Applications of Rat WEC for Teratogenic Assessment of Test Compounds

Vicki Sutherland, PhD
Bristol-Myers Squibb

Objective:

To establish and optimize the use of the rat whole embryo culture model in evaluating the potential teratogenic liability of test compounds.

- Developed a refined approach for capturing single calls (malformations) in the overall morphological assessment.
- Developed predictivity models using the test set of compounds and applied these models to our screening assays.

Rat Embryo Culture Process:

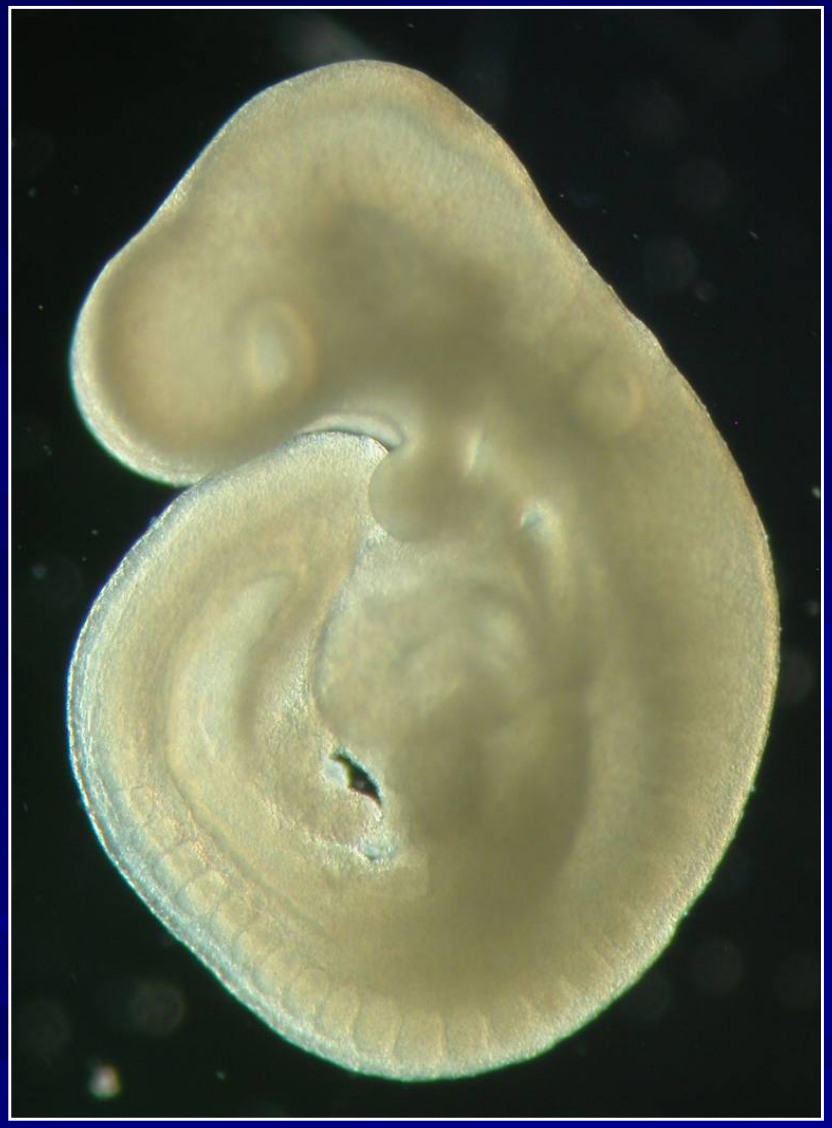
1. Embryos were removed on Gestation Day 9. The decidual tissue, parietal yolk sac, and Reichert's membrane were excised.

2. Embryos were evaluated for developmental stage and transferred into pre-warmed vial of culture medium. Untreated, vehicle-control (DMF), &/or specific concentrations of test compound were included in each experiment. The stage of the embryo was recorded.

3. Each vial was oxygenated, stoppered, & placed on a rotator inside a 37°C incubator. Embryos received additional oxygen supplementation, at increasing concentrations, at ~ 16, 24 and 40 hours post-culture.

