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Validation of Postimplantation Rodent Whole Embryo Culture for *In Vitro* Teratogenicity Testing

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Summary

Teratogenicity data obtained from rat and mouse whole embryo culture (WEC) concerning the agents compiled in the Smith list were collected from the literature. Following analyses of these data, each technique for an *in vitro* teratogenicity testing was validated for its ability to evaluate teratogenic potential.

The rodent WEC techniques were variable between investigators with regard to culture medium, exogenous metabolizing source, and endpoint measurements. Of 34 agents tested in cultured rat embryos, two showed variable results. The remaining 32 agents had accuracy values of 90.0, 84.4, and 66.7% when the teratogenic potentials *in vitro* were compared with those available from rats, mammals, and humans *in vivo*, respectively. Of 15 known mammalian teratogens *in vivo* tested in cultured mouse embryos, 14 were positive and one was variable.

The results indicated that the rat WEC assay has poorer predictive value of teratogenic potential *in vivo* in mammals or humans than in rats. Also, the validity of the results obtained by the mouse WEC assay is unknown because agents known to be nonteratogenic in mammals *in vivo* have not been tested. Standardization of rodent WEC techniques is critically important before further validation efforts are made.

Introduction

Whole embryo cultures (WEC) of postimplantation rodent embryos have been used to study the mechanisms of teratogenesis. These techniques have also been proposed as an *in vitro* teratogenicity test to reduce or replace whole animal experiments[1,2].

The potential of WEC to serve as a teratogenicity assay can only be evaluated through carefully designed validation studies[3–5]. In the mouse WEC assay, few agents have been validated[6]. On the other hand, the rat WEC assay has been validated using various agents to show the high predictability of teratogenic potential and potency *in vivo*[7–10]. However, the selection of agents to test validation is critical, and potential bias in their selection may influence the performance of the rat WEC assay[3].

To avoid the subjective selection of agents, a Consensus Workshop on *In Vitro* Teratogenicity Testing selected a list of 47 candidate agents (Smith list) for *in vitro* test validation[11]. No laboratories have previously validated the rodent WEC assays based upon testing with all the agents compiled in the Smith list. Reviews by Brown[3] and Faustman[12] have also indicated that too few agents in the Smith list have been tested to validate rodent WEC assays. Since the publication of their reviews, additional data have accumulated in the literature.

In this study, the performance of the rat and mouse WEC assays was evaluated with re-

spect to teratogenic potential by analyzing the available data collected from literature searches on the Smith list agents. The literature cited was also used to elucidate the technical difficulties remaining to be resolved before further validation of *in vitro* teratogenicity testing.

Materials and Methods

All agents compiled in the Smith list[11] were classified as either known mammalian teratogens or nonteratogens *in vivo* in rats, mice, mammals, or humans according to the original article. The Catalog of Teratogenic Agents edited by Shepard[13] was also used where no teratogenicity data from rats and/or mice *in vivo* were available in the above literature[11]. Agents were assigned to have unknown teratogenic potentials in rats and/or mice *in vivo* when there was no data available in the literature[11,13].

Teratogenicity data of the Smith list agents, tested by rat or mouse WEC, were taken from references within the author's knowledge (Table I). The following types of data were not included in the analyses: those reported in abstract form only, those indicating biochemical or cellular changes with no description of morphological abnormalities, those obtained by a combination of WEC and organ culture, and those obtained by microinjection. In addition, only *in vitro* metabolic biotransformation procedures were taken into account, and data obtained by exposure either *in vivo* followed by observations *in vitro*, or to serum prepared from drug-dosed animals were also excluded.

The teratogenic potential of each agent reported by individual investigators was designated as positive (+) or negative (−). Agents were designated as variable (±) when the test results were inconsistent between different investigators. The teratogenic potential of each agent was compared between rat or mouse embryos *in vitro* and homologous animals, mammals, or humans *in vivo*.

Following a comparison of the teratogenic potentials between *in vitro* and *in vivo*, the values of sensitivity, specificity, and accuracy of each WEC assay were computed to estimate the validation performance. Sensitivity or specificity was defined as the number of agents properly identified as teratogens or nonteratogens *in vitro*, respectively, divided by the number of agents tested. Accuracy was defined as the number of correct responses obtained *in vitro* divided by the number of agents tested. Agents found to be variable *in vitro* or untested *in vivo* were not included in the analyses.

Results

Table I shows the 47 agents compiled in the Smith list. Some teratogenicity data were unavailable from either rats or mice *in vivo*. The teratogenic potentials of hexahydrophthalimide glutarimide and phthalimide were unknown in both animal species *in vivo*.

