TERATOLOGY 54:12–19 (1996)

Interactive Dysmorphogenic Effects of all-*trans*-Retinol and Ethanol on Cultured Whole Rat Embryos During Organogenesis

HAO CHEN, HSUEH-YING L. YANG, MOSES J. NAMKUNG, AND MONT R. JUCHAU
Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98165

ABSTRACT Whole rat conceptuses (10.5 gestational days) were explanted into a culture medium containing all-*trans*-retinol (t-retinol, vitamin A_1), ethanol, or combinations of the two alcohols at various concentrations, and were cultured at 37°C for 24 hr. Parameters emphasized in morphological analyses were branchial arch development, closure of neural tube, axial rotation, and development of otic vesicles and of optic cup. Additions of t-retinol alone to the culture medium resulted in significant decreases in viability at concentrations of 7.0 μM and above. A primary target site affected by t-retinol was the second branchial arch. With initial culture medium concentrations of 3.5 μM, 28% of embryos exhibited an underdeveloped second branchial arch, and the effect was concentration dependent. Incubations with t-retinol alone also caused failure of closure of neural tubes, underdevelopment/absence of otic and optic vesicles, and failure of normal axial rotation, but these effects were statistically significant only at the higher concentrations (10.5–14.0 μM). Incubations of conceptuses with ethanol alone resulted in statistically significant decreases in viability and increases of incidence of embryonic abnormalities at 50 mM but not at 10- or 20-mM concentrations. The embryotoxicity of ethanol appeared less site-specific than that of t-retinol. However, ethanol-elicited developmental abnormalities included underdevelopment of the first and second branchial arches, abnormally open neural tubes, abnormally small or absent otic and optic vesicles, and incomplete axial rotation in common with effects elicited by t-retinol. In general, embryos incubated with combinations of t-retinol and ethanol showed lower survival rates and higher incidences of developmental abnormalities when compared to the calculated values expected for simple additive effects; i.e., interactive effects were most frequently greater than additive and probably synergistic but not antagonistic. To assist in the elucidation of possible mechanism(s) for the greater than additive/synergistic dysmorphogenic effects observed, concentrations of all-*trans*-retinoic acid (t-RA) and all-*trans*-retinal (t-retinal) in cultured conceptal tissues were determined by high-performance liquid chromatography (HPLC). HPLC analysis showed increases in conceptal tissue levels of both t-RA and t-retinal after conceptuses were exposed to t-retinol (10.5 μM) plus various quantities of ethanol for 24 hr. These observations, in combination with those of previous studies, suggested that the observed greater-than-additive/synergistic dysmorphogenic effects were not due to the inhibition by ethanol of conceptal biosynthesis of t-RA. Whether the increased levels of t-RA and t-retinal caused the observed greater than additive/synergistic dysmorphogenic effects remains to be elucidated.

Retinoids are highly potent embryotoxins and are established human teratogens (Shepard, '95; Schardein, '93; Juchau, 1993). Studies have suggested that the embryotoxic and dysmorphogenic effects of all-*trans*-retinol (t-retinol, vitamin A_1) are due, at least in part, to conversion of the retinoid alcohol to all-*trans*-retinoic acid (t-RA), an active metabolite of t-retinol (Kochhar and Penner, '87; Eckhoff et al., '89; Creech Kraft and Juchau, '92, '93a,b). Retinoic acids are known to regulate gene expression by activating nuclear retinoid receptors (RARs and RXRs), with subsequent binding of the retinoid–receptor complex to retinoid receptor response elements in the 5'-upstream regulatory regions of genetic DNA (Petkovich et al., '87; Levin et al., '92). It is now clear that stringently regulated homeostasis of retinoic acids is crucial for normal embryonic development. Either excess or deficiency of retinoic acids, as expected, are each likely to produce developmental abnormalities.

Ethanol is also a recognized human teratogen. Consumption of ethanol during pregnancy can cause serious birth defects, often manifested as fetal alcohol syn-

Received October 1, 1995; accepted April 3, 1996.

Address reprint requests to M.R. Juchau, Department of Pharmacology, School of Medicine SJ-30, University of Washington, Seattle, WA 98165.

drome (FAS). Recently, two retinoid-related mechanisms have been proposed for ethanol-induced embryotoxicity. Zachman and Grummer ('91) have suggested that embryotoxicity of ethanol may be due to an elicited elevation of the levels of retinoic acid in fetal tissues, since increased levels of t-retinol were observed after ingestion of ethanol in vivo. By contrast, Duester ('91, '95) and Pullarkat ('91) independently proposed that ethanol-induced dysmorphogenic effects may be the result of retinoic acid deficiency because ethanol could act as a competitive inhibitor of the biosynthesis of retinoic acid from retinol. The first-mentioned hypothesis of Zachman and Grummer would suggest that, in the case of retinol excess, the presence of ethanol would tend to exacerbate the retinol-induced dysmorphogenic effects, since ethanol would cause an increase in tissue levels of retinoic acid. On the other hand, if ethanol inhibits biosynthesis of retinoic acid from retinol, as suggested by the last-mentioned hypothesis of Duester and Pullarkat, the presence of ethanol should tend to reduce the retinol-induced dysmorphogenic effects by inhibiting the biosynthesis of RA from retinol, thus reducing tissue levels of the ultimate embryotoxin. Recently we discovered that rat conceptal retinol and retinal dehydrogenases are the primary enzymes in the catalysis of conversions of t-retinol and t-retinal to t-RA (Chen et al., '95). This observation provides a foundation for the possible interaction between ethanol and retinol.