4. After ~ 44 hours of culture, embryos were transferred into small dishes of pre-warmed Tyrode's buffer for morphologic evaluation.

Ten Morphological Endpoints of Interest:



~ 44 hrs post-culture

Scores were assigned for the following:

1. Yolk Sac
2. Embryo Rotation
3. Crown Rump Length
4. Caudal Development
5. Somites
6. Cranial Neural Tube
7. Presumptive Spinal Cord
8. Heart
9. Pharyngeal Arches
10. Facial structures

In-House Score System

The In-House evaluations were performed on embryos cultured for approximately 44 hours (GD11 stage). Scores were based on the morphologic appearance of embryonic structures.

Scoring criteria were as follows:

5 = structure is entirely normal for developmental stage,

4 = structure is within range of normal for developmental stage,

3 = structure has mild anomaly/malformation,

2 = structure has moderate anomalyy/malformation,

1 = structure has severe anomaly/malformation, and

0.5 = structure not evident.

Typical WEC Screen:

- Four day experiment:

  Days 1 & 2 - Harvest and culture embryos.

  Days 3 & 4 - Score embryos.

- Three to four compounds at ~3 concentrations (0.1, 1 & 10 µM)

- Six embryos per concentration (including vehicle and untreated controls)

- Morphological evaluations are compiled into datasheets. Single call data are tabulated (embryo/treatment) and group average scores are compiled for assessment.

Example of Morphological Score Tabulations:

Structure	Observation	Compound X				Control
	Concentration	0.1uM	1uM	10uM	25uM	DMF
Yolk Sac	SCORE	4.8	4.7	4.0	3.8	5.0
Rotation	SCORE	5.0	4.7	4.1	3.8	4.7
Size	Crown-Rump Length (mm)	3.0	2.9	3.0	2.5	2.9
Caudal	SCORE	5.0	5.0	5.0	3.7	5.0
Somites	SCORE	5.0	4.5	4.3	3.7	4.8
Neural Tube	SCORE	5.0	4.7	4.4	3.7	4.7
Spinal Cord	SCORE	5.0	4.5	5.0	3.8	4.8
Heart	SCORE	4.7	4.3	4.0	3.0	5.0
Arches	SCORE	4.8	4.3	4.1	3.8	4.8
Face	SCORE	4.8	4.5	4.1	3.3	5.0

Example of Specific Malformations Call Recordings:

Structure	Observation	Compound X				Control
	Concentration	0.1uM	1uM	10uM	25uM	DMF
Yolk Sac	SCORE	4.8	4.7	4.0	3.8	5.0
	Chorion - Pooled Blood	1	1	1	1	
	Allantois/Umbilicus - Thin	1	1		2	
	Blood Islands			1		
	Vasculature, Decreased				2	
	Vasculature, Abnormal					
	Bleb/Blister(s)					
	Abnormal Expansion/Collapsed			1	1	
	Ectoplacental Cone detached					
	Ectoplacental Cone inside YS				2	
	Ectoplacental Cone - Small					
	Ectoplacental Cone - Swollen					
☠	Embryo Lethal					
	NOT SCORED					

Example of Specific Malformations Call Recordings:

Structure	Observation	Compound X				
	Concentration	**0.1uM**	**1uM**	**10uM**	**25uM**	**DMF**
Yolk Sac	SCORE	4.8	4.7	5.0	3.8	4.3
Rotation	SCORE	5.0	4.7	4.7	3.8	4.7
Size	Crown-Rump Length (mm)	3.0	2.9	3.0	2.8	2.9
Caudal	SCORE	5.0	5.0	5.0	3.7	5.0
Somites	**SCORE**	**5.0**	**4.5**	**4.3**	**3.7**	**4.8**
	Initial Somite Stage					0
	Final Somite Count	**22.7**	**22.9**	**22.3**	**20.5**	**23.9**
	Irregular Shape					
	Rounded					
	Short			1	2	
	Small				1	
	Wedged Shape					
	Not Well Defined				3	
	Fused					
	Compressed					
	Narrow					
	Irregular Borders					
	Serrated					
	Missing					
	Cobblestone (asymmetric)					
Neural Tube	**SCORE**	**5.0**	**4.7**	**5.0**	**4.7**	**4.7**
Spinal Cord	**SCORE**	**5.0**	**4.5**	**5.0**	**4.8**	**4.8**
Heart	**SCORE**	**4.7**	**4.3**	**4.0**	**4.0**	**4.7**
Arches	**SCORE**	**4.8**	**4.3**	**5.0**	**4.8**	**4.8**
Face	**SCORE**	**4.8**	**4.5**	**5.0**	**4.3**	**4.8**

