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Zebrafish as a model for acetylcholinesterase-inhibiting organophosphorus agent exposure and oxime reactivation

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Abstract

The current research progression efforts for investigating novel treatments for exposure to organophosphorus (OP) compounds that inhibit acetylcholinesterase (AChE), including pesticides and chemical warfare nerve agents (CWNAs), rely solely on *in vitro* cell assays and *in vivo* rodent models. The zebrafish (*Danio rerio*) is a popular, well-established vertebrate model in biomedical research that offers high-throughput capabilities and genetic manipulation not readily available with rodents. A number of research studies have investigated the effects of subacute developmental exposure to OP pesticides in zebrafish, observing detrimental effects on gross morphology, neuronal development, and behavior. Few studies, however, have utilized this model to evaluate treatments, such as oxime reactivators, anticholinergics, or anticonvulsants, following acute exposure. Preliminary work has investigated the effects of CWNA exposure. The results clearly demonstrated relative toxicity and oxime efficacy similar to that reported for the rodent model. This review surveys the current literature utilizing zebrafish as a model for OP exposure and highlights its potential use as a high-throughput system for evaluating AChE reactivator antidotal treatments to acute pesticide and CWNA exposure.

Keywords

zebrafish; acetylcholinesterase; organophosphate compound; oxime reactivator

Introduction

Organophosphorus (OP) pesticides and chemical warfare nerve agents (CWNAs) belong to the same class of compounds, whose primary toxic mechanism of action is through irreversible inhibition of the enzyme acetylcholinesterase (AChE). AChE inhibition leads to a buildup of the neurotransmitter acetylcholine (ACh) at the synapse and subsequent overstimulation of cholinergic neurons. This process leads to numerous toxic effects: miosis, hypersecretions, respiratory depression, seizures, and, if a significant, acute exposure is left untreated, death.^{1,2} The presence of prolonged seizure activity is the primary contributor to neuropathology following OP exposure.³

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Conflicts of interest

The authors declare no conflicts of interest.

A number of treatment options are available to combat the effects of OP exposure. Prophylactically, reversible AChE inhibitors, such as pyridostigmine bromide, or bioscavenger compounds, such as butyrylcholinesterase (BChE), can be administered.¹ Reversible AChE inhibitors are capable of preemptively binding to AChE and preventing more permanent OP agent inhibition, while bioscavengers can bind to the free OP agent before it is able to inhibit endogenous AChE. Anticholinergic drugs, such as atropine sulfate, can be utilized to antagonize the effects of ACh at muscarinic receptors.^{1,2} Anticonvulsant drugs, such as benzodiazepines (diazepam or midazolam), act as GABA receptor agonists and can be administered to terminate seizure activity and reduce subsequent neuropathology.^{2,3}

All of the above treatments target the secondary effects of OP exposure but do not rescue the inhibited AChE enzyme. Oxime compounds are capable of reactivating OP-inhibited AChE through a nucleophilic attack on the phosphorus atom of the nerve agent. This forms a phosphoryloxime complex, which breaks away from AChE, allowing it to resume its normal enzymatic function.^{1,4} The oximes currently fielded by various nations are pyridinium or bispyridinium compounds such as 2-PAM (pralidoxime), HI-6, or obidoxime.⁵ The positively charged quaternary nitrogen of each allows for alignment with the negatively charged subsite on the AChE enzyme, placing the oxime in close proximity to the phosphorus.^{1,4} This quaternary nitrogen structure, however, restricts their passage through the blood–brain barrier (BBB), limiting their reactivation capabilities to only the peripheral tissues. Additionally, the currently available oximes lack broad-spectrum efficacy against different OP agents.^{6,7}

This has led to continued investigation into new oxime compounds and novel AChE reactivators. Bispyridinium oximes, such as HI-6, HLö7, and MMB-4, have demonstrated broader and more potent efficacy than 2-PAM.^{7–9} Additionally, oximes possessing a tertiary amine are under evaluation. Monoisonitrosoacetone (MINA), one of these tertiary oximes, was originally investigated in the 1950s but was disregarded because its reactivation capabilities were less than those of quaternary oximes at equimolar concentrations.¹⁰ Recent research has further demonstrated the weak affinity of MINA for inhibited AChE and its slower reactivation kinetics when compared to the pyridinium oximes.¹¹ Its ability to cross the BBB, however, has led to renewed investigation. Following nerve agent exposure, MINA has demonstrated an ability to reactivate inhibited AChE in the CNS, improve survival, and terminate active seizure activity.^{12–14}

