Oxidized derivatives of 7-dehydrocholesterol induce growth retardation in cultured rat embryos: a model for antenatal growth retardation in the Smith-Lemli-Opitz syndrome

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Abstract 7-Dehydrocholesterol accumulates in fetuses affected by the Smith-Lemli-Opitz syndrome as a result of a deficit in the ultimate step of cholesterol synthesis catalyzed by 7α-reductase. Rat embryos explanted at gestation day 10 and cultured for 48 h in the presence of the 7α-reductase inhibitor AY 9944 were used as a model to discriminate between the beneficial effect of supplementation with cholesterol and the deleterious effect of supplementation with 7-dehydrocholesterol. Cholesterol supplementation in the form of mixed cholesterol/lecithin liposomes added to serum serving as the culture medium restores the growth of embryos which is markedly decreased in the presence of the inhibitor. 7-Dehydrocholesterol under identical conditions does not restore growth and impairs the beneficial effect of cholesterol added simultaneously. UV-photooxidation of 7-dehydrocholesterol-supplemented culture medium enhances its embryotoxicity, which suggests uptake by the embryo of toxic by-products formed from 7-dehydrocholesterol. By contrast photooxidation of cholesterol-supplemented culture medium does not induce embryotoxicity. α-Tocopherol reduces the toxicity of photooxidized 7-dehydrocholesterol supplementing the culture medium. We conclude that 7-dehydrocholesterol does not fulfill the cholesterol requirement of the developing embryos and exerts an additional embryotoxic effect probably via oxidized by-products. This could explain the antenatal growth retardation of SLOS by a blockage of the maternal compensatory cholesterol influx.—Gaoua, W., F. Chevy, C. Roux, and C. Wolf. Oxidized derivatives of 7-dehydrocholesterol induce growth retardation in cultured rat embryos: a model for antenatal growth retardation in the Smith-Lemli-Opitz syndrome. J. Lipid Res. 1999. 40: 456-463.

Supplementary key words AY9944 • cholesterol/lecithin liposomes • UV-photooxidation • vitamin E

The Smith-Lemli-Opitz syndrome (SLOS) (1) has been diagnosed with more certainty since the biochemical description of the associated perturbation in cholesterol synthesis (2, 3). It is a feared and relatively common (estimated at 1/20,000 in Caucasians), often lethal, birth defect. Children with the SLO syndrome are affected by severe mental retardation and an unusual failure to thrive related to digestive system malformations and multiple organ anomalies (brain, cardiac, renal). A set of craniofacial defects and genital abnormalities in males are characteristic symptoms (1–4). Antenatal growth retardation was also constantly found in a preliminary series of seven patients (4) similar to the reduced weight of the offspring of rats dams treated by a distal cholesterol synthesis inhibitor (5, 6). Antenatal growth retardation is now considered for the diagnosis in utero for the SLO syndrome in the absence of a placental abnormality as an obvious cause. The explanation for the slow growth of the fetus has received little attention and prompted the complementary observations reported below.

SLO is associated with reduced activity of 7-dehydrocholesterol 7α-reductase (7DHC reductase) due at least in some cases to mutations in chromosome 11q12–13 which result in a complete blockage of cholesterol synthesis for the most severely affected group II patients (7). Low cholesterol levels and abundant aberrant sterols are seen in serum and tissues of all affected children (8–12). The prominent aberrant sterols found are: 7-dehydrocholesterol (7DHC, cholesta-5,8-dien-3β-ol), 8-dehydrocholesterol (8DHC, cholesta-5,8-dien-3β-ol) (13) and nortrienol (19-nor-cholesta 5,7,9(10)-tri-en-3β-ol) (14). The slow enzymatic formation of 8DHC from 7DHC has been demonstrated (15) but the origin of nortrienol has not been definitively established. One study indicated that nortrienol was not an artifact formed during the chemical isolation of sterols (16) but another study showed that 8DHC un-
nervous system malformations. The role of cholesterol in association with cholesterol (19–23) as one putative cause of central nervous system malformations occurring early in pregnancy or slow embryonic development recorded later by antenatal imaging. The relevance for therapy to estimate independently the impact of cholesterol and aberrant sterols appears clear.

