Limb malformations of rat fetuses exposed to a distal inhibitor of cholesterol biosynthesis

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Abstract **Triparanol, an inhibitor of desmosterol Δ24 reductase, produces a high rate of limb malformations in rat fetuses exposed at gestational day 10 (gd 10) to a single oral dose (150–200 mg/kg) given to the pregnant dam. AY9944, another efficient distal inhibitor of cholesterol biosynthesis that blocks dehydrocholesterol Δ7 reductase, produces a similar degree of cholesterol depletion but fewer malformations. Gas liquid chromatography–mass spectrometry (GC-MS) profiling of the sterols in the serum of the dams and in extracted embryos shows that in addition to desmosterol Δ24 reductase inhibition the conversion of Δ8 to Δ7 unsaturated sterols is also blocked by Triparanol. Therefore, the inhibitor induces the accumulation of desmosterol (Δ8 cholesten-3β-ol, 8-dehydrocholesterol) and zymosterol (Δ8, Δ24 cholestadien-3β-ol) in embryo tissues. The high concentration of the teratogenic drug assayed in the embryos at three successive gestational days (10–30 μg/g) is thought to cause the blockade in both Δ24 reductase and Δ8-Δ7 isomerase, which results in the particular profile of aberrant sterols. Comparison of the animal model with human syndromes, including limb osseous and skeleton perturbations, suggests a combination of desmosterol and Δ8 unsaturated sterols as being involved in the deleterious influence on limb bone formation.**—Chevy, F., F. Illien, C. Wolf, and C. Roux. **Limb malformations of rat fetuses exposed to a distal inhibitor of cholesterol biosynthesis.** ***J. Lipid Res.*** **2002.** 43: **1192–1200.**

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Among the numerous malformations characterizing the Smith-Lemli-Opitz syndrome (SLOS: MIM 270400) that results from a deficit in the enzyme 7-dehydrocholesterol (7-DHC) reductase (EC 1.3.1.21), limb anomalies are frequent but limited, especially in the form of syndactyly of toes 2–3. These distal limb anomalies occur together with the major holoprosencephaly syndrome with facial dysmorphia and microcephaly (1). These malformations have been related to a deficit in the patterning of the morphogen Sonic Hedgehog (Shh), which requires cholesterol for its full maturation and expression. Besides the developmental deficit due to the shortage of cholesterol in the early embryo, the possibility that the aberrant sterols that accumulate above the enzyme blockade can be embryotoxic has been less investigated (2–4). Reduced cholesterol concentration while at the same time the accumulation of multiple precursors is considered confounding in this instance.

The teratogenic potency of various inhibitors of the distal steps in cholesterol biosynthesis was demonstrated in 1964 (5). Triparanol (4-chloro-α-[4-[2-(diethylamino) ethoxy]phenyl]-α-(4-methylphenyl)benzeneethanol) inhibits the reduction of the Δ24 double bond in the lateral chain of sterol and causes hypocholesterolemia and accumulation of desmosterol (5). AY 9944 (trans-1,4-bis(2-chlorobenzyl-aminoethyl)cyclohexane dihydrochloride) and BM 15766 inhibit the ultimate enzyme of the biosynthetic pathway, dehydrocholesterol Δ7 reductase, and cause hypocholesterolemia and accumulation of 7DHC (6). At high doses, AY 9944 inhibits also in cultured embryos sterol Δ7-Δ8 isomerase, which causes the accumulation of cholest-8-en-3β-ol (7, 8). The inhibitory activity of Triparanol was reexamined in this study in order to identify specific deleterious effects of the inhibitor, as compared with AY 9944 causing a similar cholesterol depletion in the embryos after treatment of the dams. Because the different "distal" inhibitors do not target the same enzyme but cause an equally efficient blockade of embryo cholesterol synthesis, the accumulation of different characteristic "aberrant" by-derivatives can be related to the specific presentations of the fetus. One of the goals of the present investigation was to pinpoint the specific impact on the fetus limbs in which the different aberrant substances accumulate after Triparanol treatment.

All "distal" inhibitors, when given early in gestation [gestational day 3 (gd 3)], induce in fetuses of responsive

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Abbreviations: GC-MS, gas liquid chromatography-mass spectrometry; gd, gestational day.

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animal species a high rate of anterior cephalic malformations of the holoprosencephaly type, mainly in the form of corpus callosum and pituitary agenesis. The responsiveness of the different animal species varies widely. Wistar rats are highly sensitive, whereas other rat species and mice are more resistant to the teratogenic effect (6, 9, 10). We have focused until now on AY 9944-induced teratogenesis as the animal model for human SLOS due to the observed decrease in cholesterol synthesis and the chemical structure of the major accumulated aberrant sterol, 7-DHC. However, when considering the rate of limb malformation, many more cases of osseous limb malformation occur in rat fetuses after prolonged treatment throughout gestation with Triparanol than with AY9944 (5).

Therefore, in the present study we investigated the teratogenic effect of Triparanol during limb formation. The possible relationship of osseous anomalies induced by Triparanol with the limb malformations observed in SLOS (11–13) or in the Conradi Hunnerman Happle syndrome (CDPX2) (14, 15) will be discussed in view of the implication of accumulated by-products during the blocked cholesterol biosynthesis.

