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Teratogenicity screening in standardized chick embryo culture: Effects of dexamethasone and diphenylhydantoin *

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Summary. Teratogenic and toxic effects of DXM and DPH were tested using a standardized chick embryo culture. Survival, growth and malformations were scored with respect to the drug concentrations used. DXM ($>10^{-8}$ mol/l) inhibited the differentiation of the extraembryonic blood circulation and induced craniofacial anomalies. DPH ($>1.5\ 10^{-5}$ mol/l) induced cardiomegaly, craniofacial and somitic anomalies. Both drugs were lethal at 10^{-3} mol/l. Comparison of results obtained with 8 drugs shows that the method has a good discriminative power and specificity and that it can be used as a simple, reliable and economical primary screening test, making it possible to reduce the use of animals in toxicological studies.
Key words. Chick embryo; teratogenicity; screening in vitro; dexamethasone; diphenylhydantoin.

Introduction

The chick embryo has been systematically used in fundamental biological research and also as a system for testing effects of chemical and infectious agents on embryonic development [23]. However, as the application of these agents had not been standardized, the true in ovo concentrations were never precisely known. Also, no standardized scheme of evaluation was used and so the results of different studies were difficult to compare. Thus, despite the fact that avian embryos are easily obtained, and the ethical constraints on their use are less than for pregnant mammalian females, the latter have been used in embryotoxicity tests.

Our experience with in vitro culture of avian embryos [18, 19] allowed us to design an 'artifical egg', i.e., a transparent chamber in which, in the presence of an adequate medium, the development of the embryo can be continuously observed for 4 days. We have defined the relevant qualitative and quantitative criteria of normal development and tested this culture system by using six relatively well-known chemicals. On the basis of this study we have proposed a simple, rapid and economical method for routine screening of chemoteratogens [20].

In the present paper, we study the effects of dexamethasone (DXM) and diphenylhydantoin (DPH), compare them to our previous results and discuss the reliability of this test.

Materials and methods

The production and choice of eggs, technique of explantation of chick embryos, conditions of culture and development of embryos in vitro have been described in detail [20].

The chick embryos were obtained from eggs (Warren variety [10]) preincubated for 20 h at 37.5 °C and 60% humidity. Figure 1 (left) shows the corresponding developmental stage (stage 5 HH [11]). The development of the embryo takes place in the central transparent area pellucida. The latter is surrounded by the area opaca, heavily loaded with yolk particles. The two areas together form the discoidal blastoderm, the periphery of which is attached to the vitelline membrane. The chick embryo at 20 h corresponds to a human embryo about two weeks old.

A large portion of vitelline membrane with the attached blastoderm was excised from the yolk and transferred to a transparent silicone chamber (fig. 1, center). The preparation was turned upside-down and spread over the ring protruding from the bottom of the chamber. Constant volumes of culture medium were injected below and poured over the preparation. The chamber was closed by a perspex lid and incubated at 37.5 °C. Development was observed under a binocular microscope and the dimensions and morphological criteria were collected in a 'curriculum vitae' systematically established for each preparation [20].

