表6 産科的適応以外で選択的帝王切開が推奨される大動脈炎症候群合併妊婦

1. IIbあるいはIII期（特にextensive typeで重症の高安網膜症を伴うもの）で分娩第1期に著しい収縮期血圧の上昇を認める場合.
2. IIbあるいはIII期で左右いずれの上肢においても正確な血圧が測定できない場合
3. Iあるいは IIa期でも治療に関わらず分娩第1期に著しい収縮期血圧の上昇を認める場合

分娩第1期の著しい収縮期血圧の上昇の定義：子宮収縮極期に繰り返し40 mmHg以上の収縮期血圧上昇を認める

表7 大動脈炎症候群の妊娠分娩におけるポイント

1. 未治療の腹部異型大動脈縮窄では，腎性高血圧から心不全，腎不全が報告されている．敗血症，妊娠高血圧腎症となることもあり予後不良である（レベルB）.
2. 異型大動脈縮窄—大動脈縮窄のガイドラインに準じる.
3. 大動脈閉鎖不全—心臓弁膜症のガイドラインに準じる.
4. 大動脈瘤（大動脈弁輪拡張症を含む）—マルファン症候群に準じる.
5. 虚血性心疾患（入口部狭窄）—外科治療後の適応を検討する.
6. 高血圧に対してはβ-ブロッカーを投与し，ACE阻害剤（ARB）は用いない.
7. ステロイド治療の継続をするが，投与量増量にいたることはまれである.
8. 自己免疫性疾患，膠原病としての病態に注意する.

心疾患患者の妊娠・出産の適応，管理に関するガイドライン，2005より改変[5]

は異なり，妊娠中期に1か月に1回，後期は1回しか行われなかったようですが，大動脈炎症候群による大動脈の拡張も妊娠中や分娩時の解離のリスクは高いのですか.

講師B マルファン症候群に比べると低いようです．publication biasはあると思いますが，検索できた限りでは大動脈炎症候群患者の妊娠中の大動脈解離の報告はありませんでした．ただし，発症した場合の死亡率が非常に高いので，「ガイドライン」に沿って慎重に管理するべきだと考えます.

シニアF 他に大動脈炎症候群で選択的帝王切開が行われるのはどういった場合になりますか.

チーフC 分娩中の頭蓋内出血の症例が報告されており，これを予防する観点で石川分類に基づいて提案されたIshikawaらの指針があります（**表6**）[4]．この指針によると陣痛開始前に選択的帝切の適応となるのは，左右どちらの上肢でも正確な血圧測定ができない妊婦だけなので，今回の症例Aはこれにはあてはまりません.

講師A AR II度というのはどうなのでしょうか.

シニアE 妊娠中は合計4回の心エコーが行われていますが，特にARの進行は見られずEFも妊娠初期が62%，最後の33週で70%と増悪はありませんでした．いずれにしてもIshikawaらの指針ではAR単独では選択的帝王切開の適応ではありませんが，陣痛開始後に収縮期血圧が著明な上昇を認める場合には適応となります.

シニアF 症例Bは妊娠初期には経腟分娩の方針となっていて妊娠36週に入ってから選択的帝王切開を決定したようですが，心機能の悪化は急速に進行したのでしょうか.

シニアE はい，この症例の場合は妊娠初期の心機能はNYHA分類Class IIですから産科的適応がなければ経腟分娩を予定していました．妊娠が進むにつれて心不全の増悪が見られ，分娩直前の心エコー所見では心嚢水が増加するとともに心機能の低下があり，数年内に大動脈弁を置換する必要があると考えられ，第二子の分娩は困難と判断されたようです．このため，心機能的には経腟分娩は不可能ではないものの，総合的に帝王切開の方が安全性が高いと判断したようです.

表8　妊娠の際，厳重な注意を要する或いは妊娠を避けるべき心疾患

1. 肺高血圧（Eisenmenger 症候群）
2. 流出路狭窄（大動脈弁高度狭窄，圧較差＞40～50 mmHg）
3. 心不全（NYHA Ⅲ度以上，左室駆出率＜35～40%）
4. マルファン症候群（大動脈拡張期径＞40 mm）
5. 人工弁
6. チアノーゼ性心疾患（酸素飽和度＜85%）

心疾患患者の妊娠・出産の適応，管理に関するガイドライン，2005年より改変[5]

教授　つまり，大動脈炎症候群の患者の分娩様式は，病変部位とその程度によって個別に検討する必要がある，ということですね．

シニアE　はい．

講師A　症例Cは2回とも経腟分娩でしたが，鉗子および吸引分娩を行っていますね．

チーフC　努責による血圧上昇を避けるために大動脈炎症候群では重症度にかかわらず，経腟分娩の際には第二期短縮を行う方がよいとされています．また分娩第二期には心拍出量が陣痛発来前の1.5倍になるといわれており[6]，心拍出量の変化を減少させるために左側臥位を保つのがいいようです．

シニアF　症例C-2では分娩前に血圧が173/79 mmHgとかなり上昇していますが，これは努責をかけていたのでしょうか．

シニアE　子宮口全開大を確認した時点での血圧ですので陣痛のみでの上昇のようです．

チーフC　疼痛による刺激でも血圧は上昇しますから，大動脈炎症候群では，経腟分娩時の疼痛管理として硬膜外麻酔も推奨されています[7]．無痛分娩で硬膜外麻酔を行っていれば，緊急帝切の対応も迅速に行うことができます．症例Cでも，状況が許せば無痛分娩を行っても良かったのではないかと思います．