MATERIALS AND METHODS

Animal maintenance

Wistar rats weighing approximately 200 g (Iffa Credo, France) were housed under standard conditions with a 12 h light/dark cycle. After a 15 day period of adaptation, females were mated with males of the same strain. The day sperm was found in the vaginal smears was designated as gd 0.

Chemicals and reagents

Triparanol (MER-29) was a gift from Marion Merrel Dow Research Institute (Cincinnati, OH). Cholesterol, (7-DHC; cholest-7-en-3β-ol), epicoprostanol (internal standard, 5β-cholestan-3α-ol), lathosterol (Δ7 cholest-3β-ol), and desmosterol (5α-cholesta-5,24-dien-3β-ol) were obtained from Sigma (Saint Louis, MO). Solvents of analytical grade were obtained from Prolabo (France). The silylation reagent (Regisil) was obtained from Chrompack (Les Ullis, Courtaboeuf, France).

Treatment

The animals were fed a stock diet obtained from "l'Union de l'Alimentation Rationnelle" (code name AO3) of the following composition: 3,100 cal/kg, 12% water, 20% protein, 4% lipid comprising less than 4 mg/day of dietary cholesterol (25% of the lipid is of animal origin, 75% of vegetable origin), 54.5% carbohydrates, 4% cellulose, and 5.5% salt mixture. The diet was composed of the major vitamins A, B, D, K, E, and other cofactors.

The dams were separated into three groups according to treatment: a control group receiving no treatment; a group receiving 150 mg/kg of Triparanol on gd 10; a group receiving 200 mg/kg on gd 10. Triparanol was dissolved in sunflower oil (20 mg/ml) and administered by oral intubation.

Maternal blood samples were collected on gd 10, 14, 16, 18, 21, and 22. Serum samples were stored frozen at –20°C. Fetuses extracted on gd 14, 16, 18, and 22 were examined. Some of the fetuses were fixed in Bouin's fluid for anatomical examination. Most fetuses were prepared for double coloration of the skeleton, ossified bone matrix and cartilage matrix. The present study followed the recommendations of FASEB for the use of animals for research.

Lipid extraction and gas chromatography-mass spectrometry

One-half milliliter of maternal serum or embryo tissues chopped and homogenized in 0.5 ml of NaCl (150 mM) were mixed in 10 vol of the solvent mixture chloroform-methanol (2:1, v/v) containing the internal standard (epicoprostanol). Lipids were partitioned in chloroform, saponified by 0.5 N methanolic potassium hydroxyde. Fatty acids were methylated by BF_3-methanol (14%) to prevent any interference with sterols during the chromatographic step. The lipids were re-extracted in hexane and sterols silylated as described in (8). The trimethylsilyl-ether of the sterols was separated by gas liquid chromatography (GLC) on a medium polarity capillary column [RTX-65, (65% diphenyl substituted dimethylsiloxane), length 30 m, diameter 0.32 mm, film thickness 0.25 μm] (Restesk, les Ullis, France). Sterols were identified by comparison of the retention time and the mass spectrum with the National Institute of Standards and Technology library. The mass spectrometer [Nermag, R10-10C, Poissy, France] in series with the GLC [Hewlett-Packard 5890, Hewlett Packard, Waldbronn, Germany] was set up for the detection of positive fragment ions. Fragment ions were produced in the electron impact mode at 70 eV as described previously (8). Sterols were quantified by the selective monitoring of the prominent ion fragments after normalization with the internal standard epicoprostanol and calibration with weighed standards. Triparanol was assayed in embryos after extraction with dimethyl-ether in the presence of 1.5 M NaOH. Assay for Triparanol accounted for the two peaks resolved during chromatography of trimethylsilyl ether derivatives of the racemic drug. The abundant positive ion *m/z* 420 [M-silanol] was used in quantification relative to an external weighed calibrator.

Statistics

Data are reported as mean ± SD. The frequency of morphological parameters of the fetuses was examined using the χ^2 test.

RESULTS

Limb malformations

Control (group A). The offspring of 14 control pregnant females were studied (**Table 1**). The rate of fetal mortality was very low at 3.3% (146 fetuses were extracted live). The average fetal weight was 5.29 ± 0.57 g. No skeletal malformation was observed, either after external examination, or after coloration of the skeleton.

Dams treated with 150 mg/kg of Triparanol on gd 10 (group B). The mortality was also very low at 3.4% (115 fetuses were extracted live) for the offspring of the 10 pregnant females treated with Triparanol 150 mg/kg (Table 1). The mean fetal weight was reduced to 4.70 g and varied within a wide interval (±1.29 g) related to a varying degree of edema. Among the live fetuses, 95 (82.6%) were malformed. Most of the fetuses displayed several malformations, with a ratio of malformations/fetus of 1:8. The details of the malformations in the proximal, intermediary, and distal segments of the limbs are summarized in **Table 2**. The most frequent limb malformations were clubfoot and clubhand (52.2% in group B) (Table 2). Malformation of the scapula, essentially shortness, was present in 20.9% of fetuses

TABLE 1. Malformation and mortality in the offspring of pregnant rats receiving the distal cholesterol inhibitor Triparanol

Treatment	Dams	Implantations[a]	Live Fetuses at gd 22	Malformed Fetuses	Number of Malformations Per Fetus
Control	14	151	96.7%	0	0
Group B Triparanol 150 mg/kg at gd 10	10	119	96.6%	82.6%	1.8
Group C Triparanol 200 mg/kg at gd 10	18	164	68.9%[b]	94.1%	2.7

[a] The total number of implantations in utero has been reported including eggs with development stopped at an early step.