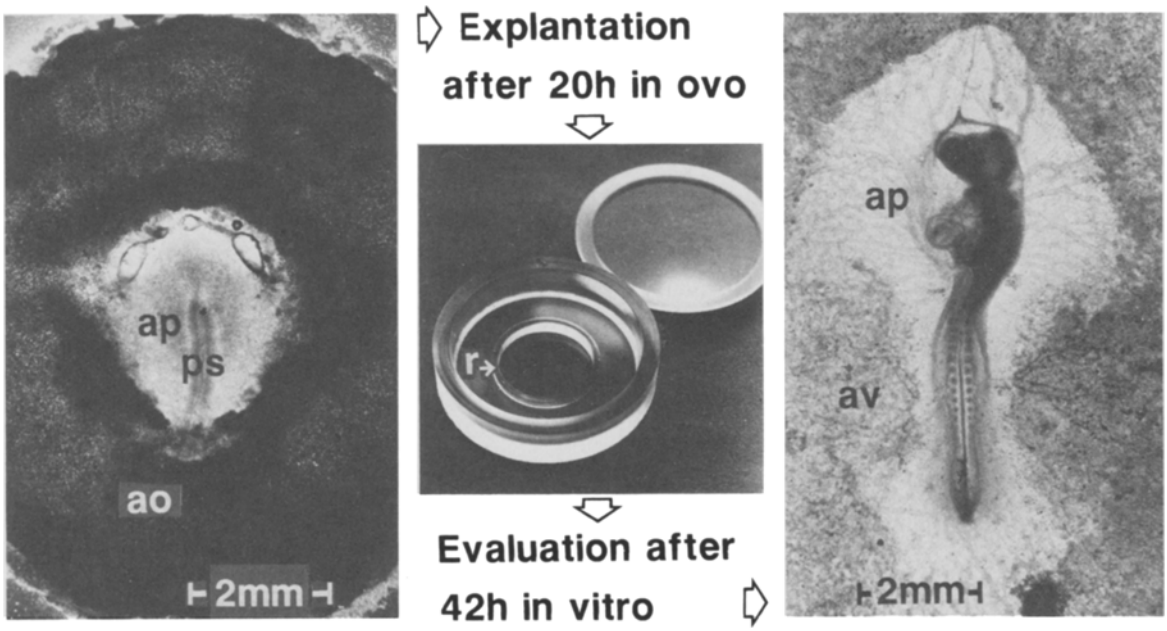

Figure 1. Chick blastoderm and the incubation chamber. *Left:* A normal Stage 5 HH blastoderm excised from an egg preincubated for 20 h. *ap:* area pellucida (place of embryonic formation), *ps:* primitive streak (axis of future embryo), *ao:* area opaca (formation of extraembryonic membranes). *Center:* The chamber consists of a silicone base and a perspex lid. The ring (*r*) protruding from the bottom of the chamber is 25 mm in diameter and serves to fix the vitelline membrane supporting the growth of the blastoderm. *Right:* a normal Stage 15 HH embryo as obtained after 42 h of incubation in the chamber is characterized by: closed neural tube, right angle cranial flexure, 1 visceral pouch, cervicothoracic flexure and rotation, 5 brain vesicles, invaginated optic and otic vesicles, 24 somites, regularly beating heart with well recognizable atrial and ventricular segments and vitelline circulaton with free erythrocytes.

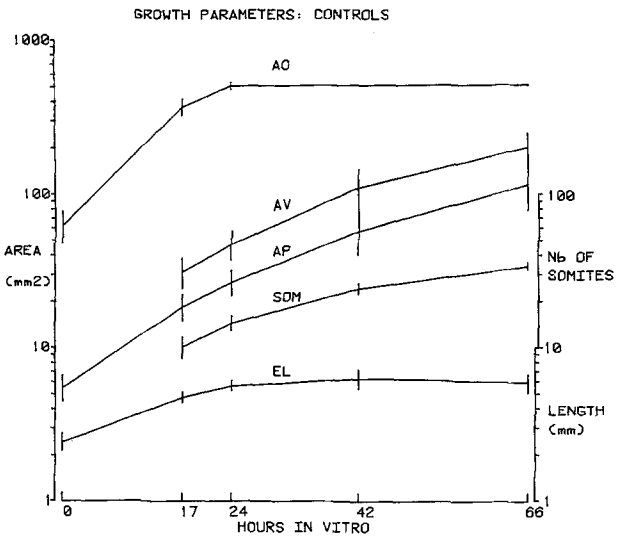

Figure 2. Growth of the blastoderm in vitro under control conditions. *AO:* area opaca, *AP:* area pellucida, *AV:* area vasculosa (*AO-AP*), *SOM:* number of somites, *EL:* length of the embryo. Based on 150 cultures, vertical bars correspond to two standard errors. The expansion of AO is limited by the ring of the chamber. The shortening of the embryo corresponds to the bending of the embryonic body.

DXM (21 dihydrogenphosphate dinatrium salt, Mepha) and DPH (5,5 diphenylhydantoin, Sigma D 4007) were dissolved in the culture medium (5.8 g NaCl, 0.15 g KCl, 0.11 g $CaCl_2 \cdot 2H_2O$, 0.16 g $MgCl_2 \cdot 6H_2O$, 0.36 g KH_2PO_4, 1.91 g $Na_2HPO_4 \cdot 2H_2O$, 1.5 g glucose in 1 l water; pH 7.4; 224 mOsm). DMX was used at 10^{-9} to 10^{-3} mol/l and DPH at $5 \cdot 10^{-5}$ to $6 \cdot 10^{-4}$ mol/l. The solutions were mixed (1:1) with thin egg albumen, which conferred a good bactericidal power on the medium. Eight to twelve embryos were used for each concentration and the control series run in parallel. The attribution of the doses to the embryos was randomized.

