In vitro assays for developmental toxicity testing

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introduction

There is increasing interest in alternative assays for in vivo developmental toxicity testing due to awareness that animal use should be reduced. The rat whole embryo culture assay, the embryonic stem cell assay, and the zebrafish embryo assay are such alternatives. Current challenges for further development of these assays are global standardization, appropriate validation, implementation in regulatory testing, and more mechanistic approaches, also feeding in to read across and category approaches as proposed part of integrated testing strategies. Some examples of ongoing research in our laboratory to address these challenges are presented here.

whole embryo culture (WEC)

principle

- post implantation rat embryos are removed at gestation day 10
- these embryos are cultured for 48 hours *in vitro*
- during culture, embryos are exposed to various concentrations of a test compound

> GD10-12 is a period of major organogenesis including closure of the neural tube, development of heart, ear and eye, brachial bars and limb buds. Disturbance during this period may lead to general retardation of growth an development, or to specific malformations in one or several organ anlagen.

- morphology of the embryos is scored after 48h of culture

scoring system *0-6 for each item* (*Brown and Fabro 1981*)

yolk sac diameter	allantois	caudal neural tube	fore limb
crown-rump length	flexion	otic system	hind limb
head length	heart	optic system	maxillaray process
initial somite number	hind brain	olfactory system	mandibular process
final somite number	mid brain	branchial bars	**total morphological score**
yolk sac blood vessels	fore brain		

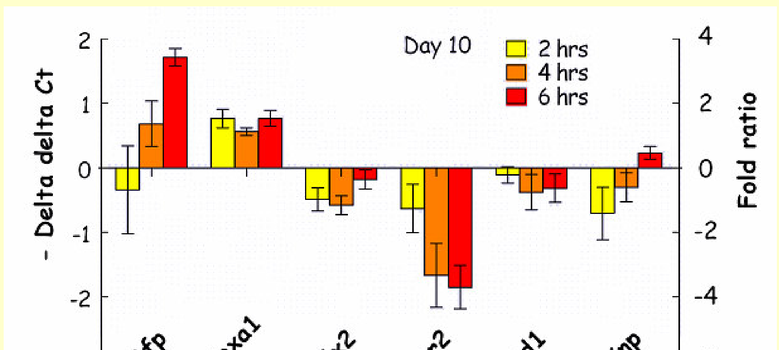


Fig. 2 – Gene expression analysis in GD10 embryos

After exposure to retinoic acid, quantitative real-time PCR shows differential expression of selected genes: Hoxa1, Cdx2, Egr2, Gad1 (developmental); Afp, Brinp (retinoic acid-responsive).

Renkens et al, 2006

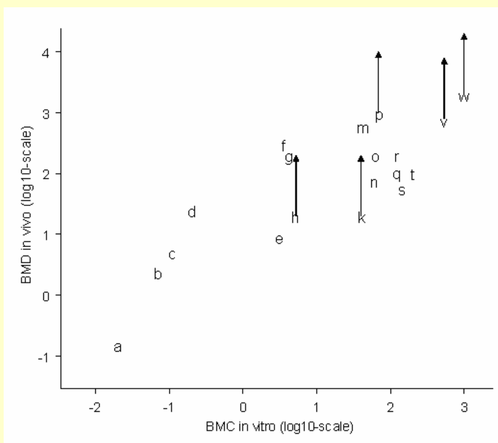
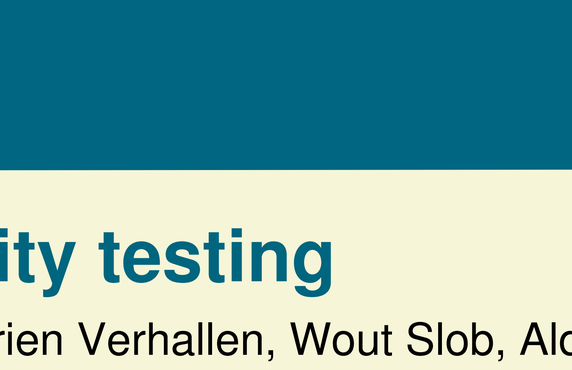
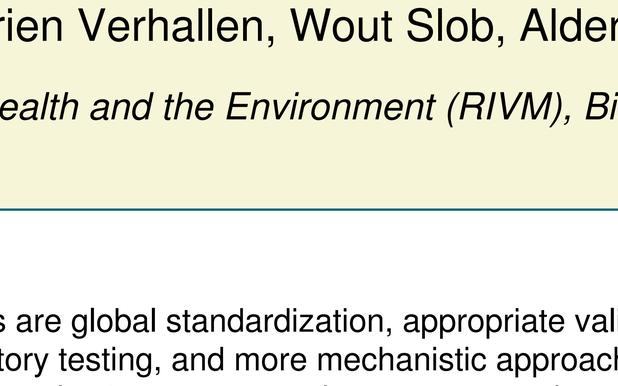