In addition, because the potential for human exposure in utero to ethanol remains very high and retinoids also have a widespread usage for treatments of various skin disorders and a potentially widespread usage for cancer chemoprevention, it is important to study the interactive effects of these two compounds on embryonic dysmorphogenesis. In this study, the direct interactive effects of ethanol and t-retinol were investigated. Whole rat conceptuses (gestational day 10.5) were incubated with t-retinol, ethanol, or combinations of both chemicals for 24 hr, and effects on growth and morphogenesis were examined. t-Retinol and ethanol-induced dysmorphogenic embryotoxic effects were used as probes to investigate the extent to which interactive effects (antagonistic, additive, or synergistic) could be observed with respect to the elicitation of such abnormalities. In addition, embryonic tissue levels of all-*trans*-retinal (t-retinal; key intermediate of retinoic acid synthesis) and t-RA were determined by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

t-Retinol, t-retinal, t-RA, and 13-*cis*-retinoic acid (13-*cis*-RA) were purchased from Sigma Chemical Co. (St. Louis, MO). 9-*cis*-Retinoic acid (9-*cis*-RA) was a gift from Dr. Sorter of Hoffmann-La Roche Inc. (Nutley, NJ). All other chemicals and reagents were of the highest purity commercially available.

Whole-embryo culture

Time-mated primagravida pregnant rats (Sprague-Dawley, Wistar-derived, 180–200 g) were obtained locally on day 9.5 of gestation from Tyler Laboratories (Bellevue, WA). All animals were allowed free access to food (Purina Rat Chow) and distilled water and were housed in plastic cages with crushed corncob material for bedding. The morning after copulation was designated as day 0.5 of gestation. Staging and explantation of embryos were conducted on the morning of day 10.5 of gestation, and the procedures used were as described previously (Faustman-Watts et al., '85; Harris et al., '89). The conceptuses were removed from ether-anesthetized dams and transferred into a culture medium previously saturated with gas mixtures of O_2–CO_2–N_2 (20:5:75). The culture media consisted of 50% female rat serum (heat-inactivated at 56°C for 30 min) and 50% Waymouth's medium. t-Retinol (1 mg/ml in acetone) or ethanol were added in varying quantities (singly and in combination) to the media immediately prior to explantation. The conceptuses were then cultured in roller bottles at 37°C for 20 hr in the dark. At the end of the 20-hr period, the roller bottles were regassed in the dark with O_2–CO_2 (95:5) and conceptuses were cultured for an additional 4 hr. At the end of the culture period (day 11.5), conceptuses were removed from the culture bottles, examined for viability (embryos with heart beat and active vitelline circulation was scored as viable) under a light microscope, and evaluated/scored for growth parameters and malformations (Harris et al., '89) without knowledge of treatment. Nonviable conceptuses were not further evaluated. Viable embryos were examined for maximal embryonic length, maximal yolk sac diameter, degree of axial rotation, branchial arch development, neural tube morphology, optic cup and otic vesicle development and other, grossly observable parameters. Immediately after evaluations and scoring, embryos were stored at −70°C for later measurements of protein content.

HPLC analyses of tissue levels of conceptal retinoids

One hundred fifty to three hundred milligrams (wet weight) of whole conceptuses (defined as consisting of the embryo proper, visceral yolk sac, ectoplacental cone, amniotic membrane, and fluid compartments inside the yolk sac and amniotic membranes) were treated with 1 ml of isopropanol, vortexed for 1 min, and homogenized at 4°C using a Sonic L converter for 10 sec at a setting of 2 (Branson Sonic Power Co., Plainview, NY). The homogenates were vortexed again and centrifuged at 4°C for 30 min at 16,000*g*. Prior to centrifugation, 50 μl of homogenates was removed for protein determinations. The mixture of standard retinoids or supernatant fraction of the homogenates after centrifugation (100 μl) were injected into the HPLC system. The HPLC apparatus consisted of two model 100A

TABLE 1. Effects of varying concentrations of *t*-retinol or ethanol on morphological parameters in cultured rat whole embryos