The growth and morphogenesis of the embryos were evaluated after 42 h and compared to the morphological criteria and dimensions characterizing the stage 15 HH (fig. 1, right and fig. 2). In uncertain cases, additional evaluations were made after 66 and 90 h. The quantitative and qualitative parameters from each "curriculum vitae" were introduced into a VAX computer and analyzed using the 'Oracle' data exploitation system. The dose-response curves were evaluated by covariance analysis [27] and were also submitted to robust regression analysis [21]. The evaluation of each drug was completed in 3 weeks.

Results

The malformations induced by DXM and DPH are illustrated in figures 3 and 4. The dose-response relationships for the growth parameters and survival are shown in figure 5.

Dexamethasone. The main target for this substance was the craniofacial region: the telencephalic overgrowth did not take place, the anterior neuropore was protruding forward and the maxillo-facial processes did not form. This effect was clearly visible at 66 h (fig. 3, left). In addition, differentiation of the extraembryonic vascular network was also altered: preferential blood channels did not form and the blood vessels remained as capillaries (fig. 3, right). At 0.1 µmol/l, the expansion of the extraembryonic area was significantly decreased (fig. 5, upper left) and the anomalies were present in 50% of embryos (fig. 5, upper right). Up to 10 µmol/l, all embryos had a functional blood circulation and were scored as living. The teratogenic effect was expressed within five orders of dilution whilst the lethal effect was complete between 0.1 and 1 mmol/l.

Diphenylhydantoin. The most important effects were seen on the heart. The fusion was not affected but the subsequent shaping was constantly abnormal. Namely, the atrial and ventricular segments became progressively dilated (increase in diameter, thinner walls). Figure 4 shows that the extent of this anomaly, present already after 24 h, was dependent on the dose. The heart was often so large that the rotation of the embryo was inverted. In addition, the branchial region was also underdeveloped and the somites were less compacted and differentiated. At 150 µmol/l, all the growth parameters were significantly decreased (fig. 5, lower left) and 50% of embryos had an anomalous although still beating heart. As shown in figure 5 (lower right), the teratogenic and lethal effects were complete between 50 and 200 µmol/l and between 400 and 800 µmol/l, respectively. Regression lines obtained for 2 experimental series (open and filled symbols)

Concentrations (in $mol \cdot l^{-1}$) affecting the development of the chick embryo in vitro

Substance	Normal survival up to	Anomalies Malform. in 50%	Perturbation extraemb. memb.	Growth embryo	Mortality LC50	LC100
Dexamethasone	10^{-10}	10^{-7}	10^{-8}	10^{-6}	$3 \cdot 10^{-4}$	10^{-3}
Methotrexate	10^{-8}	$2 \cdot 10^{-7}$	10^{-7}↑	$2 \cdot 10^{-7}$	$2 \cdot 10^{-7}$	10^{-6}
Cadmium Cl	10^{-7}	$4 \cdot 10^{-7}$	10^{-6}	10^{-5}	$2.5 \cdot 10^{-5}$	10^{-4}
Diphenylhydantoin	$5 \cdot 10^{-5}$	10^{-4}	10^{-4}	10^{-4}	$6 \cdot 10^{-4}$	10^{-3}
Phenobarbital	10^{-4}	$5 \cdot 10^{-4}$	$2.5 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$8 \cdot 10^{-3}$	10^{-2}
Caffeine	10^{-4}	$7 \cdot 10^{-4}$	10^{-3}	$5 \cdot 10^{-3}$	$4 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
Aspirin	10^{-4}	$5 \cdot 10^{-4}$	NS+	NS+	NS+	NS+
Saccharin	$5 \cdot 10^{-4}$	$2.5 \cdot 10^{-3}$	NS++	NS++	NS++	NS++

↑ increase in dimensions; NS no significant effect; + max. concentration tested 10^{-3}; ++ max. concentration tested 10^{-2}.