Indeed, the remarkable malformations of the anterior part of the brain in SLOS have recently drawn most of the interest and have allowed the definition of the role of one developmental gene, Sonic Hedgehog (shh), in conjunction with cholesterol (19–23) as one putative cause of central nervous system malformations. The role of cholesterol in shh transduction has recently been demonstrated along with a number of other potential critical targets, including the receptor Patched, which comprise a Sterol-Sensing-Domain (24).

A manifestation of SLO may be holoprosencephaly (25), a rare malformation in humans but found with an extremely high frequency in the offspring of animals (rat or rabbit) treated with inhibitors of the distal enzymes of cholesterol synthesis such as AY 9944 (trans-1,4-bis(2-chlorobenzyl-amino-methyl)cyclohexane dihydrochloride) or BM15.766, inhibitors of Δ7 reductase and Δ8,7-sterol isomerase or Triparanol, an inhibitor of desmosterol-Δ24 reductase (5, 26). A high cholesterol diet administered simultaneously with AY 9944 treatment prevented the maternal level of cholesterol from decreasing below the critical threshold estimated at 30 mg/dl (half of the normal level) and suppressed holoprosencephaly in rat fetuses (26, 27). The defective cholesterol “packaging” by the yolk sac of embryos deficient in apolipoprotein B-100 (28) exposed the mice offspring to an enhanced teratogenic activity of cholesterol synthesis inhibitors (29). This supports the occurrence of a compensatory mechanism for the fetal cholesterol synthesis defect by an increased maternal supply. Holoprosencephaly has also been reported in mice offspring lacking megalin, a member of the LDL receptor family expressed on the apical surface of yolk sac and neuroepithelium (30) which takes part in the uptake of the lipoproteins. Experimental pathology has now brought attention not only to defects of cholesterol synthesis but also to defects of cholesterol transfer to embryos as the possible causes of holoprosencephaly.

The fact that embryonic defects can be prevented in the animal model when a high cholesterol diet is fed to the pregnant rats treated with AY 9944 does not conclusively demonstrate that a low cholesterol level is the only cause of malformations (26) because the restoration of the cholesterol level also down-regulates the synthesis of aberrant sterols possibly involved in embryotoxicity.

DeFabiani et al. (31) have shown that 7DHC, which accumulates in tissues of SLO patients, is oxidized to a toxic sterol hydroperoxide which probably adversely affects these patients. This prompted the examination of the involvement of oxidized by-products of 7DHC in fetal growth retardation as compared to oxidized by-products formed from cholesterol under similar conditions. One of the by-products of cholesterol (25-OH cholesterol) that down-regulates cholesterol synthesis (32, 33) has already been shown not to share the deleterious properties of sterol-like Veratrhum calif. teratogens on neural plate explants (24).

While a deleterious effect of 7-dehydrocholesterol is due, at least partially, to the strict down-regulation exerted on HMG-CoA reductase (34) that critically compromises the residual cholesterol synthesis of SLO embryos, another distinct effect of 7DHC anticipated by the present study is the reduced influx of cholesterol from the normocholesterolemic heterozygote mother. At 3 months of pregnancy, the cholesterol flux rate into the human fetus has been estimated to be as high as 458 μg per day (35), whereas in term newborns of uncomplicated pregnancies, the contribution of maternal lipoprotein-cholesterol to the fetal cholesterol pool appears of minimal quantitative importance (36). The maternal contribution to fetal cholesterol can be expected to be relevant in humans during the early development especially when the fetal cholesterol synthesis rate is decreased. Due to animal species particularities, the rodent model used currently emphasizes the high dependence on the cholesterol influx of cultured embryos, which favors the observation of the deleterious influence of 7DHC on cholesterol transfer.

