though it cannot eliminate the experiments on mammals it can considerably reduce the number of animals used.

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Postimplantation embryo culture for the assessment of the teratogenic potential and potency of compounds

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Summary. Whole rat embryos cultured during the early stages of organogenesis were subjected to a panel of selected chemicals. Of seventeen known in vivo teratogens, seventeen also induced specific malformations in embryos grown in culture. Of ten chemicals which were reported to be negative in in vivo rat teratogenicity studies, eight also did not provoke dysmorphogenic effects in vitro. Of five additionally tested retinoids, all induced multiple malformations. However, concentrations used to induce these effects varied considerably, isotretinoin inducing malformations at 10^{-5} M and arotinoid at 10^{-11} M. The results indicate qualitatively as well as quantitatively a high predictability of this in vitro system and suggest that the postimplantation embryo culture system may also be useful in the prospective testing of new drugs and environmental chemicals.
Key words. Postimplantation rat embryo culture; whole embryo culture; teratogenicity in vitro; validation procedures in teratology; alternative teratogenicity testing; retinoids.

Introduction

Traditional predictive tests for teratogens in humans use rodent and non-rodent species as models. A number of factors make the prospect of an alternative attractive. The tests require significant animal numbers and husbandry resources, and are expensive. A test for the detection of malformations has been developed using rat embryos in culture.

This test, the postimplantation embryo culture system, was originally described by New[21]. It has been used predominantly in studying mechanisms of teratogenicity[1-5] because it mimics in vitro, under controlled conditions, the embryonic development during early organogenesis.

By choosing appropriate conditions, such as culture media[6,7], metabolizing sources[8,9], and drug-delivery systems[10,11], the technique to culture mammalian embryos in vitro has found wider application in the testing of various chemicals to discriminate teratogenic compounds from substances which are devoid of such an effect[12,13].

In the process of validating whole rat postimplantation embryo culture as a system for in vitro teratogenicity testing, we selected a series of chemicals from a list recently proposed as a basis for validation trials for this particular purpose[14]. These compounds represent chemicals of various characteristics (e.g. natural amino acids, drugs, dyes, herbicides, hormones) and include well-known or suspected teratogens for animals and/or humans. Some of the results obtained with these chemicals have also been published recently elsewhere[15].

In order to also obtain information on the technique's usefulness to discriminate on the potency of a series of closely related molecules we chose the class of retinoids; during the last years a large number of retinoids has been synthesized with marked differences in their pharmacological and teratogenic activities.

Materials and methods

The experimental procedures have already been described elsewhere[15]; therefore, only a brief description will be given in this paper.

Animals

10-week-old rats of the RAI strain were obtained from Ciba-Geigy AG (Basel, Switzerland). The animals had food and water ad libitum and were mated in our facilities. Mating was achieved by housing one nulliparous female overnight with one male. Successful copulation was assessed the next day by microscopic observations of sperm in the vaginal smear, and this day was designated as day 0 of gestation.

Culture conditions

Whole rat embryos were explanted on day 9.5 of gestation. They were dissected free of maternal decidua and Reichert's membrane, and embryos which had between 3 and 7 somites were used. 2–3 embryos were then randomly transferred to 30-ml glass bottles containing 4 ml of heat-inactivated male rat serum. The bottles containing the embryos were gassed for 3–4 min with a mixture of 5% O_2, 5% CO_2 and 90% N_2. 16 h later the O_2 concentration in the mixture was increased to 10%, then to 20% 24 h later. As no antibiotics were used, all operations were carried out aseptically. The culture vessels were incubated at 37 °C under continuous rotation (21 rpm) for a total of 48 h using the roller apparatus incubator (Heräus, type B5060 EK/CO2).

Treatment

The chemical compounds tested were obtained from Sigma Chemical Co. (USA), except cyclophosphamide and ethylenethiourea which were purchased from Koch-Light (England), and fluorouracil which was from Serva (Germany). The retinoids were a kind gift of Dr Kistler, Hoffmann-La-Roche (Switzerland). They were always kept in the dark, and the bottles were wrapped in aluminium foil during the culture and treatment period.

