

Original Article

# Overdose of D-serine Induces Movement Disorder and Neuromuscular Changes of Zebrafish Larvae

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**Abstract:** D-serine is a well-known activator of N-methyl-D-aspartate receptors; however, little is known about the teratogenic effects of D-serine overdose during early embryonic development. Here, we used zebrafish as a model to test toxicity and teratogenicity, since they have transparent eggs, making the organogenesis of zebrafish embryos easier to be observed. After D-serine injection (100–1000 ppm), the most evident defective phenotypes were bent trunk phenotypes, including malformed somite boundary, twisted body axis and shorter body length. As the injection dosages increased, the rates of embryos with bent trunk phenotypes decreased (0% for 0 ppm, n=573; 59.9–84.3% for 100–1000 ppm of D-serine, n=383–451). In addition, D-serine-injected embryos exhibited significantly reduced the frequencies of spontaneous in-chorion contraction (21.7 for 0 ppm vs. 18.3–0.9 for 100–1000 ppm D-serine, n=30) in comparison with mock-treated controls (0 ppm). Subtle changes are easily observed by staining with specific monoclonal antibodies F59, Znp1, Zn5 and  $\alpha$ -bungarotoxin to detect morphological changes in muscle fibers, primary motor axons, secondary motor axon projections and neuromuscular junctions, respectively. Our data show that overdose of D-serine leads to misalignment of muscle fibers and motor neuron defects, especially secondary motor neuron axonal growth defects. (DOI: 10.1293/tox.2013-0032; J Toxicol Pathol 2014; 27: 19–24)

**Key words:** D-serine, developmental toxicity, NMDA receptor

## Introduction

The standard amino acids are of the L-form, but their enantiomers, D-amino acids, are found in some proteins, such as peptidoglycan cell walls of bacteria<sup>1,2</sup>. It has been reported that D-amino acids accumulated in different tissues, which might represent different physiological conditions. For example, accumulation of D-aspartate and D-hydroxyproline in dentin, tooth enamel and the crystalline lens can be used as aging index<sup>3,4</sup>. Also, a large amount of D-serine accumulation was found in the frontal brain, cerebellum, cortex, hippocampus and microglia<sup>5–7</sup>. These findings indicate that the distributions of D-amino acids are diverse and may have different physiological roles.

D-serine is highly associated with neurodegenerative diseases such as schizophrenia and amyotrophic lateral sclerosis (ALS)<sup>8–13</sup>. Importantly, it was reported that D-serine could act as a potent activator of N-methyl-D-aspartate (NMDA)-type glutamate receptors<sup>14–16</sup>, indicating that D-

serine is an important neurotransmitter. In mammals and zebrafish, blockage of NMDA receptors induces some neurological defects, such as seizures and impairment of learning and memory. This means that the biological roles of D-serine might be conserved between zebrafish and mammals<sup>17–21</sup>. In this regard, D-serine-induced toxicity is worthy of study.

In rats, D-serine exposure resulted in changes in a number of pathways that may be associated with neuronal dysfunction<sup>22</sup>. In addition, administration of D-serine induced oxidative stress and resulted in renal tubular necrosis and hyperaminoaciduria<sup>23–25</sup>. These observations indicated that an excess of D-serine caused severe adverse effects such as neurotoxicity and nephrotoxicity in adult animals. However, the developmental toxicities of D-serine have not been fully clarified. Thus, development of an alternative model to study D-serine-induced developmental toxicities is essential.

Zebrafish are a good model for toxicological experiments because they produce a large number of transparent embryos and have well-characterized developmental stages. To develop a zebrafish model for studying D-serine-induced developmental toxicities, we generated a series of time- and dose-dependent D-serine exposure experiments. By staining with specific monoclonal antibodies, subtle changes in neuronal axon formation and myofibril alignment can be easily observed. This strategy is efficient for studying D-serine-induced developmental toxicities.