To delineate the respective roles of low cholesterol availability and of toxicity of aberrant sterols and oxidized by-products in the animal model of Smith-Lemli-Opitz syndrome, rat embryos were explanted at gestation day 10 and cultured in media supplemented with either cholesterol or 7-dehydrocholesterol, the conversion of 7DHC being blocked by AY 9944. UV irradiation was used to enhance oxidation of cholesterol or dehydrocholesterol, whereas the antioxidant α-tocopherol was added to culture media to counteract the deleterious effects induced by oxidized by-products.

**MATERIALS AND METHODS**

**Chemicals and reagents**

AY (trans-1,4-bis(2-chlorobenzylamino-ethyl) cyclohexane dihydrochloride was a generous gift from Ayerst Laboratories (New York, NY). Cholesterol, 7-dehydrocholesterol, epiprostanol (5β-cholestan-3α-ol), 7-lathosterol (cholestan-7-en-3β-ol), egg lecithin, and α-tocopherol were obtained from Sigma (St. Louis, MO).

**Rat embryo culture**

Female Wistar rats (200 g) were mated with males of the same strain. The day that spermatozoa were detected in the vagina, was designated as gestation day 0. Rat embryos were explanted at day 10 at +2 to +4 h within the intact visceral yolk sac and amnions and were placed in 25-ml round-bottom flasks containing 2 ml of...
culture medium. Flasks were placed on a rotatory incubator at 38°C. Rat embryos were cultured during 48 h according to the method used by New (37). The culture atmosphere was initially 5% O₂ + 5% CO₂ in N₂, and was changed with gas mixtures of increasing O₂ concentrations and 5% CO₂ in N₂ at regular intervals. The culture medium consisted of serum that was collected from adult Wistar male rats, pooled, and heat-inactivated at 56°C for 30 min. Cholesterol and/or 7-dehydrocholesterol and/or α-tocopherol were added to the culture medium in the form of mixed liposomes freshly prepared in an aqueous buffer by sonication (30 mW for 3 min: Vibracell tip probe [Bioblock]) with egg lecithin serving as the stabilizer carrier lipid (20 mg sterol/20 mg lecithin). The absence of toxicity of the egg lecithin was checked in a control experiment where embryos exposed to a high concentration (2.4 mg/ml) of pure lecithin liposomes. Culture media supplemented with sterols were U.V.-irradiated to induce the formation of oxidized by-products under aerobic conditions. This pre-treatment of the culture medium was performed prior to the introduction of the embryos. The rotatory incubator was irradiated with a UVC lamp (UVSL-25 Mineralight lamp, Ultra-Violet Products Inc., San Gabriel, CA) at 38°C. The lamp was located approximately 5 cm under the lowest part of the vessel and the radiometer measured the intensity to be 25 μW/cm² inside the 25-ml round-bottom flask which takes account of the radiant energy absorption by the thin glass wall of the vessel. The total irradiation during the 8 h was estimated to be 720 mJ/cm².

Growth and morphological analysis of cultured rat embryos

Measurement of head length with a micrometer incorporated in the lens of microscope was a reliable index of embryo development at gestation day 10 in the presence of a cholesterologenesis inhibitor. Global morphological scoring of growth and differentiation was also performed according to Brown and Fabro (38). Embryos were scored freed of amnios and the yolk sac.