Example of Specific Malformations Call Recordings:

Structure	Observation	Compound X				Control
	Concentration	0.1uM	1uM	10uM	25uM	DMF
Heart	SCORE	4.7	4.3	4.0	3.0	4.7
	Outflow Tract - Kinked		1		2	
	Outflow Tract - Narrow		2	2	2	
	Outflow Tract - Swollen					
	Outflow Tract - Looping Defect					
	Outflow Tract - Short	1				
	Outflow Tract filled with blood					
	Blebbing					
	Pericardial Sac - Swollen					
	Pericardial Sac - Filled w Blood					
	Pericardial Sac - Not Evident					
	Ventricle - Enlarged				2	1
	Ventricle - Small	1		1		
	Ventricle - Compressed					
	Atrium - Enlarged			1	3	
	Atrium - Small			1		1
	Atrium - Compressed					
	Retrograde Circulation					
	Dextrocardia	1			1	
	Clotted Blood in Chambers					
	Chambers not well Defined					
	A-V bridge				1	
	Damaged During Processing				1	1

Generating a Predictive Model

Rationale for Selection of Morphological Endpoints:

- Neural tube and Somites were chosen as they are highly conserved in both embryonic development and molecular pathways across vertebrates.
- Brain and Somites were chosen as they present high sensitivity when cultured with known teratogens.
- The primitive spinal cord score was used as a correction value in the model (affects on the primitive spinal cord (non cranial neural tube) tend to be associated with in vivo teratogens).
- Morphological score assessment of these structures was explored to determine their relative predictivity.

Generating a Predictive Model con't

Respective morphological scores were evaluated against the *in vivo* teratogenic criteria and a score formula was generated as follows:

Formula = (Mean morphological score of Brain + Somites) – Spinal Cord Deviation (SCD)

- **SCD is calculated as follows:**
  Mean spinal cord score is >4.0 but <5.0, then SCD = 1
  Mean spinal cord score is >3.0 but <4.0, then SCD = 2
  Mean spinal cord score is >2.0 but <3.0, then SCD = 3
  Mean spinal cord score is >1.0 but <2.0, then SCD = 4

- **Criteria for Classification of Teratogenic Potency:**
  Score >8.0 characterized as non teratogenic
  Score ≤ 8.0 characterized as a teratogen

Score analysis was conducted on all concentrations. It was concluded that a concentration of 0.1uM was maximally effective in producing results with high concordance.

Concordance Summary Data at [0.1μM]

	In vivo	*In vitro*	Concordant	
Compound	**Classification**	**Prediction**	**Rats**	**Others**
Endothelin R Agonist	Rats: Teratogen Rabbits: Teratogen	Teratogen	Yes/CD	Yes/ Yes
Cox 2 inhib-1	Rats: Teratogen Rabbits: CC	Teratogen	Yes/No	CD
Cox 2 inhib-2	Rats: Non Teratogen Rabbits: Teratogen	Non Teratogen	Yes/ Yes	No/No
CCR3 R antag-1	Mice: Teratogen	Teratogen	NA	Yes/Yes
CCR3 R antag-2	Mice: Non Teratogen Rabbits: CC	Teratogen	NA	No/No
CCR3 R antag-1	Mice: Teratogen	Teratogen	NA	Yes/No
Antifungal	Rats: Non Teratogen Rabbits: Non Teratogen	Non Teratogen	Yes/Yes	Yes/Yes
MTP inhib	Rats: Teratogen Rabbits: Teratogen	Teratogen	Yes/Yes	Yes/Yes
MMPI	Rats: Teratogen Rabbits: Non Teratogen	Teratogen	Yes/No	No/No
Clozapine	Non Teratogen	Non Teratogen	Yes/Yes	Yes/Yes
Olanzapine	Rats: Non Teratogen Rabbits: Non Teratogen	Non Teratogen	Yes/Yes	Yes/Yes
Risperidone	Rats: Teratogen Rabbits: Non Teratogen	Teratogen	Yes/Yes	No/No
Cyproheptadine	Rats: Teratogen	Teratogen	Yes/CD	NA
Pergolide	Non Teratogen	Non Teratogen	Yes/Yes	Yes/Yes
Norfenfluramine	Mice: Non Teratogen	Non Teratogen	NA	Yes/Yes