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## Materials and Methods

### *Fish care, embryo collection and D-serine administration*

Mature zebrafish (AB strain) were raised at the zebrafish facility of the Life Sciences Development Center, Tamkang University. Embryos were produced using standard procedures<sup>26</sup> and were staged according to standard criteria (hours post fertilization, hpf)<sup>27</sup> or by days post fertilization (dpf). D-serine (Sigma) was dissolved in sterile distilled water to the desired concentrations (0, 100, 500, 1000 ppm), and was microinjected with a Nanoliter 2000 (World Precision Instruments, Sarasota, FL, USA) into the cytoplasm of one-cell stage embryos (2.3 nl/embryo). After microinjection, embryos were cultivated at 28.5°C, and survival rates were determined at 27 and 48 hpf.

### *Spontaneous embryonic contractions*

The spontaneous in-chorion contraction of zebrafish embryos was analyzed as previously described<sup>28,29</sup>. Briefly, zebrafish embryos at 24 hpf without or with injection of different concentrations (100, 500 and 1000 ppm) of D-serine were collected and recorded. Spontaneous in-chorion contractions were defined based on the angle of the tail displacement relative to the body axis. Embryos with tail movements from one side to the other at any angles were classified as having in-chorion contraction.

### *Antibody labeling, acetylcholine receptor clustering and microscopy*

F59 monoclonal antibody (Hybridoma Bank; 1:10), Znpl (Hybridoma Bank; 1:200) and Zn5 (Hybridoma Bank; 1:200) staining and acetylcholine receptor clustering were performed as previously described, except for the fact that 27- and 48-hpf zebrafish embryos were collected<sup>30–33</sup>. After labeling, all embryos were observed at specific stages under a microscope (DM 2500, Leica) equipped with Nomarski differential interference contrast optics and a fluorescent module having a GFP or DsRed filter cube (Kramer Scientific). Photographs of embryos at specific stages were taken with a CCD (DFC490, Leica).

### *Statistical analysis*

All analyses in this study were carried out using the MATLAB software (version 7.7 R2008b). The two-way ANOVA (analysis of variance) was applied to test the effects of factors (exposure time, dosage level) on the mean of the outcome variable (survival rate or malformation rate). The P-value for each factor, reported by two-way ANOVA, was associated with the null hypothesis that samples at all levels of the factor are drawn from the same population. The Tukey-Kramer HSD (honestly significant difference) test was further used to compare the population marginal means for one factor, adjusted by removing the effect of other factors. The one-way ANOVA and Tukey-Kramer HSD test were employed to compare the average number of in-chorion contractions between dose groups. A significance level

of 0.05 was used in all statistic analyses, and a familywise error rate of 0.05 was controlled for in the Tukey-Kramer HSD test.

## Results

### *Visible and defective phenotypes of embryos after injection of D-serine and survival rates analysis*

In order to study the exposure time and dosage effects of D-serine on zebrafish larvae, we injected zebrafish embryos with different dosages of D-serine (100, 500 and 1000 ppm) and calculated their survival rates at 27 hpf or 48 hpf. As shown in Table 1 and Table 2, around 55.7–67.9% of the embryos injected with D-serine were alive at 27 hpf, and the survival rates decreased to 15.6–48.6% at 48 hpf. The two-way ANOVA revealed that the P-values for exposure time and dose effects on survival rate were 0.0754 and 0.0417, respectively. The former indicated the survival rates decreased as the time of exposure increased but not to a significant degree. The latter indicated a significant difference in survival rates between dosage groups. The Tukey-Kramer HSD test was thus used to pairwise compare the marginal mean survival rates for dosage level groups, adjusted by exposure time effect. The adjusted mean survival rates for the 0, 100, 500 and 1000 ppm dosage groups were 93.54%, 58.26%, 51.97% and 35.63% with a common standard error of 7.49%, and the difference in survival rate between the 1000 ppm D-serine-injected group and mock-treated group (0 ppm) was significant. Consequently, D-serine injection led to a reduction in the survival rates of zebrafish embryos.

We further examined the phenotypic defects caused by D-serine. Compared with mock-treated embryos, D-serine-injected embryos (100–1000 ppm, 27 hpf) displayed some defective phenotypes (bent trunk phenotypes), such as a malformed somite boundary, twisted body axis and shorter body length (Fig. 1A vs. 1B, 1C, 1D).