Lipid extraction and gas chromatography-mass spectrometry (GC–MS)

Embryos were transferred to 3 ml of chloroform–methanol 2:1 (vol/vol) with epicoprostanol added as an internal standard (100 μg). Short repeated cycles of sonication (5 times 30 sec) ensured appropriate homogenization of the embryonic tissues. Three embryos were pooled for sterol analysis. Saponification of sterol esters was carried out in 0.5 N methanol KOH (1 h at 56°C) and the fatty acids were converted to methyl esters with BF₃ in methanol (20%) to prevent contamination of the GC after repeated injections of free fatty acids. Sterols extracted in hexane were derivatized to trimethylsilyl ethers with BSTFA-TMCS 10% (Chrompack, Middelburg, Netherlands) in 15 min at 55°C. Sterol trimethylsilyl ethers were chromatographed on a medium polarity RTX 65 capillary column (internal diameter 0.32 mm; length 30 m; film thickness 0.25 μm) (Restek-France). Two μl of the TMS-sterol ethers in cyclohexane (50–100 μl) was injected in the splitless mode (Inactivated Focusliner insert from SGE Sarl, France) by the Hewlett-Packard Automatic Sampler 7673 from amber-glass vials thermostated at 6°C. The temperatures for chromatography were: injector 280°C, GC–MS interface 280°C. The initial oven temperature was 60°C and maintained for 1 min after injection. When the split was open, the oven temperature was raised quickly to 240°C (40°C/min). The sterol derivatives were eluted along a linear temperature gradient (at 2°C/min) between 240°C and 280°C. Helium, the carrier gas, was at a pressure of 55 kPa to give an average linear velocity of about 30 cm/sec in this temperature range.

Identification of sterols was obtained by comparison of their mass spectra (positive fragment ions produced in the Electron Impact Mode at 70 eV (Nermag R10-10C)) to the NIST library or to commercially available standards or to spectra that were previously published in the literature (13, 14). Sterols were quantified after normalization with an internal standard (epicoprostanol) and calibration was done with weighted standards for cholesterol and 7-dehydrocholesterol.

Statistics

Data are reported as the mean ± SEM. The statistical significance of the difference between the data was evaluated with the Mann-Whitney test and significance was accepted at the level of P < 0.05.

RESULTS

Table 1 shows that the normal rat serum cholesterol (around 0.6 mg/ml) could not maintain the usual growth rate of embryos over 48 h in the presence of AY 9944 (1 μg/ml) (P < 10⁻⁴). The severe growth retardation confirms previous observations (17, 39). When the serum was supplemented with cholesterol (1.2 mg/ml) the morphological score, which reflects the development of twenty different items, was essentially unaltered. The head length, the most sensitive index for embryos exposed to AY 9944, was restored to 93% of the control. Mixed cholesterol/lecithin liposomes with a high concentration of cholesterol (1:1, w/w) were used here because this enrichment procedure did not result in toxicity to embryos as did solvent addition. The procedure permitted easy variation of sterols that the embryos were exposed to, which was not convenient with the procedure using hypercholesterolomic rat sera (17).

To discriminate between the effects of 7-dehydrocholesterol and cholesterol, we supplemented the culture media with 7DH C and maintained a high concentration of AY 9944 (1 μg/ml) estimated from a previous study to completely inhibit Δ7-reduction to cholesterol in the embryo (17). This high concentration insured that no beneficial reduction of 7DH C to cholesterol could occur during the culture. Table 1 showed that in comparison with the beneficial cholesterol supplementation in the presence of AY 9944, 7DH C supplementation resulted, on the contrary, in an additional toxic effect leading to a highly significant growth retardation. GC–MS confirmed that the exogenous 7-dehydrocholesterol was taken up by embryos from the supplemented medium and accumulated, its conversion to cholesterol being blocked by AY 9944. 7DH C increased slightly from 0.2 ± 0.1 μg/embryo (n = 8) to 0.4 ± 0.1 μg/embryo (n = 8) treated with AY 9944 and supplemented with 7DH C. In the cholesterol plus 7-dehydrocholesterol-supplemented medium, 7DH C remained at 0.2 ± 0.1 μg/embryo in proportion with the growth retardation of the embryo (Table 1). As seen in Fig. 1, whereas the cholesterol of embryos treated with AY 9944 in cultures supplemented with cholesterol (scattered around 5.8 μg/embryo) was close to the level of controls (around 7 μg/embryo), the cholesterol of embryos in cultures supplemented with 7-dehydrocholesterol or 7-dehydrocholesterol plus cholesterol remained very low (around 2 and 3 μg/embryo). In the absence of synthesis due to the high AY 9944 concentration, these results confirmed the suppression of the compensatory chole-
terol influx from the surrounding serum to embryos exposed to 7-dehydrocholesterol.