Rodent WEC varied among investigators. The developmental stage of embryos at explantation ranged from 9 to 11.5 days in rats and from 8 to 11.5 days in mice. Vehicles including dimethyl sulfoxide, ethyl alcohol, and gelatin were used as water-insoluble drug-delivery systems. The culture media included whole serum (rat, human, rat +human), and mixtures of serum (rat, rat+human, rat+fetal bovine) and water, physiological saline (Tyrode's, Earle's), or chemically defined medium (Waymouth's, HEPES-buffered Eagle's MEM).

The exogenous metabolizing source most commonly used was the hepatic rat S9 fraction and cofactors (S9 mix). A few agents were tested in the presence of cofactors and hepatic S9 fraction prepared from mice, hamsters, rabbits, monkeys, or humans. Hepatic rat S9 fraction, microsome, cytosol, and monolayer or suspension culture of intact hepatocytes prepared from rats, hamsters, and rabbits were also used as the exogenous metabolizing sources.

Table 1. Comparison of the teratogenic potentials of the Smith list agents tested between *in vitro* and *in vivo*

Compound	Response *in vitro*[a]				Response *in vivo*[a]			
	Rat[b]	References	Mouse[b]	References	Rat	Mouse	Mammals[c]	Humans
1. Acetazolamide	+	14	ND		+	+	+	
2. Amaranth	−	9,10	ND		−	−	−	
3. 6-Aminonicotinamide	+	9,10	+	15	+	+	+	
4. Aspirin	+(+)	9,10,16,17	ND		+	+	+	−
5. Caffeine	+(+)	9,10	ND		+	+	+	−
6. Carbon tetrachloride	ND		ND		−	ND	−	
7. Chlorambucil	+(+)	9,10,18	+	6	+	+	+	+
8. Coumarin	(+)	7,8	ND		ND	−	+	+
9. Cyclophosphamide	−(+)	2,7,9,10,19–23	ND		+	+	+	+
10. Cytochalasin D	+(−)	24	ND		−	+	+	
11. Dexamethasone	+(+)	7–10	ND		+	+	+	
12. Diazepam	−(−)	9,10	ND		−	+	+	+
13. Diethylstilbestrol	+(+)	7,9,10,25	ND		+	+	+	+
14. Dilantin[d]	+(+)	9,10	±(+)	26,27	+	+	+	+
15. Diphenhydramine hydrochloride	ND		ND		−	−	−	−
16. Doxylamine succinate	ND		ND		−	ND	−	−
17. EM 12	ND		ND		+	ND	+	
18. Ethyl alcohol	+(+)	7,8,28	+	29	+	+	+	+
19. Ethylenethiourea	+(+)	9,10,30	+(+)	30	+	+	+	
20. N-Ethyl-N-nitrosourea	+	31	ND		+	+	+	
21. 5-Fluorouracil	+(+)	7–10	ND		+	+	+	+
22. Formaldehyde	ND		ND		−	−	−	
23. Hexahydrophthalimide glutarimide	ND		ND		ND	ND	−	
24. Hydroxyurea	ND		+	26	+	+	+	
25. Hyperthermia	+	32	+	33	+	+	+	+
26. Isoniazid	ND		ND		−	−	−	−
27. Meprobamate	+	9	ND		−	−	−	−
28. Methotrexate	+(+)	7–10	ND		+	+	+	+
29. Methyl mercury chloride	+	2	+	34	+	+	+	+
30. Mirex	ND		ND		+	+	+	
31. Nitrilotriacetate	+	9	ND		−	−	−	
32. Penicillin G sodium	−	9,10	ND		ND	−	−	−
33. L-Phenylalanine	−	35	+	36	−	ND	+	+
34. Phthalimide	ND		ND		ND	ND	−	
35. Procarbazine	−(−)	37	ND		+	+	+	+
36. Retinoic acid-all trans	+	2,9	+	38	+	+	+	
37. Retinoic acid-13 cis	+	2,9	+	38,39	+	+	+	
38. Saccharin sodium	−(−)	7,9,10	ND		−	−	−	−
39. Sodium arsenate	+	9	+	40	+	+	+	
40. Sodium cyclamate	−	9,10	ND		−	−	−	
41. Testosterone propionate	ND		ND		+	+	+	+
42. Thalidomide[d]	±(+)	2,10	ND		−	−	+	+
43. Trichloroethylene	ND		ND		−	−	−	
44. Trichlorophenoxyacetic acid[d]	±(±)	8–10,41,42	+(+)	42	−	+	+	−
45. Urethane	+	9,10	+	43	+	+	+	
46. Vincristine sulfate	+	9,10	+	6	+	+	+	
47. Vinyl chloride	ND		ND		−	−	−	−

[a]ND indicates that the testing results were not demonstrated.
[b]Parentheses indicate the responses tested in the presence of exogenous metabolizing sources.
[c]Mammals including rats, mice, hamsters, guinea pigs, rabbits, cats, dogs, swines, monkeys, and humans.
[d]± indicates that testing results were inconsistent between different investigators.

