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Short Communication

A MOLECULAR METHOD FOR ASSESSING THE EFFECTS OF POTENTIAL CONTAMINANTS ON THE RATE OF ZEBRAFISH (*DANIO RERIO*) DEVELOPMENT

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Abstract: Monitoring expression of the developmentally regulated genes *shh*, *sox2*, and *tnnt1* by reverse transcriptase polymerase chain reaction (RT-PCR) allows determination of the rate of embryogenesis in zebrafish (*Danio rerio*) embryos without direct visual observation. The utility of combining this approach and morphological methods during toxicity studies was demonstrated with embryos developing at either 28.5 °C or 24.5 °C and with embryos exposed to sublethal doses of silver nanoparticles. *Environ Toxicol Chem* 2014;33:238–242. © 2013 SETAC

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INTRODUCTION

Assessing the effects of a potential contaminant on a developing organism can provide insights that may never come to light if applied only to a fully developed adult. Toxicity studies using adult organisms typically involve observations of endpoints (e.g., mortality) at set intervals after a one-time, pulsed or continuous dosing. Use of early life stages is advantageous; it allows greater opportunity to investigate the mode of action of a potential contaminant, greater sensitivity to the presence and effect of a potential contaminant, the ability to observe potential teratogenicity, and the ability to use developmental rate as a valuable biomarker [1].

Zebrafish are an appealing vertebrate model for toxicological testing, particularly during embryogenesis. The external development of large translucent embryos allows immediate, direct exposure to contaminants and noninvasive observation [2–4]. Females typically spawn according to light cycle, producing hundreds of nearly synchronous embryos weekly. Zebrafish are also attractive for high-throughput testing and –omics scenarios [5,6] because embryogenesis is brief (~72 h to hatch). Furthermore, a completely sequenced genome provides resources to assess the impact of contaminants at the molecular level [7–9].

In zebrafish, rate of development is commonly used to assess the potential effects of a contaminant [10–14]. Assessment of developmental rate is typically accomplished by observation of morphology at defined intervals and then measurement of the time required to meet certain morphological milestones (Table 1) [1]. Several recent studies using zebrafish embryos have suggested developmental delays at sublethal concentrations of various contaminants, but the characterization of those delays has been minimal [1,10–14]. When using traditional observational techniques, detailed characterization of rate delay can be difficult and labor-intensive. Variables such as position or movement of the embryo at time of observation can hamper accurate measurements unless the embryo is anesthetized or sacrificed. These concerns highlight the need for an expanded tool set for the assessment of developmental rate in zebrafish.

In the present study, a molecular method for the measurement of developmental rate was described, demonstrated, and compared with a comprehensive morphological milestone measurement technique. The expression patterns of 3 developmentally regulated genes were analyzed using reverse transcriptase polymerase chain reaction (RT-PCR) to provide a set of molecular milestones that appear over time and can be plotted to show relative rates. The usefulness of this molecular method was demonstrated using 2 stressors: temperature known to slow development without causing abnormalities [1] and silver nanoparticles. Silver nanoparticles are of particular interest because they have been studied and used extensively for commercial application [15,16] in large part because of their antimicrobial properties [2,17]. Exposing zebrafish embryos to silver nanoparticles has been shown to cause behavioral changes [18,19], teratogenicity [4], and toxicity [10].

The specific objectives of the present study were 1) to ascertain the expression pattern of 3 developmentally regulated genes (*sox2, shh, tnnt1*) to establish a complementary molecular milestone method for assessing developmental rate in zebrafish; 2) to evaluate the efficacy of this method by measuring the developmental rate of zebrafish embryos under thermal stress (24.5 °C) using molecular milestones versus morphological milestones; and 3) to demonstrate the potential usefulness of the molecular milestone method by assessing delay patterns of zebrafish embryos exposed to silver nanoparticles versus controls, then comparing measurement of this delay using both molecular and morphological methods.

MATERIALS AND METHODS

Zebrafish culture

Zebrafish (*Danio rerio*) were obtained from local sources, separated by gender, and cultured in 38-L tanks containing system water. System water consisted of deionized water containing 0.06 g/L Instant Ocean, pH 7.0 ± 0.2, maintained at 28.5 ± 0.5 °C. Fish were kept on a 14:10-h light:dark cycle and fed TetraMin flake food twice daily, supplemented with brine shrimp. All treatments of adult and embryonic zebrafish and

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Table 1. Description of morphological milestones[a]