[b] $P < 0.001$ significance between groups B and C.

in this group. The delayed ossification of the metatarsal bones or toes was observed in 17.4% of fetuses. But the shortness and the distortions of limb long bones (femur, tibia, or ulna) were present in only 5.2% of fetuses, and the defects ectrodactyly and syndactyly (1.7%) were rare in group B. When the inhibitor was administered at gd 10, the defects observed in the forelimb were considerably less severe than those in the hind-limb. For example, only 0.9% of fetuses showed delayed ossification of the digits in the forepaw after the dams received Triparanol at 150 mg/kg at gd 10 (data not shown).

Dams treated with 200 mg/kg of Triparanol on gd 10 (group C). At the dose of 200 mg/kg Triparanol, 18 pregnant females were treated in order to collect 113 live fetuses. At 200 mg/kg, the fetal mortality rose sharply to 31.1% (Table 1). The mean fetal weight of fetuses extracted live was 5.05 ± 0.32 g. A number of fetuses were infiltrated with edema, which precluded weight as a reliable criteria for growth. In the 113 fetuses examined, 94.1% were severely malformed, with the ratio of limb malformations per fetus reaching 2.7, versus 1.8 in the group treated with 150 mg/kg (Table 1). The frequency of clubfoot and clubhand increased slightly with the dose of Triparanol. The frequency of malformations of the scapula (17.5%) was not statistically different from that of group B. Agenesis or delayed ossification of the metatarsal bones or toes, and shortness and distortions of long bones were considerably increased at Triparanol 200 mg/kg compared with 150 mg/kg. The differences, 41.6% versus 17.4% and 21.4% versus 5.2%, respectively, were highly significant (Table 2). Ectrodactyly and syndactyly were observed with a frequency of 8.4% and 20.8%, respectively, suggesting that a threshold teratogenic concentration of Triparanol lies between 150 mg/kg and 200 mg/kg for these limb anomalies (**Fig. 1**). Malformations were rarely observed in the forelimb, even at the highest dose of Triparanol (200 mg/kg) administered at gd 10: clubhand (2.6%), delayed ossification of the digits (9.1%) or of the metacarpal bones (2.6%). The results show that administration at gd 10 favors malformations of the hind-limbs.

Analysis of maternal circulating sterols

Gas liquid chromatography–mass spectrometry (GC-MS) profiling of the maternal serum (**Fig. 2A**) showed the appearance of aberrant sterols characterized by their distinct retention time and mass spectrum (not shown). The prominent and characteristic molecular or fragment ions were *m/z* 458 for cholesterol ($[M]^+$), 456 and 327 for desmosterol ($[M]^+$ and $[M\text{-}129]^+$) and 456 for zymosterol ($[M]^+$), which did not give the cleavage of the fragment 129 due to the presence of a Δ5 double bond in the B- ring, in contrast to desmosterol. Lathosterol was also detected by the ion current of *m/z* 458 at 17.6 min, a possible indication of the accelerated rate of sterol biosynthesis in the treated dam. A rapid decrease in the cholesterol level after a single oral administration of Triparanol at gd 10 was observed. The cholesterol depletion lasted throughout gestation (**Fig. 3A**). On gd 14, cholesterol decreased by 53% in the group treated with Triparanol 200 mg/kg, compared with control dams. The physiological cholesterol level has been known to decrease slightly in the rat from 0.61 to 0.53 g/l in the control dams between gd 10

Table 2: Limb anomalies observed in the offspring of pregnant rats receiving a single dose of Triparanol

Treatment	Short Deformed Scapula	Short Distorted Long Bones of the Limbs	Metatarse and Toes Delayed Ossification	Clubfoot and Clubhand	Ectrodactyly	Syndactyly
Triparanol 150 mg/kg at gd 10 N = 115	20.9%	5.2%	17.4%	52.2%	1.7%	1.7%
Triparanol 200 mg/kg at gd 10 N = 154	17.5%	21.4%[a]	41.6%	67.5%[b]	8.4%[c]	20.8%[d]

[a, b, c, d] $P < 0.001$, 0.10, 0.02, and 0.001 for the difference between groups 150 and 200 mg/kg.