When investigating candidate oxime compounds, the current research approach begins with either *in silico* molecular modeling or *in vitro* cell cultures. Compounds that show significant promise can then be moved into an *in vivo* rodent model. While the rodent model has been an effective tool, it lacks the high-throughput capabilities necessary to test a large library of compounds. Additionally, there are significant species differences in the oxime reactivation kinetics when compared to human AChE *in vitro*, raising concerns for the use of rodents as an accurate model for OP inhibition and oxime reactivation.⁹

Zebrafish model

The zebrafish (*Danio rerio*) is an already well-established model organism in biomedical research, with a number of publications discussing its role as an important nonmammalian model for neurotoxic and teratogenic high-throughput studies.^{15–18} Zebrafish offer an enticing option for modeling OP exposure and oxime reactivation.¹⁹ Zebrafish are genetically similar to humans, possess high fecundity, and develop as easily observable, transparent embryos.^{20,21} Transparency allows direct observation of the internal embryonic development of the zebrafish with little more than a microscope. Accessibility allows for the embryos to be genetically and embryologically manipulated through microinjection. All of the facts listed above make the zebrafish an excellent complementary research model for human disease and development.^{22–24} Additionally, they exclusively express AChE, with no detectable butyrylcholinesterase (BChE) activity, which could potentially confound AChE inhibition and reactivation experiments.^{25,26} AChE activity levels steadily increase during early development, with an approximately 75-fold increase from 1 to 6 days postfertilization (dpf) (Fig. 1).^{27,28} The human and zebrafish AChE enzymes also share 62% similar amino acid sequences, with key amino acid residues remaining conserved in the acyl- and choline-binding pockets and the catalytic triad.²⁵ Recent work has demonstrated similar 2-PAM reactivation kinetics for human and zebrafish AChE *in vitro* following OP pesticide inhibition.¹⁹

This review will provide an overview of the current research utilizing zebrafish as a model for OP agent exposure. As the laboratory research up until this point has almost exclusively evaluated the developmental consequences of subacute OP pesticide exposure and not acute nerve agent exposure, a portion of this review will focus on that aspect. There will only be a brief discussion of the preliminary work completed with OP nerve agents.

OP pesticide–induced developmental toxicity and behavioral deficits in zebrafish

The risk of household or agricultural exposure to OP pesticides remains a real danger, with children exposed *in utero* or at a young age being particularly susceptible to their effects.^{29–31} However, the underlying mechanisms of teratogenic *in utero* exposure to AChE-inhibiting OP agents are still not well understood. A number of reviews have postulated that these impairments are the result of altered neural outgrowth and connectivity, and that maintaining normal AChE activity is critical during these developmental periods.^{32,33} Up until now, the rodent has been the go-to *in vivo* model for evaluating the effects of developmental exposure to OP compounds. However, the difficulty of investigating the effects of these agents *in utero* makes this model unwieldy and labor intensive. The external fertilization, embryonic accessibility, transparency, and ease of behavioral monitoring of zebrafish make this species an ideal model for this line of research.^{16–18,22}

A number of publications have described the developmental consequences for exposure to subacute concentrations of AChE-inhibiting OP pesticides in embryonic, larval, and adult zebrafish, such as chlorpyrifos/chlorpyrifos oxon,^{34–41} azinphos–methyl,^{42,43} parathion/