Endpoint measurements that designated the test agents as teratogens or nonteratogens also varied among investigators. Many investigators designated the agents to be teratogens based upon the induction of concentration-related morphological abnormalities and growth inhibition in cultured embryos. In attempts to distinguish specific teratogenic

effects from general toxicity, some investigators designated the agents as teratogens when morphological abnormalities were induced in cultured embryos at drug concentrations without decreases in the crown-rump length and number of somites.

Thirty-four of 47 agents (72.3%) were tested by rat WEC (Table I). These techniques without an exogenous metabolizing source directly gave 12 positives and 4 negatives, whereas those with and without the metabolizing sources gave similar responses in 10 positives and 3 negatives. Coumarin was tested only in the presence of a metabolizing source and gave a positive response[7,8]. Cyclophosphamide was found to be negative or positive in the absence or presence of a metabolizing source[2,7,9,10,19,21-23], respectively, whereas the reverse was true for cytochalasin D[24]. Thalidomide was variable between different laboratories, although similar degrees of embryotoxicity were observed at similar concentrations of the drug[2,10]. In trichlorophenoxyacetic acid (2, 4, 5-T) tests, one laboratory found negative responses with and without rat S9 mix and a positive response with mouse S9 mix[42], while an other laboratory found similar degrees of embryotoxicity with and without mouse or hamster S9 mix, and decreased embryotoxicity with rat, rabbit, or monkey S9 mix[41].

A comparison of the teratogenic potentials between rat embryos developed *in vitro* and *in vivo* revealed values of 95.5% sensitivity, 75.0% specificity, and 90.0% accuracy. However, the validation performance of this assay became poorer, resulting in values of 88.5% sensitivity, 66.7% specificity, and 84.4% accuracy when dichotomous classification was analyzed with mammals *in vivo*. This low level of validation performance also occurred when analyzed with humans *in vivo* (Table II).

Only 15 known *in vivo* mammalian teratogens of 47 agents (31.9%) were tested by mouse WEC (Table I). These techniques without an exogenous metabolizing source gave direct 12 positives. The remaining three agents were tested in the presence of metabolizing sources. With and without the metabolizing sources, ethylenethiourea[30] and 2, 4, 5-T[42] were found to be teratogens. One laboratory found a negative response for dilantin in the absence of a metabolizing source[26], whereas the other found positive responses in both the presence and absence of a metabolizing source[27]. The variable responses of dilantin tested in the absence of a metabolizing source were likely due to pharmacokinetic variables. The validation performance of the mouse WEC assay was virtually meaningless because neither sufficient numbers of known human teratogens and nonteratogens nor known *in vivo* mammalian nonteratogens have been tested in this assay system (data was not shown).

Table II. Validation performance of the rat whole embryo culture assay evaluated on the basis of available test data with the Smith list agents

Validation	Reference animals *in vivo*		
performance	Rat	Mammals[a]	Human
Teratogens Correct/total (% sensitivity)	21/22[b] (95.5)	23/26[b] (88.5)	10/13[b] (76.9)
Nonteratogens Correct/total (% specificity)	6/8[c] (75.0)	4/6[d] (66.7)	2/5 (40.0)
Accurate/total (% accuracy)	27/30 (90.0)	27/32 (84.4)	12/18 (66.7)

[a]Mammals including rats, mice, hamsters, guinea pigs, rabbits, cats, dogs, swines, monkeys, and humans.
[b]Positive response of cyclophosphamide was used.
[c]Negatic response of cytochalasin D was used.
[d]Positive response of cytochalasin D was used.

Discussion

Some teratogenicity data in rats and/or mice *in vivo* of the agents compiled in the Smith list were unavailable[11,13]. Adequate *in vivo* teratogenicity data from rats or mice may be important in the selection of agents to test the validity of WEC techniques because these

animals are used in standard teratogenicity testing *in vivo*. Welsh[4] and Schwetz et al.[5] also noted a similar difficulty with the Smith list, and development of a new list of agents is now being considered for further validation efforts.