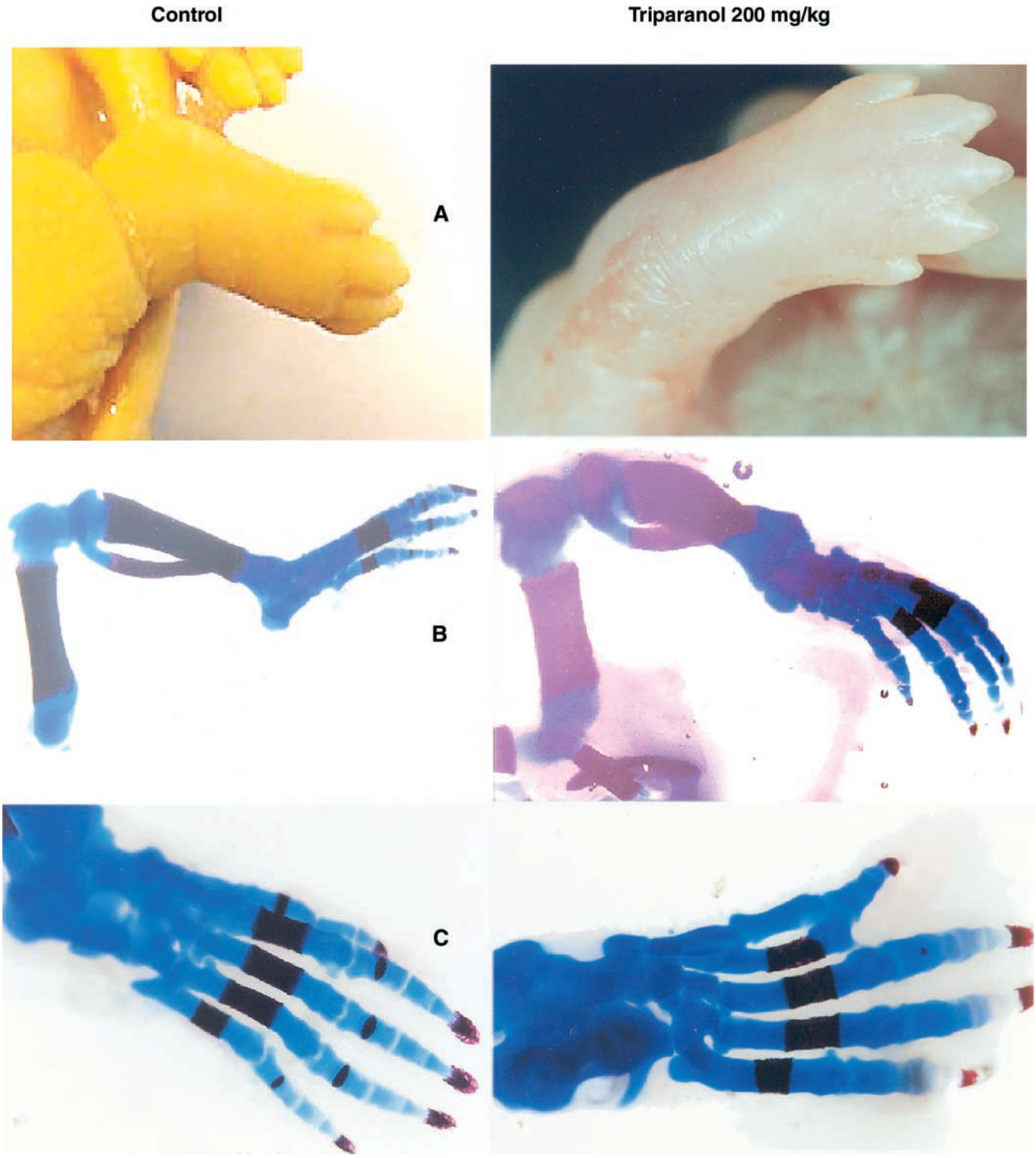
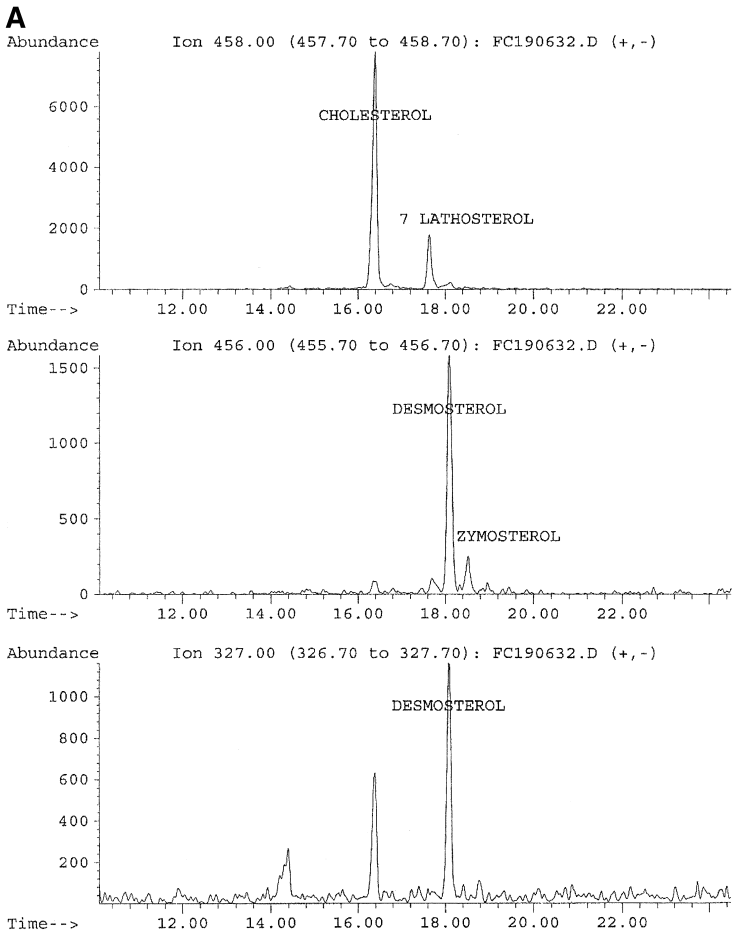