Milestone	Description	Still image
256 Cells	Mass of 256 cells on yolk sac	
Sphere	Cell mass compacts to form a sphere including yolk sac; cell mass appears to have a flat surface at junction with yolk sac	
30% epiboly	Cell mass forms a cap that envelops ~30% of yolk sac	
Shield	Cell mass cap that envelops ~50% of yolk sac; 1 leading edge of moving cells begins to involute	
80% epiboly	Cell mass cap that envelops ~80% of yolk sac	
Bud	Cell mass appears as a ring around yolk sac; head and tail buds emerge at poles of embryo	
4 somites	4 clearly defined somites on trunk when viewed from the side	
10 somites	10 clearly defined somites on trunk when viewed from the side	
13 somites	13 clearly defined somites on trunk when viewed from the side	
Movement	First discernable muscle contraction	
17 somites	17 clearly defined somites on trunk when viewed from the side	
22 somites	22 clearly defined somites on trunk when viewed from the side	
Pigmentation	Appearance of 20 pigment spots on head and trunk	

[a]Adapted from Beasley et al. [1].

experimental protocols were conducted in accordance with the approved Middle Tennessee State University Institutional Animal Care and Use Committee protocol 09-008.

Embryo collection

Matings were staged twice weekly in separate holding tanks (Carolina Biological) with perforated floors that allowed embryos to fall out of reach of the adults. Embryos were collected, washed for 1 min with a 0.05% bleach solution, and serially rinsed with system water to eliminate microbial contamination. Collected embryos were screened to ensure fertilization and verify approximate age. Recently fertilized embryos were placed in system water (or treatment) in 10-cm Petri dishes and incubated in an environmental chamber (Model 2015; Sheldon) set to 28.5 ± 0.1 °C or 24.5 ± 0.1 °C. At specific time points after fertilization, 30 treated (or control) embryos were removed and then flash-frozen as a pooled group for RNA extraction. This pooled group became 1 replicate. Pooling embryos in this way ensures sufficient quantities of RNA for subsequent gene expression analysis. Replicates in figure legends refer to the number of pooled embryo samples from independent experiments.

Gene expression studies

Pooled groups of 30 embryos under various treatment conditions and at various times were flash-frozen in liquid nitrogen. Then RNA was extracted from each group of embryos using the TriZol–chloroform extraction and column purification techniques outlined by Lan et al. [20]. The resulting RNA was analyzed using a NanoDrop spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies) to assess concentration, purity, and integrity. Only RNA that surpassed an RNA Integrity Number of 8.0 was further processed into complementary DNA (cDNA). Synthesis of cDNA was performed using oligoDT primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. For each sample, cDNA synthesis reactions that excluded reverse transcriptase were performed to ensure that genomic DNA contamination was insufficient to produce a PCR product.

Three previously characterized developmentally regulated genes (*shh, sox2*, and *tnnt1)* were chosen for analysis [21–23]. The housekeeping gene *ef1a* was included in the analysis as an internal control [24,25]. For each gene, RT-PCR was performed using published gene-specific primers, synthesized cDNA, and Platinum *Taq* polymerase (Invitrogen) according to the manufacturer's recommendations. The RT-PCR products were visualized with the Agilent 2100 Bioanalyzer to determine the expression pattern of each target gene and to verify the size of each product. We detected *ef1a* transcripts in every sample. Onset of expression of *shh, sox2*, and *tnnt1* was defined as the earliest time point (at 1-h intervals) at which the transcript could

be detected from each biological replicate (pool of 30 embryos generated in independent experiments). Variability between replicates was observed in only 1 instance: *sox2* expression at 24.5 °C was observed in 3 of 6 replicates at 7 h postfertilization (hpf) and in all 6 replicates at 8 hpf. It was therefore assigned an onset value of 7.5 ± 0.22 (standard error) hpf.

Onset of expression data (in hpf) at each temperature or treatment condition was used to assess relative rates of development. The molecular milestones were positioned along the y-axis (assigned ordinate values) according to when their onset of expression occurred at 28.5 °C, our standard conditions. Because of this positioning, a plot of the expression data at 28.5 °C yields a straight line with a slope of 1.0 and y-intercept of 0. The slope of this line represents the standard rate of development. Expression data at 24.5 °C or in the presence of silver nanoparticles were then assigned the same ordinate values as those assigned to the 28.5 °C data set. Plotting the measured onset of expression in hpf (x-axis value) versus the ordinate values generated at standard conditions allows visualization of the change in timing with respect to standard conditions. Furthermore, the slopes of the resulting linear trend lines should reveal the overall rate of development under treatment conditions relative to the rate at standard conditions.

Silver nanoparticle exposure

Silver nanoparticles were provided by nanoComposix and synthesized via methods explained by Park et al. [26]. Briefly, silver nanoparticles were synthesized using an aqueous reduction technique from silver salts to a final concentration of 2 mM in phosphate buffer. Nanoparticle size and shape were determined via transmission electron microscopy by examining 100 random particles. All nanoparticles were approximately spherical in shape and had an average diameter of 53.1 ± 4.1 nm. All zebrafish nanoparticle exposures occurred in 10-cm Petri dishes at a concentration of 0.5 mg/L and a volume of 10 mL in an environmental chamber (Model 2015; Sheldon) set to 28.5 ± 0.1 °C. All nanoparticle solutions were probe-sonicated immediately prior to use to ensure even dispersal of nanoparticles according to Bowman et al. [12].

