A review of the contribution of whole embryo culture to the determination of hazard and risk in teratogenicity testing

WILLIAM S. WEBSTER[1]*, PATRICIA D.C. BROWN-WOODMAN[2] and HELEN E. RITCHIE[2]

[1]Department of Anatomy and Histology and [2]Department of Biomedical Sciences, University of Sydney, Lidcombe, Australia

ABSTRACT   Whole embryo culture appears to be an excellent method to screen chemicals for teratogenic hazard. Compared to *in vivo* testing it is cheap and rapid and does not involve experimentation on live adult animals. Also in the important area of risk estimation whole embryo culture offers distinct advantages over *in vivo* teratogenicity testing. Adverse embryonic outcomes (malformations or embryotoxicity) are directly related to the serum concentration of the compound being tested and can be compared to the serum concentration in the human. A similar comparison is not possible after *in vivo* testing because for most compounds there are major pharmacokinetic differences between humans and experimental animals. *In vivo* testing is also limited by the possibility that metabolites that occur in the human do not occur in the test animal. This problem can be overcome in the *in vitro* system by adding the metabolite directly at the desired concentration either with or without the parent compound. There is only one major disadvantage to *in vitro* testing and that is the limited period of embryogenesis that is undertaken in the commonly used culture system. This restricts the range of malformations that can be induced and may render the testing system unsuitable for compounds that are likely to exert their major toxicological effect late in gestation. Any evaluation of whole embryo culture for hazard and risk assessment in teratology must take into account the limited value of currently used *in vivo* methods. Over 2000 chemicals have been reported to be teratogenic in experimental animals exposed *in vivo* (Shepard, *Catalog of Teratogenic Agents,* 1989). In comparison only about 20 chemicals are known to cause birth defects in the human. This large number of *in vivo* false-positive cannot easily be distinguished from true-positives. In this respect *in vivo* testing is severely deficient. The embryo culture testing system would also be expected to produce many false-positives; but by comparing effective drug concentrations with human therapeutic concentrations they can be differentiated from true-positives. The most serious deficiency for an *in vivo* or *in vitro* teratogenicity testing system would be false-negatives. This has not been a problem in the validation of *in vitro* testing so far (except perhaps procarbazine), but difficult drugs such as thalidomide were not included. Thalidomide remains an important index chemical because it is not teratogenic in rats or mice but is teratogenic in the rabbit and human. It is likely that these species differences are due to metabolic differences between species and it is possible that if the proximate teratogen/s of thalidomide were identified they would be teratogenic in rat embryo culture. Whole embryo culture remains a very powerful technique that should continue to contribute to the determination of the safety of drugs and other chemicals during pregnancy.

KEY WORDS: *whole embryo culture, teratogenicity testing, teratology, risk, hazard, birth defects*

Introduction

The development of a reliable and technically easy method for the *in vitro* culture of rodent embryos during their organogenic period (New, 1978), presented a great opportunity to expand and improve teratology testing methods. Denis New and his colleagues developed and refined the procedures to the extent that *in vitro* development of early somite rat embryos was equivalent to that seen *in vivo*.

Since the early 1970s whole embryo culture has been used in many laboratories for the study of various aspects of teratology. This has been driven, at least in part, by the efforts to replace, reduce and refine toxicity testing (Piersma, 1993). It has proved to be particularly popular in the study of teratogenic mechanisms in the absence of maternal effects (Cockroft, 1980; Cockroft and Steele, 1987; New, 1990) and this remains a major use of the technique not covered in this review. Another use for embryo culture, first proposed by Denis New, was as a cheap method to

screen chemicals for potential teratogenicity (New, 1978). It has been estimated that there are 65,000 chemicals in the general environment (Schardein, 1993) with hundreds or thousands of new chemicals being added each year. Only a few thousand of these chemicals have been examined for potential teratogenicity.

It is a popular belief, often encouraged by uncritical newspaper reporting, that chemicals in the environment are responsible for many birth defects. This idea originated from two events in the 1960s. The first was the marketing of thalidomide and the resultant 10,000 severe birth defects seen around the world. The second was the publication of the book *Silent Spring* by Rachel Carson (Carson, 1962). In this book she documented how pesticides were entering the food chain and interfering with the reproduction of birds. By implication, there were potentially similar effects in the human. Both the thalidomide disaster and Rachel Carson's book received widespread media coverage and created a heightened awareness of the potential for chemicals to cause birth defects.

Current methodology for testing drugs and other chemicals for teratogenicity (segment II testing) was introduced in the United States in the 1960s in response to the outcry which followed the marketing of thalidomide; indeed many of the parameters of the test are based on the ability to demonstrate the teratogenicity of thalidomide. The usual protocol involves daily administration of the test substance to pregnant animals during their organogenic period and examination of the products of gestation shortly before parturition. Two species are required, in practice this is usually the rat and the rabbit, and three dosage levels such that the highest dose causes some signs of maternal toxicity.

This testing procedure is good at detecting hazardous chemicals but it is very expensive. Hence, whole embryo culture, as suggested by New was attractive as a potentially cheap and simple way of exposing rodent embryos to test chemicals during their critical organogenic period. The proposed method was to add chemicals to the culture medium and to use morphological endpoints to detect abnormal development.