Fig. 1. Hind-limb malformations in Triparanol treated fetuses [200 mg/kg administered to the dam at gestational day 10 (gd 10)]. The treated animals (right column) are compared with control displayed in the left column. A: Photo of the external aspect of a club-foot detected by the extension and rotation of the distal segment of the limb in a treated fetus extracted at gd 21. B: Skeleton examination of the hindlimb after a double-step coloration by the dyes alizarin/alcyan blue. In the treated animal (right), the shortness and distortion of the long bones are observed. The extension of the distal segment confirms the club-foot. C: Skeleton of the paw (alizarin/alcyan blue dyes): the fusion of the first and second metatarsal bones results in syndactyly and ectrodactyly (a reduction to four digits).



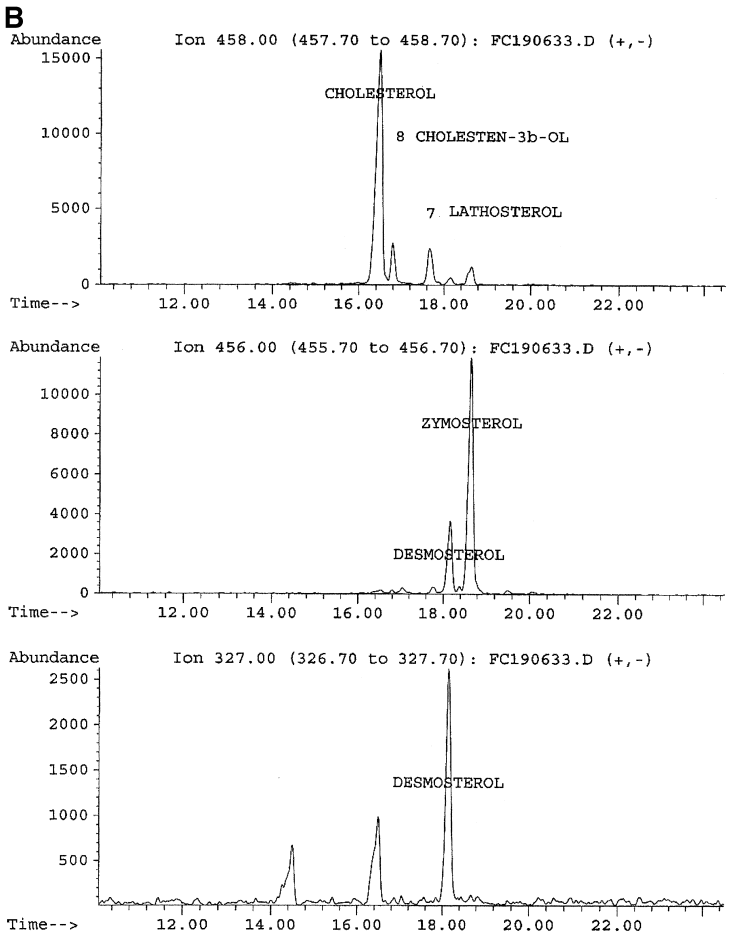


Fig. 2. A: Sterol profiling by gas liquid chromatography–mass spectrometry (GC-MS) of pregnant rat serum. Rats were treated with a single oral dose of Triparanol at gd 10 (150 mg/kg). The chromatogram for *m/z* 458, 456, and 327 were obtained at gd 14. B: Sterol profiling of embryos exposed to Triparanol after the treatment of the pregnant dam at gd 10. Embryos were extracted at gd 14, and sterols were characterized by GC-MS. The proportions of Δ8 unsaturated sterols (Δ8 cholesten-3β-ol and zymosterol) are considerably increased with respect to the maternal serum profile.

and gd 14 and then to increase steadily up to 0.75 g/l at gd 22 (16, 17). On gd 22, 12 days after the single dose of Triparanol, the cholesterol level of the treated dam was less than half of the control (0.75 g/l at gd 22).

The circulating levels of desmosterol and zymosterol increased in the dam serum from undetectable levels at gd 10 and to 0.24 and 0.09 g/l, respectively after 8 days (Fig. 3B, C). These sterols were not detected in the control dams. The aberrant sterols peaked at gd 18, whereas the maximum cholesterol depletion measured in the serum was reached at gd 16, an indication that circulating levels were occurring with a delay different from that of the metabolic alterations induced on the synthesis sites.