Statistics

Linear regression analysis was performed to determine the existence of a significant linear relationship ($\alpha = 0.05$) for the molecular milestones at 24.5 °C and silver-exposed zebrafish. Analysis of covariance (covariate of time and fixed factor of temperature/silver) was performed to determine whether significant differences ($\alpha = 0.05$) in developmental timing using the molecular milestone method existed between zebrafish exposed to different temperatures and between silver-exposed and untreated embryos.

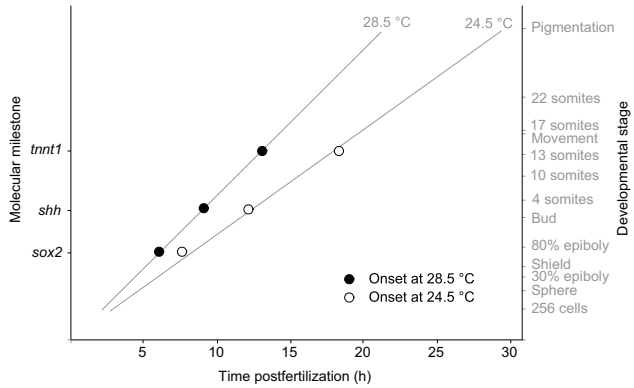

Figure 1. Zebrafish development in hours postfertilization. Solid circles indicate mean onset of expression of *sox2, shh*, and *tnnt1* at 28.5 °C ($n = 3$). Open circles indicate mean onset of expression of *sox2, shh, and tnnt1* at 24.5 °C ($n = 3$). Solid lines indicate developmental rate at 28.5 °C and 24.5 °C as measured by Beasley et al. [1].

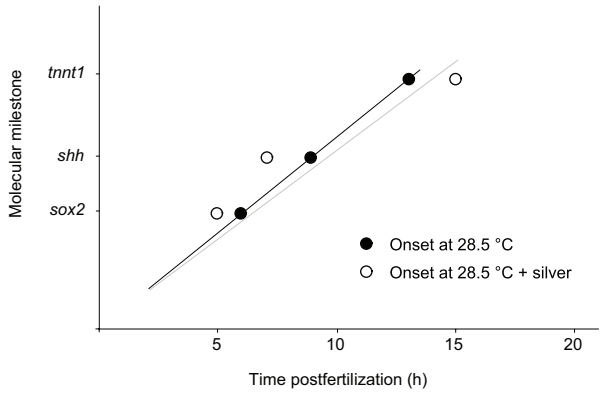

Figure 2. Onset of *sox2, shh*, and *tnnt1* (hours postfertilization) in zebrafish embryos incubated at 28.5 °C. Open circles indicate mean onset of expression of *sox2, shh*, and *tnnt1* in embryos exposed to silver nanoparticles ($n = 3$). Solid circles indicate mean onset of expression of *sox2, shh*, and *tnnt1* in untreated embryos ($n = 3$).

RESULTS

Timing of the onset of detectable expression for each gene at 28.5 °C was established using RT-PCR as described in *Materials and Methods*. The *sox2* transcripts were first detectable at 6 hpf, *shh* transcripts at 9 hpf, and *tnnt1* transcripts at 13 hpf (Figure 1). These molecular milestones were then used to establish a baseline of expression timing with a slope of 1, representing the rate of development at 28.5 °C.

When incubation temperatures were lowered to 24.5 °C, *sox2* transcripts were first detectable at 7.5 ± 0.22 hpf, *shh* transcripts at 12 hpf, and *tnnt1* transcripts at 18 hpf (Figure 1). A comparison of the time to achievement of molecular milestones at 24.5 °C and 28.5 °C revealed a significant developmental delay ($F_{1,3} = 13.37$; $p = 0.021$) at the colder temperature (Figure 1). This comparison revealed that the 1.5-h delay in the expression of *sox2* widened to a 5-h delay for *tnnt1*, and the overall rate was slowed to 73% of that at 28.5 °C. These results were remarkably similar to those previously reported [1] using morphological milestones (Figure 1).

In embryos exposed to silver nanoparticles, the onset of detectable expression of *sox2* was observed at 5 hpf, that of *shh* at 7 hpf, and that of *tnnt1* at 15 hpf (Figure 2). Compared with results for untreated embryos, *sox2* and *shh* transcripts were detectable at earlier time points in the presence of silver nanoparticles, whereas *tnnt1* transcripts were detectable 2 h later. A comparison of time to achievement of molecular milestones between silver-exposed embryos and control embryos (28.5 °C) revealed no significant delay in development ($F_{1,3} = 0.06$; $p = 0.816$; Figure 2).