Treatment	No. of embryos (n)	Viab. loss (%)	Total defects[1] (%)	Underdeveloped branchial arches		Open neural tube		Abnormal vesicle development		Abnormal rotation (%)
				1st (%)	2nd (%)	Ant. (%)	Post. (%)	Optic (%)	Otic (%)	
Control	20	0	0	0	0	0	0	0	0	0
Retinol (3.5 μM)	25	4	28[2]	0	28[2]	0	0	0	0	0
Retinol (7 μM)	16	25[2]	33[2]	0	33[2]	0	0	0	0	0
Retinol (10.5 μM)	25	28[2]	78[2]	11	38[2]	11	6	0	12	17
Retinol (14 μM)	19	58[2]	63[2]	13[2]	50[2]	25[2]	25[2]	13[2]	50[2]	13[2]
EtOH, 10 mM	15	0	7	0	7	0	0	0	0	0
EtOH, 20 mM	22	14	11	0	0	0	0	0	0	11
EtOH, 30 mM	21	10	26[2]	0	11	5	0	16	0	11
EtOH, 50 mM	19	42[2]	82[2]	9	36[2]	45[2]	45[2]	45[2]	73[2]	36[2]

[1]Embryos exhibiting any observed abnormality.
[2]The values were significantly different from those of the corresponding controls ($P<0.05$).

dual piston Beckman pumps linked together for activation of a binary gradient. The system was interfaced with a Shimadzu SPD-10A UV-VIS detector (set at 354 nm) and a Shimadzu C-R5A Chromatopac data processor for quantitation. HPLC was equipped with a Beckman mixing chamber and manual injector. A Zorbax octadecylsilane (ODS) column (0.46 × 15 cm) (MacMod Anal, Chadds Ford, PA) was employed for retinoid analyses and the method was described by Kim et al. ('92). The eluents consisted of solvent A (acetonitrile–H_2O–acetic acid, 49.75:49.75:0.5, v/v/v) and solvent B (acetonitrile–H_2O–acetic acid, 90:10:0.04, v/v/v), both containing 10 mM ammonium acetate. The mobile phase was programmed with a gradient from 25% mobile phase B to 80% mobile phase B in 10 min, and a second gradient of 80% mobile phase B to 25% mobile phase B in 15 min, at a flow rate of 1.5 ml/min. Duplicated experiments were performed and the values of t-RA or t-retinal were expressed as the means of four to six HPLC measurements with standard deviations.

Protein determinations

The method of Lowry et al. (1951) was used to determine quantitatively the concentration of protein in whole cultured conceptuses. Bovine serum albumin (BSA) was used as a standard protein for the quantitation.

Statistical analyses

Analyses of variances (ANOVA) with Dunnett's test were conducted with a Microexcell statistics package (Microsoft, Redmond, WA) to test for the statistical significance of differences between mean values measured on a continuous or graded scale (embryonic length, yolk sac diameter, protein concentration). Contingency tables with the chi-square criterion were utilized to statistically analyze differences in quantally assessed parameters such as viability/survival, abnormally open neuropores, abnormally formed otic and optic vesicles, and abnormal rotation of embryos. The level of significance chosen was $P<0.05$. All methods for statistical evaluations have are described in the literature (Zar, '84).

RESULTS

In Table 1, data are presented for the individual effects of t-retinol or ethanol on viability and morphogenesis of cultured rat embryos in vitro. Additions of the higher concentrations of t-retinol (≥7.0 μM) to the culture medium resulted in statistically significant decreases in viability and statistically significant increases in the incidence of abnormal development. Additions of ethanol alone (50 mM) produced a statistically significant decrease in viability as well as statistically significant increases in morphologic defects. Dysmorphogenic effects produced by t-retinol alone and ethanol alone each exhibited concentration-dependent patterns.

The individual effects of t-retinol or of ethanol on growth parameters in cultured whole rat embryos grown in vitro are presented in Table 2. Additions of t-retinol or of ethanol alone to the culture medium resulted generally in decreases in maximal yolk sac diameter and maximal embryonic length as well as in embryonic protein, although the effects were somewhat variable, possibly due to preferential survival of the largest embryos (analyses were performed only on viable embryos). In general, however, the changes in growth parameters appeared to be concentration-dependent for both t-retinol and ethanol.

Table 3 shows the effects of combinations of t-retinol plus ethanol on viability, total defects, and specific morphogenic abnormalities observed in cultured rat embryos in vitro. Explantation of embryos with combinations of ethanol plus t-retinol appeared to result generally in greater than additive effects. For instance, additions of ethanol alone (10.0 mM) did not cause a