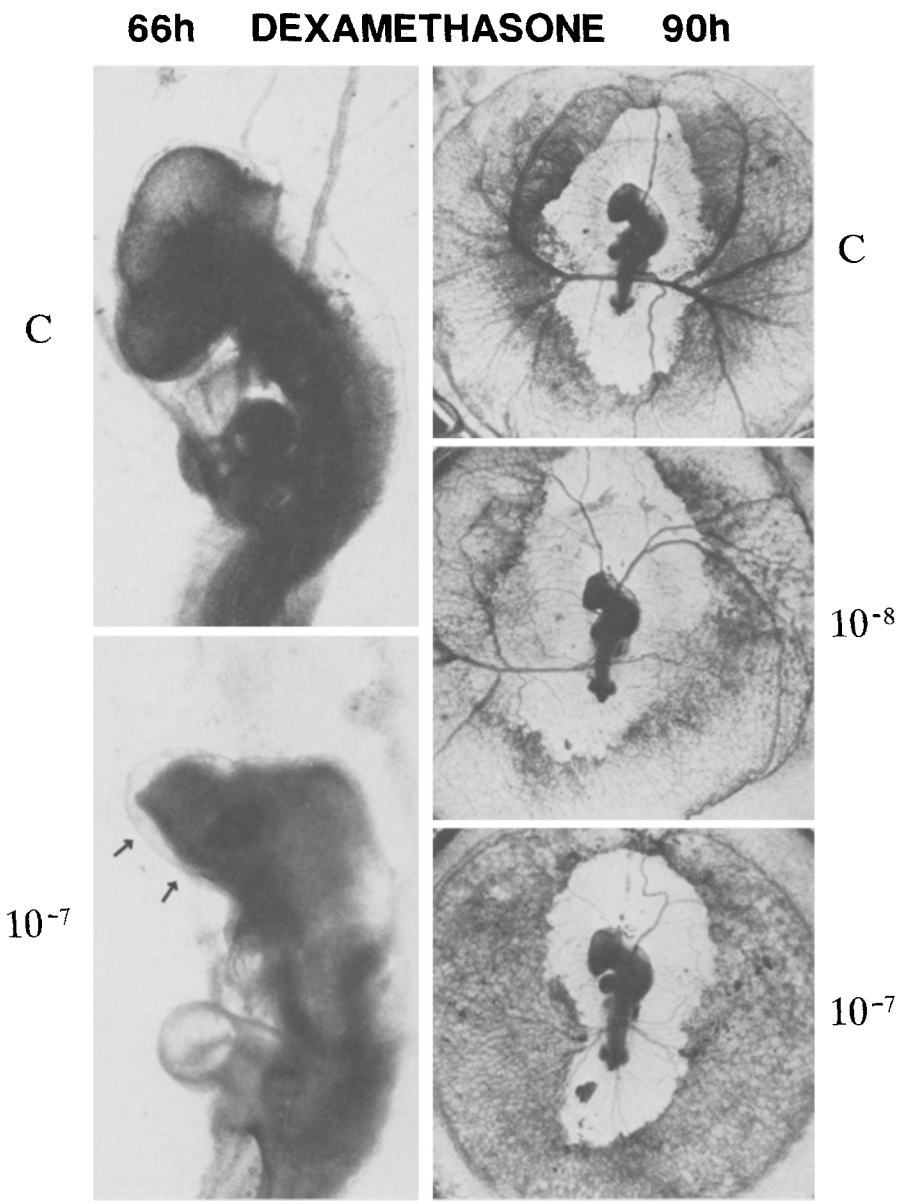

Figure 3. Developmental anomalies produced by dexamethasone. *Left:* C: Control embryo; 10^{-7}: Characteristic craniofacial defect present in 50% of embryos at this concentration. *Right:* at 10^{-8}, the differentiation of extraembryonic vascular network is already inhibited although the embryos are still normal.

were superimposable (maximal values of F obtained for differences between slopes and elevations were respectively $F_{1/55} = 1.8$ and $F_{1/56} = 0.1$).