Embryo culture and the determination of hazard

Initial validation studies

In the early 1980s several teratology groups considered using embryo culture for chemical screening and there was considerable discussion about the need for validation (e.g. Kimmel *et al.*, 1982). This was generally interpreted as whether use of the technique would allow the investigator to accurately predict known teratogenic and non-teratogenic chemicals. Schmid at the Sandoz Laboratory in Switzerland attempted such a validation by testing 39 chemicals in whole rat embryo culture using the effects on embryonic growth (crown-rump length), differentiation (somite number) and morphology (neural tube, brain, visceral arches, heart, otic, optic and olfactory systems and limbs) as the endpoints (Schmid, 1985). Eighteen of the test substances were "undisputed" animal teratogens and the remaining 21 substances were classified from whole animal teratology testing as non-teratogens. The test compounds were added to the culture medium (5 ml heat-inactivated rat serum with added S-9 mix as a metabolic activating system) and 9.5 day rat embryos (3-5 somites) were cultured for 48 h. Each compound was tested at 3-5 different concentrations.

The results showed that each of the known teratogens caused malformations *in vitro*, although the types of malformations were

not detailed. The non-teratogens induced two types of response in the culture system. Ten of the compounds had no effect on embryonic morphology even at "high" concentrations. Ten of the remaining compounds showed deviations from controls (undefined morphological changes), but these changes were accompanied by effects on growth and differentiation of the embryo as well as reduced growth and vascularization of the yolk sacs. o'-Tolidine (a metabolite of trypan blue) was the only non-teratogen which induced malformations at concentrations that did not affect other embryonic or extra-embryonic sites (i.e. it was an apparent false-positive). This important study suggested that rat embryo culture could be successfully used for chemical screening, but it also identified some problems.

Test compound solubility

First was the difficulty of testing compounds that were not soluble in water. In Schmid's study they were suspended in gelatin, but it was not known how this affected their biological availability. In later studies it was shown that water insoluble compounds could be dissolved in other solvents before addition to the culture medium but this added the confounding variable of potential toxicity of the solvent (Kitchin and Ebron, 1984).

Toxicological endpoints

A second problem was the definition of teratogenic. This is an issue for both *in vivo* and *in vitro* teratogenicity studies since some compounds cause toxicity (e.g. embryonic growth retardation) without causing malformations. Hence it is necessary to differentiate between a true teratogenic effect and an embryotoxic effect. A positive teratogen was defined by Schmid (1985) as a compound which caused more than 10% of the *in vitro* tested embryos to have "malformations" at a concentration having no effect on overall embryonic growth and differentiation. It was reported that the "teratogenic compounds" caused malformations at lower concentrations than those required to cause changes in growth and differentiation, while the "non-teratogens", with one exception, only showed changes at concentrations affecting growth and differentiation. In a later paper from the same laboratory a teratogen-positive compound was defined as a chemical inducing concentration-dependent specific dysmorphogenic effects in ≥30% of the embryos without affecting their growth, differentiation or extra-embryonic sites (Cicurel and Schmid, 1988). The reason for this definition is to avoid positive results due to general toxicity, but in view of the frequent association between growth retardation and teratogenicity in *in vivo* studies, the definition may not be appropriate. Other studies testing known human teratogens in rat embryo culture have shown that malformations, growth retardation and reduced somite number can occur at the same concentration (e.g. Webster *et al.*, 1986). Schmid also chose to exclude embryos from assessment if they had no heart beat or if they had failed to fully rotate into the concave position, although these can be clear signs of a toxic effect. Failure to turn, double hearts and abnormalities of the caudal part of the embryo can also be caused by physical damage to the explant during the preparation phase and investigators may exclude such embryos as "artifacts" (Klug, 1991).

Other investigators have also discussed the difficulty of differentiating between a teratogenic and an embryotoxic effect in cultured rat embryos (e.g. Brown-Woodman *et al.*, 1994a,b, 1995). When a range of solvents was tested using rat embryo culture a common

finding was general embryonic growth retardation with the telencephalon of the brain being most affected. Should this be interpreted as a general embryotoxic effect with the differential effect on the forebrain resulting from different rates of growth at this stage of development? Or does it represent a teratogenic effect that might be evident as microcephaly *in vivo*?

Metabolism

The Schmid study (1985) also brought up a third problem. It is recognized that teratogenic activity is not always due to the parent compound, but may be caused by one or more metabolites formed by maternal metabolism (Fantel, 1982). If the metabolites are known, and are available, they can be added to the culture medium and tested as any other compound (e.g. Webster *et al.*, 1986). Where the metabolites are unknown, or poorly characterized, the addition of metabolic activating systems has been proposed. Tested systems have included microsomes, postmitochondrial supernatants and hepatocytes (e.g. Schmid, 1985; Kitchin *et al.*, 1986; Oglesby *et al.*, 1986). In the Schmid study (1985) all chemicals were tested in the presence of a rat liver microsomal preparation.

The most serious failure of any teratogen screening system would be so-called false-negatives. How confident can we be that compounds identified as non-teratogens in rat embryo culture do not have potentially teratogenic metabolites? The addition of various metabolic activating systems to the culture system partly solves the problem (Kitchin *et al.*, 1986). In some instances, such activating systems have been shown to metabolize pro-teratogens into their active metabolites. For example, cyclophosphamide is only teratogenic to rat embryos in culture when it is added with a microsomal metabolic activation system (Fantel *et al.*, 1979; Kitchin *et al.*, 1981; Schmid *et al.*, 1981). However, a rat postmitochondrial metabolic system in rat embryo culture was not able to activate procarbazine into a teratogenic compound (Schmid *et al.*, 1982).

