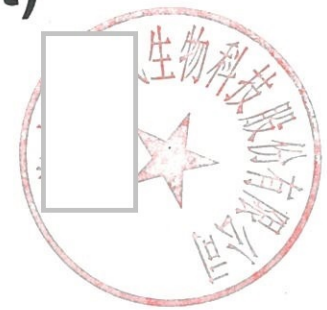
Zebrafish Model for Assessing *Super GelRed* Toxicity and Safety

(Study Report)



Confidential

Toxicity and Safety of *Super GelRed* in Zebrafish Model

Purpose

- **To assess the toxicity and safety of *Super GelRed* in zebrafish model.**

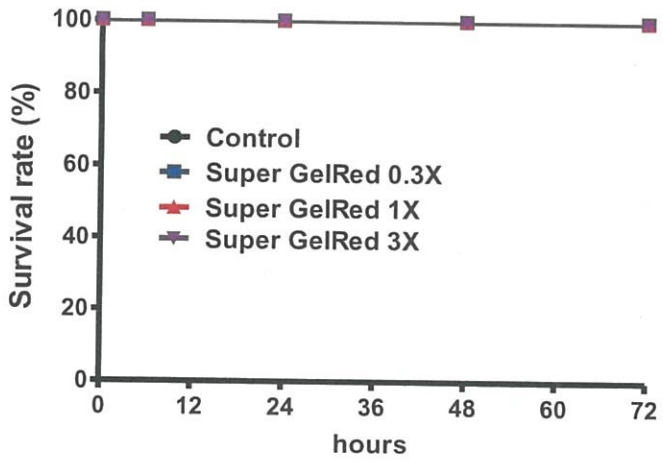
Treatment Window	Endpoints
6hpf-72hpf	Survival rate; Phenotypes; Macrophage migration;
Super GelRed	0.3X, 1X, 3X
Vehicle Control	Fish water

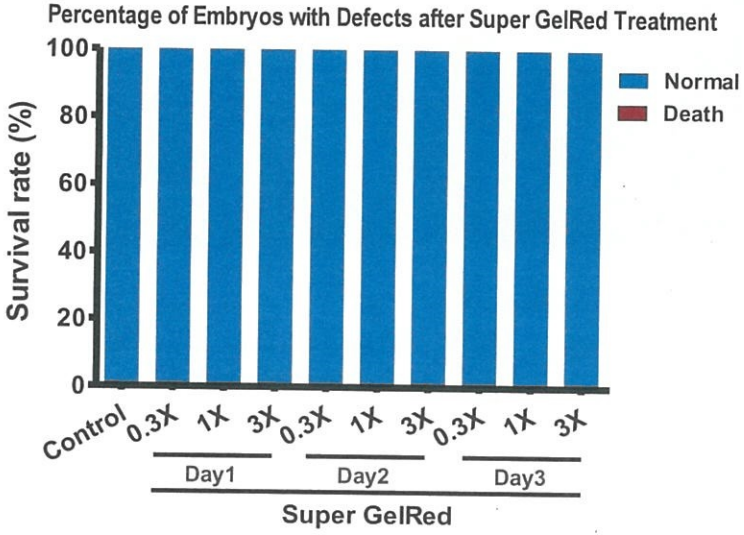
hpf, hours post fertilization.

Parameters

- **Zebrafish Strain:** ***TG(zlyz:EGFP)*** **transgenic lines**
- **Route of Administration:** Soaking in fish water
- **Animal Number:** 60 total embryos for each condition.

Result





	Time (hours)	Vehicle Control	Super GelRed 0.3X	Super GelRed 1X	Super GelRed 3X
Survival rate(%)	6	100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
	24	100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
	48	100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
	72	100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00
		100.00	100.00	100.00	100.00

Time(hours)	Vehicle Control			Super GelRed 0.3X			Super GelRed 1X			Super GelRed 3X		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0	100%	0	60	100%	0	60	100%	0	60	100%	0	60
6	100%	0	60	100%	0	60	100%	0	60	100%	0	60
24	100%	0	60	100%	0	60	100%	0	60	100%	0	60
48	100%	0	60	100%	0	60	100%	0	60	100%	0	60
72	100%	0	60	100%	0	60	100%	0	60	100%	0	60

Figure 1. A time-course plot of percent survival ± SEM in vehicle control vs.Super GelRed for 72 h, n = 20 (n refers to the number of independent experimental observations, each evaluating the survival of approx.60 embryos).

Zebrafish treated with *Super Gelred* present normal development.