Fig. 3 – Correlation between WEC "total morphological score" and in vivo results

WEC and vivo data show good correlation. Arrows indicate underestimation of BMDs due to dosing limits. Letters indicate respectively methotrexate, 6-aminonicotinamide, all-trans retinoid acid, 5-fluorouracyl, methylmercury, bromodeoxy-uridine, hydroxyurea, diphenydramine, acrylamide, boric acid, lithium, dimethadione, d-camphor, pentyl-VPA, valproic acid (VPA), methoxyacetic acid, salicylic acid, dimehtylphthalate, saccharine.

Piersma et al. submitted





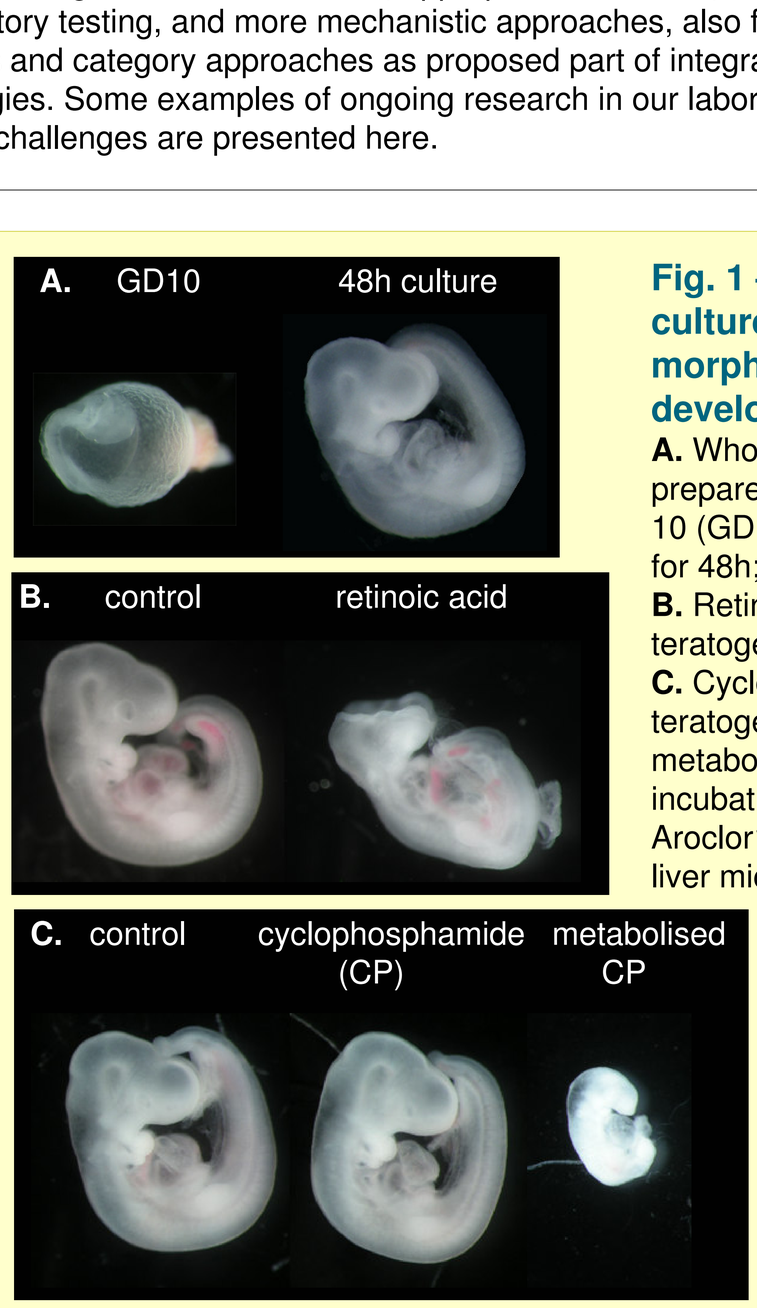


Fig. 1 – Rat embryo culture and effects on morphological development

A. Whole embryos prepared at gestation day 10 (GD10) can be cultured for 48h;
B. Retinoic acid is a teratogen;
C. Cyclophosphamide is teratogenic after metabolisation (by pre-incubation with Aroclor1254-induced rat liver microsomes).

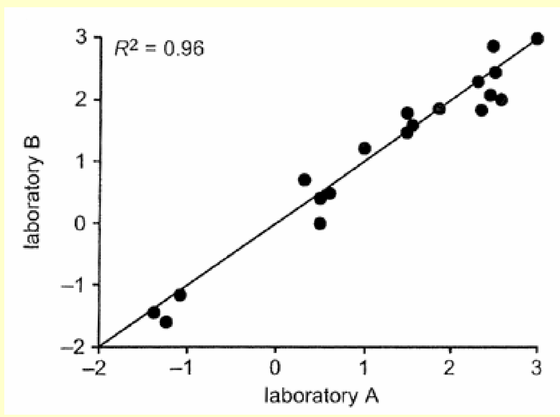


Fig. 4 – Inter-laboratory validation for "total morphological score"

Twenty substances were tested in a ring study in four laboratories for validation of the WEC. This ECVAM validation test set included the compounds listed in Fig. 3 plus isobutyl-ethyl-VPA. Interlaboratory correlations r^2 were always >0.82.

Piersma et al, 2004

embryonic stem cell test

principle