Analysis of fetal sterols

Figure 4A compares the rapid accumulation of cholesterol in embryos between gd 10 and gd 18 with the considerably slower increase observed when the dams received Triparanol on gd 10. Assuming a mono-exponential regression for the accumulation of cholesterol by embryos, it was found to be reduced 5.6-fold at gd 18 after treatment with 200 mg/kg Triparanol. The accumulation of aberrant sterols mirrored the depletion in cholesterol in treated fetuses, and the final total sterol content of treated embryos reached 90% of the control at gd 18. Accumulation of desmosterol (Fig. 4B) was considerably increased in treated animals as a result of Δ24 reductase inhibition. This accumulation exceeded substantially the accumulation found in a normal fetus, where desmosterol is considered to be a marker of the maturity of the central nervous system, peaking at less than 3% of the normal cholesterol level at gd 21 (18). The accumulation of zymosterol (Fig. 4C), 8-DHA (134 μg/embryo at gd 18; not shown) and 8 cholesten-3β-ol (177 μg/embryo at gd 18; not shown) was assessed by sterol profiling (Fig. 2B). These levels were extremely significant compared with control, where zymosterol reached 51 μg/embryo and 8-DHC 27 μg/embryo at gd 18, while 8 cholesten-3β-ol was not detected. Interestingly, the Δ8 unsaturation of these three aberrant sterols pointed to the accumulation of precursors above the step of sterol Δ8-Δ7 isomerase inhibited by Triparanol in addition to the blockade of Δ24 reductase. This inhibition has been previously reported at high concentrations of Triparanol (19). This agrees with the present assays for the drug in exposed fetuses, which show that Triparanol peaked 6 days after administration: 23.3 μg/g at gd 14, 30.6 ± 4.0 μg/g at gd 16, and 11.4 μg/g at gd 18 (data not shown).

DISCUSSION

The present observations complement the previous studies of two teratogenic inhibitors of cholesterol synthesis in rats, AY9944 (16, 17) and BM15766 (20, 21). These inhibitors displayed a distinct spectrum of malformation with respect to Triparanol that included a higher rate of limb malformations for a similar depletion in cholesterol. The detailed sterol profiling suggested that in addition to the major cholesterol depletion that was observed with all distal inhibitors, the accumulation of specific aberrant sterols could be responsible for a particular embryotoxicity for each inhibitor. The present work focuses on limb bone malformations occurring after exposure in vivo of the embryo to Triparanol.

The multiple by-derivatives appearing in treated animals can be formed by three alternative (and complementary) mechanisms, the first of which is the lack of specificity of the inhibitor. In addition to the main target enzyme with the highest affinity for the teratogenic compound, other steps in a biosynthetic pathway can be inhibited at higher concentrations. In cultured rat hepatoma cells, Triparanol causes the accumulation of different intermediates as a function of the concentration (19). This is interpreted as resulting in the inhibition of Δ24 reductase, Δ8-Δ7 isomerase and Δ7 reductase at increasing concentrations, successively. At the Triparanol concentration of

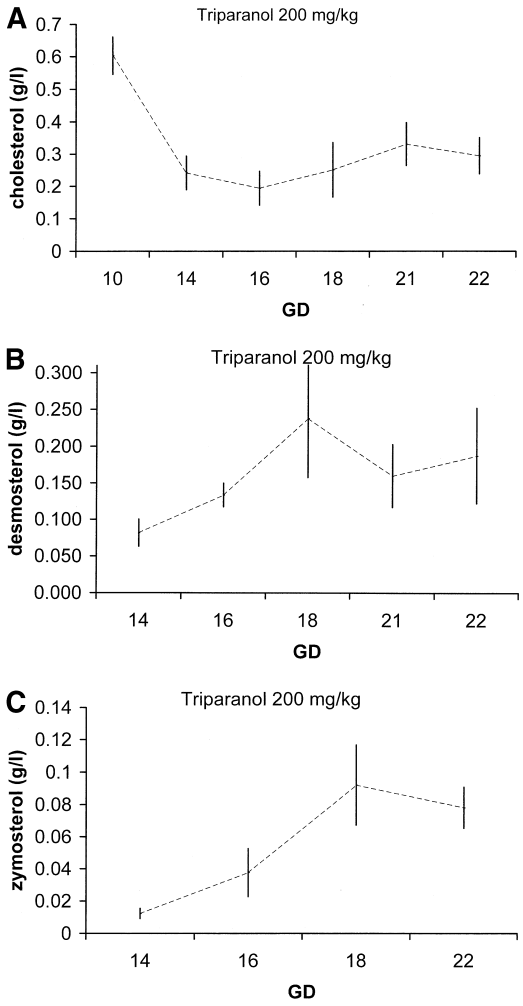


Fig. 3. Sterol level in the pregnant rat dam serum as a function of the time after the administration of a single oral dose of Triparanol (200 mg/kg) at gd 10. A: Cholesterol (the vertical bar represents 1 SD. The number of animals (N) varied between 8 and 12 according to the gd). B: Desmosterol (Δ5, Δ24 cholestadien-3β-ol). C: Zymosterol (Δ8, Δ24 cholestadien-3β-ol).

4.5 μM, the authors found desmosterol as the end-product of the cholesterol biosynthesis, but at 9 μM desmosterol, cholesta-5,7,24-trien-3β-ol and zymosterol were found. At 45 μM Triparanol, only zymosterol was evidenced, indicating that the whole sequence of the pathway from Δ8-Δ7 isomerase was blocked. Similarly, in *Zea mays* seedlings, AY9944 inhibits Δ7-Δ8 isomerase, Δ7-DHC reductase, and Δ14 dehydrosterol reductase at an increasing $IC_{50\%}$ of 0.5, 1.2, and 40 μM, respectively (22). A common carbocationic intermediate appearing during the reduction or the isomerisation of sterols is thought to be targeted by the positively charged analog (AY9944 or Triparanol positively charged amino group) in the catalytic site of the different enzymes (7).