Water-soluble chemicals were dissolved in Waymouth medium (Gibco, Switzerland), whereas water-insoluble compounds were normally dispersed in gelatin (0.2% final concentration). Exceptionally, the retinoids were dissolved in 10 µl of absolute ethanol as the drug vehicle. They were applied in a 100-µl volume, and the controls received the identical volume of the solvent administered. Chemicals requiring metabolic activation were tested in culture medium supplemented with S_9-mix, an Aroclor 1254-induced rat liver microsomal extract and its cofactor NADPH[1].

The compounds were always added at the beginning of the culture period and remained there for 48 h. They were tested usually at 5 concentrations, each including an average of 10 embryos. With the exception of the retinoids, factor 3 was used in between the individual concentrations. Because extremely low concentrations of compounds had to be used for the retinoids, factor 10 was chosen in between the individual concentrations. A minimum of three individual experiments were performed to evaluate each compound.

Endpoint measurements

At the end of the 48-h culture period, the embryos were transferred to phosphate-buffered saline, pH 7.4, to be examined under a dissecting microscope. Embryonic growth and differentiation were evaluated only when a beating heart and a full rotation were apparent.

Crown-rump and head length measurements were taken as growth indicators, and somite numbers were used as markers of differentiation. Morphological features of the embryos were evaluated to assess the nature and the extent of the abnormalities. These included: flexion, heart, fore-, mid-, and hindbrain, otic, optic, and olfactory systems, branchial bars, mandibular and maxillary processes, limb buds, somites. Yolk-sac vascularization and size were evaluated in order to assess possible adverse effects of the chemical at extra-embryonic sites.

Statistical analysis

Data on growth (crown-rump length), differentiation (somite number), and yolk-sac size were analyzed by the Student t-test.

Results

The concentrations at which various compounds affected the different developmental parameters assessed are shown in table 1. Compounds which induced embryonic malformations at concentrations lower than those required to affect either growth or differentiation parameters (crown-rump length and somite number, respectively) were considered as in vitro teratogens. The chemicals considered as non-teratogenic in vitro yielded two main types of effects on rat embryos in culture. Most of the negative compounds induced no malformations and did not affect any other developmental parameters even at the highest concentrations tested (e.g. amaranth, cyclamate, cysteine). Some compounds, however, such as diazepam, theophylline and trichlorophenoxyacetic acid induced dysmorphogenic effects in embryos at concentrations also affecting growth and/or differentiation and reducing yolk-sac size and/or vascularization, thus indicating a general toxic effect on embryonic development in vitro at high concentrations (100–1000 µg/ml).

Table 2 presents comparative results of the teratogenicity of chemicals tested in vitro with the whole embryo culture system and in vivo during conventional teratogenicity studies in rats. As can be seen, the in vitro system identified 17 out of 17 teratogens and thus exhibits a sensitivity of 100% for the compounds tested. Eight chemicals evaluated as non-teratogens in rat in vivo studies did not induce specific malformations in cultured rat embryos. No correlation was found for meprobamate and nitrilotriacetate since they appeared as teratogenic in vitro and non-teratogenic in vivo. The systems' specificity to recognize non-teratogens was thus 80% for the compounds tested.

Figure 1 illustrates some of the malformations which were encountered in embryos cultured in the presence of the selected compounds. Two main target sites for teratogenic effects of chemicals are the craniofacial and the rump regions. The compound N,-N′-ethylenethiourea, for example, selectively induced blister formation in the hind-limb bud region only. In any case, each of the 17 individual teratogens induced a particular pattern of malformations which was characteristic for a given compound.

Table 1. Concentrations affecting developmental parameters (µg/ml)[a]