To obtain complementary insight into the toxic influence of 7-dehydrocholesterol, further experiments were performed to examine the hypothesis that oxidized derivatives formed from 7DHC could play a role.

The present observation led us to consider the possibility that toxic contaminants accumulated in the commercial preparation of 7DHC during aerobic storage. This reflected the possibility that toxic oxysterols (hydroperoxide (31) or hydroxylated sterols (32)) could have been introduced along with 7-dehydrocholesterol in supplemented media.

### Table 1. Growth measurement and morphological scoring of explanted 10-day-old rat embryos after 48 h of culture supplemented media

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Head Length (mm)</th>
<th>Morphological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 38)</td>
<td>2.06 ± 0.23</td>
<td>45.84 ± 0.31</td>
</tr>
<tr>
<td>Treated with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AY (1 μg/ml) (n = 23)</td>
<td>1.28 ± 0.10a</td>
<td>27.65 ± 1.14a</td>
</tr>
<tr>
<td>AY + cholesterol 1.2 mg/ml (n = 9)</td>
<td>1.91 ± 0.61b</td>
<td>44.61 ± 0.57c</td>
</tr>
<tr>
<td>AY + cholesterol + UV irradiated 8 h (n = 8)</td>
<td>1.95 ± 0.46b</td>
<td>43.31 ± 0.51c</td>
</tr>
<tr>
<td>AY + 7DHC 1.2 mg/ml (n = 33)</td>
<td>0.87 ± 0.50d</td>
<td>19.37 ± 1.05d</td>
</tr>
<tr>
<td>AY + cholesterol 1.2 mg/ml + 7DHC 1.2 mg/ml (n = 5)</td>
<td>1.06 ± 2.2e</td>
<td>15.90 ± 0.36e</td>
</tr>
<tr>
<td>AY + 7DHC + UV 4 h (n = 7)</td>
<td>0.81 ± 0.63f</td>
<td>16.35 ± 0.66f</td>
</tr>
<tr>
<td>AY + 7DHC + UV 6 h (n = 6)</td>
<td>0.55 ± 0.34g</td>
<td>10.90 ± 0.70g</td>
</tr>
<tr>
<td>AY + 7DHC + UV 8 h (n = 8)</td>
<td>0.46 ± 0.32h</td>
<td>11.25 ± 0.95h</td>
</tr>
<tr>
<td>AY + 7DHC + α-tocopherol (n = 7)</td>
<td>1.50 ± 0.00i</td>
<td>29.70 ± 0.22i</td>
</tr>
<tr>
<td>AY + 7DHC + α-tocopherol + UV 8 h (n = 3)</td>
<td>1.00 ± 0.00j</td>
<td>19.00 ± 0.00j</td>
</tr>
</tbody>
</table>

Data (mean ± SEM) were analyzed with the Mann-Whitney test.

- aP < 0.0001: head length and morphological scoring are significantly different from control.
- bP = 0.03 for head length and P = 0.07 for scoring are not significantly different from control.
- cP = 0.05 for head length and scoring are not significantly different after irradiation of cholesterol-supplemented media.
- dP < 0.0006 and eP < 0.0001, respectively, for head length and scoring are significantly lower than in non-supplemented serum.
- fP = 0.5 for both head length and scoring are not significantly different from AY-treated embryos cultured in 7DHC-supplemented medium.
- gP < 0.05 and hP < 0.0006, respectively, for head length and scoring are significantly lower than for AY-treated embryos cultured in non-irradiated 7DHC-supplemented serum.
- iP < 0.0004 and jP < 0.0008, respectively, for head length and scoring are significantly lower than for AY-treated embryos cultured in non-irradiated 7DHC-supplemented medium.
- kP < 0.005 and lP < 0.005, respectively, for head length and scoring are significantly higher than for AY-treated rat embryos cultured in (non-irradiated) 7DHC-supplemented media.
- mP < 0.05 and nP < 0.05, respectively, for head length and scoring are significantly higher than for AY-treated embryos cultured in 7DHC-supplemented media irradiated for 8 h.