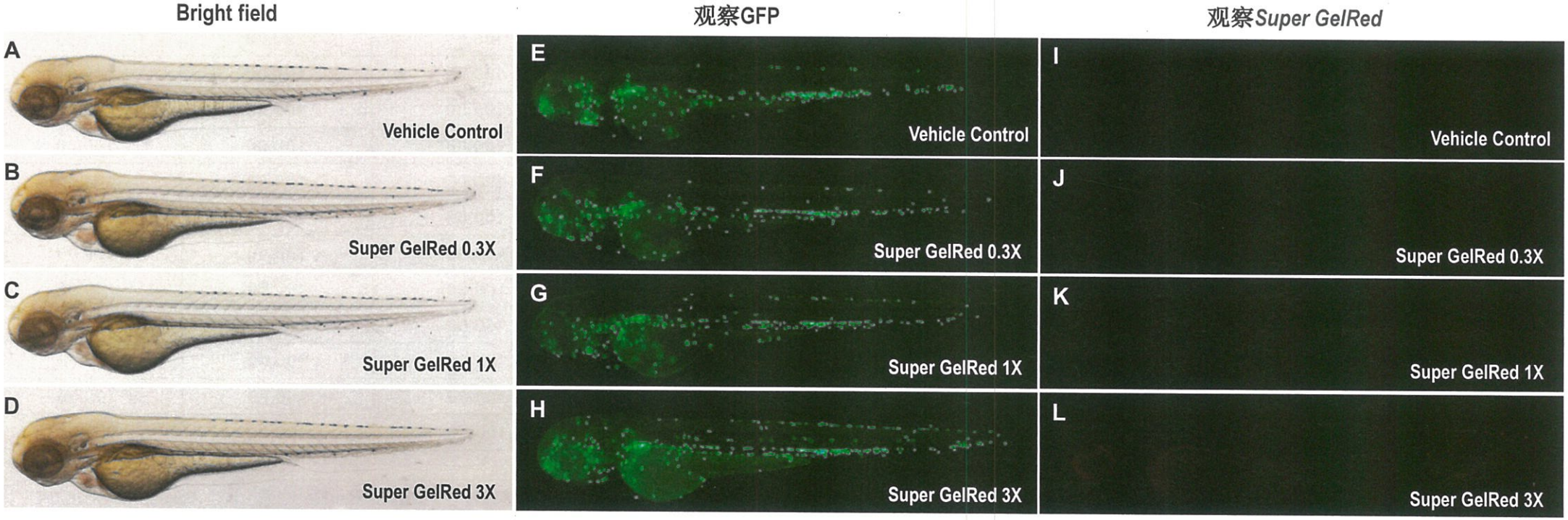


Figure 2. Zebrafish treated with *Super Gelred* present normal development.

（A-L）在四种***Super GelRed***浓度（0×、0.3×、1×和3×）的水中孵育Tg(zlyz:mGFP) zebrafish embryos 72h后进行显微镜观察。

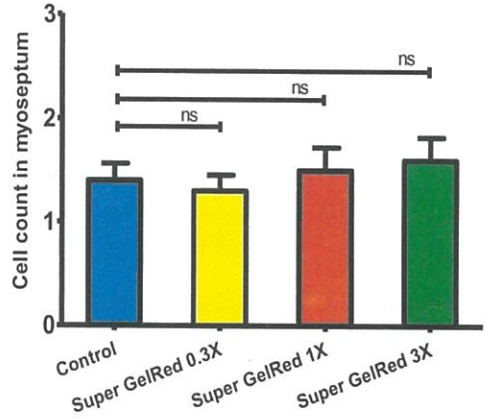
(E-H)所有Tg(zlyz:mGFP) zebrafish embryos巨噬细胞数量和位置几乎没有发生变化。

(I-L)≤1×***Super GelRed***的Tg(zlyz:mGFP) zebrafish embryos体内不含***Super GelRed***染料。

(M) Quantification of the macrophage number in myoseptum. Columns, mean; bars, SEM (n =10; ANOVA; ns, not significant.). dpf, days post fertilization.