The use of rat metabolic activating systems does not ensure that the same metabolites that occur in the human will be formed; the use of human derived metabolic activating systems may avoid this problem (Rogiers *et al.*, 1995). Another potential problem is the concentrations of the various metabolites. Some metabolites accumulate during normal human dosing (e.g. 4-oxo-isotretinoin during isotretinoin dosing) and the concentration of the metabolite may be greater than the parent compound and may be the cause of teratogenicity. In the culture system, even with a metabolic activation system it is unlikely that the metabolites will achieve the concentrations seen in the human. These quantitative and qualitative problems with metabolites are not restricted to the *in vitro* system but are the same if not greater in the *in vivo* system. Optimization of conditions for the various activating systems, and time course studies of their activity are important areas that must be standardized before these systems can be routinely used in whole embryo culture.

Other tested alternatives for examining the potential teratogenicity of metabolites include using sera from dosed rats as the culture medium, reasoning that the sera will contain the metabolites. For example the drug procarbazine needs to be metabolized into carbazine to exhibit teratogenic activity. Procarbazine was not teratogenic when tested *in vitro* with a S-9 mix (Schmid, 1985) but teratogenicity was demonstrated when embryos were grown in serum from procarbazine-treated animals (Schmid *et al.*, 1982).

Several investigators have used the same approach with human serum as the culture medium. This was pioneered in Norman Klein's laboratory where it was demonstrated that sera from five subjects undergoing chemotherapy and six subjects receiving anticonvulsants was either lethal or teratogenic to rat embryos (Chatot *et al.*, 1980). Although this approach seems theoretically sound, it is limited by the inconsistent growth of rat embryos in human sera (Cockroft, 1991). Nevertheless, it offers tremendous possibilities and has recently provided intriguing data on isotretinoin teratogenicity (Van Maele-Fabry *et al.*, 1993).

Further validation studies

Brown (1987) criticized the test chemicals used by Schmid in his initial validation studies, claiming that only one of the non-teratogens used was an acceptable test compound. The reasoning for this criticism was that the chosen non-teratogens were essentially non-toxic and would be negative in any system while the commonly tested "teratogens" were highly biologically active antimetabolites which would be positive in virtually any system. A list of "appropriate" test compounds has been published as part of a consensus workshop on *in vitro* teratogen testing (Smith *et al.*, 1983).

Perhaps with Brown's constructive criticism in mind Cicurel and Schmid (1988) did a further validation test with 27 chemicals selected from the consensus list of test compounds (Smith *et al.*, 1983). Seventeen of the chemicals were known *in vivo* teratogens and 10 chemicals were negative *in vivo*. Chemicals requiring metabolic activation were tested in culture medium supplemented with S9-mix, an Aroclor 1254-induced rat liver microsomal extract and its cofactor NADPH. Rat embryos (9.5 days) were cultured for 48 h. All 17 of the known teratogens were positive in the *in vitro* system while 8 out of 10 of the non-teratogens were negative. Meprobamate and nitrilotriacetate were apparent false-positives as they caused malformations at concentrations that did not affect growth and differentiation. From these results the authors concluded that whole embryo culture can be used to estimate the possible teratogenicity of drugs. The system affords reliability, sensitivity and specificity with the added advantages of reduced animal numbers, costs and time over conventional methods. The system was also used to assess four antimycotic drugs. The results, which separated the drugs into two with relatively high teratogenic potential and two with none, were in agreement with those of *in vivo* studies (Bechter and Schmid, 1987). The technique has also been incorporated into the registration package of new drugs by the Sandoz Company (Bechter and Schon, 1988).

Mouse embryos can also be cultured successfully (Sadler and New, 1981) and were used by Van Maele-Fabry and Picard (1987) to examine 10 chemicals ranging from an alkylating agent to an amino acid. Preliminary experiments were performed to determine lethal and non-lethal concentrations and subsequently batches of 20 embryos were cultured for 26 h at intermediate concentrations. The culture medium was 80% human serum and 20% rat serum. Five of the compounds were clearly teratogenic; one, dimethyl sulphoxide, was rated as a weak teratogen and four were weakly embryotoxic. The authors concluded that the results showed no significant discrepancies from published teratological data on the compounds.

The largest application of whole embryo culture in toxicity screening appears to be by Tesh at Life Sciences Research in England (Tesh, 1988). He reports that over 150 experimental compounds have been examined in their laboratories in addition to

known teratogenic agents. Their methodology involved culture of 9.5 day rat embryos for 48 h with and without a rat S-9 activation system in 50% rat serum: 50% Eagle's minimum essential medium. For selection of concentrations a range finding study was performed followed by the main study using concentrations determined to be under the lethal range. Cyclophosphamide was used as a positive control to confirm activity of the S-9 preparation. Unfortunately the author states that the majority of the work is subject to strict confidentiality and the results cannot be discussed!

In a review of the use of postimplantation embryos to evaluate teratogens it was reported there had been more than 200 studies by 1986 (Flynn, 1987). The author, perhaps reflecting his background in the FDA, criticized the lack of uniformity in experimental conditions between laboratories. This should be viewed more as a frustrated inability to evaluate the studies than a criticism of the method as a screen.

Advantages and disadvantages

Kitchin and colleagues (1986) listed the advantages of whole embryo culture as a screening technique as: (1) the ability to control exposure to the test chemical without interference from maternal absorption, distribution, metabolism and excretion, (2) embryos of different species (mainly the rat and mouse) can be exposed to identical test concentrations of a test substance, (3) litter specific effects are circumvented as explanted embryos can be cultured individually, and (4) the ability to add metabolic activating systems.