**Fig. 1.** Cholesterol content of rat embryos explanted at day 10 and cultured for 48 h in supplemented sera. Cholesterol was quantitated by ion abundance at m/z 329 by GC–MS with reference to a calibrated standard and epicoprostanol as the internal recovery standard. Each point represents the value from a single assay of three pooled embryos. The horizontal bar indicates the mean value. n (number of independent experiments) = 12 for control embryos, n = 12 for AY-treated embryos, n = 7 for AY-treated embryos cultured in medium supplemented with cholesterol, n = 9 for AY-treated embryos cultured in medium supplemented with 7-dehydrocholesterol, and n = 9 for AY-treated embryos cultured in medium supplemented with cholesterol and 7-dehydrocholesterol.
media prepared for embryo culture. Experiments were conducted with media supplemented with 7-dehydrocholesterol or cholesterol (control) and pre-conditioned under controlled oxidative conditions by UV irradiation.

The oxidation of 7-dehydrocholesterol after exposure for 6 or 8 h of the culture medium to UV irradiation (25 \( \mu \)W/cm\(^2\)) reinforced significantly (4.10 \( \times \) 10\(^{-4} \) < \( P \) < 5.10\(^{-2} \)) the deleterious effects of 7-dehydrocholesterol on head length (Table 1). As a control, the growth of embryos was not affected in 8-h irradiated medium supplemented with cholesterol instead of 7-dehydrocholesterol (Table 1). The suppression of the deleterious influence of AY 9944 by cholesterol irradiated for 8 h was still very efficient and compared similarly with non-irradiated cholesterol-supplemented media. This contrasted with the embryotoxicity that increased with the length of irradiation of 7-dehydrocholesterol-supplemented growth media (head length increased by 72% relative to the head length reduced by 47% when the 7DH C-containing medium was irradiated for 8 h) (Table 1).

Previous studies have shown that 7-hydroperoxycholesta-5,8-dien-3-ol could be obtained in vitro from 7-dehydrocholesterol exposed to \( \Delta_5,7 \) radical photooxidation (31). Such a hydroperoxide was suspected to be one of the very toxic by-products formed in biological tissues under prooxidant conditions. Photooxidation of \( \Delta_5,7 \) diene sterols was also recognized as a self-perpetuating mechanism generating highly toxic singlet molecular oxygen (39). Indeed, 7-hydroperoxide decomposed to cholest-5,7,9(11)-trien-3-ol, a cholestatrienol detected in plasma of patients with SLO (40) and in the embryo culture medium as a trace of ion \( m/z \ 364 \) (M\(^+\)-silanol) by GC-MS in the selective ion monitoring mode. Along with cholestatrienols, 19-norcholesta-5,7,9(10)-trien-3-ol was also detected in the embryo culture medium after AY 9944 treatment of rats as in plasma from Smith-Lemli-Opitz patients (41). Under the present experimental conditions, UV-irradiation as checked by GC-MS did not induce the photolysis of 7DH C to pre-vitamin D and to vitamin D.

