;23:216–22.
[25] Noda T, Nakamura T, Simizu M, Yamano T, Morita S. Critical gestational day of teratogenesis by di-*n*-butyltin diacetate in rats. Bull Environ Contam Toxicol 1992;49:715–22.
[26] Noda T, Morita S, Baba A. Teratogenic effects of various di-*n*-butyltins with different anions and butyl(3-hydoroxybutyl)tin dilaurate in rats. Toxicology 1993;85:149–60.
[27] Noda T, Morita S, Baba A. Enhanced teratogenic activity of di-*n*-butyltin diacetate by carbon tetrachloride pretreatment in rats. Food Chem Toxicol 1994;32:321–7.
[28] Noda T, Yamamo T, Shimizu M. Effects of maternal age on teratogenicity of di-*n*-butyltin diacetate in rats. Toxicology 2001;167:181–9.
[29] Ema M, Kurosaka R, Amano H, Ogawa Y. Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats. J Appl Toxicol 1995;15:297–302.
[30] Ema M, Kurosaka R, Amano H, Ogawa Y. Comparative developmental toxicity of di-, tri-, and tetrabutyltin compounds after administration during late organogenesis in rats. J Appl Toxicol 1996;16:71–6.
[31] Ema M, Iwase T, Iwase Y, Ogawa Y. Dysmorphogenic effects of di-*n*-butyltin dichloride in cultured rat embryos. Toxicol In Vitro 1995;9:703–9.
[32] Ema M, Iwase T, Iwase Y, Ohyama N, Ogawa Y. Change of embryotoxic susceptibility to di-*n*-butyltin dichloride in cultured rat embryos. Arch Toxicol 1996;70:724–8.
[33] Guideline for Animal Experimentation Issued by Japanese Association for Laboratory Animal Science (1987).
[34] Law Concerning the Protection and Control of Animals (LAW No. 105, October 1, 1973).
[35] Standards Relating to the Care and Management of Experimental Animals (Notification No. 6, March 27, 1980 of the Prime Minister's Office).
[36] Hendrickx AG, Cukierski MA. Reproductive and developmental toxicology in nonhuman primates. In: Graham CE, editor. Preclinical Safety of biotechnology products intended for human use. Proceeding of a Satellite Symposium to the IV International Congress of Toxicology. 1986. p. 78–88.
[37] Dawson AB. A note on the staining of the skeleton of cleared specimens with alizarin red S. Stain Technol 1926;1:123–5.
[38] Staples RE, Haseman JK. Commentary: selection of appropriate experimental units in teratology. Teratology 1974;9:259–60.
[39] Snedecor GW, Cochran WG. Statistical Methods. 7th ed. Ames: Iowa State University Press; 1980.
[40] Dunnett CW. A multiple comparison procedure for comparing several treatments with control. J Am Statis Assoc 1996;50:1096–121.
[41] Miller Jr RG. Simultaneous Statistical Inference. 2nd ed. Berlin: Springer-Verlag; 1981.
[42] Hendrickx AG, Binkerd PE. Primate teratology: selection of species and future use. In: advances in the study of birth defects, teratological testing, Vol. 2. Baltimore: University Park Press; 1979. pp. 1–23.
[43] Schardein JL. Hormones and hormonal antagonists. In: chemically induced birth defects, revised and expanded. 3rd ed. New York: Marcel Dekker Inc.; 2000. pp. 281–357.
[44] Poggel HA, Günzel P. Necessity of using nonhuman primates in assessing prenatal toxicity. View of a scientist from the industry. In: Neubert D, Merker H-J, Hendrickx AG, editors. Non-human primates- developmental biology and toxicology. Wien: Ueberreuter Wissenschaft; 1988. p. 585–97.
[45] Hendrickx AG, Binkerd PE. Fetal deaths in nonhuman primates. In: Porter IH, Hook EB, editors. Embryonic and fetal death. NewYork: Academic Press; 1980. p. 45–69.
[46] Binkerd PE, Tarantal AF, Hendrickx GH. Embryonic/fetal loss and spontaneous malformations in nonhuman primates. In: Neubert D, Merker H-J, Hendrickx AG, editors. Non-human primates- developmental biology and toxicology. Wien: Ueberreuter Wissenschaft; 1988. p. 115–28.

[47] Korte R, Vogel F, Osterburg I, Bell DA. Prenatal waste and spontaneous malformations in Macaques. In: Neubert D, Merker H-J, Hendrickx AG, editors. Non-human primates- developmental biology and toxicology. Wien: Ueberreuter Wissenschaft; 1988. p. 141–50.

[48] Harazono A, Ema M. Suppression of decidual cell response induced by dibutyltin dichloride in pseudopregnant rats: as a cause of early embryonic loss. Reprod Toxicol 2003;17:393–9.

[49] Pepe GL, Rothchild I. A comparative study of serum progesterone levels in pregnancy and in various types of pseudopregnancy in the rat. Endocrinology 1974;95:275–9.

[50] Stabenfeldt GH, Hendrickx AG. Progesterone studies in the *Macaca fascicularis*. Endocrinology 1973;92:1296–300.

[51] Leemans R, Harms G, Timens W. The utility of the accessory spleen: a spare part after accidental) splenectomy. In: the human spleen after trauma: saving techniques and autotransplantation. Leeuwarden: Grafisch Bedrijf Hellinga bv; 1999. pp. 103–114.

[52] Solecki R, Bürginb H, Buschmann J, Clark R, Duvergere M, Fialkowskif O, et al. Harmonisation of rat fetal skeletal terminology and classification. Report of the third workshop on the terminology in developmental toxicology Berlin, 14–16 September 2000. Reprod Toxicol 2001;15: 713–21.

[53] Chahoud I, Buschmann J, Clark R, Druga A, Falke H, Faqi A, et al. Classification terms in developmental toxicology: need for harmonization. Reprod Toxicol 1999;13:77–82.

Available online at www.sciencedirect.com
ScienceDirect
Reproductive Toxicology 22 (2006) 672–678
Reproductive Toxicology
www.elsevier.com/locate/reprotox

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

Makoto Ema[a,*], Sakiko Fujii[b], Mariko Matsumoto[a], Akihiko Hirose[a], Eiichi Kamata[a]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Safety Research Institute for Chemical Compounds Co., Ltd., Sapporo Japan*

Received 28 December 2005; received in revised form 26 April 2006; accepted 4 May 2006
Available online 16 May 2006

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-

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

0890-6238/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2006.05.003

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 ($p < 0.01$).