Disadvantages were (1) the limited number of embryos that can be prepared by one person in a day (estimated at 50 embryos by New, 1990), and (2) the limited period of embryogenesis undertaken in culture which limits the range of abnormalities that can be observed. This latter point may become less significant with the use of techniques for culturing late gestation rat embryos (Barber *et al.*, 1993) and adjunct procedures such as aggregating embryonic brain cell cultures (Kucera *et al.*, 1993).

Some investigators consider that the system is too costly, too time-consuming and requiring too much expertise for routine screening (Brown and Fabro, 1983; Brown and Freeman, 1984). In contrast others claim that large numbers of embryos can be maintained at costs considerably below those presently employed in typical *in vivo* testing procedures (Sadler *et al.*, 1982, 1984). Even so they acknowledged that rat serum is an expensive component with costly requirements in time and personnel for serum collection. Use of human (Chatot *et al.*, 1980; Steele, 1985) or bovine sera (Klug *et al.*, 1985) as alternatives has been investigated and totally artificial sera may be available in the future.

In summary, there is now considerable evidence to support the use of rat embryo culture to screen chemicals for teratogenic activity. Most authors consider this to be a relatively inexpensive technique and Schmid and co-workers have done much to standardize the parameters and validate the technique. It should be recognized that this form of testing determines hazard, i.e. whether a chemical has the potential for causing birth defects. The extrapolation of the results to the human population is about risk. The estimation of risk is perhaps the most difficult aspect of teratology testing and the least objective.

Embryo culture and the determination of risk

When a chemical is identified as a teratogen, in either segment II teratology testing or in embryo culture, it is considered a hazard-ous chemical. Such a classification is often misinterpreted by the popular press or by various pressure groups who imply that all hazardous chemicals cause birth defects or miscarriage in the human. The likelihood that a particular hazardous chemical will cause birth defects in the human at anticipated exposure levels is known as "risk".

The problem of risk determination using in vivo models

The difficulty in calculating risk can be best appreciated by considering several examples.

Retinyl palmitate

The common form of vitamin A (retinyl palmitate) is teratogenic in segment II testing. The lowest teratogenic dose in rats was ~163,000 IU/kg/day and in the rabbit ~9,000 IU/kg/day (Kamm, 1982). These results clearly indicate that retinyl palmitate is a hazard when taken during pregnancy; but they do not tell us what dose would be teratogenic in a human pregnancy and therefore provide no information regarding risk. There is no unequivocal evidence that vitamin A has caused birth defects in the human but it is desirable that a safe level of intake is established. For the most sensitive species, the rabbit, the no-effect dose was 3,600 IU/kg/day. For a 50 kg pregnant women 3,600 IU/kg/day would represent a daily intake of 180,000 IU. In an attempt to extrapolate animal data to the human, regulators often apply safety factors to animal doses, 10-fold for species differences and a further 10-fold for inter-human differences. If this common, but arbitrary, 100-fold safety factor is applied then a daily intake of 1800 IU for a 50 kg woman is the upper safe limit. This is below the recommended daily vitamin A intake for pregnant women and well below the average intake in pregnancy. Hence this method is not appropriate for calculating the dose of vitamin A that might cause birth defects in the human.

Isotretinoin

Another example is the drug isotretinoin, a vitamin A analog used for the treatment of severe cystic acne. In segment II testing this drug was teratogenic in rats at 150 mg/kg/day and in rabbits at 10 mg/kg/day. The respective no-effect doses were 50 and 3 mg/kg/day (Kamm, 1982). For this drug, there is reliable human data demonstrating that the drug is teratogenic at doses as low as 0.5 mg/kg/day (Lammer *et al.*, 1985; Rosa *et al.*, 1986). This is 300-fold less than the teratogenic dose for rats and 20-fold less than the teratogenic dose for rabbits.

Caffeine

A third example is caffeine which is teratogenic in rats at a single dose of 80 mg/kg with a no-effect dose of 40 mg/kg (Collins *et al.*, 1981). Caffeine consumption in the human can be very high, up to 19-30 mg/kg/day from coffee alone (Jacobson *et al.*, 1981), although more normal intake levels are about 2 mg/kg/day. What safety factor should be applied to the rat data?

Pharmacokinetics

This difficulty in comparing doses (mg/kg) between experimental animals and humans is a major problem in the interpretation of all toxicology data. The application of arbitrary safety factors is basically unscientific although it does tend to err on the side of safety. The reason that dose is such a poor index for comparison across species is that there are major pharmacokinetic and some-

times metabolic differences between experimental animals and humans. The most obvious of these differences is serum or plasma half-life of the drug. Usually, but not always, the half-life is much shorter in rats and rabbits than in the human. For example, in the isotretinoin example the half-life of this drug in the human is between 7 and 22 h (Brazzell *et al.*, 1983) while in the rat it is 56 min (Shelley *et al.*, 1982). This more rapid metabolism means that experimental animals can tolerate much higher doses than the human. As a result, the daily dosing regimen used in segment II testing, leads to a series of precipitous peaks and troughs in the animal's blood drug levels. For most therapeutic drugs this is unlike the relatively constant blood levels achieved and required therapeutically in the human.