Similar results were also observed at 48 hpf (Fig. 1E vs. 1F, 1G, 1H). The D-serine-induced malformation rates were 59.9%–84.3% and 61.6–68.6% at 27 and 48 hpf, respectively (Tables 1 and 2). Statistically, the two-way ANOVA indicated that the D-serine effect on the malformation rate was significant (P-value=0.0027). Furthermore, the Tukey-Kramer HSD test revealed that the mean malformed rates, adjusted by exposure time effect, for the 0, 100, 500 and 1000 ppm dosage groups were 0%, 60.90%, 66.00%, and 76.46% with a common standard error of 4.07% and identified that each of the D-serine injected groups (100, 500, and 1000 ppm) differed significantly from the control group (0 ppm) at a familywise error rate of 0.05, but no significant difference existed among the doses.

### *Spontaneous in-chorion contraction was reduced in D-serine-injected embryos*

We also noted that D-serine-injected embryos seemed to have less mobility at early larval stages. Thus, spontaneous in-chorion contractions in 27-hpf embryos were examined. The times of in-chorion contraction for each group

**Table 1.** Morphological Phenotypes of 27-hpf Zebrafish Embryos Derived from Fertilized Eggs Injected with Different Concentrations of D-serine

Injection dose (ppm)	Injected embryos	Survival embryos	Survival rates <sup>#</sup>	Malformed embryos	Malformed rates <sup>§</sup>
0	600	573	95.5%	0	0%
100	664	451	67.9%	270	59.9%
500	653	394	60.3%	276	70.0%
1000	688	383	55.7%	323	84.3%

<sup>#</sup>(Surviving embryos/injected embryos×100%); <sup>§</sup>(Malformed embryos/surviving embryos×100%).

**Table 2.** Morphological Phenotypes of 48-hpf Zebrafish Embryos Derived from Fertilized Eggs Injected with Different Concentrations of D-serine

Injection dose (ppm)	Injected embryos	Survival embryos	Survival rates <sup>#</sup>	Malformed embryos	Malformed rates <sup>§</sup>
0	700	641	91.6%	0	0%
100	638	310	48.6%	192	61.9%
500	844	368	43.6%	228	62.0%
1000	776	121	15.6%	83	68.6%

<sup>#</sup>(Surviving embryos/injected embryos×100%); <sup>§</sup>(Malformed embryos/surviving embryos×100%).

were recorded for 3 min. As shown in Fig. 2, the average number ( $\pm$  standard error) of in-chorion contractions in the mock-treated control (0 ppm of D-serine) embryos was  $21.7 \pm 0.69$  (3 min per embryo;  $n = 30$ ). On the other hand, the average numbers of in-chorion contractions in the embryos injected with 100, 500 and 1000 ppm of D-serine were  $18.3 \pm 0.97$ ,  $12.7 \pm 0.83$  and  $0.9 \pm 0.23$  ( $n = 30$ ), respectively. The one way ANOVA test revealed a highly significant difference ( $P$ -value $<0.0001$ ) in the average number of in-chorion contractions between dose groups, and the Tukey-Kramer HSD test identified all pairwise differences as significant at a familywise error rate of 0.05. This demonstrated that D-serine treatment reduced significantly the motilities of zebrafish embryos.

#### *Injection of D-serine results in disorganized muscle fibers alignments*

To further investigate the molecular mechanisms resulting in the reduced spontaneous in-chorion contraction of D-serine-injected embryos, the monoclonal antibody F59 was used to visualize the alignments of muscle fibers in mock-treated control and D-serine-injected zebrafish embryos. In the mock-treated control embryos (27 hpf), muscle fibers aligned well in the V-shaped somites (Fig. 3A). In contrast, muscle fibers aligned disorderly after injection of 100–1000 ppm of D-serine (Fig. 3B–3D). Similar results were observed but were more severe at 48 hpf (Fig. 3E vs. 3F–3H). These observations strongly indicate that injection of D-serine results in dose- and time-dependent defects of disorganized muscle fiber alignment.