In the second mechanism, multiple sterols can also appear in the profile when a substrate accumulates to such a high concentration that it is retro-converted to precursors above the blockade of the biosynthetic pathway.

In the third mechanism, a variety of by-products can also appear if alternative pathways, which are usually ineffective at physiological concentrations of the substrate, are re-opened. An example is the accumulation of 8-dehydrocholesterol in AY9944-treated rats (23) under the influence of the liver sterol Δ7-Δ8 isomerase. Usually the enzyme converts 8 cholesten-3β-ol to lathosterol instead of 7-DHC to 8-DHC. Finally, the possibility that a few aberrant sterols could be artifacts produced during the separation procedure has also been considered by Ruan et al., who compared GLC and HPLC (24). In humans, the children affected by a genetic enzyme deficit also display a variety of circulating aberrant sterols (which orients the molecular diagnosis), and complex malformations take place. The spectrum of malformations in children largely overlaps the effects of a single dose of a teratogenic inhibitor in animals. The kinetics and the distribution of the teratogenic drug in rat embryos limit the spectrum of malformations in the animal model. The timing (gd 10) and doses (150–200 mg/kg) given in the present investigation were chosen, after preliminary studies, to maximize the limb osseous defects. The experimental data were validated for the Wistar strain, but it should be underlined that other animals or even rat strains have been shown to display a lesser sensitivity to inhibitors (9, 10, 25, 26). The present experimental design was chosen to show that in addition to the developmental defect related to the cholesterol shortage, embryos also contain a variety of potentially embryotoxic sterols. An indication of this possibility has been suggested by the positive influence of antioxidants on the growth of cultured embryos in the presence of 7-DHC (3). The present study aimed to identify among the non-oxidized aberrant sterols that accumulated in utero the compounds with a potential toxicity on skeletal limb development. Comparison of exposed animal fetuses and human syndromes can also be helpful to focusing on this goal. In SLOS, which is caused by a deficit in dehydrocholesterol Δ7 reductase, 7-DHC, cholesta-5,7,9(11)-trien-3β-ol, but also 8-DHC accumulate. Neither desmosterol, nor 8 cholesten-3β-ol, zymosterol, or other Δ8 sterols (except 8-DHC) have been observed in human SLOS or in the animal

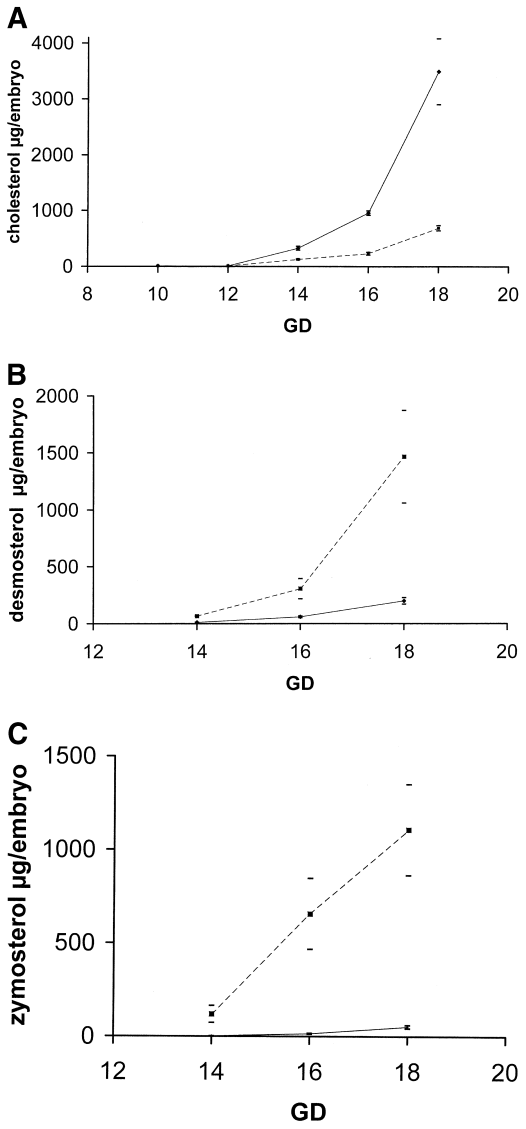


Fig. 4. A: Increase in cholesterol in the embryo between gd 10 and gd 18. [Solid line, control; dashed line, embryos exposed to Triparanol (200 mg/kg given to the pregnant rat dam at gd 10).] The vertical bar represents 1 SD. ($3 < N < 6$). B: Desmosterol accumulated in the rat embryo (solid line, control: dashed line, embryos exposed to Triparanol). C: Zymosterol accumulated in the rat embryo. (Solid line, control: dashed line, embryos exposed to Triparanol).

model exposed to AY9944 or BM15766. The children or the treated rat embryos show major nervous, cranio-facial, digit, and visceral malformations. Growth is limited, but skeletal development remains harmonious, except for the syndactyly 2-3 or post-axial polydactyly. The dysfunction of the patterning protein Shh resulting from a severe cholesterol shortage during early pregnancy is able on its own to explain these abnormalities. In the deficit of sterol Δ8-Δ7 isomerase that causes CDPX2, or in the mouse mutant "tattered" that serves as a model for chondrodysplasia, one observes the accumulation of Δ8 unsaturated sterols such as cholest-8(9)-en-3β-ol, in addition to 8-dehydrocholesterol (14). The syndrome includes severe skeletal abnormalities with shortened limbs and asymmetric rhizomelia. The deficit in Δ24 reductase (27) observed in des-

mosterolosis also includes a prominent osterosclerosis and limb shortening (28). In this case, no other aberrant sterol than desmosterol (23% relative to total sterol) was reported. Therefore, a comparison of human syndromes with the present teratogenic activity of Triparanol at high doses points to Δ8 and/or Δ24 unsaturated sterols as potential toxic compounds for the limb bone development.