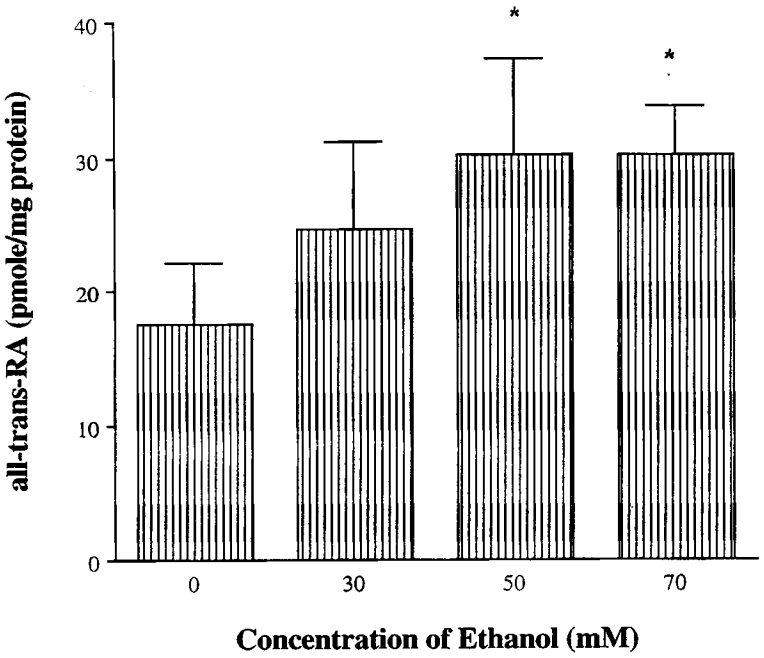

Fig. 1. Effects of ethanol on tissue levels of t-RA after embryos were cultured with *t*-retinol (10.5 μM) plus various concentrations of ethanol. The values (means ±SD) with asterisks indicate that the tissue levels of t-RA in treated embryos were significantly higher than those of the corresponding controls (at the 95% confidence level). For experimental details, see Methods.

statistically significant increase in incidence of morphologic defects of any type when compared to that of the corresponding controls for any of the parameters investigated (Table 1). Additions of t-retinol alone (3.5 μM) caused statistically significant increase in incidence of defects for only two of the parameters investigated (Table 1). However, a combination (Table 3) of ethanol (10.0 mM) plus t-retinol (3.5 μM) caused statistically significant increases in incidence of defects in all but three parameters. The combination effects at these concentrations were generally greater than additive and were statistically greater than the predicted (simple additive) effects (see Table 5) in three cases. Similar observations were applicable to other combinations; the greater than additive effects were particularly prominent with combinations of ethanol (30 mM) plus t-retinol (7.0 μM) and with ethanol (30 mM) plus t-retinol (10.5 μM).

The interactive effects of t-retinol and ethanol on growth parameters in cultured rat whole embryos are shown in Table 4. In general, incubations with both t-retinol and ethanol tended to cause greater than additive effects on the growth of the embryos. For example, explantations with t-retinol (10.5 μM) or with ethanol (30.0 mM) alone did not result in statistically significant decreases in embryonic protein content, but a combination of the two at the same concentrations caused a statistically significant greater-than-additive decrease in protein content.

Analyses of interactive effects on quantally assessed parameters such as viability/survival, open neuropores, open otic vesicles, and abnormal rotation of embryos were performed with contingency tables using the chi-square criterion and are given in Table 5. Embryos incubated with t-retinol or ethanol alone usually had higher viability rates and lower incidences of abnormal development than those expected on the basis of results obtained with embryos incubated with combinations of two chemicals at the same concentrations. For example, embryos incubated with a combination of t-retinol (10.5 μM) plus ethanol (30.0 mM) exhibited a

TABLE 2. Effects of *t*-retinol or ethanol on growth parameters in cultured rat whole embryos[1]

Treatment	Maximal yolk sac diameter (mm)	Maximal embryonic length (mm)	Protein (μg/embryo)
Control	3.7 ± 0.2	3.3 ± 0.3	354 ± 52
Retinol (3.5 μM)	3.4 ± 0.3	3.4 ± 0.3	297 ± 15
Retinol (7 μM)	3.4 ± 0.3	3.5 ± 0.3	222 ± 30[2]
Retinol (10.5 μM)	3.3 ± 0.4	3.0 ± 0.3	192 ± 21[2]
Retinol (14 μM)	3.5 ± 0.6	3.0 ± 0.4	200 ± 5[2]
Ethanol (10 mM)	3.2 ± 0.4[2]	3.2 ± 0.3	337 ± 22
Ethanol (20 mM)	3.3 ± 0.1[2]	3.5 ± 0.3	344 ± 35
Ethanol (30 mM)	3.1 ± 0.1[2]	3.2 ± 0.4	303 ± 13[2]
Ethanol (50 mM)	3.0 ± 0.2[2]	2.9 ± 0.4[2]	183 ± 9[2]

[1]The values presented were obtained from the same set of animals listed in Table 1.
[2]The values (means ±SD) were significantly smaller than those of the corresponding controls at the 95% confidence level ($P<0.05$).

TABLE 3. Interactive effects of *t*-retinol and ethanol on morphogenesis of cultured rat whole embryos