・麻酔方法

シニアF　症例Aの帝王切開では当初脊椎麻酔が行われたようですが，これは何か理由があるのでしょうか．

シニアE　当初は硬膜外麻酔と脊椎麻酔の併用を予定していましたが，BMI 30と肥満があり，術後ヘパリン静注による血栓症予防療法を行うことになったため，硬膜外血腫形成のリスクを考慮し，脊椎麻酔のみの方針となりました．さらに，脊椎麻酔の効果がやや不良であることから全身麻酔となりました．

チーフC　大動脈炎症候群の患者の帝切時の麻酔方法は，局所麻酔と全身麻酔ではどちらがよいとは言えないようです[8,9]．大動脈の拡張病変がある場合，「ガイドライン」では全身麻酔が推奨されていますが，硬膜外麻酔で意識レベルを確認しながら安全に手術を行えたという報告もあります[10]．現時点では硬膜外麻酔による局所麻酔がよく行われているようです．

講師A　実際，症例Bではswan-Ganzカテーテルを入れつつ硬膜外麻酔で帝王切開を行っていますね．

チーフC　経腟分娩における血圧上昇に伴う脳出血の危険に対して，麻酔時は血圧低下に伴う臓器血流の低下や頸部過伸展に伴う脳血流の低下に注意する必要があるのですが[8]，局所麻酔で手術を行うことによって患者の意識・呼吸状態を把握し急激な血圧低下に伴う脳循環の悪化をモニターすることができます．また，ARがある場合は末梢血管抵抗の増加を避けるという意味でも硬膜外麻酔のメリットは大きいようです．

・妊娠の可否について

チーフD　話が戻りますが，症例Aでは大動脈の拡張は妊娠前から指摘されていたようですが，妊娠の可否について本人と主治医の間で話はあったのでしょうか．

講師B　それは今回の症例の大きな問題点です．妊娠前の心エコーでは大動脈径は44 mmでした．「ガイドライン」によると，マルファン症候群女性では大動脈の拡張が44 mm以上ある場合は妊娠をしないように勧めることになります（表8）．症例Aでは発症から1年で妊娠され，未婚だったということもあり，適切な妊娠出産に関する助言が主治医から本人とパートナーにされていなかったようです．分娩半年後に造影CTにて病変を評価する予定ですが，結果によっては外科的

表9 分娩時心内膜炎感染予防を必要とする心疾患

1. 特に重篤な感染性心内膜炎を引き起こす可能性が高い心疾患で，予防が必要である患者
   - 生体弁，同種弁を含む人工弁置換後
   - 感染性心内膜炎の既往
   - チアノーゼ性先天性心疾患（未手術，姑息術，修復術後）
   - 体肺短絡術後
2. 感染性心内膜炎を引き起こす可能性が高く予防が必要である患者
   - 多くの未修復先天性心疾患，術後遺残病変のある先天性心疾患
   - 後天性弁膜症
   - 閉塞性肥大型心筋症
   - 弁逆流をともなう僧帽弁逸脱
3. 以下の病態では感染性心内膜炎を引き起こす可能性が高いとの証明はないが，予防を行う方がよいとの説もある
   - 人工ペースメーカあるいは除細動器植込み後
   - 長期にわたる中心静脈カテーテル留置

心疾患患者の妊娠・出産の適応，管理に関するガイドライン，2005年より改変[5]

治療が必要と考えられ，今後の妊娠については現時点では不可とお話ししています．

チーフD 症例Bでは，妊娠前から心不全はあったようですが，この方の場合は妊娠は許可されていたのでしょうか．

シニアE はい．NYHAではII度と，一応，妊娠・分娩は許可されていて，ステロイドを完全に中止した状態で計画的に妊娠しています．本人にとってもラストチャンスの覚悟で臨んだようです．

・妊娠が大動脈炎症候群に与える影響

シニアF 妊娠が大動脈炎症候群を増悪させるということはありますか？

チーフC さまざまな意見があって，Ishikawaらの83例の報告では，61.4%が妊娠によって悪化，38.6%は特に問題なかったとされています[1]．一方で，改善するというデータもあり，16人の大動脈炎症候群患者の妊娠1年前から妊娠1年後までのCRPを追ったデータでは軽快が見られています[11]．

シニアF 症例Aでは妊娠初期に炎症所見が上昇していますが．

シニアE 症例Aの場合は，寛解期とは言えない時期に妊娠していますので，初期のコントロールに苦慮したようです．妊娠初期の原疾患の重症度と妊娠後期の血圧上昇の程度が大動脈炎症候群合併妊娠の管理方針を決定するのに最も重要とされています[12]．

・予防的抗菌薬投与

チーフD その他，大動脈炎症候群の分娩時に注意を要する点はありますでしょうか．

チーフC 大動脈炎症候群ではARだけでなく大動脈とそこから分岐する動脈の狭窄病変によって感染性心内膜炎と敗血症のリスクがあることから分娩時は全例抗菌薬を予防投与をすべきとする意見があります[4]．

講師B 感染性心内膜炎を予防するために，分娩時に抗菌薬の投与を必要とする循環器疾患があり（**表9**），本症例はこれにあてはまります．標準的なプロトコールは**表10**のようになりますが，今回の症例は第1世代セフェムを使用しています．

・プレドニゾロンの内服について

チーフD 症例Aでpreterm PROMとなったのは大動脈炎症候群と関連があるのでしょうか．

シニアE 原疾患との直接の関連は不明ですが，PSLを22.5 mg内服していますので，これが易感染性あるいは羊膜の脆弱化といったpreterm PROMのrisk factorをもたらした可能性はあると思います．