paraoxon,^{41,42,44,45} malathion,^{38,46,47} dichlorvos,^{39,48} and diazinon.^{39,41} The major observations from a number of these studies are summarized in Table 1. Exposure to 300 nM chlorpyrifos oxon during early developmental stages (1–3 dpf) significantly inhibited AChE activity, yet the majority of zebrafish larvae displayed normal gross morphology, with only about 15% possessing an enlarged yolk sack with edema. However, Rohon-Beard sensory neurons, which express high levels of AChE activity, displayed disorganized axonal extension at 1 dpf.³⁵ This suggests that only partial AChE activity is necessary to maintain normal developmental morphology, yet the development of certain cell types may be more sensitive to changes in AChE activity. There are contradicting results from a number of publications on this topic. In a study with paraoxon, significant AChE inhibition (36% of control) was achieved, yet there was no effect on the development on secondary motor neurons.⁴⁵ In another study, however, significant AChE inhibition (< 10% of control) produced by similar concentrations of chlorpyrifos oxon significantly hindered axonal growth of sensory, primary, and secondary neurons.⁴⁰ These differences are most likely attributable to the degree of AChE inhibition achieved in these exposure models. It suggests that a critical level of AChE activity must be maintained during development to maintain normal neuronal morphology. It is interesting to note that another study investigating the effects of subacute exposure to carbamate compounds, such as pyridostigmine bromide, that temporarily inhibit AChE produced similar gross morphological changes to the OP pesticides but required a 1000-fold higher concentration.⁴⁹

Exposure to chlorpyrifos at the sublethal concentration of 10 μ M at 1–3 dpf produced kyphosis and a decrease in heart rate, while exposure to 100 μ M diazinon induced cardiac edema and decreased blood flow.³⁹ Malathion exposure at sublethal concentrations produced decreased body length, increased abdominal cavity area, and decreased heart rate.^{46,47} Interestingly, when larvae were exposed from 1–5 dpf to concentrations of malathion not capable of significantly inhibiting AChE activity, a decrease in forebrain and hindbrain size was still observed.³⁸ This appears to suggest the presence of secondary effects of some OP compounds beyond AChE inhibition. A model to aid in identification of these targets has been developed utilizing zebrafish, which were discovered to possess a nonfunctioning *ache* mutation.⁵⁰ Phenotypic changes produced through exposure to a number of AChE-inhibiting OP compounds were observed that were not present in the *ache* mutant.⁵¹ More research must be done to identify the mechanisms of action producing these phenotypes.

The behavioral effects of exposure to OP pesticides have also been investigated. Following exposure to chlorpyrifos oxon or diazinon early in development, larval zebrafish demonstrated a significant decrease in overall swim activity and an increase in spontaneous movements.³⁷⁻⁴¹ Malathion exposure produced an opposite effect, by increasing overall swim activity in larval zebrafish. This is possibly attributable to the lack of AChE inhibition produced by malathion in this particular exposure experiment.³⁸

Behavioral deficits are maintained in adult fish that were exposed only during early developmental stages. After being exposed to approximately 300 nM chlorpyrifos during early developmental stages (1–5 dpf), 20- to 38-week-old zebrafish that were conditioned in a three-compartment maze performed significantly less well, and the exposure had a

significant effect on later overall survival (26 weeks or more).³⁶ An increase in tap-elicited startle response was observed in adult fish with the same exposure model.³⁴

Acute exposure (30–120 min) to paraoxon concentrations ranging from 0.064–1000 μM produced a time- and concentration-dependent AChE inhibition and mortality (Table 2).^{27,28} Behavioral testing demonstrated a significant reduction in overall swim activity and light-induced startle response 24 h after paraoxon exposure.²⁷

Oxime reactivation of OP pesticide–inhibited AChE

Although a fair amount of research into OP-induced toxicity utilizing zebrafish has been reported in the literature, unfortunately very few publications describe the efficacy of known and novel antidotes in this model, and even fewer have evaluated oxime reactivators. A recent study attempted to establish a high-throughput screen of antidotes to OP toxicity, and discovered a number of known and novel compounds that were found to be efficacious.⁴² These compounds utilized various mechanisms of action, from anticholinergic activity to reversible AChE inhibition. A combination of 2-PAM and atropine was also demonstrated to significantly improve survival following exposure to parathion or azinphos–methyl.⁴² The results presented in this study demonstrate the powerful high-throughput drug screening capabilities possessed by this model, with this study discovering several previously unknown efficacious compounds.

A recent publication from Schmidt *et al.*¹⁹ took an extensive look at the differences in inhibition and reactivation kinetics for zebrafish versus human AChE *in vitro* and the efficacy of oxime reactivation of OP-inhibited AChE in the *in vivo* larval zebrafish model. In an *in vitro* evaluation, 2-PAM and the novel tertiary amine oxime RS-194B demonstrated similar rates of reactivation in both zebrafish and human AChE following inhibition by chlorpyrifos oxon or dichlorvos. For the *in vivo* studies, both 2-PAM and RS-194B were efficacious in reducing spontaneous movements induced by chlorpyrifos oxon or dichlorvos toxicity. Interestingly, the chorion was observed to reduce penetration of 2-PAM but not RS-194B into the embryo, possibly offering a model for evaluating the ability of novel oximes to penetrate the BBB. This study offered strong evidence for the use of zebrafish in OP exposure and oxime treatment modeling.