Treatment	No. of treated (n)	Viab. loss (%)	Total defect[1] (%)	Underdeveloped branchial arches		Open neural tube		Abnormal vesicle development		Abnormal rotation (%)
				1st (%)	2nd (%)	Ant. (%)	Post (%)	Optic (%)	Otic (%)	
Control	23	0	8	0	4	4	0	0	0	4
Retinol (3.5 μM) + EtOH (10 mM)	24	21[2]	37[2]	0	21[2]	21[2]	21[2]	7	7	21[2]
Retinol (3.5 μM) + EtOH (20 mM)	18	23[2]	57[2]	7	44[2]	7	7	0	7	7
Retinol (7 μM) + EtOH (10 mM)	20	15	65[2]	0	65[2]	6	6	0	24[2]	12
Retinol (7 μM) + EtOH (30 mM)	18	50[2]	100[2]	22[2]	89[2]	33[2]	33[2]	11	22[2]	0
Retinol (10.5 μM) + EtOH (10 mM)	18	34[2]	75[2]	17	67[2]	8	8	8	33[2]	25[2]
Retinol (10.5 μM) + EtOH (30 mM)	19	74[2]	100[2]	60[2]	80[2]	100[2]	80[2]	80[2]	80[2]	60[2]
Retinol (10.5 μM) + EtOH (50 mM)	20	80[2]	100[2]	60[2]	100[2]	60[2]	60[2]	60[2]	60[2]	60[2]

[1]Embryos exhibiting any observed abnormality.
[2]The values were significantly different from those of the corresponding controls ($P<0.05$).

TABLE 4. Interactive effects of *t*-retinol and ethanol on growth parameters in cultured rat whole embryos[1]

Treatment	Maximal yolk sac diameter (mm)	Maximal embryonic length (mm)	Protein (μg/embryo)
Control	3.7 ± 0.2	3.3 ± 0.3	342 ± 52
Retinol (3.5 μM) + ethanol (10 mM)	3.6 ± 0.4	3.4 ± 0.3	328 ± 21
Retinol (3.5 μM) + ethanol (20 mM)	3.4 ± 0.3	3.2 ± 0.2	266 ± 52[2]
Retinol (7 μM) + ethanol (10 mM)	3.5 ± 0.3	3.4 ± 0.3	358 ± 21
Retinol (7 μM) + ethanol (30 mM)	3.1 ± 0.2[2]	3.3 ± 0.3	207 ± 40[2]
Retinol (10.5 μM) + ethanol (10 mM)	3.4 ± 0.1	3.0 ± 0.4	312 ± 6
Retinol (10.5 μM) + ethanol (30 mM)	3.0 ± 0.1[2]	2.5 ± 0.4[2]	206 ± 15[2]
Retinol (10.5 μM) + ethanol (50 mM)	2.6 ± 0.4[2]	2.4 ± 0.5[2]	258 ± 7[2]

[1]The values presented were obtained from the same set of animals listed in Table 3.
[2]The values (means ±SD) were significantly different from those of the corresponding controls at the 95% confidence level ($P<0.05$).

significantly decreased rate of viability as well as an increased incidence of developmental abnormalities when compared with the calculated, combined effects obtained in experiments with embryos incubated with t-retinol or ethanol alone at the same concentrations. Greater than additive effects were observed in the majority of cases and were particularly noted in experiments in which ethanol concentrations were at 30 mM (Table 5).

Figure 1 presents the effects of added ethanol at initial culture medium concentrations ranging from 30 to 70 mM on t-RA levels in conceptal tissues after the whole conceptuses were incubated with t-retinol (10.5 μM) and ethanol for 24 hr. As concentrations of ethanol increased, increases in conceptal tissue levels of t-RA were observed. Statistically significant increases in conceptal tissue levels of t-RA were observed when the concentrations of ethanol were increased to 50.0 and 70.0 mM.

Figure 2 exhibits the effects of varying concentrations of ethanol on conceptal tissue levels of t-retinal after whole conceptuses were incubated with combinations of t-retinol (10.5 μM) plus ethanol for 24 hr. The tissue levels of t-retinal increased as the concentrations of ethanol in culture medium were raised. Incubations of conceptuses with ethanol at the higher concentrations (50–70 mM) resulted in a threefold increases in conceptal tissue levels of t-retinal after a 24-hr culture period.

DISCUSSION

Both retinoids and ethanol are recognized as highly important human teratogens and are also known to adversely affect embryonic development in several species. In these studies with cultured whole rat embryos in vitro, additions of either t-retinol or ethanol alone to the culture medium resulted in severe craniofacial abnormalities and abnormal neuropore development. These observations are consistent with results from other studies (Campbell and Fantel, '83; Shepard, '95; Giavini et al., '92; Schardein, '93). In the system used, the dysmorphogenic effects produced by t-retinol were observed at concentrations approximately three orders of magnitude lower than the concentrations of ethanol required to produce similar abnormalities. For example, additions of t-retinol (14.0 μM) significantly affected the normal development of branchial arches and neural tubes as well as of otic vesicles and optic cups, while more than a 3,000-fold higher concentration of ethanol (50.0 mM) was required to produce a similar incidence of dysmorphogenic effects on the cultured

TABLE 5. Direct comparisons of individual versus combined actual and observed versus predicted[1] interactive effects of *t*-retinol and ethanol on morphogenesis of cultured rat whole embryos