チーフD 妊娠中のPSL内服について他に何か注意すべきことはありますか．

表 10　分娩時の感染性心内膜炎予防法

	対象	抗菌薬	投与方法
特に重篤な感染性心内膜炎を引き起こす可能性が高い心疾患を対象とした予防法	通常	ABPC +GM	ABPC 2.0 g と GM 1.5 mg/kg (120 mg を超えない) の筋注または静注を処置前 30 分以内に併用．6 時間後に ABPC 1.0 g 筋注／静注または AMPC 1.0 g 経口投与
	ABPC/AMPC にアレルギー	VCM +GM	VCM 1.0 g 静注(1-2 時間かけて)と GM 1.5 mg/kg (120 mg を超えない) の筋注／静注を併用．処置前 30 分以内に投与を終了させる．
それ以外の予防法	経口投与可能	AMPC	2.0 g を処置 1 時間前に経口投与 (体格に応じ減量可能)
	経口投与不能	ABPC	2.0 g を処置前 30 分以内に筋注／静注
	ABPC/AMPC にアレルギー	VCM	VCM 1.0 g 静注(1-2 時間かけて)．処置前 30 分以内に投与を終了させる．

ABPC：アンピシリン，GM：ゲンタマイシン，VCM：バンコマイシン，AMPC：アモキシシリン

心疾患患者の妊娠・出産の適応，管理に関するガイドライン，2005 年より改変[5]

講師 B　催奇形性については，マウスやウサギを用いた動物実験では口唇裂や口蓋裂を引き起こすとの報告がありますが，ヒトの報告はありません．疾患の治療に必要であれば，妊娠中も内服を継続するのが望ましいようです．副腎皮質ホルモンの中では，PSL はベタメサゾン，デキサメサゾンと比較すると胎児への移行は少ないため，胎児への影響は少ないと言えます．もちろん使用する際は必要最低限の量を用いていくことになります．周術期には，外科的侵襲を考慮してステロイドカバーをする必要があります．症例 A でも行っています．

・高血圧と降圧剤について

チーフ D　症例 B のように妊娠前より高血圧がある場合は降圧剤を使うことになるわけですが，血圧の評価と管理は，どのようにしたら良いのでしょうか．

講師 B　一番重要なのは，正確な血圧の評価です．患側では低くなりますから，四肢で血圧を測定すること，両上肢で正確な血圧が測定できない場合には，分娩時に下肢で血圧を評価するのは困難なため，Ishikawa らの指針にのっとって帝王切開がすすめられます[4]．また，この場合，中心動脈圧・静脈圧が測定できるように，大腿動脈からの中心動脈圧測定カテーテルおよび Swan-Ganz カテーテルを挿入するのが理想的となります[13]．初回妊娠時に経腟分娩後に脳内出血を起こした妊婦が，第二子の分娩で中心動脈圧をモニターしながら安全に帝王切開を行うことができた，という報告もあります[14]．ただ，下肢足背での動脈圧測定も可能ですので必ずしも侵襲的な中心血圧測定は必要ないという意見もあります[10]．治療薬としては欧米ではαメチルドーパあるいは塩酸ヒドララジン，ニフェジピン，βブロッカーなどが用いられるようですが，本邦ではニフェジピンやαβブロッカーのラベタロールなどは妊娠中の投与は禁忌となっています．妊娠初期から管理すればこれらの降圧剤によるコントロールは比較的良好に行うことが可能です．症例 B でも妊娠初期からαメチルドーパが使われていますが，妊娠 36 週まで増量しながら比較的良好にコントロールができました．

・抗凝固療法

シニア F　症例 B では術後に肺梗塞が疑われていましたが，大動脈炎症候群とやはり関連するのでしょうか．

チーフ C　大動脈炎症候群では，肺動脈の狭窄病変もかなりの頻度で認められるようですが，長期予後にはあまり関連がなく，臨床的に肺機能が問題となることは少ないようです[3]．分娩に関連

表11　大動脈炎症候群合併妊婦の新生児の予後スコア

スコア	腹部大動脈の罹患	適切な治療を開始した時期	妊娠後期の最高平均血圧(mmHg)	加重型妊娠高血圧症が発症した時期
0	なし	妊娠初期	＜100	なし
1	あり	妊娠中期	101－130	妊娠末期
2	あり＋腎血管病変	妊娠末期	＞130	妊娠初期～中期

スコア4点以上の場合，子宮内発育遅延のリスクが高いと考えられる．
Wongら，1983年より改変[15]

した肺梗塞や抗凝固療法に関しても報告は少なくIshikawaらの報告でanisiodineという抗凝固剤を4人の妊婦が使用していて，血尿が認められたのが1例，分娩後出血が認められたのが1例となっています[4]．今回は3症例とも妊娠中に抗凝固療法は行われておらず，症例AではBMI 30と肥満を認めることから当院での帝王切開後の肺塞栓症予防プロトコールにのっとってダルテパリンナトリウムの持続静注を行いました．症例BではBMI 21とむしろやせであり，リスクは低いと考えられたのですが，臨床的に肺梗塞が否定できない状況であり，ヘパリンの持続静注を行いました．また，術後には予防的にアスピリンとワーファリンの内服を開始しています．

チーフD　この場合，母乳はどうするのでしょうか．

シニアE　薬剤の母乳への移行性とリスク及び母乳の利点を説明したところ，本人の希望により停乳しました．

・加重型妊娠高血圧症

講師A　妊娠高血圧症候群の合併は多いのでしょうか．

チーフC　加重型妊娠高血圧症の頻度は高いようです[15]．大動脈炎症候群では血管壁の柔軟性が低下していることが多く，妊娠中の循環血漿量の増加，心拍出量の増加に適応しきれないことから高血圧症の悪化やARの悪化を来す可能性があります．また，腎動脈に病変が及ぶ場合には，腎機能の悪化を来すこともあるので，注意が必要です．ただし，妊娠後期の発症が多く，それほど産科的予後を不良にはしていないようです．