In this respect embryo culture offers distinct advantages over *in vivo* testing (Webster *et al.*, 1986, 1996; Webster and Valois, 1987; Webster, 1988, 1992, 1995; Cicurel and Schmid, 1988; Brown-Woodman *et al.*, 1994a,b). Using embryo culture it is possible to expose embryos to a drug or its metabolites at constant serum concentrations. This raises the possibility that the concentration associated with teratogenicity or embryotoxicity *in vitro* may be the same concentration that would be teratogenic or embryotoxic in the human if it were achieved at a critical period of gestation. Although this aspect of risk estimation has not been studied to the same extent as the hazard identification, there are some preliminary results to support this contention.

Risk determination using rat embryo culture

Isotretinoin

The human teratogen isotretinoin was teratogenic *in vitro* at serum concentrations of 500 ng/ml with 250 ng/ml the no-effect concentration. The main human metabolite 4-oxo-isotretinoin was also teratogenic at the same concentration (Webster *et al.*, 1986; Ritchie and Webster, 1991). Both compounds caused underdevelopment of the second visceral arch which is the precursor of the external ear. A reduced and abnormal external ear is the most common external malformation seen in the human isotretinoin embryopathy. During normal dosing with this drug in the human trough plasma levels of isotretinoin are 132-186 ng/ml and 4-oxo-isotretinoin, 610-791 ng/ml (Brazzell *et al.*, 1983). The combined plasma trough concentrations of 742-977 ng/ml in the human are greater than the teratogenic threshold of 500 ng/ml for either compound in rat embryo culture. Hence, it is predictable from the rat embryo culture studies, that isotretinoin would be a human teratogen at normal therapeutic doses if used during pregnancy; furthermore the culture results predict that the most likely severe malformation would be abnormal external ears.

Valproic acid

The anticonvulsant, valproic acid, is another human teratogen that is teratogenic in rat embryo culture. In the human, valproic acid is associated with 1% risk of spina bifida when used in pregnancy (Robert and Guibaud, 1982; Lammer *et al.*, 1987). Rat embryo culture studies showed that valproic acid was teratogenic to early somite rat embryos at serum concentrations of 144 μg/ml, inducing a high incidence of exencephaly. At 86 μg/ml the embryos had irregular neural suture lines and irregular somites (Kao *et al.*, 1981). Normal serum levels in the human are 50-150 μg/ml (Browne, 1980). Again the embryo culture studies predicted that the upper range of valproic acid plasma levels seen in the human

during normal dosing would be teratogenic if they occurred during pregnancy, and that abnormalities of neural tube closure might occur.

Etretinate

A third example is etretinate. Use of this drug in pregnancy has been associated with a number of birth defects in the human including meningomyelocele, encephalocele, dysmorphic facies including abnormal external ears, multiple synostoses and syndactyly (Happle *et al.*, 1984; Rosa *et al.*, 1986). Rat embryo culture studies showed that etretinate was teratogenic at 30,000 ng/ml while its metabolite etretin (acitretin) was teratogenic at 100 ng/ml (Bechter and Hall, 1987). The malformations observed in culture included abnormalities of the rhombencephalon, visceral arches and tail hypoplasia. Etretinate is present in the plasma at trough values of 100-500 mg/ml during normal long term human dosing (Lucek and Colburn, 1985) while etretin is present at 80-200 ng/ml (Larsen *et al.*, 1988). Hence the metabolite etretin was teratogenic *in vitro* at concentrations which are known to occur in human plasma during normal therapeutic dosing. The parent compound etretinate was not teratogenic *in vitro* at therapeutic concentrations. Hence, the observed teratogenicity of etretinate in the human is likely to be due to the metabolite etretin.

Solvents

Recently the embryo culture system was used to examine a wide range of solvents for teratogenic/embryotoxic activity (Brown-Woodman *et al.*, 1991, 1994a,b, 1995). For each solvent an effect and no-effect concentration was established. In general, each solvent was thought to cause embryotoxicity rather than teratogenicity, although the distinction can be difficult to justify. As can be seen from Table 1, although all of the solvents caused embryotoxicity (hazard) the effective concentration for each solvent was much higher than the concentration seen in human

TABLE 1

EFFECT AND NO-EFFECT LEVELS OF SOLVENTS AND SOLVENT METABOLITES COMPARED TO AVERAGE HUMAN BLOOD LEVELS REPORTED IN THE LITERATURE

Solvent	Effect level (μmol/mL)	No-effect level (μmol/mL)	Examples of blood concentration following human exposure (μmol/mL)	
			Controlled	Accident/abuse
Styrene oxide	0.038	0.024	0.00005	-
Dibromoethane	0.33	<0.18	-	-
Styrene	1.00	0.63	0.021	-
Benzene	1.56	0.82	0.0026	-
Xylene	1.89	1.08	0.068	0.377 (fatal)
Chloroform	<2.06	1.05	0.034	1.01 (fatal)
Octane	2.21	2.05	-	-
Toluene	2.25	1.46	0.0395	0.358 (fatal)
Heptane	<2.83	2.53	-	-
Dichloromethane	6.54	3.46	0.174	6.00 (fatal)
Formic acid	<18.7	3.7	0.1717.1	
Triethylene glycol dimethyl ether	50.1	24.0	-	-
Ethylene glycol monoethyl ether	75.5	42.8	-	-
Acetone	93.6	61.1	0.44	43.04
Ethylene glycol	253.6	169.9	-	104.7
Methanol	286.5	211.7	0.22	182.0 (fatal)

plasma during controlled exposure (risk). In most instances, where there were appropriate data, it appeared the LD_{50} for the mother would be exceeded before embryotoxic concentrations were reached.