Following acute exposure to paraoxon in 6-dpf zebrafish larvae, 2-PAM and MMB-4 produced significant dose-dependent reactivations of inhibited AChE, returning to 55% and 34% of control values, respectively, at a concentration of 400 μM (Fig 2D). MINA proved incapable of significant reactivation at these equimolar concentrations.^{27,28} These results are interesting in that the bispyridinium oxime MMB-4 proved to be a lesser reactivator than 2-PAM, a result that contradicts what has been observed in rodent models of nerve agent exposure.⁷ This could possibly be attributed to differences in rodent and zebrafish AChE reactivation kinetics for these particular oximes or in the paraoxon–AChE complex when compared to nerve agent–inhibited AChE.

Chemical warfare nerve agent toxicity, AChE inhibition, and oxime reactivation

In addition to the work done with the OP paraoxon, we have completed the preliminary work in evaluating the use of 6-dpf larval zebrafish to model exposure to acute doses of the more toxic class of OP compounds, the CWNAs. Exposure to sarin (GB), soman (GD), or cyclosarin (GF) generated time- and concentration-dependent mortality, summarized in Table 2. The relative toxicity of these agents was $GD > GF > GB$, with calculated LC_{50} (median lethal concentration) values of 4.0, 21.0, and 65.4 μM , respectively, for an acute 60-min exposure.^{27,28,52} The relative toxicity of these CWNAs appears to be much greater than what has been observed with OP pesticide compounds, and is in general comparable to what has already been established in the rodent model.^{39,42,43,53} It might be interesting in future experiments to investigate the age-dependent toxicity of these CWNAs at stages of development besides 6 dpf.

The concentration-dependent efficacy of three oximes (2-PAM, MINA, and MMB-4) to reactivate CWNA-inhibited AChE *in vivo* was also evaluated and is presented in Figure 2. For GB exposure (Fig. 2A), all three oximes were able to reactivate inhibited AChE in a concentration-dependent manner, with 2-PAM and MMB-4 proving more effective reactivators than MINA; only MMB-4 and MINA were capable of reactivating GF-inhibited AChE (Fig. 2B). Following GD exposure (Fig. 2C), the reactivation produced by oxime treatment, although it reached significance with MMB-4, remained below 2% of control values and is thus unlikely to have therapeutic benefit.^{27,28} The lack of reactivation against GD can possibly be attributed to the propensity for this agent to rapidly “age” AChE, a process by which the loss of an alkoxy group leaves a more stable GD–AChE complex, which is resistant to oxime reactivation.^{1,4} The oxime specificity data obtained here all correlate well with previous rodent model data.^{7,14,54} This lends more evidence for the use of zebrafish for modeling CWNA exposure and AChE reactivation by oxime reactivators.

Preliminary behavioral and physiology observations following CWNA exposure have revealed a phenotype similar to that observed for OP pesticide exposure. Heart rate and blood flow were measured via high-speed microscopy, and significant reductions in both were observed following acute CWNA exposure (unpublished observations). There was also a significant reduction in swim activity and light-elicited startle response.²⁷

Future directions and considerations

The flexibility and large number of tools available in the zebrafish model offers several future research opportunities. For continuation of current experiments, additional oxime compounds currently fielded and under investigation (i.e., HI-6, obidoxime, HLö7) should be evaluated and verified in zebrafish to offer more concrete support for the use of this exposure model. And while preliminary work noted overall oxime specificity similar to that seen in the rodent model, the translational accuracy moving from the zebrafish into a mammalian model should be addressed. Such concerns have already been raised about species differences in AChE inhibition and reactivation kinetics between humans and nonhuman primates, as well as among different rodent species,⁹ and notable differences

between human and zebrafish AChE-inhibition kinetics for chlorpyrifos oxon and dichlorvos have been demonstrated.¹⁹ Further *in vitro* investigations comparing human, rodent, and zebrafish AChE kinetics against both OP pesticides and CWNAs would offer valuable insight.