CC = Cannot calculate in vivo maternal toxicity: fetal toxicity dose ratio
CD = Cannot determine
NA = Not applicable

Predictivity of Fetal Effects Summary

	Systems Affected		
Compound	***in vivo***	***in vitro***	**Predictive Precision**
Endothelin R antagonist	craniofacial	brain, spinal cord, heart, arches, craniofacial	Y
Cox 2 inhib-1	craniofacial, neural tube, limb, resorptions, litter size reductions	brain,arches, craniofacial, viablility (83%)	Y
Risperidone	Some fetal body weight reduction		N
CCR3 antag-1	craniofacial, limbs, litter size reductions	somites, brain, viability (50%)	Y
CCR3 antag-2	resorptions	brain, arches, heart, craniofacial, viability(50%)	Y
CCR3 antag-1	limbs, neural tube	brain, heart, craniofacial, viability (50%)	Y
MMPI	skeletal, craniofacial, neural tube, cardiovascular	brain, heart, arches, craniofacial	Y
MTP inhib	posterior axis, cardiovascular, limbs, resorptions	caudal, somites, brain, spinal cord, heart, arch, craniofacial	Y

a. Predictive Precision = at least 1 system affected

Summary of Preliminary Results

Parameter	Species	Predictivity of Fetal Effects of In Vivo Teratogens
Cumulative Concordance		16/17 = 94%
Concordance	Rats Others	11/12 = 92% 10/14 = 71%
Concordance for Teratogens		10/11 = 91%
Concordance for Non Teratogens		5/6 = 83%
	Concentrations	
Predictivity of Fetal Effects	0.1μM	8/10 = 80%

Predictive Model Conclusions

- A predictive model was developed that is potentially more robust in predictivity than using the standard Total Morphological Score approach.
- This screen was also evaluated for predictivity of fetal effects by comparing target tissue and viability effects *in vitro* to tissue and viability effects *in vivo*. The percent of compounds for which there was at least partial agreement between these data was 80% at 0.1 µM concentrations.
- The assay provides the capability of running an abbreviated assessment of 3 structures (i.e., somites, cranial neural tube, and presumptive spinal cord) for a predictivity assay, or to expand the assessment to the full battery of endpoints for a precision assay.

Optimizing the Predictive Model

Predictive Model could be further enhanced by placing a stronger emphasis on single call data:

- Specific malformations can be diluted in the predictive model.

- Single call data enhances our ability to identify compounds with very specific malformation(s) and allows for accurate categorization.

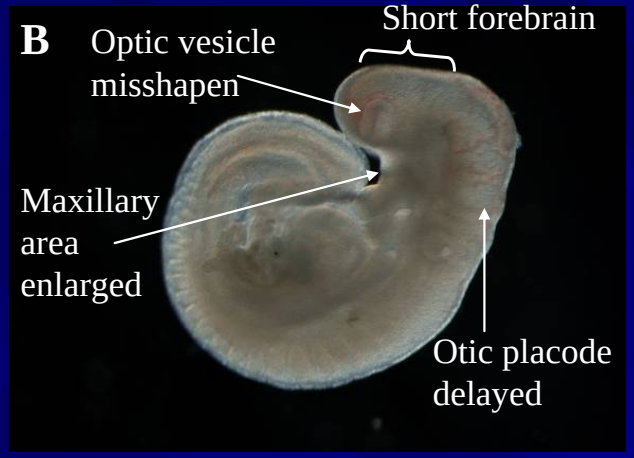
- Neural tube/somite predictive outcomes for the at the 0.1uM concentration vs. additional assessments based upon concentration responsiveness of single calls:

Example of Specific Malformations Call Recordings:

Structure	Observation	Compound X				Control
	Concentration	0.1uM	1uM	10uM	25uM	DMF
Face	SCORE	4.8	4.5	4.1	3.3	5.0
	MF - Small/Narrow				1	
	MF - Misshapen					
	MF - Not Evident					
	Nasal Prominence - Hypoplastic					
	Nasal Prominence - Not Evident					
	Otic Placode - Delayed Develop				1	
	Otic Placode - Small					
	Otic Placode - Misshapen					
	Otic Placode - Hypoplastic					
	Otic Placode - Not Evident					
	Otic Placode - Not Well Defined					
	Optic Vesicle - Delayed Devel					
	Optic Vesicle - Small					
	Optic Vesicle - Misshapen					
	Optic Vesicle - Hypoplastic					
	Optic Vesicle - Not Evident				1	
	Optic Vesicle - Not Well Defined			1	2	
	Maxillary Area - Excess Cells		1	2	5	
	Loss of Cells Below Eye	1		2	2	
	Hypoplasia					
	Blister					

Effects of Cleft Palate Producing Compounds:

Untreated

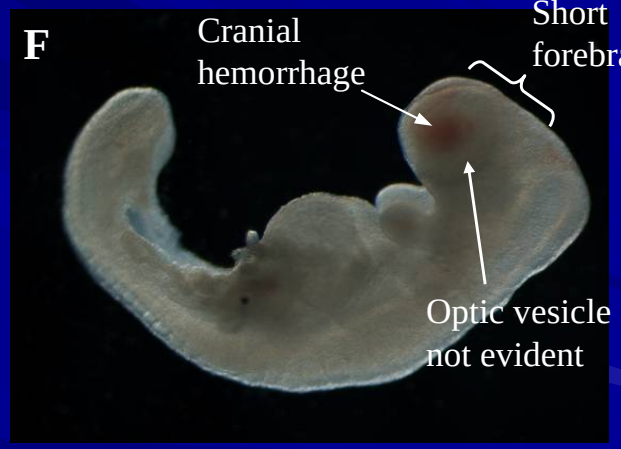


10uM Alprazolam

10uM Hydrocortisone

10uM Diazepam

10uM Phenobarbital



10uM Picrotoxin

Overall Conclusions

- A novel morphological score system and predictive model were developed using the rat WEC screen to allow for teratogenic categorization of test compounds.
- Advantages:
  - increased statistical predictivity when compounds are run at one concentration.
  - follow-up screens with expanded concentration ranges can be utilized to increase predictivity and concordance.
  - provides the capability of running an abbreviated assessment of 3 structures (i.e., somites, rotation and arches) for a predictivity assay, or to expand the assessment to the full battery of endpoints for a precision assay.
  - concentration-response assessment of single call findings can enhance assessment
  - lessons learned
- A validated morphological assessment approach can serve as a predictive teratogenicity screen to support proactive identification of potential leads with low teratogenic liability.
- Additional work includes an expanded test set with statistical

Acknowledgements & References

- Copp, A.J., Cockroft, D.L., *Postimplanation Mammalian Embryos A Practical Approach.* IRL Press 1990.
- Piersma, A. H., et al. Validation of the Postimplantation Rat Whole-embryo Culture Test in the International ECVAM Validation Study on Three *In Vitro* Embryotoxicity Test. *Atla* 2004 (32): p.275-307.
- Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development*. 1993 (118): p.1255-1266.
- Replacement of ether with alternate volatile anesthetics for collection of rat serum used in embryo culture. *Tox in Vitro.* 2004 (18): 719-724.

Special thanks to everyone in the Bristol-Myers Squibb Department of Reproductive Toxicology for assistance and support in the conduct of this project.