・子宮内胎児発育遅延と新生児予後について

講師A　今回の3症例はいずれも児の予後は良好でしたが，症例Bは子宮内胎児発育遅延が見られたようです．これは大動脈炎症候群と関連するのでしょうか．

チーフC　文献的には，高血圧や腹部大動脈の罹患があると子宮内胎児発育遅延が生じやすいと言われています[15-17]．症例Bはこのリスクファクターが二つともあてはまります．また，Wongらは13人の大動脈炎症候群患者の30妊娠（発症前の11妊娠と発症後の19妊娠）から，新生児の予後をスコア化しています(**表11**)．腹部大動脈の罹患，適切な治療を開始した時期，妊娠後期の平均血圧，加重型妊娠高血圧の合併の4項目で評価し，スコア4点以上の患者の児は全て30パーセンタイル以下の出生体重，スコア4未満は全て30パーセンタイル以上だったとして，スコア4点以上を子宮内胎児発育遅延のハイリスクと提案しています[15]．

シニアF　症例Aの児はちょっと大きめですね．

シニアE　はい．妊娠中に超音波検査による推定胎児体重が90パーセンタイルを上回り糖尿病の家族歴もあるため，妊娠糖尿病を疑い，妊娠31週2日に75g経口血糖負荷試験を施行しましたが，結果は83（前値）～147（1時間値）～130（2時間値）g/dlと正常でした．また，出生後もIRDSや低血糖を発症することなく経過は良好で，日齢8に母とともに退院しています．症例AはWongらのスコアではロウリスクに分類されます．

・禁忌薬剤について

シニアF　症例C-2では弛緩出血によって輸血を行ったようですが，分娩直後にマレイン酸メチルエルゴメトリンを使っていないのはなぜですか．

チーフC　薬剤添付文書では禁忌というわけではないようですが，血管閉塞性病変がある場合は，慎重投与となっています．呼吸困難やチアノーゼが生ずることがあるので，使用を避けるか厳重にモニターしつつ使用するべきであるとされています[4]．症例C-2でマレイン酸メチルエルゴメトリンを使用することで輸血を回避できた可能性はないとは言えませんが，虚血性心疾患などが生ずるリスクを考えると，使用しない方がよいと思います．

・長期予後について

チーフD　症例Aの様な方は，今後は妊娠は許可できないということですが，例えば症例Cの様な方が何度も妊娠を繰り返すことで，大動脈炎症候群の長期予後に何か影響は出てくるのでしょうか．

チーフC　長期予後については症例が少ないことから，なかなか結論は出せないようです．症例Cの方は，第二子が8か月でSIDSとなっていますので挙児の希望があり，内科主治医からは妊娠の許可が出ているようです．ただ，第二子の分娩時に輸血までしていることからご本人が，まだ決心がついていないというところです．

講師A　C先生，E先生，ありがとうございました．今回提示していただいた3症例は，同じ疾患でも病態が様々であるため，それぞれに適切なガイドラインを参考にしながら，妊娠分娩管理を行う必要がありました．

教授　今回の3例は診断後に妊娠をされていますが，本邦での疫学を考えると，妊娠中に偶然発見される可能性もあるわけですね．妊娠そのものの予後や長期的な健康管理を考えると，妊婦健診は女性にとっての健康診断のチャンスとなります．特に大動脈炎症候群は脈が触れないことや左右の上肢の血圧の差が診断の手がかりとなりますので，特に初回の妊婦健診ではしっかりとした身体所見をとることが重要でしょう．また，医療の進歩に伴って心疾患をはじめとした様々合併症をもつ女性が妊娠・分娩を希望されるケースが増えてきています．ただ危険だからということで一概に妊娠を禁ずるのではなく，正確に疾患の状態を評価した上で内科医・産婦人科医・小児科医・麻酔科医などが協力して適切な妊娠分娩管理を行っていくことが，大学病院に期待される責務だと思います．

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

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

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

2.1. Animals

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

2.2. Chemicals and dosing

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

2.3. Observations

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

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, weight of the gravid uterus, food consumption, numbers of corpora lutea, implantations and live fetuses, fetal weight and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalences, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. The incidences of pre- and postimplantation embryonic loss and fetuses with malformations and variations and sex ratio of live fetuses were analyzed using Wilcoxon's rank sum test. The rates of pregnancy, 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 ($p < 0.01$).

Table 4
Skeletal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
Total no. of fetuses (litters) examined	184(24)	176(24)	170(24)	130(20)
Total no. of fetuses (litters) with malformations	1	1	13(6)	26(12)*
Split cartelige of thoracic centrum	0	0	1	1
Fused cartilage of cervical vertebral arches	0	1	1	1
Fused cartilage of ribs	1	0	0	0
Absence, fusion or malposition of caudal vertebrae	0	0	8(3)	10(8)
Absence or fusion of phalanges	0	0	5(3)	18(9)
Fusion of metacarpal/metatarsal and phalanx	0	0	0	2(2)
Absence or fusion of metacarpals	0	0	0	4(4)
Shortening of tibia and fibula	0	0	0	1
Total no. of fetuses (litters) with variations	10(7)	16(9)	16(11)	12(8)
Bipartite ossification of thoracic centrum	0	2(1)	1	0
Dumbbell ossification of thoracic centrum	0	1	0	0
Unossified thoracic centrum	1	1	0	1
Variation of number of lumbar vertebrae	1	0	0	2(1)
Wavy ribs	0	1	1	0
Short supernumerary rib	9(6)	12(7)	14(10)	4(4)
Short 13th rib	0	0	0	2(2)
Sacralization of lumbar vertebra	0	0	0	2(1)
Bipartite ossification of sternebra	0	0	1	1
Asymmetry of sternebra	0	0	0	1
Degree of ossification[a]				
No. of sacral and caudal vertebrae	7.3 ± 0.5	7.5 ± 0.5	7.5 ± 0.5	7.0 ± 0.6
No. of sternebrae	4.6 ± 0.4	4.8 ± 0.5	4.6 ± 0.4	4.2 ± 0.4**
No. of metatarsals	8.0 ± 0.0	7.9 ± 0.3	7.8 ± 0.4	6.7 ± 1.4