This model offers unique options to address these translational issues. Zebrafish possessing a nonfunctioning mutant *ache* gene have already been discovered.⁵⁰ It should be possible to drive expression of the human *ACHE* gene in this mutant background. A stable, transgenic strain of zebrafish expressing human AChE enzyme would offer a powerful tool in evaluating OP-induced inhibition and oxime reactivation. The ability to utilize a model with such translational relevance in a high-throughput study would allow for a more accurate representation of human AChE inhibition and more efficient drug discovery.

Beyond the acute CWNA exposure model discussed here, it would also be beneficial to investigate the effect of subacute developmental and long-term consequences following exposure to low levels of CWNA compounds, similar to the models discussed with OP pesticides. Comparable phenotypes would be expected; however, any noted differences could aid in the investigation of off-target effects for these more highly toxic substances. It might also be interesting to investigate the age-dependent toxicity of acute exposure to CWNA compounds utilizing the zebrafish model. Similar studies done in the rodent model have demonstrated an age-related effect on toxic signs, with increasing toxicity as the rodent ages.⁵⁵

Finally, careful consideration should be given to possible differences between the various wild-type strains of zebrafish when moving forward with this model and comparing the results of different studies. It has been demonstrated that the two most commonly used research strains (AB and Tubingen) display different responses to light/dark challenges and overall locomotion in both adults and larvae.⁵⁶ It is interesting to note that behavioral differences were also seen when evaluating the short-fin wild-type and leopard (*leo*) strains.⁵⁷

Conclusions

Having been already firmly established as a model organism for biomedical research, the zebrafish offers an exciting option for modeling OP exposure. Its embryonic clarity and external fertilization allow for easy visualization of developmental processes and consequences of OP exposure, and its exclusive expression of AChE lends itself well to modeling enzyme inhibition and reactivation. Most research studies up until this point have focused solely on OP pesticide-induced developmental toxicity and behavioral deficits in zebrafish embryos/larvae, attempting to model the types of exposures expected in household and agricultural settings. The results showed, in general, detrimental effects on gross morphology, neuronal development, and behavior.

Few studies have investigated the effectiveness of antidotes against acute OP pesticide exposure using zebrafish as a model. One utilized this model's high-throughput capabilities to screen a library of chemical compounds, discovering several with known and unknown

mechanisms of action.⁴² Another took a more in-depth look at differences in the inhibition and oxime reactivation kinetics of human and zebrafish AChE with both a quaternary and tertiary oxime, determining that the enzyme reactivation kinetics are overall similar. Additionally, it was noted in this study that the chorion was capable of limiting penetration of the quaternary oxime 2-PAM, offering an exciting possible *in vivo* model system for evaluating the BBB-penetrating capabilities of novel oximes.¹⁹ However, certain considerations should be addressed before utilizing this as a BBB model. The BBB and chorion membranes are similar in that low molecular weight and lipid solubility are two of the main properties that dictate the effectiveness of transmembrane diffusion.^{58–60} However, the BBB can utilize additional processes to facilitate crossing, such as saturable transporters and adsorptive endocytosis, that are not present in the chorion membrane.⁵⁸ The choice of solvent for drug delivery can also have dramatic effect on diffusion across the chorion, with dimethyl sulfoxide concentrations as low as 0.1% greatly increasing membrane permeability.⁵⁹ Additionally, the chorion hardens with age, lessening permeability and adding a possible confounding variable to future studies.⁶¹

Our recent studies of acute exposure to the CWNAs GB, GD, and GF and the pesticide paraoxon have demonstrated relative toxicity and oxime (2-PAM, MMB-4, and MINA) reactivation efficacy similar to what has already been established in the rodent model, supporting the translational relevance of zebrafish. We additionally demonstrated the much greater toxicity and rapid AChE inhibition provided by CWNAs when compared to OP pesticides.^{27,28}

Overall, the research presented here demonstrates the effectiveness and flexibility of the zebrafish model system and validates its use as a suitable vertebrate animal model of OP exposure, complementary to *in vitro* cellular systems and rodent studies. Future development of a human AChE-expressing zebrafish strain would further strengthen this model and would greatly improve its translational relevance for OP exposure and AChE reactivation, allowing for more accurate high-throughput screening during drug discovery.