#### *D-serine causes motor axons and neuromuscular junctions defects*

To address whether the projections of motor axons and the formation of neuromuscular junctions were affected

by injection of D-serine, monoclonal antibody Znp1 and  $\alpha$ -bungarotoxin labeling were carried out. The antibody Znp1 labeled the axonal bundles of primary motoneurons (pre-synapses) and revealed the common axonal path as well as the projections into ventral and dorsal somitic muscle blocks in mock-treated control embryos at 27 hpf (Fig. 4A). In addition,  $\alpha$ -bungarotoxin bound to acetylcholine receptors (AChRs; post-synapses) (Fig. 4B) and revealed clusters of AChRs. The merged signals suggested that axonal projections correlated well with the clusters of AChRs (Figs. 4C–4D, yellow signals), indicating that the motor axons innervated to the muscle fiber functionally. Interestingly, we found that only 8.1% (5/61, numbers of defective embryos/total number of D-serine-injected embryos) of D-serine-injected zebrafish embryos displayed defective primary motoneuronal pre-synapses and clusters of AChRs (Fig. 4D–4F). These observations suggest that overdose of D-serine seems to have little effects on primary motor neuron projection.

Axons of secondary motor neurons enter the common path set out by the primary neurons and complete migration as one nerve. When the development of primary motor neurons is impaired, the outgrowth of secondary motor axons is disrupted as well<sup>34</sup>. We labeled secondary motor neurons with monoclonal Zn5, and the results revealed that secondary motor neurons completed their axonal migration along the common path and reached the trajectory point at 48 hpf (Fig. 5A). However, secondary motor neuron axonal growth was impaired by injection of 100 ppm of D-serine (30.7%, 65/212; Fig. 5B). At higher concentrations (500 and 1000 ppm), secondary motor neuron axonal growth was nearly abandoned (Figs. 5C–5D). Taken together, we suggest that overdose of D-serine can cause motor neuron defects, especially for secondary motor neuron axonal growth.

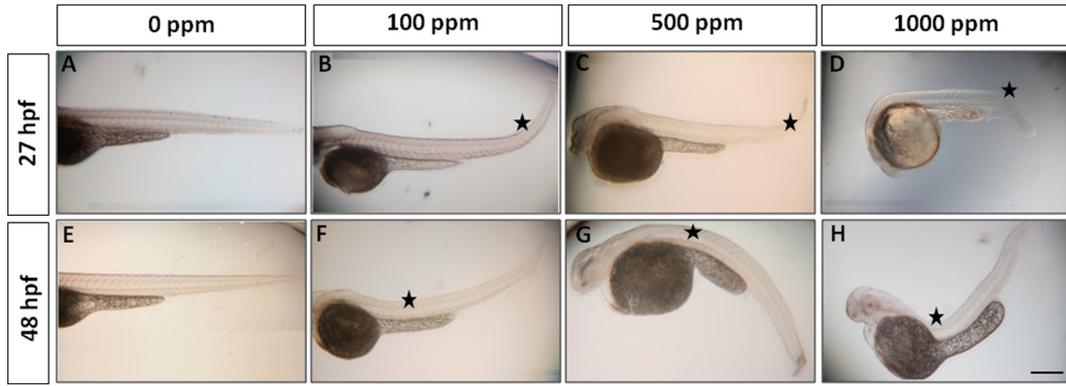


Fig. 1.

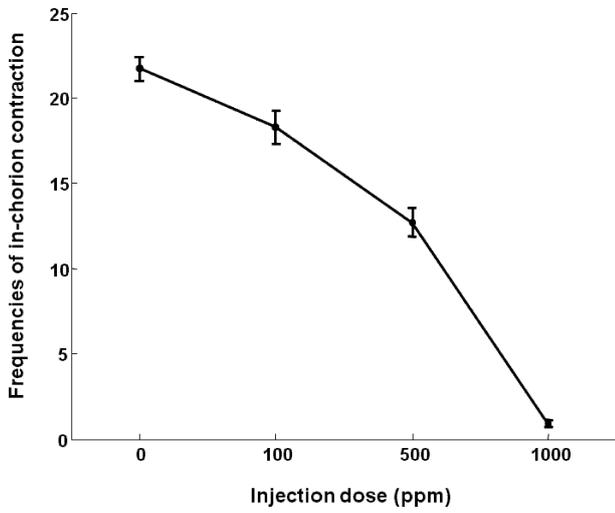


Fig. 2.