Compounds	Growth (crown-rump length)[a]	Differentiation (somite number)[a]	Morphology (malformations)[b]	Yolk sac (size, vascularization)[a]
Acetylaminofluorene	>30	>30	10[d]	>30
Acetylsalicylic acid	400	400	150	400
Amaranth	>1000	>1000	>1000[e]	>1000
6-aminonicotinamide	0.3	0.3	0.1	3
Arsenate	20	20	10	20
Cadmium chloride	0.05	0.02	0.01	0.05
Caffeine	300	100	30[d]	600
Chlorambucil	10	10	3	30
Cyclamate	>800	>800	>800[e]	>800
Cyclophosphamide[c,d]	>30	>30	30	>30
D-Cysteine	>1000	>1000	>1000	>1000
Dexamethasone	810	270	90	810
Diazepam	100	100	100[d]	100
Diethylstilboestrol	100	30	10	300
Diphenylhydantoin	100	100	50	300
N,N′-ethylenethiourea	300	100	30	1000
Fluorouracil	0.6	0.6	0.3	0.6
Meprobamate	1000	1000	300	1000
Methotrexate	0.8	0.8	0.4	0.8
Nitrilotriacetate	1000	1000	300	1000
Phenobarbital	1000	1000	300[d]	1000
Penicillin-G	>1000	>1000	>1000[e]	>1000
Saccharine	>800	>800	>800[e]	>800
Theophylline	100	300	100	100
Trichlorophenoxyacetic acid	900	900	900[d]	900
Urethane	>300	>300	100	>300
Vincristine sulfate	0.03	0.03	0.01	0.03

[a] Statistically different from controls at $p \geq 0.05$; [b] in a least 30% of the embryos; [c] tested only at one concentration of 30 µg/ml; [d] tested in the presence of the S_9-metabolic source; [e] malformations independent of the concentration were occasionally observed.

Table 2. Comparison of teratogenicity in vivo[a] and in vitro in the rat

		In vitro	
		Positive	Negative
In vivo	Positive	Acetylaminofluorene Acetylsalicylic acid 6-aminonicotinamide Arsenate Cadmium chloride Caffeine Chlorambucil Cyclophosphamide Dexamethasone Diethylstilboestrol Diphenylhydantoin Fluorouracil Methotrexate N,N'-ethylenethiourea Phenobarbital Urethane Vincristine sulfate	
	Negative	Meprobamate Nitrilotriacetate	Amaranth Cyclamate D-Cysteine Diazepam Penicillin-G Saccharine Theophylline Trichlorophenoxyacetic acid

[a] See Shepard[16] and Smith et al.[14].

Table 3 shows the summarized results obtained during the validation of the embryo culture system presented as a comparison between minimal teratogenic concentrations determined in rat embryos in vitro and human maximal plasma levels obtained from published data. Acetylsalicylic acid, for example, induces specific malformations in $\geq 30\%$ of the treated rat embryos at 150 µg/ml, whereas its concentration in human plasma levels off at about 15 µg/ml. Cadmium chloride on the other hand has been reported to reach plasma peak levels of 0.3 µg/ml after occupational exposure of workers to this heavy metal; a concentration of 0.01 µg/ml cadmium was found to be teratogenic to rat embryos in culture.

As can also be seen in table 3, the range of teratogenic concentrations in vitro lies between 3 and 100 times the human maximal plasma levels for most of the chemicals tested (except for cadmium, cyclophosphamide, methotrexate and vincristine, the teratogenic concentrations of which are similar or lower to that measured in human plasma). Maximal concentrations of chemicals tested in vitro include and/or overtake the maximal plasma levels in humans 3–7000 times (theophylline and dexamethasone, respectively). However, the concentrations tested in vitro remain below the human maximal plasma concentrations for the anti-cancer drugs cyclophosphamide, fluorouracil and methotrexate.

Table 3. Comparison of the concentrations (µg/ml) teratogenic in vitro in rat with maximal plasma concentrations (µg/ml) in vivo in man