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Figure 1. Developmental AChE activity in zebrafish. Zebrafish were collected at 24-h intervals from 1 dpf to 6 dpf. An extra collection was made at 2 dpf for embryos that still possessed their chorion. $n = 3$ per time point, with each n pooling 20 individual fish; error bars = SEM. Adapted from Refs. 27 and 28.

Figure 2.

Oxime reactivation following OP agent exposure. Zebrafish larvae (6 dpf) were exposed to (A) sarin (GB), (B) soman (GD), (C) cyclosarin (GF), or (D) paraoxon, for the indicated concentration and time before treatment with various concentrations (25–400 μ M) of MINA, 2-PAM, or MMB-4. * P < 0.05, *** P < 0.001 vs. control. n = 3 per oxime concentration, with each n pooling 20 individual fish; error bars = SEM. Adapted from Refs. 27 and 28.

Table 1
Summary of morphology and behavioral observations following developmental OP exposure in zebrafish larvae.

OP Agent	Concentration (μM)	Exposure duration (hpf) ^a	Significant AChE inhibition?	Observations	Calculated LC ₅₀ (μM)	Ref.
Chlorpyrifos	0.1	24–120	Yes	Decrease in swim activity	—	38
	1	24–72	No	Normal gross morphology and motor neuron development	—	40
	10	2–72	—	Increased kyphosis Decrease in swim activity and heart rate	16 at 5 dpf	39
	0.3	0–120	—	Impairment to spatial discrimination (20–38 weeks)	—	36
Chlorpyrifos oxon	0.3	6–120	—	Decrease in swim activity	—	37
				Increase in startle response (adults) Decrease in brain dopamine levels (adults)	—	34
				Decrease in swim activity, no significant mortality	—	41
				Inhibited axonal growth of sensory and motor neurons Decreased touch-induced swim distance	—	40
Malathion	0.3	3–72	Yes	Disorganized axon extension of Rohon-Beard sensory neurons (1 dpf) Overall normal gross morphology	—	35
	1	24–120	No	Increase in swim activity Decrease in forebrain and hindbrain size	—	38
	150	10 min ^b	—	Decrease in heart rate	—	47
	≈ 7	3–120	—	Decrease in body length and eye diameter Increase in abdominal area	—	46
Azinphos-methyl	2–79	0–48	—	—	13.2 at 2 dpf	43
	0–100	24–72	—	No significant mortality	—	42
		16 h ^c	—	—	53.8 at 8 dpf	
Paraoxon	0.25	5–96	Yes	Normal gross morphology and motor neuron development	—	45
Parathion	0.5	5–96	Yes	Decrease body length and trunk malformations	—	41
	10	6–120	Yes	Increase in mortality, no movement, developmental malformations	—	
	0–100	16 h ^c	—	—	13.5 at 8 dpf	
Diazinon	100	2–72	—	Pericardial edema and decreased blood flow (1 dpf)	22 at 5 dpf	39

OP Agent	Concentration (μM)	Exposure duration (hpf) ^a	Significant AChE inhibition?	Observations	Calculated LC ₅₀ (μM)	Ref.
	10	6–120	Yes	Decrease in swim activity, no significant mortality	—	41

^a Hours postfertilization; unless otherwise noted, observations were made at the end of the exposure timeframe.

^b 10-min acute exposure at 52, 72, or 96 hpf.

^c 16-h acute exposure at 8 dpf.

Table 2

Calculated LC₅₀ values (μM with 95% CI) following acute exposure to OP compounds in 6-dpf zebrafish larvae.^{27,28}

Exposure time (min) ^a	GB	GD	GF	Paraoxon
30	229.7 (151.7–378.1)	21.8 (13.1–38.8)	30.6 (19.4–50.8)	451.9 (361.9–566.2)
60	61.5 (45.9–83.2)	4.0 (2.8–5.5)	21.1 (14.0–32.8)	165.8 (134.2–202.3)
90	18.9 (13.6–26.7)	2.3 (1.1–4.2)	15.4 (10.8–22.3)	82.9 (63.2–109.2)
120	6.9 (4.7–10.0)	1.3 (0.7–2.3)	6.6 (3.9–12.0)	108.7 (86.4–137.2)

^aExposures were conducted for the indicated time, and mortality was recorded at 24 h as indicated by cessation of heartbeat.