[a] Values are given as the mean ± S.D.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

characterize the effects of DTG on embryonic/fetal development. The findings of the present study confirmed the results of a previous screening study and extended the understanding of the reproductive and developmental toxicity of DTG. The present data showed that the prenatal oral administration of DTG produced maternal toxicity, as evidenced by deaths, neurobehavioral changes, decreased body weight gain and reduced food consumption, and developmental toxicity, as evidenced by a high incidence of postimplantation loss, a decreased number of live fetuses and lower weight of fetuses, and teratogenicity, as evidenced by a higher incidence of fetuses with external and skeletal malformations.

DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,21,22]. The systemic injection of DTG has been reported to cause neurobehavioral changes in rats [4,6,7,9,22]. The present study shows that the oral administration of DTG also induced neurobehavioral changes at 20 and 40 mg/kg bw/day in pregnant rats. Lowered body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were also observed in pregnant rats. These findings indicate that DTG is maternally toxic at 20 mg/kg bw/day and higher.

The sex ratio (males/females) was significantly lowered in all DTG-treated groups. The values for sex ratio were 0.429–0.521 in the background control data for the last 6 years in the laboratory performed present study. Statistically significant changes in the sex ratio observed in the present study were considered to be unrelated to the administration of DTG, because the values for sex ratio in the DTG-treated groups were within the range of the historical control data, no increased embryonic/fetal deaths were detected at 10 and 20 mg/kg bw/day and the control value for the sex ratio was very high in the present study. A decreased number of live fetuses, increased incidence of postimplantation loss, and reduced weights of fetuses and placentae were detected at 40 mg/kg bw/day. A decreased number of live fetuses and increased incidence of postimplantation loss indicate embryonic/fetal lethality, and reduced weights of fetuses and placentae indicate intrauterine growth retardation. These findings indicate that DTG is toxic to embryonic/fetal survival or fetal growth at 40 mg/kg bw/day when administered during the time of implantation to the term of pregnancy.

In our previous reproductive and developmental screening test [15], the total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg. At this dose, oligodactyly and tail anomalies were frequently observed, and the teratogenic effect of DTG was strongly suggested. No malformed fetuses were found at 20 mg/kg bw/day in our previous study. In the present study, morphological examinations in the fetuses of exposed mothers revealed increased incidence of fetuses with external and skeletal malformations at 20 and 40 mg/kg bw/day.

Fetuses with external, internal and/or skeletal malformations and/or variations were found in all groups. The malformations and variations observed in the present study are of the types that occur spontaneously among the control rat fetuses [23–26]. At 40 mg/kg bw/day, significantly higher incidences of the total number of fetuses with external and skeletal malformations were detected, and significantly higher incidences of individual types of external and skeletal malformation were also noted. At 20 mg/kg bw/day, the incidence of the total number of fetuses with skeletal malformations was significantly higher than that of control group. Although the incidence of individual types of skeletal malformation was not significantly increased at 20 mg/kg bw/day, types of external and skeletal malformations observed at this dose were the same as those observed at 40 mg/kg bw/day. Consideration of the sum of these findings suggests that a conservative estimate of the LOAEL for the teratogenic dose of DTG is 20 mg/kg bw/day in rats when administered during the time of implantation to the term of pregnancy. DTG caused suppression of body weight gain and neurobehavioral changes in dams and abnormally morphological development and developmental delay in the offspring of rats at 20 and 40 mg/kg bw/day. Therefore, the teratogenic effects of DTG at doses without maternal toxicity, a selective teratogenicity of DTG, was not found in the current study. There are no available reports in which the developmental toxicity of DTG is assessed in any other animal species. Further studies are needed to confirm the reproductive and developmental toxicity of DTG in additional species. Developmental neurotoxicity and multigeneration studies are also required to support the conclusion of the prenatal hazard of DTG.

In conclusion, DTG caused maternal neurobehavioral changes and decreased body weight gain at 20 mg/kg bw/day and higher, embryonic/fetal deaths and lowered fetal weight at 40 mg/kg bw/day, and increased incidence of fetuses with malformations at 20 mg/kg bw/day and higher when administered during the time of implantation to the term of pregnancy in rats.

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Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and viability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.