Treatment	No. of treated (n)	Viab. loss (%)	Total defects[2] (%)	Underdeveloped branchial arches		Open neural tube		Abnormal vesicle development		Abnormal rotation (%)
				1st (%)	2nd (%)	Ant. (%)	Post. (%)	Optic (%)	Otic (%)	
Retinol (3.5 μM)	25	4	28	0	28	0	0	0	0	0
EtOH (10 mM)	15	0	7	0	7	0	0	0	0	0
Combination	24	21	37	0	21	21[3]	21[3]	7	7	21[3]
Predicted[3]		4	35	0	35	0	0	0	0	0
Retinol (3.5 μM)	25	4	28	0	28	0	0	0	0	0
EtOH (20 mM)	22	14	11	0	0	0	0	0	0	11
Combination	18	25	57	7	44	7	7	0	7	7
Predicted		18	39	0	28	0	0	0	0	11
Retinol (7 μM)	16	25	33	0	33	0	0	0	0	0
EtOH (10 mM)	15	0	7	0	7	0	0	0	0	0
Combination	20	15	65	0	65	6	6	0	24[3]	12
Predicted		25	40	0	40	0	0	0	0	0
Retinol (7 μM)	16	25	33	0	33	0	0	0	0	0
EtOH (30 mM)	21	10	26	0	11	5	0	16	0	11
Combination	18	50	100[3]	22[3]	89[3]	33[3]	33[3]	11	22[3]	0
Predicted		35	59	0	44	5	0	16	0	11
Retinol (10.5 μM)	25	28	78	11	38	11	6	0	12	17
EtOH (10 mM)	15	0	7	0	7	0	0	0	0	0
Combination	18	34	75	17	67	8	8	8	33	25
Predicted		28	85	11	45	11	6	0	12	17
Retinol (10.5 μM)	25	28	78	11	38	11	6	0	12	17
EtOH (30 mM)	21	10	26	0	11	5	0	16	0	11
Combination	19	74[3]	100	60[3]	80[3]	100[3]	80[3]	80[3]	80[3]	60[3]
Predicted		38	100	11	49	16	6	16	12	28
Retinol (10.5 μM)	25	28	78	11	38	11	6	0	12	17
EtOH (50 mM)	19	42	82	9	36	45	45	45	73	36
Combination	20	80	100	60[3]	100[3]	60	60	60	60	60
Predicted		70	100	20	74	56	51	45	85	53

[1]Effects predicted for combinations with the assumption of simple additivity.
[2]Embryos exhibiting any observed abnormality.
[3]Observed combination effects differed statistically from the predicted combination effects ($P<0.05$).

embryos. In addition, the dysmorphogenic effects of t-retinol were more target site-specific than those produced by ethanol. For example, as the concentrations of t-retinol were raised to as high as 10.5 μM, the only significant abnormality consistently observable was underdevelopment of the second branchial arch. On the other hand, at higher concentrations (30–50 mM), ethanol affected virtually all the evaluated target parameters in the embryos. The mechanism(s) behind the specificity of retinol toxicity and the generality of ethanol toxicity is not clear at this time but is probably related to interactions of t-retinol oxidation products (retinoic acids) to specific cellular retinoid receptors.

Recently, the developmental effects of combined exposures in vivo to ethanol and vitamin A_1 on fetuses of pregnant rats have been reported (Whitby et al., '94; Sundaresan et al., '94). In those studies, ethanol appeared to potentiate the deleterious developmental effects of vitamin A_1 excess. For example, treatments of pregnant dams with ethanol combined with vitamin A_1 at 80,000 IU/kg resulted in a greater than additive incidence of cleft palate relative to either treatment alone. In addition, the incidences of misshapen zygomatic arches and enlarged renal pelvises also were reportedly increased in a greater than additive fashion in the groups treated with combinations of vitamin A_1 and ethanol. The statistical analyses suggested a *synergism/potentiation* in the interactive effects of vitamin A_1 and ethanol in vivo. As shown in our studies, direct exposures of embryos to combinations of t-retinol and ethanol resulted generally in greater-than-additive incidences of abnormal development. The interactive effects of t-retinol and ethanol appeared to be greater than additive and possibly *synergistic,* but not *antagonistic.* These observations suggested that inhibition by ethanol of biotransformation of t-retinol to t-RA in conceptal tissues, if there is any, is probably not the cause for the ethanol-elicited embryonic dysmorphogenesis since the inhibition by ethanol of biosynthesis of t-RA would counteract the dysmorphogenic effects produced by excess of t-retinol shown in this study.

Recent studies with cultured embryos have demonstrated that retinoic acids, such as t-RA and 9-*cis*-RA, are direct acting and more powerful teratogens than their respective precursors or metabolites (Creech Kraft and Juchau, '93a,b). For example, approximately 16-