In this respect, the possibility of obtaining more frequent skeleton defects by administrating Triparanol rather than AY9944 and BM15766 has been confirmed. Triparanol is known to inhibit the reduction of the Δ24 (25) unsaturated lateral chain of sterol, an obligatory step in the biosynthetic pathway leading to cholesterol (29) which takes place usually at the step of 5α-chola-7,24-dien-3β-ol, straight after sterol Δ8-Δ7 isomerization. However, after a single oral high dose of Triparanol, the end-products observed in the embryos are not only desmosterol but also zymosterol, Δ8 cholesten-3β-ol, and Δ8-DHC. This suggests that the high dose of Triparanol administered presently produces the concentration range of 10–30 μg/g in embryo tissues, which far exceeds the level inhibiting only Δ24 reductase. At this high concentration, Triparanol would inhibit also the Δ8-Δ7 isomerase activity which results in the production of a whole variety of Δ8 unsaturated sterols as described previously in X-linked human *chondrodysplasia* or in "tattered" mutant mouse with prominent limb malformations. The combination of the Δ8 unsaturated compounds with desmosterol is particularly suspected to be involved in the multiple osseous defects observed in the present animal model produced by Triparanol. It is not possible at the moment to decipher which one of the aberrant sterols has the most deleterious activity. Among the Δ8 unsaturated compounds, we speculate that cholest-8(9)-en-3β-ol (quoted also as Δ8 cholesten-3β-ol) deserves special attention because it is absent in the SLOS or in AY9944 intoxicated animals with rare osseous limb defects other than digit anomalies, while 8-DHC is abundant in this case. By difference, cholest-8(9)-en-3β-ol is present in CDPX2 and in Triparanol-intoxicated animal with major abnormalities of the limb long bones.

The mechanism of digit anomalies frequently observed in SLOS should be assessed with caution because these defects are more prone to result of a patterning deficit. Indeed, the enzyme deficit causes a constant cholesterol shortage for the embryo. In turn, the cholesterol shortage is known to limit the expression of the morphogenetic protein Shh, which requires a posttranslational autocleavage mediated by cholesterol (30). For instance, AY9944 has been proven to limit the expression of Shh and downstream morphogenetic proteins in the nervous system while a cholesterol-enriched diet reestablishes its expression in treated fetuses (4). Because Shh behaves as a crucial patterning protein active in the limb zone of polarizing activity (ZPA) which determines antero-posterior and proximo-distal axes, the digit reduction due to syndactyly or ectrodactyly can be observed in SLOSs. Because Shh is also responsible for controlling negatively cell proliferation in the post-axial part of the limb, the post-axial polydactyly could be linked to the cholesterol depletion. Therefore digit anomalies should be considered as an index of the cholesterol deficit as holoprosencephaly that parallel the severity of cholesterol depletion in the animal model (16, 31). A different interpretation should be given for specific limb bone abnormalities where we assume that Δ8 and Δ5 unsaturated sterols could play a role.

For similar cholesterol depletion, Triparanol-treated embryos as compared with AY9944 treated embryo display many more abnormalities in the long bones of the limbs (8, 17, 23). The presence of frequent limb abnormalities was already reported more than four decades ago after the initial observation of the teratogenic effect of Triparanol (5). In contrast, a lower rate of skeletal malformations was observed with AY9944. In a study conducted with 75 mg/kg given at gd 10, one delayed ossification of the extremities, one clubfoot, and two cases of scapula anomalies in 207 fetuses (data not shown), and rare scapula anomalies were reported (four cases in 115 fetuses) (32). If it is assumed that the wide difference in osseous limb defects relies on the sterol profile engendered by the inhibitor, either AY9944 or Triparanol, the differences point to desmosterol, Δ8-cholesten-3β-ol and zymosterol as the causative Δ24 and Δ8 unsaturated sterols for limb defects. A like cholesterol, desmosterol, was found to elicite the autocleavage of Shh (33, 34), which is required for its activity on embryo limb patterning. However the biological activity of the precursor-derived adducts of the N-terminal sequence of Shh has possibly a different potency as regard to the cholesterol adduct of Shh (2, 35). Eventually, Indian Hedgehog (Ihh), another protein of the hedgehog family, could be the target for aberrant sterols with osseous toxicity. Ihh is involved in chondrocyte proliferation and differentiation (36, 37). If these Δ24 and Δ8 aberrant sterols interfere with the processing of Ihh, it could explain the particular limb osseous toxicity of Triparanol. At the moment, this suggestion, made on the basis of a comparison of the sterol profiles during inhibitor-induced teratogenesis holds in good agreement with deductions which implicate the sterol Δ8-Δ7 isomerase defect in the impaired function of Ihh (36).

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