Zebrafish Models for Human Acute Organophosphorus Poisoning

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Acute organophosphorus poisoning, as a major public health concern some facts

**Developing countries**

Self-poisoning

around 3 million cases

300,000 deaths

**Developed countries**

Intentionally (terrorist attack)/ chemicals released from transportation or storage facilities after an accident or a natural disaster

Acute Organophosphorus Poisoning: looking for new therapeutic strategies

Although pathophysiology of OPP is complex....

- AChE inhibition
- ACh accumulation
- Nicotinic & muscarinic AChR Activation
- EAA release
- Seizures
- Cholinergic neurones overstimulation
- NMDA-receptor activation
- Ca$^{2+}$ disruption
- Inflammatory response
- Mitochondrial respiration
- Oxidative stress
- Immune response
- Cell death

...standard therapy mainly targets:

- AChE reactivators
- Muscarinic AChR antagonist
- Anticonvulsants

“Multifunctional drug therapies”
Manifestations of Organophosphate Poisoning

**Optic System**
- Pupil Constriction
- Blurred Vision
- Lacrimation

**Respiratory System**
- Bronchospasm
- Bronchial Secretion
- Pulmonary Edema
- Tightness of Chest
- Wheezing
- Cough
- Difficulty Breathing

**Brain**
- Headache
- Dizziness
- Vertigo
- Anxiety
- Apathy
- Confusion
- Anorexia
- Insomnia
- Lethargy
- Fatigue
- Inability to Concentrate
- Memory Impairment
- Convulsion
- Coma

**Gastrointestinal Tract**
- Salivation
- Nausea
- Cramps
- Abdominal Pain
- Vomiting
- Diarrhea
- Fecal Incontinence

**Cardiovascular System**
- Bradycardia, Tachycardia
- Increased Blood Pressure

**Musculature**
- Weakness
- Tremor
- Fasciculations
- Twitching
- Cramps
- Increased Sweating

**Urinary - Genital**
- Urinary Incontinence
- Impotence
- Uterus Contraction
ANTIDOTES against the cholinergic toxidrome

1- Atropine: competitive antagonist of muscarinic cholinergic receptors in both the CNS and the PNS (improves respiratory function by decreasing secretions)

2- Pralidoxime (2-PAM): prevents aging of AChE and reverse muscle paralysis with OP poisoning

3- Benzodiazepines: Depresses all levels of CNS (e.g., limbic and reticular formation) by increasing activity of GABA. Used for treatment of seizures.
DEVELOPMENT OF A ZEBRAFISH MODEL OF CHOLINERGIC TOXIDROME, AS A TOOL FOR IDENTIFICATION OF ANTIDOTES
Acute Organophosphorus Poisoning: some facts

Identification of new medical countermeasures against OPP by the development and validation of *in vivo* animal models for rapid screening of molecular libraries

Zebrafish, vertebrate model of human diseases suitable to *in vivo* medium and high-throughput screening of chemicals

NIH CounterACT program
Objectives

To develop and validate new OPP mechanistic models suitable for in vivo medium and high throughput screening with drugs of therapeutic value.

1. Development of chemical models of OPP, with different grades of severity, in zebrafish larvae by using chlorpyrifos-oxon as a prototypic OP compound
2. Characterization of the models, by analysing the adverse effects at different levels of organization (transcriptional, biochemical, ultrastructural, cellular & tissular, organismal and behavioural)
3. Deciphering the pathophysiological pathways involved in OPP development in our models by using a pharmacological approach and the analysis of the perturbed KEGG pathways
Methods

• Biochemical determination: AChE activity (individual fish), SOD, CAT, GSH (pools of 5 larvae)
• LPO determination
• In vivo detection of ROS generation
• Histopathology
• Behavior: basal locomotor activity, visual motor response, and touch-evoked escape response
• RNAseq
• Oxygen consumption
• Adenine nucleotide levels (AMP, ADT, ATP)
Grading OPP severity in zebrafish larvae

IC50: 9.6 nM
LC50: 3.97 µM

AChE: 4.18%
AChE: 0.13%
AChE: 0.02%
Grading OPP severity in zebrafish larvae
Concentration-response: in vivo inhibition of zebrafish AChE activity by CPO

1 h IC50: 64.34 nM CPO

24 h IC50: 9.58 nM CPO
Visual motor response (VMR) is impaired in larvae exposed to low concentrations of CPO.
VMR parallels AChE inhibition in larvae exposed to low concentrations of CPO

- Behavioral phenotype in larvae exposed to low concentrations of CPO (1-100 nM) is fully explained by the inhibition of AChE activity

AChE activity and speed (% control values). Average speed was measured between 30-32 min of the assay.
AChE activity in larvae exhibiting P2 and P3 is lower than 1% of the control values.
Larvae exposed to the same concentration of CPO exhibiting a similar degree of AChE residual activity can exhibit different phenotypes.