Compounds	Teratogenicity in vitro (rat) Concentrations (µg/ml) Maximum tested	Minimum teratogenic[a]	Kinetics in vivo (man) Maximum plasmatic
Acetylaminofluorene	30	10	ND[d]
Acetylsalicylic acid	800	150	10–50
Amaranth	1000	NT[b]	ND
6-Aminonicotinamide	800	0.1	ND
Arsenate	100	10	8×10^{-4}–23×10^{-4}
Cadmium chloride	0.4	0.01	0.002–0.3
Caffeine	600	30	5–10
Chlorambucil	30	3	0.4
Cyclamate	800	NT	
Cyclophosphamide[b]	30	30	3–52
D-Cysteine	1000	NT	ND
Dexamethasone	1000	90	0.02–0.13
Diazepam	100	100 T[c]	0.5–2.5
Diethylstilboestrol	100	10	0.04–0.7
Diphenylhydantoin	1000	50	3–45
N,N'-ethylenethiourea	1000	30	ND
Fluorouracil	1	0.3	0.8–50
Meprobamate	1000	300	10–15
Methotrexate	10	0.4	31
Nitrilotriacetate	1000	300	ND
Phenobarbital	1000	300	6–40
Penicillin-G	1000	NT	0.06–1.2–32
Saccharine	800	NT	10–35
Theophylline	300	100 T	20–100
Trichlorophenoxyacetic acid	900	900 T	ND
Urethane	300	100	ND
Vincristine sulfate	0.03	0.01	ND

[a] Specific malformations observed in $\geq 30\%$ of the embryos; [b] not teratogenic; [c] toxic; [d] No information available for these compounds; [e] Tested only at 30 µg/ml.

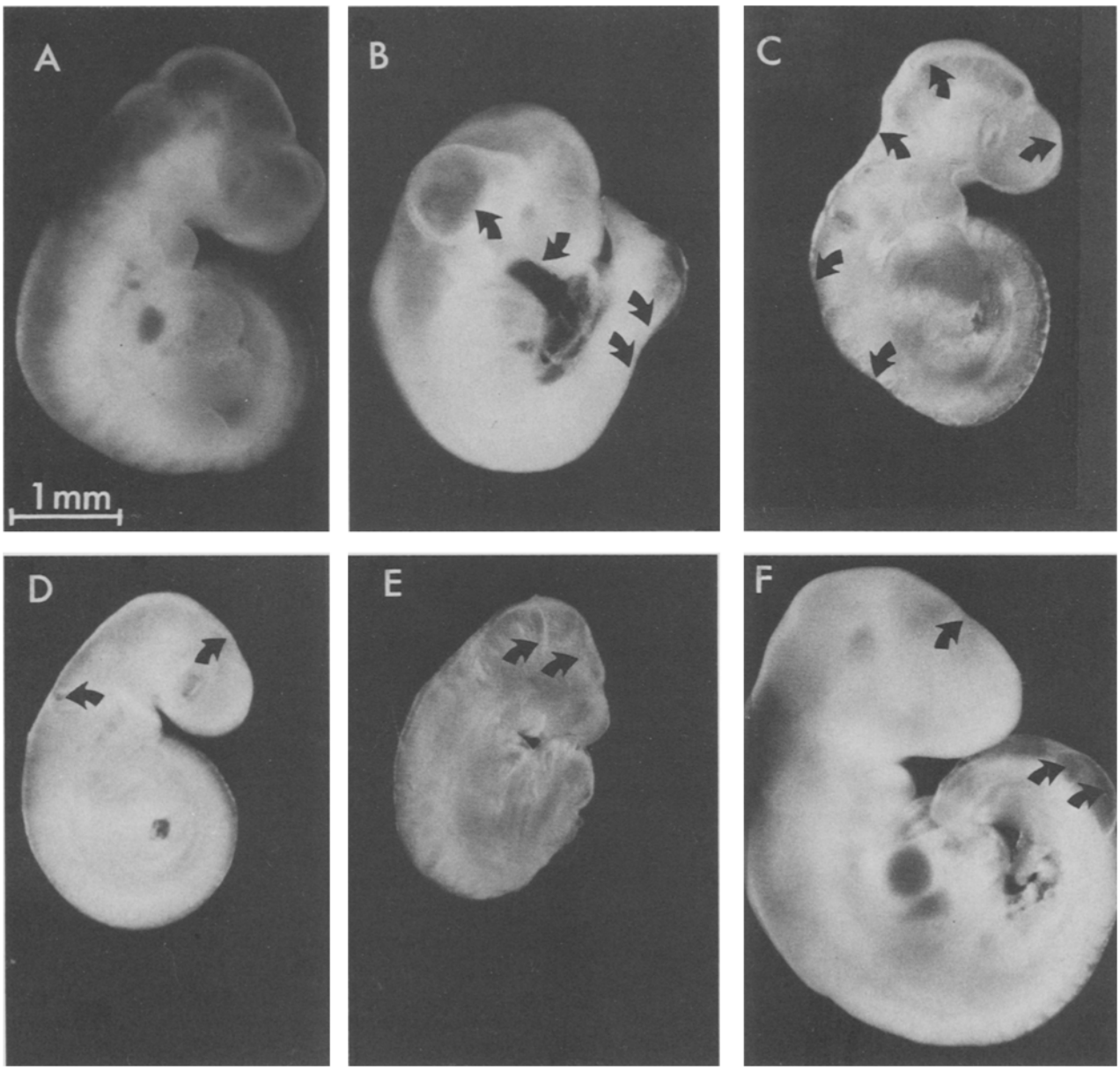
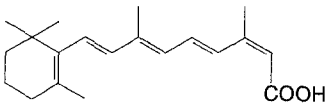
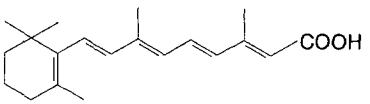
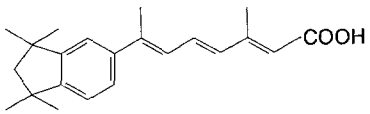
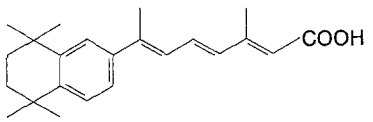
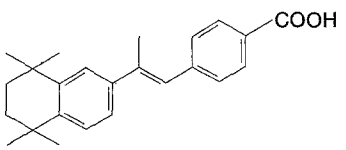