Other drugs

When Cicurel and Schmid (1988) tested 27 drugs for validation they also considered using the data to estimate risk. They compared the minimal teratogenic concentration determined using rat embryos *in vitro* and human maximal plasma levels obtained from published data. For most tested chemicals (including caffeine) the range of teratogenic concentrations *in vitro* was 3 to 100 times the maximal plasma levels and hence these chemicals presumably do not present much of a teratogenic risk to the human. Three chemicals, cyclophosphamide, methotrexate and vincristine were all teratogenic within the maximal plasma concentration range and all are suspected human teratogens. Cadmium was also reported to be teratogenic *in vitro* at concentrations below maximal human plasma levels but the quoted maximal plasma level in humans was unreferenced and seems very high. The known human teratogen phenytoin had virtually no safety margin while the human teratogen and transplacental carcinogen, diethylstilbestrol, appeared to have a huge margin of safety. This latter drug, which is hormonally active during development of the genital system, may indicate a limitation of the culture system, since this period of development cannot be adequately examined *in vitro*.

Protein binding and other variables

Although these results showed a good correlation between the teratogenic concentrations of a drug or metabolite in rat embryo culture and the maternal plasma concentration associated with teratogenicity in the human, a note of caution should be added. An additional factor which is important is the extent to which a drug binds to plasma proteins. If there are differences in protein binding of the drug in rat serum and human serum this will influence the relative effective or "free" concentration (Nau *et al.*, 1988; Neubert, 1988). If the percentages of protein binding of a drug in rats and humans are known then the appropriate "free" concentrations can be easily calculated. Alternatively, the drug can be tested on rat embryos cultured in human serum.

Some investigators claim that the only valid way to extrapolate to an *in vivo* situation is to measure the concentration of the test chemical in the embryonic tissues (Klug, 1991). While this may theoretically increase the accuracy of the extrapolation (although tissue binding may also vary between species) it does not help in a practical sense. There is an immediate need to improve risk assessment; but there is no foreseeable possibility of measuring the embryonic concentrations of drugs in humans during the organogenic period. Currently maternal blood concentrations of test compounds can be accurately measured in humans and in experimental animals and these measurements must be related to subsequent embryonic concentrations. It is proposed that comparison of human maternal serum concentrations with "effect" and "no-effect" concentrations observed in embryo culture (adjusted for protein binding and other identifiable variations) is the most objective method for estimating teratogenic risk currently available. As mentioned previously the short half-life of most drugs in experimental animals makes comparison of pharmacokinetic parameters between humans and *in vivo* models more difficult to interpret.

References

BARBER, C.V., CARDA, M.B. and FANTEL, A.G. (1993). A new technique for culturing rat embryos between gestation days 14 and 15. *Toxicol. In Vitro 7*: 695-700.

BECHTER, R. and HALL, L. (1987). *In vitro* embryotoxicity and teratogenic potential of etretinate and etretin. *Toxicol. In Vitro 1*: 225-231.

BECHTER, R. and SCHMID, B. (1987). Teratogenicity *in vitro* - A comparative study of four antimycotic drugs using the whole embryo culture system. *Toxicol. In Vitro 1*:11-15.

BECHTER, R. and SCHON, H. (1988). Use of whole embryo culture system in drug safety assessment. *Toxicol. In Vitro 2*:195-203.

BRAZZELL, R.K., VANE, F.M., EHMANN, C.W. and COLBURN, W.A. (1983). Pharmacokinetics of isotretinoin during repetitive dosing to patients. *Eur. J. Pharmacol. 24*: 695-702.

BROWN, N.A. (1987). Teratogenicity testing *in vitro*: status of validation studies. *Arch. Toxicol. 11 (Suppl.)*:105-114.

BROWN, N.A. and FABRO, S. (1983). The value of animal teratogenicity testing for predicting human risk. *Clin. Obstet. Gynecol. 26*: 467-477.

BROWN, N.A. and FREEMAN, S.J. (1984). Alternative tests for teratogenicity. *Altern. Lab. Anim. 12*: 7-23.

BROWNE, T.R. (1980). Valproic acid. *N. Engl. J. Med. 302*: 661-666.

BROWN-WOODMAN, P.D.C., HUQ, F., HAYES, L., HERLIHY, C., PICKER, K. and WEBSTER, W.S. (1995). *In vitro* assessment of methanol and the metabolite formic acid on embryonic development of the rat. *Teratology 52*: 233-243.

BROWN-WOODMAN, P.D.C., WEBSTER, W.S., PICKER, K. and RITCHIE, H.E. (1991). Embryotoxicity of xylene and toluene: an *in vitro* study *Ind. Health 29:* 139-151

BROWN-WOODMAN, P.D.C., WEBSTER, W.S., HUQ, F., HERLIHY, C., HAYES, L. and PICKER, K. (1994a). *In vitro* studies of induction of birth defects by exposure to solvents. *Worksafe Report*, pp. 1-134.

BROWN-WOODMAN, P.D.C., WEBSTER, W.S., PICKER, K. and HUQ, F. (1994b). *In vitro* assessment of individual and interactive effects of aromatic hydrocarbons on embryonic development of the rat. *Reprod. Toxicol. 8*: 121-135

CARSON, R. (1962). *Silent Spring*. Fawcett Publications, Inc., Greenwich.

CHATOT, C.L., KLEIN, N.W., PIATEK, P. and PIERRO, L.J. (1980). Successful culture of rat embryos in human serum: use in the detection of teratogens. *Science 207*:1471-1473.