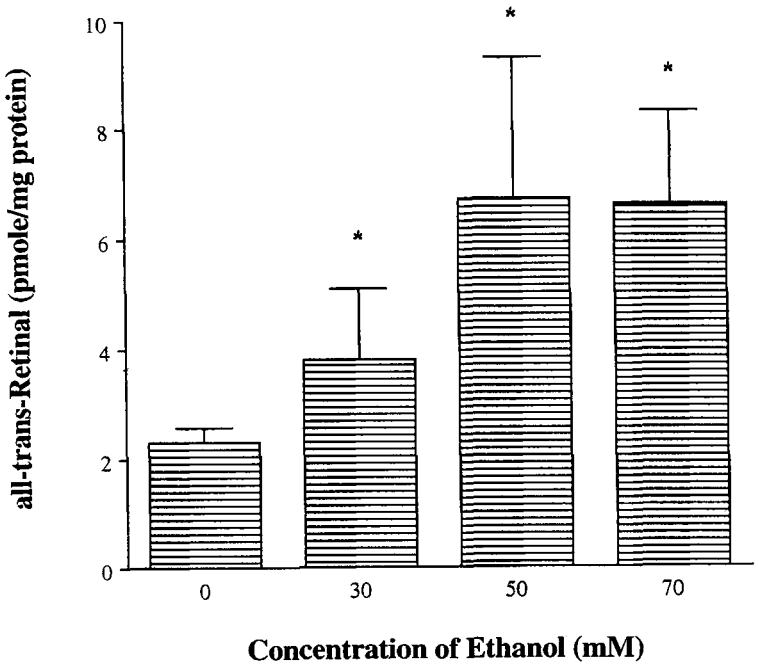

Fig. 2. Effects of ethanol on tissue levels of t-retinal after embryos were cultured with *t*-retinol (10.5 μM) plus various concentrations of ethanol. The values (means ±SD) with asterisks indicate that the tissue levels of t-retinal in treated embryos were significantly higher than those of the corresponding controls (at the 95% confidence level). For experimental details, see Methods.

fold higher concentrations of t-retinol were required to produce the same incidence/severity of dysmorphogenesis as that produced by t-RA in cultured whole rat embryos (Creech Kraft and Juchau, '93b). Interestingly, the dysmorphogenic effects of retinoid excess are somewhat similar to those produced by ethanol, especially in the craniofacial region and on neural crest cell derivatives (Zachman and Grummer, '91), while retinoid deficiencies tend to produce more extensive ocular, urogenital, diaphragmatic and cardiac anomalies (Shepard, '95). Those studies and observations, therefore, tended to suggest that the interactive dysmorphogenic effects of ethanol/retinol may be due, at least in part, to increases in conceptal tissue levels of retinoic acid. Indeed, Zachman and Grummer ('91) have suggested that a possible increase of retinoic acid levels in fetal tissues produced by ethanol may be one of the causes for the fetal alcohol syndrome. This hypothesis was supported by their observations that maternal ethanol ingestion resulted in an increase of retinol levels in fetal tissues (Grummer and Zachman, '90; Grummer et al., '93; Sundaresen et al., '94). Even though the increased retinol levels observed are likely to increase the receptor ligand (retinoic acid) levels, the possibility that the elevated levels of retinol may be due to an inhibition by ethanol on conversion of retinol to retinoic acid also exists because the levels of retinoic acid were not examined in those studies. Thus, to test the hypothesis of Grummer and Zachman, the determination of conceptal tissue levels of t-RA under the influence of ethanol becomes crucial. In this study, the conceptal tissue levels of t-RA and t-retinal (the latter is an obligate intermediate in the conversion of t-retinol to t-RA) were determined by HPLC after rat embryos were cultured with t-retinol alone or with combinations of t-retinol plus ethanol. After 24-hr incubation with t-retinol and various concentrations of ethanol, embryonic tissues exhibited no decreases in either t-RA or t-retinal produced by simultaneous incubations with ethanol. Instead, there were *increases* of both t-RA and t-retinal. The positive correlation between the increased levels of t-RA and exacerbation of the dysmorphogenic effects due to the combination of ethanol and t-retinol suggested that ethanol-elicited increases in conceptal t-RA levels could play an important role in the embryotoxicity produced by ethanol.

In summary, we have studied the interactive effects of t-retinol and ethanol on embryonic development during organogenesis. Combinations of t-retinol plus ethanol resulted in a higher than expected incidence of abnormal development than that hypothetically expected from antagonistic or from simple additive effects. The analyses of tissue levels of t-RA and t-retinal also suggested that ethanol did not inhibit the biotransformation of t-retinol to t-RA. Based on these observations, we tentatively conclude that *inhibition* by ethanol of the conceptal biotransformation of t-retinol to t-RA appears not to play a significant role in the interactive dysmorphogenesis elicited by ethanol and t-retinol in rat embryonic development.

ACKNOWLEDGMENTS

We thank Trina Irico for her assistance in this study. We appreicate Drs. A. Levin and P. Sorter from Hoffmann-La Roche for their kind contribution of the retinoids. This work was supported by NIEHS grants ES-04041 and ES-05861. H.C. was supported by NIEHS training grant ES-07032.

LITERATURE CITED

Campbell, M.A., and A.G. Fantel (1983) Teratogenicity of acetaldehyde in vitro: Relevance to the fetal alcohol syndrome. Life Sci., *32*:2641–2647.

Chen, H., M.J. Namkung, and M.R. Juchau (1995) Biotransformation of all-*trans*-retinol and all-*trans*-retinal to all-*trans*-retinoic acid in rat conceptal homogenates. Biochem. Pharmacol., *50*:1257–1264.