即在该实验条件下，3×Super GelRed不影响斑马鱼胚胎的健康，≤1×Super GelRed不可进入斑马鱼胚胎体内。

M



Supplementary Figure1

Materials and Methods

Zebrafish Care and Maintenance

Adult zebrafish were maintained at 28.5 ℃on a 14 h light/10 h dark cycle (***ref1***). Five to six pairs of zebrafish were set up for nature mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28.5℃ in fish water (0.2% Instant Ocean Salt in deionized water). The embryos were washed and staged according to (***ref2***). The establishment and characterization of the *TG(zlyz:EGFP)* transgenic lines has been described elsewhere (***ref3***). The zebrafish facility at Shanghai Research Center for Model Organisms is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Zebrafish Embryo Toxicity Testing

During toxicity experiments, zebrafish embryos were treated with uremic solution from 6-hpf (hours post fertilization) to 72-hpf and mortality was recorded every 24h. Dead zebrafish was defined as the absence of heartbeat under a dissecting stereomicroscope (Nikon SMZ745; Japan). Mortality curves were generated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data from at least three independent experiments were used for statistical analysis.

Zebrafish in vivo macrophage migration assays

TG(zlyz:EGFP) transgenic zebrafish embryos (6-hpf) were treated with vehicle control (fish water) and Super Gelred (0.3X, 1X and 3X) for 72h in a 12-well plate format (twenty embryos per well) (BD Falcon). All embryos were incubated at 28.5 ℃. After treatment, embryos were washed with fish water three times and anaesthetized with 0.016% MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO) and the number of macrophages recruited to the body trunk was counted.

Image acquisition

Embryos and larvae were analyzed with Nikon SMZ18 Fluorescence microscope and subsequently photographed with digital cameras. A subset of images was adjusted for levels, brightness, contrast, hue and saturation with Adobe Photoshop 7.0 software (Adobe, San Jose, California) to optimally visualize the expression patterns. Quantitative image analyses processed using image based morphometric analysis (NIS-Elements D4.6, Japan) and ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/). Inverted fluorescent images were used for processing. Positive signals were defined by particle number using ImageJ. 10 animals for each treatment were quantified and the total signal per animal was averaged.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis and graphical representation of the data were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was performed using a Student's t test, ANOVA, or χ^2 test as appropriate. Statistical significance is indicated by *, where $P < 0.05$, and ***, where $P < 0.0001$.

Supporting References

[1]. Westerfield M. The Zebrafish Book: A Guide for the Laboratory use of Zebrafish. Eugene: The University of Oregon Press ,1993.

[2]. Kimmel CB, Ballard WW, Kimmel SR, et al. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995 Jul;203(3):253-310.

[3]. Zhang Y, Bai XT, Zhu KY, et al. In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury. *J Immunol.* 2008 Aug 1;181(3):2155-64.

Reference

1. Brannen KC, Panzica-Kelly JM, Danberry TL, et al. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res B Dev Reprod Toxicol. 2010 Feb;89(1):66-77.
2. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. 2012 Jul 2;122(7):2337-43.
3. Truong L, Harper SL, Tanguay RL. Evaluation of embryotoxicity using the zebrafish model. Methods Mol Biol. 2011;691:271-9.
4. Zhang Y, Bai XT, Zhu KY, et al. In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury. J Immunol. 2008 Aug 1;181(3):2155-64.
5. d'Alençon CA, Peña OA, Wittmann C, et al. A high-throughput chemically induced inflammation assay in zebrafish. BMC Biol. 2010 Dec 22;8:151

主要试剂耗材、仪器设备

英文名称（English Name）	中文名称 （Chinese Name）	分子量（MW）	货号 （Cat#）	供应商（Supplier）
12-well plate	12孔培养板	无	#353043	BD Falcon
100-mm petri dish	100mm培养皿	无	#353003	BD Falcon
10μL Filter Tips	10微升进口滤芯枪头	无	TF-300-R-S	Axygen
200μL Filter Tips	200微升进口滤芯枪头	无	TF-200-R-S	Axygen
1000μLFilter Tips	1000微升进口滤芯枪头	无	TF-1000-R-S	Axygen
1.5mL microcentrifuge tubes	进口1.5mL离心管	无	MCT-150-C	Axygen
Methyl cellulose	甲基纤维素	无	M0262	Sigma
Ethyl 3-aminobenzoate methanesulfonate salt	3-氨基苯甲酸乙酯 甲磺酸盐	261.29	A5040	Sigma

中文名称 （Chinese Name）	型号	供应商（Supplier）
体式荧光显微镜	SMZ-18	日本尼康（Nikon）
体式显微镜	SMZ-745	日本尼康（Nikon）
光照恒温培养箱	GZP-250S	上海精宏实验设备有限公司
0.1-2μl单道移液枪	P2	法国吉尔森（GILSON）
1-10μl单道移液枪	P10	法国吉尔森（GILSON）
50-200μl单道移液枪	P200	法国吉尔森（GILSON）
200-1000μl单道移液枪	P1000	法国吉尔森（GILSON）