Figure 1. Malformations observed in rat embryos after a culture and treatment period of 48 h (day 9.5 to day 11.5 of gestation). *A* Untreated embryo. *B* Diethylstilboestrol (30 µg/ml). Blister formation in the head region and reduction of the lower telencephalic area. In addition, curvature of the rump region as well as the rump size is affected. *C* Caffeine (100 µg/ml). Abnormal curvature of the neural tube as well as abnormalities in the rhomben-, mesen- and telencephalic area. *D* Acetylsalicylic acid (400 µg/ml). This concentration affected the size of the embryo and induced abnormalities of the optic system and the telencephalic region. *E* Cadmium chloride (0.02 µg/ml). Reduced size and abnormal formation of the head region. *F* N,N′-ethylenethiourea (100 µg/ml). Indentation between the telencephalic and the rhombencephalic region absent. Blister formation in the hind-limb bud area.

The evaluation of the teratogenicity of vitamin A and 4 structural analogs is presented in table 4. Teratogenic concentrations inducing malformations in embryos were found to vary between 10^{-11} M and 10^{-5} M depending on the molecule tested. Comparing our in vitro results with those obtained from in vivo teratogenicity studies in rats, we observed that all retinoids are teratogenic in vivo and in vitro to a very similar extent: Ro 4–3780 was the least active compound (10^{-5} M) and Ro 13–7410 was found to be active at 10^{-11} M. The same ranking orders were obtained in rat teratology studies as reported by Kistler[17].

All retinoids induced comparable malformations in the embryos. As it is shown on figure 2, vitamin A and its structural analogs affected mainly the angle of head inclination and the rhombencephalon and there were atrophic mandibular arches and malformed optic and otic systems.

Table 4. Potency of the individual retinoids in vitro and in vivo in the rat

Code number	Chemical structure	Lowest teratogenic dose/concentration in vitro [M][a]	in vivo [mg/kg/day][b]
Ro 4-3780		10^{-5}	150.0
Ro 1-5488		10^{-7}	0.4-2.0
Ro 13-4306		10^{-10}	0.3
Ro 13-6307		10^{-10}	0.3
Ro 13-7410		10^{-11}	0.01

[a] Malformations observed in ≥30% of the embryos; [b] according to the values presented by A. Kistler[17].

Discussion

The use of the postimplantation whole embryo culture for in vitro teratogenicity testing of chemicals has been described recently [12, 15]. The results obtained and reported in the present paper confirm and extend the previous findings on the excellent correlation observed on the teratogenicity/non-teratogenicity in vitro of chemicals with their teratogenic potential in vivo.