Analyses of the literature cited here revealed that there are at least three technical difficulties remaining to be resolved before rodent WEC can be further validated for *in vitro* teratogenicity testing. The first is the variability in culture media used among investigators. Recent interlaboratory evaluations have shown similar degrees of normal development by culturing postimplantation rodent embryos in culture medium composed of rat, human, or rat+human serum samples[44]. However, embryotoxic responses by exposure to the agents are variable depending on the binding or competition with serum proteins[45] and decomposition by serum enzymes[16]. These findings indicate that standardization of culture medium is difficult when it contains a significant amount of serum. Thus, development of a standard chemically defined medium should be considered.

Although recent investigations of xenobiotic metabolism have revealed the ability of *in vitro* cultured postimplantation rodent embryos to bioactivate certain proteratogens[27], WEC is usually supplemented with an exogenous metabolizing source to detect the teratogenic effects of such agents. The addition of S9 mix to culture medium has previously shown a false negative response for procarbazine[37], different abnormalities to those observed *in vivo* with ethylenethiourea[30], and the inability to enhance diallate embryotoxicity[46]. Tests with some chemicals have also shown that the teratogenic potentials or potencies *in vitro* vary depend on both the absence or presence of S9 mix and the origin of S9 fractions[17,19,24,27,30,36,41,42]. Furthermore, cellular fractions reportedly show such disadvantages as embryotoxicity[21,27,30], limited period of activity[20], potential interaction between the test chemical and the S9 fraction or cofactors[21], as well as high embryotoxicity induced by supplementation with an NADPH-regenerating system[21] or organic solvents[46]. Although cultured intact hepatocytes have some advantages over cellular fractions[47], they have been used only in cyclophosphamide tests[21-23]. There have been no reports of WEC assays supplemented with purified enzyme components of the drug-metabolizing system. These findings indicated that standardization of rodent WEC is currently required regarding use of exogenous metabolizing sources to test chemicals with unknown teratogenic potential *in vivo*.

The third difficulty is that no endpoints have been established to classify the testing agents to be teratogens or nonteratogens *in vitro*. The reason for the variable responses of 2, 4, 5-T[9,10,41] is unknown, but that of thalidomide may result from differences in the endpoint measurements[2,9,10]. Based upon the concentration-related induction of morphological abnormalities and growth inhibition in cultured embryos, Tesh et al.[2] found that thalidomide was a teratogen. In contrast, Cicurel et al.[9,10] reported that thalidomide was not teratogenic, since they designated the test agents to be teratogens when morphological abnormalities were induced at drug concentrations without inhibition in embryonic growth and differentiation. Although the latter designation may be more objective for *in vitro* teratogenicity testing, it has been shown to give false positives with podophyllotoxin, meprobamate, and nitrilotriacetate[6,9]. More promising endpoint(s) capable of giving a validation performance as high as possible should be developed to distinguish specific teratogenic effects from general toxicity.

The available teratogenicity data for compounds on the Smith list suggest that the rat WEC assay is a poorer predictor of teratogenic potential *in vivo* in mammals or humans than in rats. Furthermore, the validation performance as determined in the present

study is poorer than that reported by Schmid and co-workers[7,9,10], who validated the rat WEC assay using their own list of agents. This difference may be attributable to the selection of agents that were validated. The possibility of poorer validation performance by biased chemical selection has been suggested by Brown[3], and has in fact been demonstrated in the rat limb bud cell culture assay[48,49]. On the other hand, similar to the review by Faustman[12], the present study showed no progress in validating the mouse WEC assay, since neither sufficient numbers of known human teratogens and nonteratogens nor known mammalian nonteratogens compiled in the Smith list have been tested in this assay system.

Validation studies based on testing with the Smith list agents have reported accuracy values of 56–59% in the mouse ovarian tumor cell assay (MOT)[50], 60–64% in human embryonal palatal mesenchymal cell assay (HEPM)[50], 73–74% in a battery of MOT/HEPM assays[50] and 75% in the rat limb bud cell culture assay[49]. The accuracy value of the rat WEC assay obtained in the present study was a little higher than those of the above *in vitro* teratogenicity assays. However, comparisons of accuracy values between these assay systems are meaningless, since there are some agents either untested in the rat WEC assay or inconsistently classified as teratogens and nonteratogens *in vivo* by different investigators.

In summary, the validity of the rodent WEC has been neither tested with all agents compiled in the Smith list nor accepted as an effective *in vitro* teratogenicity assay. Furthermore, to establish the usefulness of the rodent WEC for *in vitro* teratogenicity testing, it is important to standardize the assay technique, validate the standardized assay based on an established validation program, and to compare the performance with those of a number of proposed *in vitro* teratogenicity assay systems.

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