CICUREL, L. and SCHMID, B.P. (1988). Postimplantation embryo culture for the assessment of the teratogenic potential and potency of compounds. *Experientia 44*: 833-840.

COCKROFT, D.L. (1980). Application of post-implantation embryo culture to problems in teratology. *Acta Morphol. Acad. Sci. Hung. 28*: 117-124.

COCKROFT, D.L. (1991). Culture media for postimplantation embryos. *Reprod. Toxicol. 5*: 223-228

COCKROFT, D.L. and STEELE, C.E. (1987). Postimplantation embryo culture and its application to problems in teratology. In *Methods in Toxicology* (Eds. C.K. Atterwill and C.E. Steele). Cambridge University Press, Cambridge, pp. 365-389.

COLLINS, T.F.X., WELSH, J.J., BLACK, T.N. and COLLINS, E.V. (1981). A study of the teratogenic potential of caffeine given by oral intubation to rats. *Regul. Toxicol. Pharmacol. 1*: 355-378.

FANTEL, A.G. (1982). Culture of whole rodent embryos in teratogen screening. *Teratogenesis Carcinog. Mutagen. 2*: 231-242.

FANTEL, A.G., GREENAWAY, J.C., JUCHAU, M.R and SHEPARD, T.H. (1979). Teratogenic bioactivation of cyclophosphamide *in vitro*. *Life Sci. 25*: 67-72.

FLYNN, T.J. (1987). Teratological research using *in vitro* systems. I. Mammalian whole embryo culture. *Environ. Health Perspect. 72*: 203-210.

HAPPLE, R., TRAUPE, H., BOUNAMEAUX, Y. and FISCH, T. (1984). Teratogene Wirkung von Etretinate beim Menschen. *Deut. Med. Wochenschr. 109*: 1476-1480.

JACOBSON, M.F., GOLDMAN, A.S. and SYME, R.H. (1981). Coffee and birth defects. *Lancet 1*: 1415-1416.

KAMM, J.J. (1982). Toxicology, carcinogenicity and teratogenicity of some orally administered retinoids. *J. Am. Acad. Dermatol. 6*: 652-659.

KAO, J., BROWN, N.A., SCHMID, B., GOULDING, E.H. and FABRO, S. (1981). Teratogenicity of valproic acid: *in vivo* and *in vitro* investigations. *Teratogenesis Carcinog. Mutagen.* 1: 367-382.

KIMMEL, G.L., SMITH, K., KOCHHAR, D.M. and PRATT, R.M. (1982). Overview of *in vitro* teratogenicity testing: aspects of validation and application to screening. *Teratogenesis Carcinog. Mutagen.* 2: 221-229.

KITCHIN, K.T. and EBRON, M.T. (1984). Further development of an *in vitro* whole embryo culture system: solvent toxicity and water insoluble compound delivery system. *Toxicology 30*: 45-57.

KITCHIN, K.T., SCHMID, B.P. and SANYAL, M.K. (1981). Teratogenicity of cyclophosphamide on a coupled microsomal activating/embryo culture system. *Biochem. Pharmacol. 30*: 59-64.

KITCHIN, K.T., SCHMID, B.P. and SANYAL, M.K. (1986). Rodent whole-embryo culture as a teratogen screening method. *Methods Find. Exp. Clin. Pharmacol. 8*: 291-301.

KLUG, S. (1991). Whole embryo culture: interpretation of abnormal development *in vitro*. *Reprod. Toxicol. 5*: 237-244.

KLUG, S., LEWANDOWSKI, C. and NEUBERT, D. (1985). Modification and standardization of the culture of early postimplantation embryos for toxicological studies. *Arch. Toxicol. 58*: 84-88.

KUCERA, P., CANO, E., HONEGGER, P., SCHILTER, B., ZIJLSTRA, J.A. and SCHMID, B. (1993). Validation of whole chick embryo cultures, whole rat embryo cultures and aggregating embryonic brain cell cultures using six pairs of coded compounds. *Toxicol. In Vitro 7*: 785-798.

LAMMER, E.J., CHEN, D.T., HOAR, R.M., AGNISH, N.D., BENKE, P.J., BRAUN, J.T., CURRY, C.J., FERNHOFF, P.M., GRIX Jr, A.W., LOTT, I.T., RICHARD, J.M. and SUN, S.C. (1985). Retinoic acid embryopathy. *N. Engl. J. Med. 313*: 837-841.

LAMMER, E.J., SEVER, L.E. and OAKLEY, G.P. (1987). Teratogen update: valproic acid. *Teratology 35*: 465-473.

LARSEN, F.G., JAKOBSEN, P., LARSEN, C.G., KRAGBALLE, K. and NIELSEN-KUDSK, F. (1988). Pharmacokinetics of etretin and etretinate during long term treatment of psoriasis patients. *Pharmacol. Toxicol. 62*: 159-165.

LUCEK, R.W. and COLBURN, W.A. (1985). Clinical pharmacokinetics of the retinoids. *Clin. Pharmacokinet. 10*: 36-62.

NAU, H., LEWANDOWSKI, C., KLUG, S. and NEUBERT, D. (1988). Pharmacokinetic aspects of drug effects *in vitro* (II). Placental transfer to the embryo and activity of some carboxylic acids structurally related to valproic acid in whole embryos in culture. *Toxicol. In Vitro 2*: 169-174.

NEUBERT, D. (1988). Significance of pharmacokinetic variables in reproductive and developmental toxicity. *Xenobiotica 18 (Suppl. 1)*: 45-58.