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

1. Introduction

The basic rubber accelerator 1,3-di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the United States [1,2]. DTG is known as a selective sigma ligand [3]. In this context, many pharmacological studies of DTG were performed [3–12]. Ligands that interact with sigma sites have been shown to produce hypothermia [4–6]. Hypothermia induced by DTG was detected following subcutaneous or intracerebroventricle injection in rats [5,6] and intraperitoneal injection in mice [4]. The intraperitoneal injection of DTG potently reduced the pain behavior in the acute but increased pain behavior in the tonic phase in the formalin test in mice [7]. Intraperitoneal injection of DTG produced significant but short-lived increases in the withdrawal latencies in mice [4]. Bastianetto et al. [8] showed that unilateral intranigral injection caused circulating behavior in rats and suggested that sigma sites play a role in movement and posture through their association with brainstem and forebrain motor control circuits. Decreased locomotor activity induced by intraperitoneal injection [9,10], increased bladder capacity induced by intravenous injection in the anaesthetized condition [11] and no change in immobility time in open field after intraperitoneal injection [12] were also reported in rats given DTG. Toxicological studies on DTG have given little information on acute animal toxicity [13]: intraperitoneal LD50 was 25 mg/kg bw in mice; oral LD50 was 500 mg/kg bw in rats; 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. At the present time, no information is available for the reproductive and developmental toxicity of DTG. It is generally assumed that the results of animal test on chemical toxicity are relevant to human health [14]. As such, the testing for reproductive and developmental toxicity

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in animal models is an important part of the overall toxicology. The present study was conducted to obtain information on the effects of DTG on reproductive and developmental parameters in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15] and in accordance with the principles for Good Laboratory Practice [16,17] and "Guidance for Animal Care and Use" of Panapharm Laboratories Co., Ltd.

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 and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Japan, Inc.).

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water ad libitum and maintained in an air-conditioned room at 24 ± 2 °C, with a relative humidity of 55 ± 10%, a 12-h light/12-h dark cycle and ventilation with 13–15 air charges per hour.

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. 30J08) used in this study was 99.6% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 8, 20 or 50 mg/kg bw. The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DTG by gavage at 0, 10, 20, 40 or 80 mg/kg bw/day, in which deaths were found at 80 mg/kg bw/day, decreased locomotor activity, mydriasis, tremor and salivation were observed at 40 and 80 mg/kg bw/day, and no adverse effects were detected at 10 and 20 mg/kg bw/day (data not shown). DTG was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. Males (12 rats/group) were dosed for a total of 49 days beginning 14 days before mating. Females (12 rats/group) were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 ml/kg body weight based on the latest body weight during the re-mating and mating period in males and females or the body weight on day 0 of pregnancy in females after copulation. Control rats were given 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 the target concentration was 96.5 to 101.4%.

2.3. Observations

All rats were observed daily for clinical signs of toxicity. The body weight was recorded twice a week in males, and twice a week during the pre-mating and mating periods, on days 0, 7, 14 and 21 of pregnancy and on days 0 and 4 of lactation in females. Food consumption was recorded twice weekly during the pre-mating period in males, and twice weekly during the pre-mating period, on days 1, 7, 14 and 21 of pregnancy and on days 1 and 4 of lactation in females. The rats were euthanized by exsanguination under anesthesia on the next day of the last administration in males and on day 4 of lactation in females. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testes and epididymides were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovaries were stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin–eosin-stained tissue sections of these organs.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period, 2 weeks, had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before noon from day 20 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 12:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded. Gender was determined on live pups examined grossly and individually weighed on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

2.4. Data analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain, food consumption, length of estrous cycles, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups and weight of live pups were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous the data were analyzed using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the DTG-treated groups were compared with that of the control group with Steel's multiple comparison test. The implantation, delivery and viability indexes, and incidence of pups with anomalies and individual anomalies were analyzed with Wilcoxon's rank sum test. The mortality, copulation, fertility and gestation indexes, and sex ratio of pups were analyzed with Fisher's exact test. The 5% level of probability was used as the criterion for significant.

3. Results

Table 1 shows the findings in male rats given DTG. At 50 mg/kg bw/day, one male died after six administrations and one male died after seven administrations. These dead rats showed mydriasis, decreased locomotor activity, bradypnea, a prone position and tremor 10–20 min after the administration of DTG. In surviving males, mydriasis, decreased locomotor activity, bradypnea and prone position on days 1–9 of the administration period, tremor during the whole period of administration and salivation on days 22–49 of the administration period were also observed at 50 mg/kg bw/day. Salivation was noted on days 28–49 of the administration period at 20 mg/kg bw/day. A significant decrease in the body weight gain was found on days 1–8 (81% decrease) and days 15–22 (48% decrease) of the administration period at 50 mg/kg bw/day. At this dose, significantly lower food consumption on days 7–8 (20% decrease) and days 14–15 (7% decrease) of the administration period was also observed.

Table 1
Findings in male rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of male rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	30 ± 7	33 ± 7	25 ± 7	6 ± 9**
Days 8–15	29 ± 5	32 ± 5	32 ± 7	24 ± 7
Days 15–22	23 ± 6	25 ± 8	23 ± 7	12 ± 11**
Days 22–29	19 ± 9	22 ± 7	25 ± 8	19 ± 5
Days 29–36	22 ± 6	22 ± 6	23 ± 7	18 ± 8
Days 36–43	15 ± 8	12 ± 9	13 ± 5	14 ± 7
Days 43–50	19 ± 8	19 ± 7	13 ± 4	13 ± 11
Food consumption (g/day/rat)[a]				
Days 7–8	25 ± 3	26 ± 3	26 ± 2	20 ± 3**
Days 14–15	29 ± 2	30 ± 2	29 ± 3	27 ± 3*
Days 29–30	27 ± 2	27 ± 3	28 ± 3	25 ± 2
Days 35–36	28 ± 2	29 ± 2	29 ± 2	27 ± 2
Days 42–43	26 ± 3	25 ± 3	27 ± 4	27 ± 3
Days 49–50	28 ± 4	29 ± 3	28 ± 2	28 ± 3