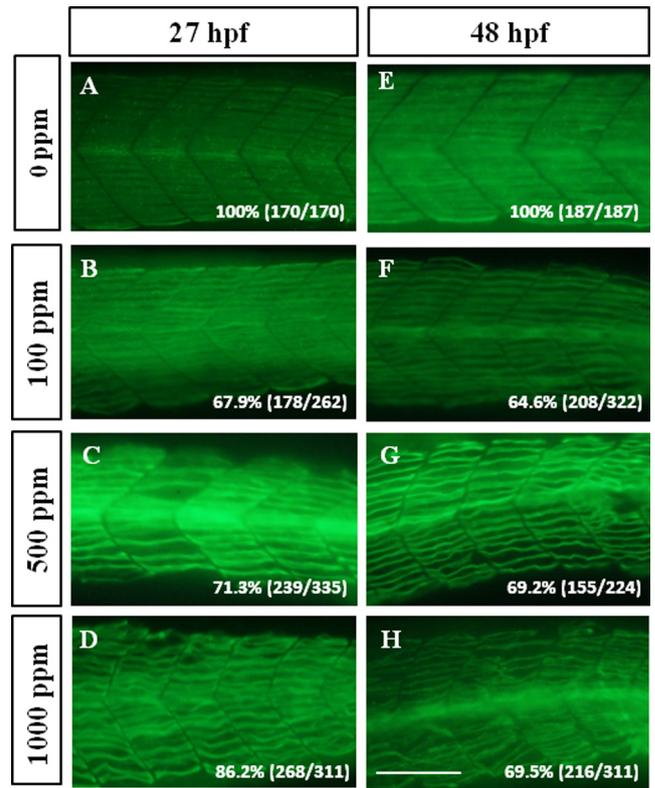


Fig. 3.

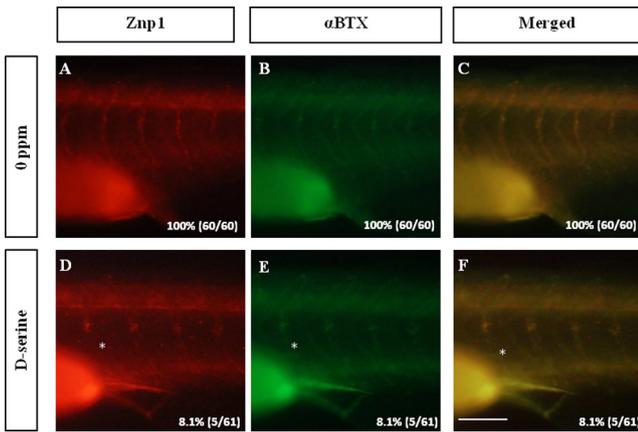


Fig. 4.

## Discussion

D-serine is a coagonist of NMDA receptors and plays a significant role in neuronal activity, including learning, memory and cell-death signaling<sup>35,36</sup>. As might be expected, increased levels of D-serine are associated with excitotoxicity of NMDA receptors<sup>32</sup>. In adult rats, injection of 50–200 mg/kg D-serine induced oxidative stress, which was thought

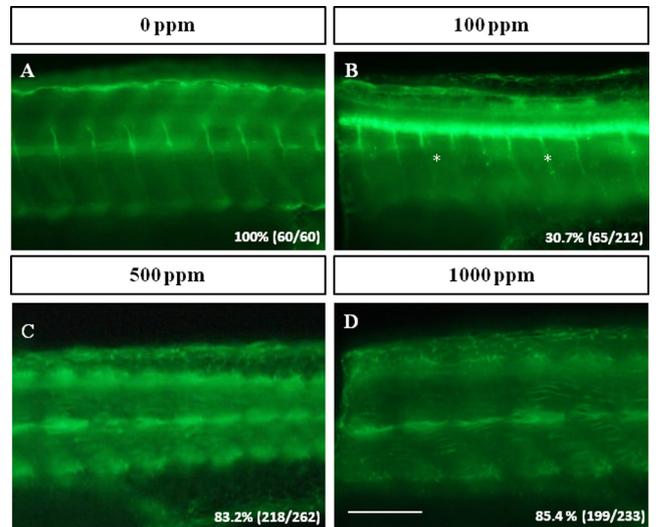


Fig. 5.