DISCUSSION

A robust toolset for the characterization of effects of potential contaminants on the rate of development in zebrafish is needed. Sublethal concentrations of contaminants have been found to alter the rate of embryogenesis in zebrafish [10,11,13,14,27–29].

Traditional microscopic observation of developing embryos is useful for measuring such effects but has limitations. Dechorionation of the embryo, use of anesthesia, and eventual sacrifice are often required; these are labor-intensive, and can render the fish unusable for continued study. Direct observation of embryos without anesthesia, in contrast, makes reliable measurements difficult because of the positioning and movement of embryos at the time of observation. Furthermore, measurement guidelines are not well standardized; Kimmel et al. [3] set forth basic guidelines, but subsequent studies have used variations on those guidelines [1,30,31].

A combined morphological and molecular approach would be ideal for evaluation of effects of potential contaminants on rate of development. Gene expression drives development; therefore, tracking the onset of gene expression of developmentally regulated genes would be appropriate when developmental rate is used as a biomarker [32]. Many developmentally regulated genes are well characterized (e.g., *shh)* [33,34] but have not been used to determine the overall rate of embryogenesis. Evaluating onset of gene expression via RT-PCR can be utilized as a binary outcome; a PCR product is visible only if the target transcript is sufficiently abundant at a specific time point, and no product is visible prior to onset [32].

A comparison of the present study with previous work shows similarities in timing of target gene expression. Previous studies showed weak sox2 expression at 5 hpf and strong expression at 9 hpf [21]. The present study first detected expression of *sox2* at 6 hpf. Expression of *shh* has been observed as early as 5 hpf to 7 hpf by various methods [33,34], but the PCR parameters were not provided for comparison. The primers used in the present study were taken from Okuda et al. [23]; and in that study, *shh* expression is absent at 6 hpf and present at 9 hpf. The present study also first detected *shh* expression at 9 hpf. Ambiguous expression of *tnnt1* has been reported starting at 9 hpf [21]; using the methods outlined in the present study, *tnnt1* transcripts were observed starting at 13 hpf.

Because temperature directly affects rate of development in exothermic animals, it is frequently used as a stressor when evaluating developmental rate. Hallare et al. [28] showed that development of zebrafish embryos under extreme cold stress (21 °C) progressed at one-half of the rate of development at control temperatures (26 °C). Likewise, Beasley et al. [1] observed that when incubation temperature was lowered to 24.5 °C, normal development progressed at 75.8% of that seen at 28.5 °C [1]. In the present study, zebrafish embryos were subjected to a lower temperature (24.5 °C) to compare this molecular method with existing morphological methods of tracking developmental rate. When molecular milestones were tracked through embryogenesis at 24.5 °C, comparison with milestones at the standard temperature of 28.5 °C corroborated a significant delay in developmental rate of 73% (Figure 1). A comparison with studies that utilized morphological methods showed striking similarity in observed delays [1,3].

As a further application of this molecular method, embryos exposed to sublethal concentrations (0.5 mg/L) of silver nanoparticles were analyzed to substantiate previous observations of developmental delay (Figure 2) [12]. Various studies have noted delays in developmental rate at similar concentrations of silver nanoparticles [12,27,32] but have not yet characterized these delays. Bowman et al. [12] observed a slight rate delay using a measurement of eye–otolith distance; however, a subsequent study by Beasley et al. [1] showed that this delay was not significant using a comprehensive morphological technique. Likewise, the present study utilizing molecular milestones confirmed that silver nanoparticles did not have a significant effect on the overall rate of embryogenesis.

It is interesting to note that the linear trend line for silver nanoparticle–treated embryos depicted in Figure 2 does not appear to be the best fit for the data. While this depiction is necessary for a simple comparison of the overall developmental rates, it assumes that changes in rate are uniform throughout development. Although this seems to be true for development at different temperatures (Figure 1 and Kimmel et al. [3]), it is certainly possible that silver nanoparticles (or other contaminants) could affect rate differently at different stages of development. An expanded set of developmentally regulated genes would be useful in such cases.

In summary, a molecular method for measuring the rate of embryonic development in the zebrafish *Danio rerio* was described. The method was as effective as those based on morphology at detecting the decreased rate resulting from incubation at lower temperature. The method was also used to demonstrate that silver nanoparticles cause no significant overall delay in developing embryos in the early stages of embryogenesis. Using a combination of time-lapse image sequences to track the appearance of a set of morphological developmental milestones [1] and RT-PCR to determine the onset of expression for specific developmentally regulated genes yields a robust toolset for measuring effects on developmental rate. These combined methods provide a strong weight-of-evidence approach for evaluating the effects of a potential contaminant on zebrafish in early life stages.

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