POSTER Nº 8:

Reducing variability of toxicity data:
Novel developmental toxicity assay with Zebrafish embryos (DTZ)

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ABSTRACT

Reducing variability of results is a main issue when developing and validating a new toxicity test. ZF BIOLABS has developed and standardized a protocol to detect developmental toxicity of compounds based on the use of Zebrafish embryos (*DTZ Test*) and one of the objectives has been to increase the test method reliability (intra-laboratory repeatability) but without increasing the complexity and the overall cost of the test.

Different issues have been introduced in the DTZ protocol in order to minimize the variability of the results obtained when testing the teratogenicity of chemical compounds. Standardized maintenance conditions for zebrafish breeders, innovative technology for controlled embryo production, strict quality control of eggs and embryos used for the test are some of the aspects that have been optimized and standardized in the DTZ test. Moreover, a well defined, described and documented Atlas of Abnormalities of Zebrafish development has been developed in order to provide specific morphological endpoints to assess the teratogenicity of the tested compound.

The intra-laboratory repeatability using the DTZ protocol has been analyzed with five teratogenic compounds. The coefficient of variation (CV) of LC50 (concentration that is expected to be lethal to 50% of the embryos at 48 hpf) and Teratogenic Index (ratio of LC50 and EC50 which is the concentration of the compound that can be expected to cause 50% malformation of the embryos at 48 hpf) has been 11,1% and 15,7% for urethane; 16,0% and 7,6% for methylmercuric chloride; 19,5% and 16,1 % for 5-fluorouracil; 22,3% and 26,0% for aspirin and 25,3% and 7,1% for caffeine. These data on intra-laboratory repeatability are compared with other *in vitro* developmental toxicity test (FETAX, EST, etc.).

Results obtained with the DTZ show good results compared with other developmental toxicity tests and thus can be used as a valid and reliable test to asses teratogenicity of chemical compounds with a variability ratio that is acceptable for this type of toxicity tests.

Keywords: toxicity, variability, Zebrafish, teratogens.

INTRODUCTION

The zebrafish embryo was originally used to study the genetics of development due to its transparency, quick embryonic development, easy collection in high numbers and similarity with human development (Page, 1990). In fact the zebrafish embryo constitutes a complete, developing vertebrate organism, and it allows testing predictability for toxicity not only in the context of cellular function, but also at the level of organ and organism toxicity (Hill *et al*, 2005). Moreover the anatomic and genomic similarity with humans (80% genetic homology), foresee tests predictability for human toxicity (Carroll & Fitzgerald, 2003).

All these characteristics make the Zebrafish especially suitable for the assessment of developmental toxicity and highly prone to yield additional information to the cell-based assays. Humans and fishes share many developmental pathways, organ systems and physiological mechanisms, making conclusions relevant to human biology (Crollius& Weissenbach, 2005). The Zebrafish embryo has already been used in the assessment of developmental toxicity, as can be ascertained with nearly 300 publications covering this topic, and recently has gained interest in the pharmaceutical industry as a promising model for the late discovery/early preclinical toxicological studies (Parng, 2005; Rubenstein, 2006).

OBJECTIVES

Reducing variability of results is an important issue when developing or/and validating a new toxicity test (Edler&Ittrich, 2003). **ZF Biolabs** has developed and standardized a protocol to detect developmental toxicity (teratogenicity) of water soluble chemical compounds based on the use of zebrafish embryos DTZ Test (Rodriguez *et al*, 2006).

The main objective of this work has been to develop an appropriate protocol and new standard operating procedures to reduce the intra-laboratory variability of the data obtained using the **ZF Biolabs** DTZ test but without increasing the number of zebrafish embryos needed and/or the complexity of the test.

METHODS

We have analyzed different biological & technological variables in order to reduce the variability of the results obtained using the DTZ protocol. Main focus has been set on:

- Maintenance conditions of zebrafish spawners, including feeding regime.
- Zebrafish embryo production methodology.
- Embryo quality control procedures.
- DTZ endpoints assessment.
- Strict DTZ protocol rules to define valid data.

To evaluate the potential teratogenity of a compound ZF Biolabs' DTZ method analyzes 23 endpoints that have been selected based on the OCDE recommendations and on practical reasons.

After incorporating several improvements in our method, the variability of the results obtained using the "*improved*" DTZ protocol has been evaluated using five teratogenic compounds frequently used in developing new developmental toxicity tests, which are indicated in the following table:

- Urethane (CAS 51-79-6).
- Methylmercuric chloride (CAS 115-09-3).
- 5-Fluorouracil (CAS 51-21-8).
- Aspirin (CAS 50-78-2).
- Caffeine (CAS 58-08-2).