- "embryoid bodies" are produced from embryonic stem cells ("hanging drops" procedure)
- lineage specific differentiation (myogenic, neurogenic etc.) is obtained through specific culture protocols
- this differentiation is disturbed by teratogens
- improvement of the method through gene expression analysis is under development
- validation with ECVAM test chemicals set (see Fig. 3-4) is planned
  - advantage: high throughput protocols
  - challenge: extrapolation to whole organisms

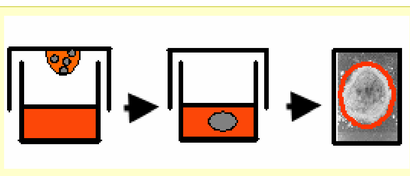


Fig. 5 – Production of embryoid bodies

zebrafish embryo assay

principle

- fertilised zebrafish eggs are exposed at sensitive stages
- analysis of morphology, gene expression, or neurobehaviour is conducted within 5 days post fertilisation

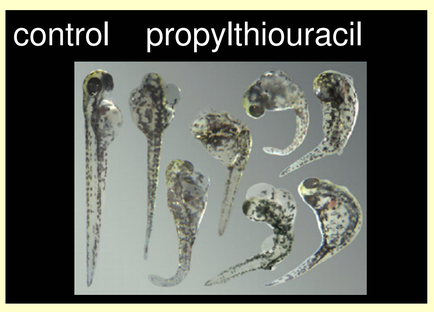


Fig. 6 – Teratogenic effects of propylthiouracil (PTU)

Zebrafish larvae show lordosis, cardiac edema, microcephaly or anencephaly, and eye dysmorphogenesis after exposure to PTU. Control specimen is approx. 5 mm.

Van der Ven et al, 2006

- validation with ECVAM test chemicals set (see Fig. 3-4) is planned
  - advantages: easily obtainable, non-licensed organisms, whole organism, high throughput
  - challenge: extrapolation non-mammalian to mammalian

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