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 2 presents the findings in female rats given DTG. At 50 mg/kg bw/day, two females died after the first administration and one female died after normal delivery of her pups on day 22 of pregnancy. Mydriasis, decreased locomotor activity, bradypnea, prone position, and tremor and salivation 10–20 min after the administration of DTG were observed in females died after the first administration. These clinical signs and salivation were found during pregnancy and on day of parturition in a female which died after parturition. In surviving females, mydriasis, decreased locomotor activity, bradypnea and prone position on day 1 of the administration period to day 0 of lactation, tremor on day 1 of the administration period to day 5 of pregnancy and salivation on day 4 of pregnancy to day 3 of lactation were observed at 50 mg/kg bw/day. Mydriasis, decreased locomotor

Table 2
Findings in female rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of female rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
No. of deaths during pregnancy	0	0	0	1
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	19 ± 8	17 ± 7	11 ± 6*	−1 ± 9**
Days 8–15	10 ± 7	15 ± 8	20 ± 5**	15 ± 10
Days 0–7 of pregnancy	34 ± 6	31 ± 6	33 ± 4	28 ± 8
Days 7–14 of pregnancy	34 ± 5	34 ± 4	36 ± 3	30 ± 10
Days 14–21 of pregnancy	85 ± 17	100 ± 14	105 ± 9*	42 ± 21**
Days 0–4 of lactation	20 ± 19	14 ± 16	22 ± 9	16 ± 13
Food consumption (g/day/rat)[a]				
Days 7–8	22 ± 3	21 ± 2	19 ± 2**	13 ± 3**
Days 14–15	20 ± 4	22 ± 3	22 ± 2	20 ± 2
Days 6–7 of pregnancy	22 ± 3	23 ± 2	23 ± 3	17 ± 3**
Days 13–14 of pregnancy	23 ± 2	24 ± 3	25 ± 2	22 ± 5
Days 20–21 of pregnancy	24 ± 4	26 ± 3	29 ± 3*	21 ± 5
Days 3–4 of lactation	41 ± 5	41 ± 3	46 ± 4*	32 ± 6**

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 3
Reproductive findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of pairs	12	12	12	10
Length of estrous cycles (day)[a]	4.0 ± 0.2	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2
Precoital interval (day)[a]	3.0 ± 1.0	2.7 ± 1.0	2.4 ± 1.1	2.2 ± 1.0
Copulation index (%)[b]				
Male	100	91.7	100	100
Female	100	91.7	100	100
Fertility index (%)[c]	100	100	91.7	100
Gestation index (%)[d]	100	100	100	90.0
Gestation length (day)[a]	22.6 ± 0.5	22.3 ± 0.5	22.5 ± 0.5	22.6 ± 0.5
Weight of testes (g)[a]	3.24 ± 0.34	3.34 ± 0.19	3.31 ± 0.28	3.30 ± 0.24
Relative weight of testes[a,e]	0.60 ± 0.05	0.62 ± 0.07	0.63 ± 0.07	0.68 ± 0.07*
Weight of epididymides (g)[a]	1.16 ± 0.10	1.21 ± 0.06	1.21 ± 0.12	1.23 ± 0.07
Relative weight of epididymides[a,e]	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.03	0.25 ± 0.02**
Weight of ovaries (mg)[a]	101 ± 8	106 ± 6	101 ± 11	102 ± 10
Relative weight of ovaries[a,e]	30 ± 2	31 ± 2	28 ± 3	32 ± 2

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females copulated) × 100.
[d] Gestation index (%) = (no. of females with parturition/no. of females copulated) × 100.
[e] Relative weight = organ weight/100 g of body weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

activity, bradypnea and prone position on days 2–3 of the administration period, and salivation on day 14 of pregnancy to day 3 of lactation were observed at 20 mg/kg bw/day. Body weight gain was significantly lowered on days 1–8 of the pre-mating period at 20 mg/kg bw/day (42% decrease) and on days 1–8 of the pre-mating period (105% decrease) and days 14–21 of pregnancy (49% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significantly higher body weight gain was observed on days 8–15 of the pre-mating period and days 14–21 of pregnancy. Food consumption was significantly reduced on days 7–8 of the pre-mating period at 20 mg/kg bw/day (14% decrease) and on days 7–8 of the pre-mating period (41% decrease) and days 3–4 of lactation (24% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significant increase in the food consumption was observed on days 20–21 of pregnancy and days 3–4 of lactation.

The reproductive findings in rats given DTG are presented in Table 3. No effects of DTG were observed on the length of estrous cycles, precoital interval and gestation length. One pair did not copulate at 8 mg/kg bw/day, one female did not become impregnated at 20 mg/kg bw/day and one female did not deliver any pups at 50 mg/kg bw/day; however, no significant differences were noted in the copulation, fertility or gestation index between the control and DTG-treated groups. The weights of the testes and epididymides, and absolute weight and relative weight of the ovaries in the DTG-treated groups did not differ from the control group. The relative weights of the testes (13% increase) and epididymides (14% increase) were significantly higher at 50 mg/kg bw/day.

The developmental findings in rats given DTG are shown in Table 4. There was no significant difference in the numbers of corpora lutea, implantations and stillborns, implantation index, sex ratio of live pups, viability index on day 0 of lactation and body weight of live pups on day 4 of lactation between the control and DTG-treated groups. The numbers of pups delivered (45% decrease) and live pups delivered (45% decrease) and delivery index (43% decrease) were significantly lowered at 50 mg/kg bw/day. At this dose, the viability index on day 4 of lactation (34% decrease) and body weight of live male (16% decrease) and female (19% decrease) pups on day 0 of lactation were also significantly decreased. Two dams with totally litter loss were observed. No poor maternal behavior or nursing was observed in dams at 50 mg/kg bw/day. No histopathological changes were found in the testes, epididymides and ovaries in the DTG-treated groups. External anomalies in pups of rats given DTG are also presented in Table 4. No fetuses with external malformations were observed in the control and groups given DTG at 8 and 20 mg/kg bw/day. At 50 mg/kg bw/day, fetuses with external malformations were found in 10 out of the 65 fetuses and in 3 out of the 9 litters. Oligodactyly was observed in four pups in two litters. A kinked tail was found in six pups in one litter and a short tail and anal atresia was observed in one pup in each letter. Although there was no significant difference in the incidence of fetuses with individual malformations between the control and 50 mg/kg bw/day groups, a significantly higher incidence of total number of fetuses with external malformations was noted at this dose.