Discussion

Effects of DXM and DPH. Glucocorticoids, when applied at early organogenesis, can induce facial coloboma, cleft palate, dwarfism and delayed ossification in rodents[8, 22, 25] and birds[13, 17]. In our culture, DXM is also clearly teratogenic: from 10^{-8} mol/l it inhibits the growth of extraembryonic adnexa and at 10^{-7} mol/l it induces craniofacial anomalies. These concentrations are comparable to those effective in tissue cultures. On murine BCL_1 cells or on isolated chick embryonic cells, DXM inhibits the DNA synthesis at 10^{-8} and 10^{-10} mol/l respectively[6, 24].

In man, malformations due to glucocorticoids seem to be rare. In newborns whose mothers were treated with DXM a cleft palate seems to occur at higher frequency than would be predicted by epidemiological studies[2, 7]. Human embryonic cells do have glucocorticoid receptors which are saturable by DXM at about 10^{-8} mol/l [8]. DXM crosses the human placenta (the mother to fetal ratio of plasma concentrations is 3 to 4[4, 5]), but it is likely

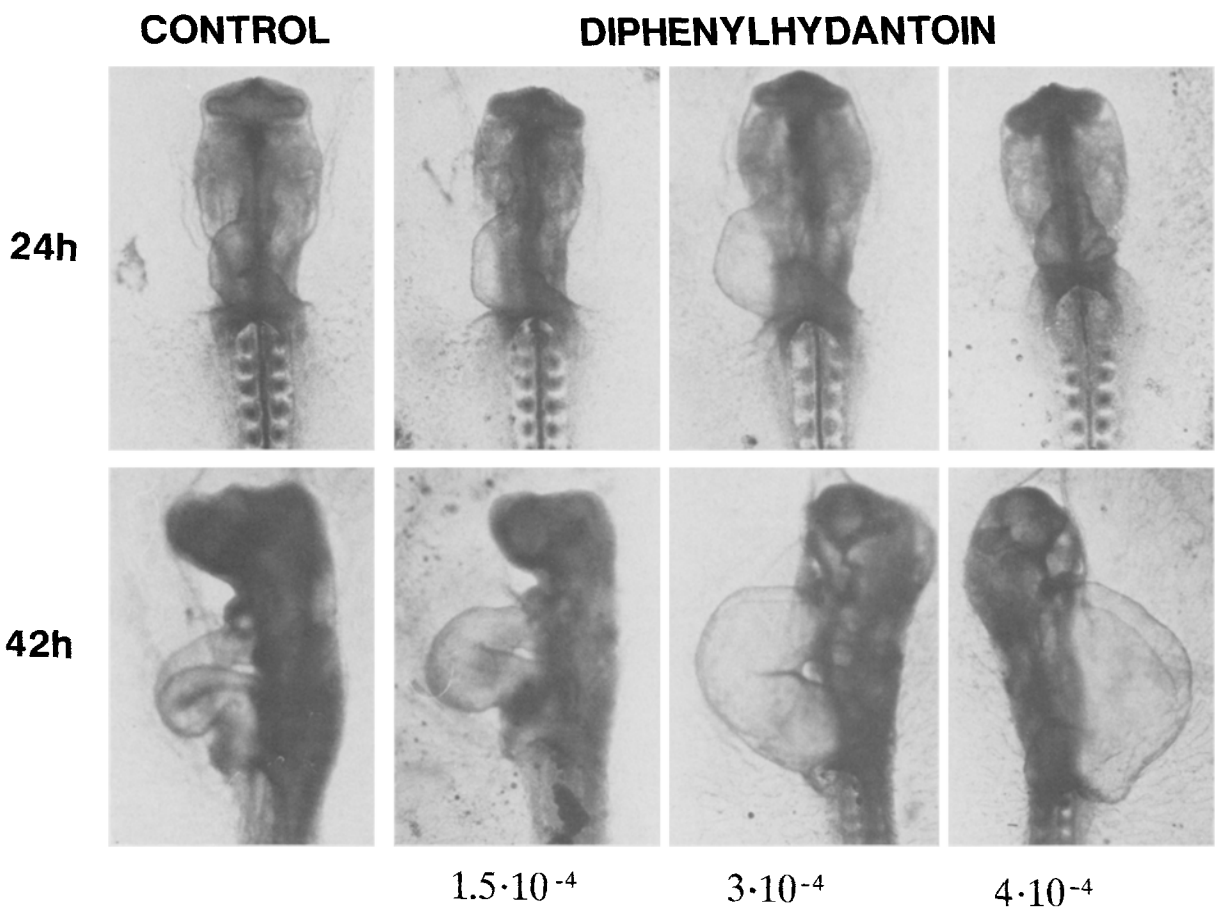

Figure 4. Developmental anomalies produced by diphenylhydantoin. *Upper panel:* Stages 10 HH showing alteration of the heart morphogenesis. *Lower panel:* Stages 15 HH with markedly dilated heart and inverted rotation of the embryo. At 0.3 and 0.4 mmol/l, the hearts were not beating. In addition, the differentiation of brain vesicles and branchial region is abnormal.

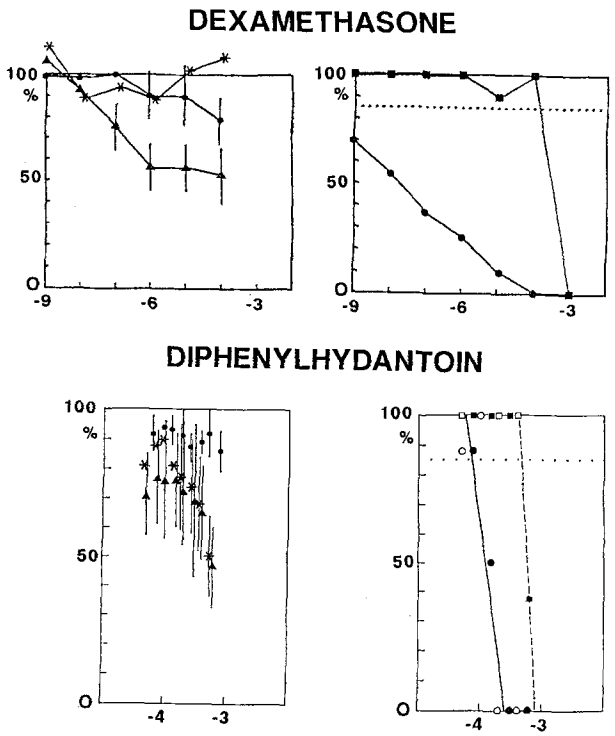

Figure 5. Growth and survival in presence of DXM and DPH. Abscissae: concentrations used in log scale. *Left panels:* Length of the embryo (●), surface of AP (▲) and surface of AV (*) expressed in percent of controls. Values significantly different from controls are indicated by bars corresponding to 2 standard errors. *Right panels:* fractions of normal embryos (circles) and live abnormal embryos (squares). Open and filled symbols: 2 determinations giving superimposable values fitted by the regression lines. Dotted line: minimal normal survival in controls.