We have attempted to define the teratogenic potential of a compound in vitro by choosing a concentration of a chemical inducing concentration-dependent specific dysmorphogenic effects in ≥30% of the embryos without affecting their growth, differentiation or extra-embryonic sites. A substance was considered non-teratogenic but toxic when a given concentration inducing malformations also reduced growth and/or differentiation and/or extra-embryonic site parameters.

All known teratogens in rats also exhibited teratogenic activity in the in vitro system of cultured embryos. Among the non-teratogens in rats eight out of ten compounds appeared as non-teratogenic in vitro.

For several chemicals, we also attempted to compare the minimal teratogenic concentrations determined in vitro in rat with the maximal plasma levels determined in humans. We observed that some teratogens in vitro are found in human plasma at concentrations similar or higher than those required to elicit malformations in vitro. Furthermore, such compounds are also teratogenic in man. Weak teratogens in vitro are found in low concentrations in the human plasma and, usually, are non-teratogenic in man (e.g. acetylsalicylic acid, meprobamate). Finally, in order to evaluate the teratogenic potency of chemicals, we tested several retinoids with structural similarities in the embryo culture system. Retinoids are well-known teratogens in vivo in several animal species [18] and man [19].

In the embryo culture system as presented in this paper, vitamin A and its analogs were teratogenic within concentrations ranging from 10^{-5} M to 10^{-11} M. The least teratogenic compound in vitro was also the one which required the highest dose to induce teratogenic effect in vivo. Two retinoids of similar reactivity in vivo gave a teratogenic response at the same concentration in vitro (Ro 13-4306 and Ro 13-6307). It is interesting to note that the 13-cis retinoic acid gave a response only at a relatively high concentration (10^{-5} M). This is in accordance with findings obtained from in vivo animal models [17] but contrasts the findings obtained in human beings [19]. Whether this species difference can be explained