Table 4
Developmental findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of litters	12	11	11	9
No. of implantations[a]	14.3 ± 2.6	16.2 ± 1.9	15.9 ± 1.4	14.2 ± 3.6
Implantation index (%)[b]	92.2	94.7	97.6	90.9
No. of pups delivered[a]	13.0 ± 2.4	15.2 ± 2.0	14.7 ± 1.4	7.2 ± 4.1**
No. of live pups delivered[a]	13.0 ± 2.4	15.1 ± 1.9	14.7 ± 1.4	7.2 ± 4.1**
No. of stillborns	0	0.1 ± 0.3	0	0
Delivery index (%)[c]	91.0	93.3	92.2	51.7**
Sex ratio of live pups (males/females)	71/85	84/82	80/82	31/34
Viability index (%)[d,e]				
Day 0 of lactation	100	99.5	100	100
Day 4 of lactation	99.4	99.4	100	65.4**
Body weight of male pups during lactation (g)[a]				
Day 0	7.4 ± 0.7	6.9 ± 0.6	7.3 ± 0.6	6.2 ± 1.0**
Day 4	11.9 ± 1.3	11.1 ± 1.0	11.7 ± 1.0	11.0 ± 2.3
Body weight of female pups during lactation (g)[a]				
Day 0	7.0 ± 0.7	6.6 ± 0.6	6.8 ± 0.7	5.7 ± 0.8**
Day 4	11.4 ± 1.3	10.5 ± 1.0	11.0 ± 0.9	10.5 ± 2.0
External examination of pups				
No. of pups (litters) with malformations	0	0	0	10 (3)*
Oligodactyly	0	0	0	4 (2)
Kinky tail	0	0	0	6 (1)
Short tail	0	0	0	1
Anal atresia	0	0	0	1

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c] Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d] Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[e] Viability index on day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

4. Discussion

The present study was conducted to obtain initial information on the possible effects of DTG on reproduction and development in rats. The data show that DTG exerts developmental toxicity and suggest that DTG possesses teratogenic potential.

DTG was given to males during the pre-mating and mating periods and to females during the pre-mating, mating, pregnancy and shortly after parturition. The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic and neurobehavioral effects. As expected, general toxicity, such as decreases in body weight gain and food consumption, was found at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females. Decreases in the body weight gain and food consumption during the early administration period, and thereafter, significant increases in body weight gain and food consumption were observed in females at 20 mg/kg bw/day. One possible explanation for increased body weight gain during late pregnancy at 20 mg/kg bw/day may be higher number of pups and higher net weight gain during pregnancy at this dose compared with the controls. Such recovery did not occur at the highest dose. Neurobehavioral effects, such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and salivation, were also observed at 20 and 50 mg/kg bw/day. DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,18,19]. It was reported that systemic injection of DTG caused neurobehavioral changes in rats [5,6,9,10]. The present study shows that the oral administration of DTG also induces neurobehavioral changes, and it is neurobehaviorally toxic at 20 and 50 mg/kg bw/day in rats.

Higher relative weights, but not the absolute weight, of the testes and epididymides were observed at 50 mg/kg bw/day. Body weights of male rats on the day of scheduled sacrifice were 537 and 485 g in the control and 50 mg/kg bw/day groups, respectively. It seems likely that the higher relative weights of the testes and epididymides at the highest dose were due to secondarily lowered body weight but not due to the direct effects of DTG on the male reproductive organs. Other male reproductive parameters were not significantly changed, even at the highest dose. These findings suggest that DTG is not reproductively toxic to male rats. It seems unlikely that DTG exerts reproductive toxicity to female rats when administered during the pre-mating, mating, pregnancy and early lactation period, because no adverse effects on the maternal reproductive parameters, including estrous cyclicity, precoital interval, copulation

index, fertility index, gestation index, gestation length and ovarian weight, were caused by the administration of DTG in females.

As for the developmental indexes, decreases in the numbers of total pups and live pups delivered, delivery index, viability on PND 4 and body weight of live pups on PND 0 were detected at 50 mg/kg bw/day. These findings indicate that DTG is toxic to the survival and growth of offspring and exerts developmental toxicity at 50 mg/kg bw/day in rats.

In the present study, the teratogenic effect of DTG is strongly suggested by the external examinations of pups. At 50 mg/kg bw/day, a significant increase in the total number of fetuses with external malformations was noted; however, incidences of fetuses with individual types of external malformations at this dose were not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]. In the present study, only external examination in the newborn rats was performed, and no internal or skeletal examinations were performed. Even animals not ordinarily carnivorous, including nonhuman primates, are likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [24]. To accurately evaluate the prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals [24,25]. The present study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15], and this screening test guideline 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. In order to further evaluate the developmental toxicity, including teratogenicity, of DTG in rats, a prenatal developmental toxicity study is currently in progress.

In conclusion, DTG caused decreased body weight gain and food consumption at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females, neurobehavioral changes at 20 and 50 mg/kg bw/day in both sexes, and changes in developmental parameters at 50 mg/kg bw/day. DTG is suggested to be teratogenic. The NOAELs of DTG for general and developmental toxicity were 8 and 20 mg/kg bw/day, respectively, in rats.

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