Optimization of the Zebrafish Embryo Developmental Toxicity Assay (ZEDTA)

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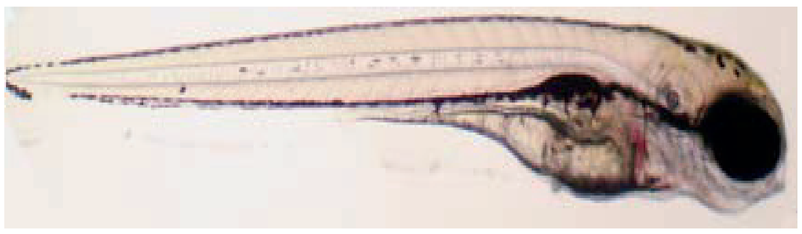
1 Introduction

The ZEDTA is a promising and innovative method with a potential to replace the screening of teratogenicity in animals (rats and rabbits) and is gaining acceptance among scientists and regulators. So far, no harmonized and validated protocol exists for the ZEDTA.

The aim of this research was to optimize the protocol, i.e. examine which combination of exposure parameters is optimal for embryonic and larval development and is at the same time most cost-effective. An optimal condition should yield normal growth and development with minimal mortality and/or malformations.



2 Materials and Methods

The OECD guideline No. 236 was used as base;
Embryos of wildtype Zebrafish in the blastula phase (2-4 hours post fertilization (hpf)) were exposed to blank medium for a total period of 96 hours (100 hpf). One embryo was exposed per well.
The following factors and their combinations were investigated:

- temperature (26 vs. 28 grade C)
- exposure vessels (24 vs. 96 well plates)
- renewal periods (static (S; no renewal) vs. semi-static (SS; 24 or 48 h renewal))
- use of solvent (0.05% v/v DMSO vs. blank medium).

Development was scored daily, using the Extended General Morphology Score (GMS). This system grades the normal development of a zebrafish embryo up to 100 hpf. Assessed endpoints consist of, but are not limited to: detachment of tail, somite formation, eye development, heartbeat and movement. The maximum score at 100 hpf is 18.
Teratogenic endpoints such as malformations of saccule/otoliths, head, hart, tail, yolk, pectoral fins and entire body were scored as 'present' or 'absent' after 96 hours of exposure.
Mean developmental and teratogenic scores were calculated and used to select the most optimal condition for each factor.
Total length of a number of surviving embryos was measured at the end of the exposure as an additional factor.

3 Results

Table 1. Development of exposed embryo's/larvae and the frequency of malformations under different exposure conditions

Treatment	Temp. (°C)	Well-plate	Refreshment period (h)	Number of examined larvae	Survival (%)	GMS score at time of exposure [1]				Frequency of malformations						
						24 h (7)	48 h (12)	72 h (15)	96 h (18)	Head	Sacculi/ otholiths	Tail	Heart	Body shape	Yolk	Total score [2]
Blank	26	24	24	386	94	7	12	15	18	0.0052	0.0026	0.047	0.11	0.039	0.047	0.25
			48	138	96	7	12	14	18	0.0072	0	0.014	0.036	0.014	0.20	0.27
			96	159	95	7	12	15	16	0	0	0.013	0.20	0.031	0.14	0.38
		96	24	37	93	7	12	15	17	0	0	0.027	0.41	0.054	0.27	0.76
	28	24	24	231	96	7	12	15	18	0.013	0.0087	0.026	0.091	0.026	0.026	0.19
			48	23	96	7	12	15	18	0	0	0	0.087	0	0.043	0.13
			96	72	100	7	12	15	17	0	0	0	0.083	0	0.125	0.21
		96	24	34	85	7	12	15	17	0.029	0	0	0.15	0.059	0.15	0.37
DMSO	26	24	24	67	93	7	12	15	18	0.015	0	0.044	0.044	0.029	0.029	0.16
		96	24	55	92	7	11	15	18	0	0	0.055	0.091	0	0.055	0.2
	28	24	24	67	93	7	12	15	18	0	0	0.030	0.030	0	0	0.06
		96	24	52	88	7	12	15	17	0.10	0	0.038	0.31	0.15	0.077	0.67

1. Between brackets the maximum score at the given time point is given
2. Sum of all malformations
3. Colors in the columns show the gradation of effect (green – lowest, red-highest)

Table 2. Growth of larvae (expressed as total body length) after 96 hours of incubation at different conditions

Temperature (°C)	Well-plate	Refreshment period (h)	Average length (mm)	Standard deviations	Number of examined larvae
26	24	24	3.04	1.08	210
		48	3.66	0.22	30
		96	1.66	0.18	70
	96	24	3.58	0.24	60
28	24	24	3.49	0.85	160
		48	3.63	0.30	30
		96	1.65	0.17	40
	96	24	3.37	0.27	60

Colors of cells show the gradation of effect (green – lowest, red-highest

The results showed consistent pattern of responses (see also Table 1 and Table 2):

- A delay of development was observed when medium was not refreshed during the exposure (static design) and in 96-well plates at both temperatures.
- Static design and 96-well plates induced more malformations than semi-static exposure in 24-well plates.
- Larvae incubated at 26 centigrade showed slightly higher frequency of malformations than larvae incubated at 28 centigrade.
- Two most common malformations were heart and yolk oedema.
- Body shape was malformed twice as frequent in 96-well plates than in 24-well plates
- Larvae were shorter when test medium was not refreshed during exposure (96h), independent of temperature.
- The effect of refreshment on larval growth (length) was stronger than the effect of the available space (well-plate).
- Larvae showed highest growth when medium was renewed once during the exposure (48 h).
- Use of DMSO did not increase frequency of malformations or affected the development.

4 Conclusions

- Temperature of 28 centigrade in combination with 24-well plate and 48 h semi-static exposure appeared to be the most optimal for growth and development with the lowest incidence of malformations.
- A delay of growth and development in combination with highest malformation rate was observed when static renewal (96h) was applied or exposure was performed in 96-well plates.
- Although not clearly shown in the presented data, the development of zebrafish is faster in the temperature of 28 than 26 centigrade. In order to prevent possibility that the larvae will reach the protected phase within the duration of the experiment, e.g. under presence of a development accelerating compound, the lower temperature was chosen as more optimal.
- Exposure in 96-well plates was less beneficial than in 24-well plates for both the growth and development. Therefore, the latter was included in the optimized procedure.
- The semi-static design (24 and 48h) was more optimal for the growth and development of zebrafish larvae than the static design (96 h). The 48h semi-static design was chosen as the more cost-effective option.
- Consequently, the following conditions were included in the optimised procedure: semi-static exposure with renewal of medium after 48 hours of exposure, in 24-well plates and at a temperature of 26 centigrade. The maximum concentration of DMSO that can be safely used is 0.5% v/v.

Historical data produced with the optimized protocol is shown on poster MO228.