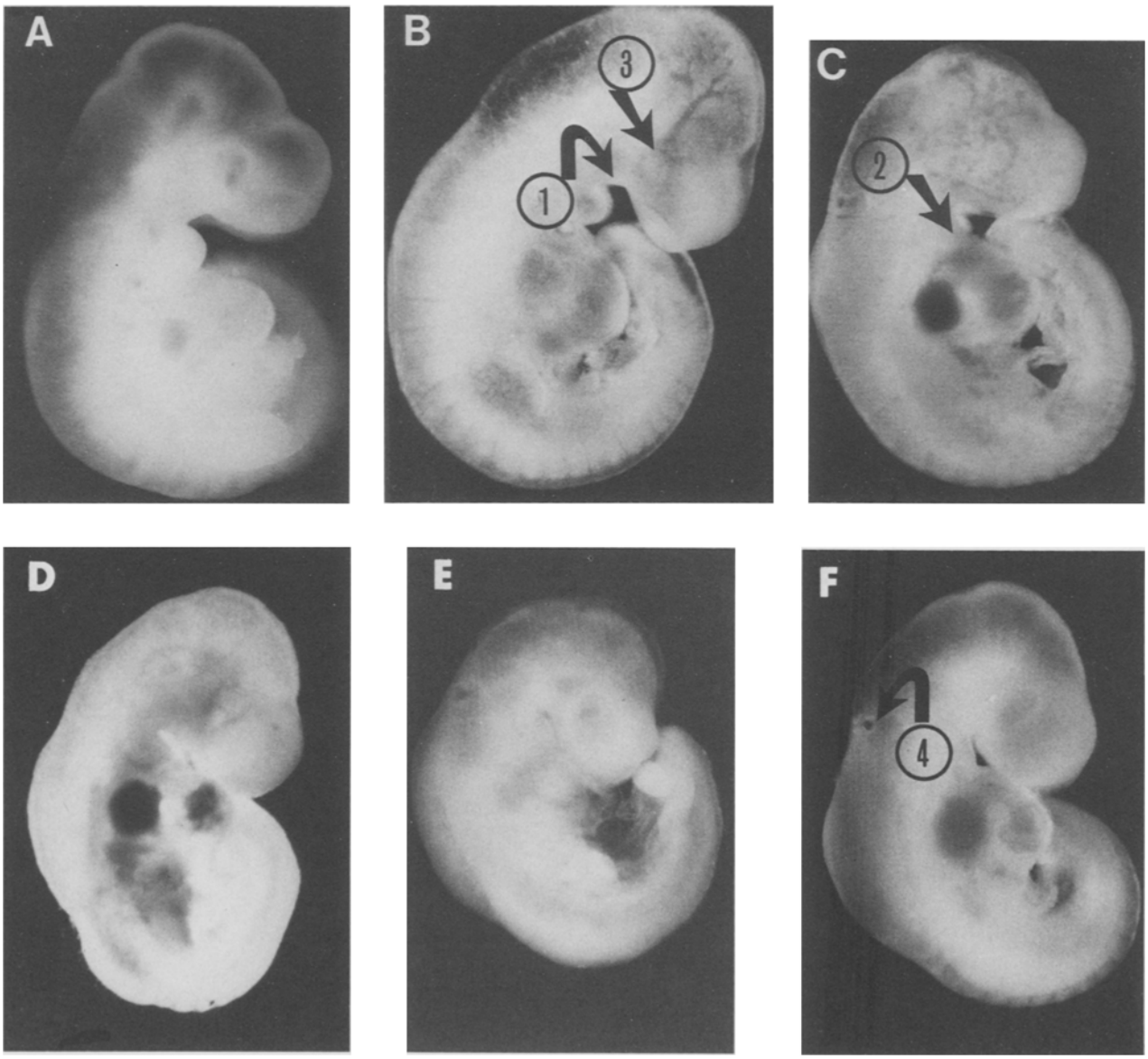


Figure 2. Malformations observed in rat embryos after treatment with various retinoids. The embryos were cultured and treated for 48 h (day 9.5 to day 11.5 of gestation). *A* Untreated embryo; *B* Ro 1–5488 (10^{-5} M); *C* Ro 4–3780 (10^{-7} M); *D* Ro 13–4306 (10^{-8} M); *E* Ro 13–6307 (10^{-8} M); *F* Ro 13–7410 (10^{-8} M). Comparable malformations were observed with all retinoids tested: 1) affected angle of head inclination; 2) atrophic mandibular arches; 3) abnormal optic system; 4) abnormal otic system.

Table 5. Teratogenicity testing:

	In vivo	In vitro
Species applied	Rat, mouse, rabbit	Rat, mouse
Treatment period (days)	6–15 (rats)	9–11; 10–12 (rats)
Endpoints evaluated	Growth (weight, crown-rump length) Differentiation (degree of ossification) Morphology (external, internal)	Growth (crown-rump length) Differentiation (somite number) Morphology (external)
No. of animals used (rat study)	20 ♀/dose 80 ♀ for four doses 80 ♂ 160 (minimal requirement)	5 ♀ = 50 embryos 5 ♀ for five concentrations 18 ♂ for mating and serum 23 (maximal requirement)
Time required	~ 90 days (serial mating)	2 days
Amount of compound required (mg)	2000–30,000	20–30
Approximate costs (sFr.)	80,000	6000

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by a low transport of the 13-cis retinoic acid in vivo to rodent embryos[20] remains an open question.

The malformations induced by retinoids in vitro were reproducibly similar: curvature of the head, transparency of the rhombencephalon, malformation of the otic and optic systems, hypotrophic mandibular arches. Using the whole embryo culture system, Steele et al.[22] compared the teratogenicity of trans-retinoic acid and synthetic retinoids. However, the concentrations used were much higher and ranged around 1.5×10^{-3} M to 3×10^{-2} M. Their observations were mainly indicative of overall embryotoxicity with reduction of growth and differentiation along with malformations.

In summary, the findings reported here strongly point towards the great value of the whole embryo culture system as a testing procedure in the assessment of a compound's teratogenic potential and/or potency. It can certainly be used for the estimation of the possible teratogenicity of drugs at an early stage of their development, since the procedure requires only a very small quantity of the compounds to be tested. Furthermore, the procedure affords not only reliability, sensitivity and specificity but the added advantages of reduced animal numbers, costs and time over in vivo conventional methods (see also table 5).

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