- Fig. 1.** Injection of D-serine leads to curved-body embryos. Zebrafish embryos were injected with water (A, E) or water containing 100, 500 or 1000 ppm of D-serine (B–D, F–H), and were recorded at 27 and 48 hpf. A star indicates the bent trunk region. Scale bar: 250  $\mu$ m.
- Fig. 2.** Effects of D-serine on spontaneous in-chorion contractions. Frequencies of spontaneous in-chorion contractions of zebrafish embryos after injection of 0, 100, 500, and 1000 ppm of D-serine were measured. The times of in-chorion contraction for each group were recorded for 3 min. The X- and Y-axes represent concentrations of D-serine and frequencies of in-chorion contraction, respectively.
- Fig. 3.** F59 monoclonal antibody-staining of the zebrafish embryos after injection of 0, 100, 500, and 1000 ppm of D-serine. (A–D) 27 hpf, (E–H) 48 hpf. Data are presented as percentages: numbers of shown embryos/total numbers of D-serine-injected embryos surviving at the check point (27 or 48 hpf). Scale bar: 100  $\mu$ m.
- Fig. 4.** Znp1 monoclonal antibody-staining (A, D) and  $\alpha$ -bungarotoxin labeling (B, E) of the 27-hpf zebrafish embryos after injection with 100 ppm of D-serine. Merged pictures: (C), panels A and B, (F) panels D and E. The yellow signals in panel C indicate that the Znp1-positive cells are also  $\alpha$ -bungarotoxin positive. Data are presented as percentages: numbers of shown embryos/total numbers of D-serine-injected embryos surviving at 27 hpf. A star indicates the defective region. Scale bar: 100  $\mu$ m.
- Fig. 5.** Zn5 monoclonal antibody-staining of the 48-hpf zebrafish embryos after injection with different doses of D-serine. (A) 0 ppm, (B) 100 ppm, (C) 500 ppm and (D) 1000 ppm. Data are presented as percentages: numbers of shown embryos/total numbers of D-serine-injected embryos surviving at 48 hpf. A star indicates the defective region. Scale bar: 100  $\mu$ m.

to be neurotoxic to the brain<sup>37</sup>. Our data showed that zebrafish embryos injected with 100, 500 and 1000 ppm of D-serine displayed a significant decrease in in-chorion contraction (Fig. 2) and few defects in primary motor neuron axonal growth but did not display severe impairment of secondary motor neuron projection (Figs. 4 and 5). Based on published information, we speculate that the D-serine-induced neural defects in zebrafish might be due to impairment of NMDA receptor function and that the injection concentrations described in this study are appropriate for exploration of D-serine-induced neurotoxicities.

In addition to neuronal malformation, we also found that embryos injected with D-serine displayed severe muscle defects, especially myofibril misalignment (Fig. 3). Here, we propose two possible causes contributing to D-serine-induced muscle defects. One possibility is that such muscle malformation in D-serine-injected zebrafish embryos might result from D-serine affecting the NMDA receptors of the pre-synapse and disturbing the release of neurotransmitters from the axon terminals. Previous studies have shown that knockdown of neuronal activity led to impairment of muscle development<sup>28,38</sup>. In this regard, D-serine-induced muscle defects might be the consequences of excitotoxicity of NMDA receptors. In other words, muscle defects are an indirect defect caused by D-serine-induced neuronal toxicity. The other possibility is that D-serine affects the muscle-type NMDA receptor or even an unknown muscle-specific receptor, and disturbs muscle development. In mouse C2C12 myoblasts, it has been demonstrated that NMDA receptors were expressed in myoblasts during muscle differentiation, and played a role in myoblasts fusion<sup>39</sup>. In rats, NMDA receptors were found to be present at the neuromuscular junctions (NMJ) of the diaphragm<sup>40,41</sup>. These observations suggested that NMDA receptors have direct effects on muscle development. Thus, whether or not NMDA receptors are present at the myoblasts of developing zebrafish embryos merits further study.

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