This variability (intra-laboratory repeatability) has been assessed by calculating coefficients of variation CV (%) of the LC50 (concentration that is expected to be lethal to 50% of the embryos at 48 hpf), of the EC50 (which is the concentration of the compound that can be expected to cause 50% malformation of the embryos at 48 hpf) and of Teratogenic Index (ratio of LC50 and EC50).

RESULTS

Standardized maintenance conditions for zebrafish breeders:

- Feeding protocol for reproductive adults, based exclusively on standardized inert food of high nutritional value and digestibility, and with very good stability in water.
- Continuous control of water quality parameters.

Innovative technology for controlled embryo production:

- Fully controlled embryo production: ovulation and spermiation hormonally induced, followed by *in vitro* insemination, obtaining better embryo survival and less test variability.
- High synchrony of the embryo developmental stage at the beginning of the test.
- No disinfection step needed and therefore no side-effects.

Table I: Comparison on embryo viability between natural spawning and induced ovulation

	Natural Spawning	Hormonally Induced
% Fertilization	70.1	**70,5**
% Survival 6-24 hr.	83.2	**92,3**
% Survival 0-48 hr.	82.4	**91,1**

Strict quality control of eggs and embryos:

Two-tier embryo quality control during first hours of development, based on defined morphological criteria. First tier concerning spawns quality (over 60% viability) at 3 hpf. Second tier, embryo quality: only "normal" embryos are selected, discarding "anomalous" and "non fertilized" embryos at 4 hpf.

Table II: Second tier embryo quality control results

	Survival 24h (%)	Survival 48h (%)	Spontaneous malformations 48h (%)	Spontaneous lethality 48h (%)
Without full 2nd quality control tier (anomalous not discarded)*	95.5 +/- 5.0	91.8 +/- 6.5	4.8 +/- 6.6 %	11.1 +/- 7.7 %
With full 2nd quality control tier	97.7 +/- 3.0	94.3 +/- 5.2	5.0 +/- 6.1 %	7.7 +/- 6.1 %

*based on more than 200 embryo data

Atlas of Abnormalities of Zebrafish:

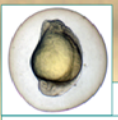
ZF Biolabs has developed an "Atlas of Developmental Abnormalities of the Zebrafish Embryo" based on our own experimental work with teratogen compounds and extensive bibliographic support already available.

The DTZ Atlas is a basic tool to provide detailed information on malformations, functional abnormalities and lethality criteria to be used in the test and therefore preventing variability among the evaluators. Atlas also provides a full protocol to maintenance, feeding, embryo production and selection, data selection and statistical analysis, covering all processes in the DTZ test.

Results obtained using the improved DTZ protocol with the 5 tested compounds is shown in the following table:

Table III: Results obtained using the improved DTZ protocol.

	LC50 (mg/l)	CV (%)	TI	CV (%)	EC50 (mg/l)	CV (%)
5 FLUOROURACIL *(n=4)*	1265,88	19,5	1,65	16,1	865,742	26,7
ASPIRIN *(n=3)*	537,51	22,3	1,18	26,0	460,414	9,8
CAFFEINE *(n=5)*	567,95	25,3	10,85	7,1	56,535	20,2
METHYLMERCURY *(n=9)*	0,30	16,0	1,70	7,6	0,168	19,8
URETHANE *(n=6)*	5182,63	11,1	2,08	15,7	2441,628	10,2
Average CV (%)		**19,0**		**14,5**		**17,3**



DISCUSSION

The variability data obtained compare favourable with variability from other embryotoxic tests: mean coefficient of variation of 40-50% (range 13-195%) have been obtained for the IC50 value (the 50% inhibitory concentration) of chemicals to embryonic stem cells and fibroblasts used in Embryonic Stem Cell Test (Bremer, 2004), CVs of more than 40% for LC50 and TI for caffeine using the FETAX method (Fort *et al*, 1998), etc.

Nevertheless we believe that the present variability could be reduced in different ways:

- Use of an alternative method to the Probit Method for estimating the LC50 and the EC50 (i.e. the Trimmed Spearman-Karber Method).
- Mathematical modelling of the test and use of the Monte Carlo method to try to define the optimum number of embryos to be used in the test.
- Further improvement of the zebrafish embryo production technology.
- Further refining of the DTZ protocol.

DTZ can be used as a valid and reliable test to asses teratogenicity of chemical compounds with a variability ratio that is acceptable for this type of toxicity tests.

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