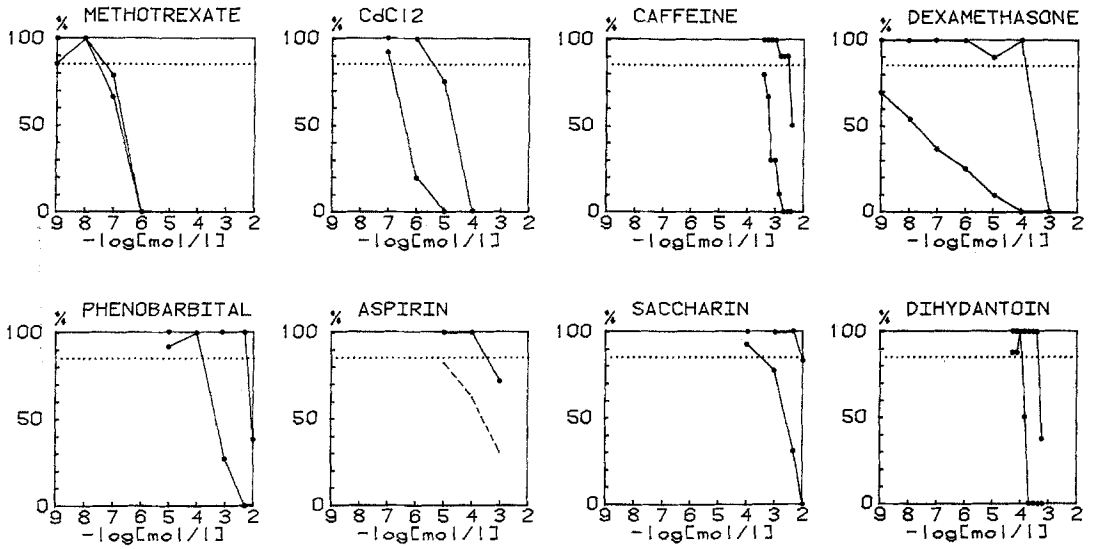

Figure 6. Dose-response curves for the chemicals tested. Abscissae: used concentrations. Ordinates: fraction of survival. Left lower curves and right upper curves represent live normal and live abnormal embryos respectively. With Aspirin, hypotrophic area vasculosa was detectable (dashed line) or well marked (full line) but no malformations were found. Caffeine and DPH were tested with dilutions grouped around threshold values estimated in preliminary experiments.

that the concentration in fetal tissues does not often reach teratogenic levels after the therapeutic doses given to pregnant women. As DXM is about 30 times more potent than natural glucocorticoids, about 0.5 µmol/l of free cortisol in the fetal plasma would be necessary for the appearance of malformations. As the maternal plasmatic corticosteroid binding in pregnancy increases[28] and the fetal plasma equilibrates to only one third of the maternal value[4,5], the total maternal plasmatic glucocorticoids should exceed about 2.5 µmol/l. Such exceptionally high levels (Cushing's syndrome) occur sometimes during the first 12 weeks of pregnancy, and do indeed induce a cleft palate[15].

DPH is teratogenic in rodents[3,25] and in man[1,12,16,25]. Therapeutical doses may lead to levels of 20 µg/ml in the maternal and fetal plasma[26]. DPH induces the fetal hydantoin syndrome, i.e., craniofacial, cardiac and limb anomalies[1,12,16]. Our culture reproduces these malformations, especially the cardiomegaly. Interestingly, 50% of embryos are malformed at about 10^{-4} mol/l which is a concentration sometimes reached in humans[26]. In addition to its teratogenic action, DPH also reproduces in the chick embryo the inhibitory effect on excitable membranes[9], i.e., cardiac arrhythmia and arrest in diastole. Because both DXM and DPH induce craniofacial defects in animals, it has been proposed that this effect is mediated via the glucocorticoid receptor IB on which DPH acts as agonist. DXM binds to both IB and II glucocorticoid receptors[14]. Our results do not contradict this hypothesis: the DXM dose-response curve extends over 5 orders of dilution as if there were multiple DXM effects occurring at different thresholds. The DPH dose-response curve of DPH (about 10 times weaker IB agonist[8]) is steep and inside the range of DXM.

Comparison with previous results: evaluation of the method. Figure 6 compares the dose-response curves for the eight chemicals tested up to now. Remarkably precise and steep variations in the response were obtained using dilutions distributed around a known threshold (fig. 6: caffeine and DPH). Each drug is characterized by two curves delimiting the living normal embryos from living abnormal ones, and the living abnormal embryos from dead abnormal ones. The closer these curves are together (e.g. methotrexate) the lower is the chance of survival of abnormal embryos, and the drug is mostly toxic. On the contrary, the more separated they are (e.g. DXM or cadmium) the more can malformations be expected, and the drug is also a teratogen.

The table lists the eight tested drugs according to their teratogenic potency in the chick embryo and reveals also that the general toxic effects are best observed in the extraembryonic membranes. This is probably because the area opaca and area vasculosa are metabolically the most active regions of the blastoderm[18,19].

Thus, the chick embryo seems to react to drugs similarly to other vertebrates. The concentrations to which it responds are comparable to those found in other in vitro or in vivo systems (see above and ref.[20]), and, when these concentrations are reached in man, they may actually induce toxic effects or malformations (methotrexate, DPH, phenobarbital and dexamethasone). Furthermore, malformations produced by a given drug in the chick are similar to those described in other species including man. In conclusion, the embryotoxicity test with the chick embryo in vitro is characterized by good sensitivity, resolution and reproducibility, and it reveals specific patterns of responses to each drug. The test is simple, economical and suitable for a rapid preliminary screening. Even

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.