NEW, D.A.T. (1978). Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol. Rev. 53*: 81-122.

NEW, D.A.T. (1990). Whole embryo culture, teratogenesis, and the estimation of teratologic risk. *Teratology 42*: 635-642.

OGLESBY, L.A., EBRON, M.T., BEYER, P.E., CARVER, B.D. and KAVLOCK, R.J. (1986). Co-cultures of rat embryos and hepatocytes: *in vitro* detection of a proteratogen. *Teratogenesis Carcinog. Mutagen. 6*: 129-138.

PIERSMA, A.H. (1993). Whole embryo culture and toxicity testing. *Toxicol. In Vitro 6*: 763-768.

RITCHIE, H. and WEBSTER, W.S. (1991). Parameters determining isotretinoin teratogenicity in rat embryo culture. *Teratology 43*: 71-81.

ROBERT, E. and GUIBAUD, P. (1982). Maternal valproic acid and congenital neural tube defects. *Lancet 2*: 937.

ROGIERS, V., BLAAUBOER, P., MAUREL, P., PHILLIPS, I., and SHEPHARD, E. (1995). Hepatocyte-based *in vitro* models and their application in pharmacotoxicology. *Toxicol. In Vitro 9*: 685-694.

ROSA, F.W., WILK, A.L. and KELSEY, F.O. (1986). Teratogen update: vitamin A congeners. *Teratology 33*:355-364.

SADLER, T.W. and NEW, D.A.T. (1981). Culture of mouse embryos during neurulation. *J. Embryol. Exp. Morphol. 66*: 109-116.

SADLER, T.W. and WARNER, C.W. (1984). Use of whole embryo culture for evaluating toxicity and teratogenicity. *Pharmacol. Rev. 36*: 145S-150S.

SADLER, T.W., HORTON, W.E. and WARNER, C.W. (1982). Whole embryo culture: a screening technique for teratogens. *Teratogenesis Carcinog. Mutagen. 2*: 243-253.

SCHARDEIN, J.L. (1993). *Chemically Induced Birth Defects*, 2nd ed. Marcel Dekker Inc., New York.

SCHMID, B.P. (1985). Xenobiotic influences on embryonic differentiation, growth and morphology *in vitro*. *Xenobiotica 15*: 719-726.

SCHMID, B.P., GOULDING, E., KITCHIN, K. and SANYAL, M.K. (1981). Assessment of the teratogenic potential of acrolein and cyclophosphamide in a rat embryo culture system. *Toxicology 22*: 235-243.

SCHMID B.P., TRIPPMACHER, A. and BIANCHI, A. (1982). Teratogenicity induced in cultured rat embryos by the serum of procarbazine treated rats. *Toxicology 25*: 53-60.

SHELLEY, R.S., JUN, H.W., PRICE, J.C. and CADWALLADER, D.E. (1982). Blood level studies of all-trans- and 13-cis-retinoic acids in rats using different formulations. *J. Pharm. Sci. 71*: 904-907.

SHEPARD T.H. (1989). *Catalog of Teratogenic Agents*. Johns Hopkins University Press, Baltimore.

SMITH, M.K., KIMMEL, G.L., KOCHHAR, D.M., SHEPARD, T.H., SPEILBERG, S.P. and WILSON, J.G. (1983). A selection of candidate compounds for *in vitro* teratogenesis test validation. *Teratogenesis Carcinog. Mutagen. 3*: 461-480.

STEELE, C.E. (1985). Human serum as culture medium for rat embryos. *Experientia 41*: 1601-1603.

TESH, J.M. (1988). The application of whole embryo culture to new product development. *Toxicol. In Vitro 2*:189-194.

VAN MAELE-FABRY, G. and PICARD, J.J. (1987). Evaluation of the embryonic potential of ten chemicals in the whole mouse embryo culture. *Teratology 36*: 95-106.

VAN MAELE-FABRY, G., THERASSE, P., LENOIR, E., DESAGER, J.P., DESPONTIN, K., GOFFLOT, G., JACOBS, M.C., LECART, C., BERTHET, P., LACHAPELLE, J.M. and PICARD, J.J. (1993). Embryotoxicity of human sera from patients treated with isotretinoin. *Toxicol. In Vitro 7*: 809-815.

WEBSTER, W.S. (1988). The use of animals models in understanding human teratogens. *Cong. Anom. 28*: 295-302.

WEBSTER, W.S. (1992). The interpretation of results from teratology and reproductive toxicity tests including comments on the new draft guidelines. *Cong. Anom. 32 (Suppl.)*: S99-S110

WEBSTER, W.S. (1995). The use of rat embryo culture in reproductive toxicity testing. In *Alternative Methods in Toxicology and the Life Sciences*, Vol. 11. Mary Ann Liebert, Inc., New York, pp. 145-152.

WEBSTER, W.S. and VALOIS, A.A. (1987). Reproductive toxicology of manganese in rodents, including exposure during the postnatal period. *Neurotoxicology 8*:437-444.

WEBSTER, W.S., BROWN-WOODMAN, P.D.C., SNOW, M. and DANIELSSON B.R.G. (1996). Teratogenic potential of drugs with potassium channel blocking activity. *Teratology 53*: 168-175

WEBSTER, W.S., JOHNSTON, M.C., LAMMER, E.J. and SULIK, K.K. (1986). Isotretinoin embryopathy and the cranial neural crest: an *in vivo* and *in vitro* study. *J. Craniofac. Genet. Dev. Biol. 6*: 211-222.