Creech Kraft, J., and M.R. Juchau (1992) Correlations between conceptal concentrations of all-*trans*-retinoic acid and dysmorphogenesis after microinjections of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoyl-β-glucuronide, or retinol in cultured whole rat embryos. Drug Metab. Disp., *20*:218–226.

Creech Kraft, J., and M.R. Juchau (1993a) 9-cis-Retinoic acid: A direct-acting dysmorphogen. Biochem. Pharmacol., *46*:709–717.

Creech Kraft, J., and M.R. Juchau (1993b) Conceptal biotransformation of microinjected retinoids. Correlations with dysmorphogenic activities. Ann. NY Acad. Sci., *678*:338–340.

Duester, G. (1991) A hypothetical mechanism for fetal alcohol syndrome involving ethanol inhibition of retinoic acid synthesis at the alcohol dehydrogenase step. Alcohol Clin. Exp. Res., *15*:568–572.

Duester, G. (1995) Involvement of alcohol-metabolizing enzymes in retinoic acid synthesis and inhibition by ethanol. In: Drug and Alcohol Abuse Reviews. vol. 6: Alcohol and Hormones. R.R. Watson, ed. Towota, NJ: Humana Press, pp. 75–95.

Eckhoff, C., B. Loefberg, I. Chahoud, G. Bochert, and H. Nau (1989) Transplacental pharmacokinetics and teratogenicity of a single dose of retinol (vitamin A) during organogenesis in the mouse. Toxicol. Lett., *48*:171–184.

Faustman-Watts, E.M., M.J. Namkung, J.C. Greenway, and M.R. Juchau (1985) Analyses of metabolites of 2-acetylaminofluorence generated by embryo culture system: Relationship of biotransformation to teratogenicity in vitro. Biochem. Pharmacol., *34*:2953–2960.

Giavini, E., M.L. Broccia, M. Prati, D. Bellomo, and E. Menegola (1992) Effects of ethanol and acetaldehyde on rat embryos developing in vitro. In Vitro Cell. Dev. Biol., *28*:205–210.

Grummer, M.A., and R.D. Zachman (1990) The effects of maternal ethanol ingestion on fetal vitamin A in rat. Pediatr Res., *28*:186–189.

Grummer, M.A., R.E. Langhough, and R.D. Zachman (1993) Maternal ethanol ingestion effects on fetal rat brain vitamin A as a model for fetal alcohol syndrome. Alcohol Clin. Exp. Res., *17*:592–597.

Harris, C., K.L. Stark, D.L. Luchtel, and M.R. Juchau (1989) Abnormal neurulation induced by 7-hydroxy-2-acetylaminofluorene and acetaminophen: evidence for catechol metabolites as proximate dysmorphogens. Toxicol. Appl. Pharmacol., *101*:432–442.

Juchau, M.R. (1993) Chemical teratogenesis. Progr. Drug Res., *41*:9–50.

Kim, C-I., M.A. Leo, and C.S. Lieber (1992) Retinol forms retinoic acid via retinal. Arch. Biochem. Biophys., *294*:388–393.

Kochhar, D.M., and Penner, J.D. (1987) Developmental effects of isotretinoin and 4-oxo-isotretinoin: the role of metabolism in teratogenicity. Teratology, *36*:67–75.

Levin, A.A., L.J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, Speck, J., Kratzeisen, C.I., Rosenberger, M., Lovey, A., and Grippo, J.F. (1992) 9-*cis*-retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature, *355*:359–361.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193*:265–275.

Petkovich, M., J.B. Nigel, A. Krust, and P. Chambon (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature, *330*:444–450.

Pullarkat, R.K. (1991) Hypothesis: Prenatal ethanol-induced birth defects and retinoic acid. Alcohol Clin. Exp. Res., *15*:565–567.

Schardein, J.L. (1993) Chemically Induced Birth Defects. 2nd Ed. Marcel Dekker, New York. pp. 540–562.

Shepard, T.H. (1995) Catalog of Teratogenic Agents, 8th Edition. Johns Hopkins University Press, Baltimore.

Sundaresan, P.R., Collins, T.F.X., Whitby, K.E., Welsh, J.J., Black, T.N., Shackelford, M., Flynn, T., Newell, R.F., and O'Donnell, M.W. (1994) Effects of ethanol and

Whitby, K.E., Collins, T.F.X., Welsh, J.J., Black, T.N., Flynn, T., Shackelford, M., Ware, S.E., O'Donnell, M.W., and Sundaresan, P.R. (1994) Developmental effects of combined exposure to ethanol and vitamin A. Food Chem. Toxicol., *32(4)*:305–320.

Zachman, R.D., and M.A. Grummer (1991) Letters to the editor. Alcohol Clin. Exp. Res., *16*:141.

Zar, J.H. (1984) Statistical Analyses. 2nd Ed. Prentice-Hall, Englewood Cliffs, NJ, pp. 1–696.