Finally, the protection afforded by \( \alpha\)-tocopherol to embryos exposed to non-irradiated and irradiated media supplemented with 7DH C confirmed the deleterious effect of oxidized by-products (including those from a partially pre-contaminated commercial source of 7DH C). Observations indicated in Table 1 suggested that oxidized by-products pre-formed in the 7DH C preparation supplementing the culture medium were counteracted. A significant improvement of the embryo’s condition caused by \( \alpha\)-tocopherol treatment was detectable for cultures in both non-irradiated (head length increased by 72% relative to the head length in the absence of vitamin E) and irradiated 7DH C-supplemented medium (head length increased by 126%). Noticeably, 7DH C level was strictly maintained in the culture medium in the presence of \( \alpha\)-tocopherol after irradiation.

**DISCUSSION**

The influence of excess 7-dehydrocholesterol on embryonic development was studied with the aim of address-
vention by cholesterol of holoprosencephaly in vivo should be multiplied 6-fold between gestation day 7 (prevention by cholesterol synthesis (i.e., in that the cholesterol supply in the absence of embryonic embryos made fully dependent by AY 9944. The serum cholesterol level at gestation day 7 when there was a high concentrations currently added, does not significantly impair the cholesterol uptake and transport through the visceral leaflet of the yolk sac which succeeds in maintaining normal growth by a compensatory influx. This contrasts with the toxic role expected for AY 9944 on the cholesterol transfer from neural plate explants experiments (24).

It was shown that tripling the cholesterol level in the culture (the serum level serving as the medium is ca 0.6 mg/ml) was required to prevent growth retardation in embryos made fully dependent by AY 9944. The serum cholesterol level at gestation day 7 when there was a high frequency of holoprosencephaly was previously estimated at 0.20–0.30 mg/ml (half of the normal level) in pregnant dams (27). If a quantitative comparison can be made between data recorded in vivo and in vitro this would suggest that the cholesterol supply in the absence of embryonic synthesis (i.e., in \( \Delta 7 \)-reductase knock-out offsprings) should be multiplied 6-fold between gestation day 7 (prevention by cholesterol of holoprosencephaly in vivo >0.30 mg/ml) and gestation day 10 (prevention by cholesterol of growth retardation in vitro >1.20 mg/ml).

The present study established that supplementation in vitro with 7-dehydrocholesterol, the immediate precursor of cholesterol that accumulates in the Smith-Lemli-Opitz syndrome, was unable to sustain the rapid growth rate achieved by embryos while cholesterol under the identical experimental conditions achieved this goal. Though the precursor has been shown to be taken up by the embryos, it amounted to only a tenth of the expected cholesterol content after 48-h culture. 7DHC appeared unable to substitute, at least quantitatively, for cholesterol. We hypothesize now that 7DHC perturbs the sterol influx, i.e., the cholesterol as well as its own transfer into the embryo. Of relevance to SLO therapy is the observation that an effective embryotoxicity of 7-dehydrocholesterol-oxidized by-products could be reproduced in the cultured embryos and that it was alleviated by \( \alpha \)-tocopherol. The particular toxicity of UV-irradiated 7DHC can be shown by contrast to the absence of embryotoxic effects of UV-irradiated cholesterol under similar experimental conditions. Whereas oxidized derivatives of cholesterol formed from the serum or the liposome cholesterol (44) are also expected to appear after irradiation, these derivatives do not display the high toxicity of oxidized 7DHC for embryos. The toxicity of 7-dehydrocholesterol per se (non-oxidized) could not be demonstrated here as no commercial preparation has been found to be devoid of any contaminant and it would always be anticipated that even pure 7-dehydrocholesterol could have been oxidized readily by \( \text{O}_2 \) concentration used throughout the embryo culture.

An estimation of the high potency of the embryotoxicity of 7DHC or oxidized by-products is obtained from the damage inflicted on the embryos supplemented with 7-dehydrocholesterol in excess (1.2 mg/ml) which was not reversed by cholesterol supplementation. At this point, the present data support the view that 7-Dehydrocholesterol impairs cholesterol influx. This complements the inhibition of the cholesterol synthesis previously reported as a cause (34) to explain the embryonic defect and the low cholesterol level sustained after birth. In the present study, no residual synthesis was expected because of the high AY 9944 concentration and the observations, indeed, focused on the reduced cholesterol influx. 7-Dehydrocholesterol is known to disturb LDL binding and internalization via receptors in cultured fibroblasts (34). In the rat model this comes in addition to the fact that a number of basic amphiphile drugs including AY 9944 are known to influence the internalization of LDL via receptors (6). In a recent observation Cooper et al. (24) suggested that AY 9944 should also be considered as a class 2 inhibitor of the cholesterol transport inside the cell. These inhibitors reduce cholesterol efflux from lysosomes after lipoprotein endocytosis (43). We suggest now that the combination of the influences of 7DHC and AY 9944 on cholesterol influx contributes to the worsened condition of embryos as a result of a severely defective cholesterol supply. This inhibition in sterol transport also explains why the accretion of 7DHC itself remains moderate as compared to the high influx for cholesterol in the absence of 7DHC.

An unexpected observation is that 7DHC oxidized derivatives are, at least partially, responsible for the toxicity of this aberrant sterol and that embryonic growth can be improved by the addition of an antioxidant such as vitamin E. The influence of vitamin D from the photolysis of 7DHC can be disregarded on the basis of the GC–MS assay which demonstrates its absence in the 7DHC supplemented and irradiated rat serum.

Taken together, these observations point to the toxic role of the accumulation of 7-dehydrocholesterol in SLO. We note that the accumulation of aberrant sterols resulting from inhibition of “distal” cholesterol synthesis might be required to obtain the full teratogenic activity which is lacking with “proximal” HMG-CoA reductase inhibitors that block an early step of the pathway where only water-soluble precursors are formed. Eventually observations with sterol-like teratogens such as Veratrum alkaloids also support a role for structural analogues of cholesterol acting as competitors of the normal signalling transduction pathway of SHH, implying cholesterol-sensing domains (24, 45).

The consequences for SLO of the up-regulation of HMG-CoA reductase in response to the decline of cholesterol and the simultaneous down-regulation exerted by 7DHC were
recently discussed (34). We now suggest that 7DHC in the embryo or in the embryonic envelope compromise the cholesterol influx from the maternal supply, which is especially critical in rodents. Whether this should be considered in the human SLO malformations should also be questioned as the maternal cholesterol is not decreased in heterozygote mothers (8, 40). The physiological relevance of the cholesterol influx has not been established in early human development as firmly as it is in the rodent model in vitro. The possibility that oxidized by-products of 7DHC could also impair the nutrient influx should be appreciated in order to qualify the beneficial effect of vitamin E. Whether such treatment extended during pregnancy could circumvent, at least partially, the toxicity of 7-dehydrocholesterol derivatives in the SLO fetus is worth being investigated as a prevention of the antenatal growth retardation associated with the malformations. This supplementation has already been debated to prevent the similar abnormalities in apoB-deficient mice where the influx of cholesterol was also compromised (28). At this point, a comparison of the rodent model with the human where normal children are born from homozygous hypobetalipoproteinemic mothers, supports a clear distinction for the role of β-lipoproteins in human versus α-lipoproteins in rodent as the preferential source of cholesterol during early development. It is worthwhile to underline that cultured rat embryos expose the visceral leaflet of the yolk sac to the external HDL-rich serum serving as the growth medium. This envelope has been recognized to display a much higher clearance rate for HDL than for LDL (46) possibly through Gp330 and SR-BI receptors highly expressed in the yolk sac as compared to the placenta, the prominent absorbing envelope in humans. In conclusion, the cholesterol precursor 7-dehydrocholesterol that accumulates in the Smith-Lemli-Opitz syndrome has been shown to be unable to substitute for the growth effect of cholesterol in cultured rat embryos and has been found to be embryotoxic and to influence cholesterol transfer negatively, at least after its partial oxidation.

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