- At high concentrations of CPO, the phenotype presented by the larvae is not explained by the degree of AChE inhibition.
Mild OPP zebrafish model

- No morphological defects at CPO concentrations below 100 nM
- Mild but significant decrease in the length of the trunk
- Histopathological assessment: any effect at CNS/PNS, retina, axial muscle fibers
- No oxidative stress
- Large-scale transcriptomic analysis (RNAseq): 80 DEGs (FDR adjusted p≤0.05)
  - 4 down-regulated KEGG pathways
Visual Motor Response is strongly impaired in the mild OPP model

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<thead>
<tr>
<th>IC50/EC50 (nM)</th>
<th>NOEC (nM)</th>
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<tbody>
<tr>
<td>AChE</td>
<td>9.58</td>
</tr>
<tr>
<td>VMR</td>
<td>8.42</td>
</tr>
</tbody>
</table>

$r^2 = 0.323$, $P < 0.001$, $n = 92$
**Visual Motor Response is strongly impaired in the mild OPP model**

<table>
<thead>
<tr>
<th>CPO (nM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>75</th>
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<tr>
<td>Speed (% control)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>AChE activity (% control)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
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<tr>
<td>r² = 0.323/ P &lt; 0.001/ n=92</td>
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**Phototransduction** (dre04744)
Although kinematic of the touch-evoked escape response is altered, mild OPP model is responsive to the touch stimulus
Visual Motor Response is strongly impaired in the mild OPP model

“Phototransduction” (dre04744)

Retina architecture impairment
Moderate OPP zebrafish model

Severe decrease in the length of the trunk (around 50%)
Moderate OPP zebrafish model

Severe decrease in the length of the trunk (around 50%!)  
Histopathological assessment: **Altered axial muscle fibers and retina, but no CNS**
Moderate OPP zebrafish model

No oxidative stress

Transcriptomic analysis (RNAseq): 4,568 DEGs (FDR adjusted p≤0.05)

30 down-regulated KEGG pathways

“Phototransduction” (dre04744)

41 up-regulated KEGG pathways

Immune and inflammatory response
- Proteosome (dre03050), toll-like receptor signaling pathway (dre04620), MAPK signaling pathway (dre04010), RIG-I like receptor (dre04622)
Moderate OPP zebrafish model

Transcriptomic analysis (RNAseq): 4,568 DEGs (FDR adjusted p≤0.05)
- 30 down-regulated KEGG pathways
- 41 up-regulated KEGG pathways

“Phototransduction” (dre04744)

Immune and inflammatory response
- Proteosome (dre03050), toll-like receptor signaling pathway (dre04620), MAPK signaling pathway (dre04010), RIG-I like receptor (dre04622)
Severe OPP zebrafish model

- Mild, but significant, decrease in the length of the trunk
- Histopathological assessment: *Altered axial muscle fibers, CNS, PNS, retina*

- In contrast with the severity of the lesions in organs with cholinergic innervation, such as CNS or muscle, non-cholinergic tissues such as liver, remained well preserved.
Severe OPP zebrafish model

Liquefactive necrosis of the brain
Spinal cord degeneration
Retina degeneration
Disrupted architecture of fast muscle fibers
Severe OPP zebrafish model

- Mild, but significant, decrease in the length of the trunk
- Histopathological assessment: *Altered axial muscle fibers, CNS, PNS, retina*
Severe OPP zebrafish model

- ROS generation, LPO increased leading to oxidative stress
- Mitochondrial respiration decreased
- Phenotype is partially rescued by modulating GSH levels
- Antioxidants are not able to rescue the phenotype
Severe OPP zebrafish model

Transcriptomic analysis (RNAseq): 4,996 DEGs (FDR adjusted p≤0.05)

9 down-regulated KEGG pathways

34 up-regulated KEGG pathways

Immune and inflammatory response

- Proteosome (dre03050)
- Salmonella infection (dre05132)
- Citokine-cytokine receptor interaction (dre04060)
- Toll-like receptor signaling pathway (dre04620)
- NOD-like receptor signaling pathway (dre04621)
- RIG-I like receptor (dre04622)
- MAPK signaling pathway (dre04010)
Severe OPP zebrafish model

NMDA-receptor antagonists induce an almost total rescue of grade 3 phenotype

NMDA-receptor activation is a key event in the severe OPP pathophysiology in zebrafish
Severe OPP zebrafish model

Intracellular Ca$^{2+}$ levels are relevant for the pathophysiology of severe OPP in zebrafish

Permeable Ca$^{2+}$ chelator BAPTA-AM induces a partial rescue of severe OPP phenotype (48% decrease)
Severe OPP zebrafish model

NMDA-receptor activation is a key event in the severe OPP pathophysiology in zebrafish

Increase in intracellular Ca$^{2+}$ is a key event in the severe OPP pathophysiology in zebrafish
**Compound**

CPO

**MIE**

AChE Inhibition <95%

**Cells**

Decreased axonal growth and neuronal cell signaling disruption

**Organ**

Nervous system disruption

**Larvae**

Suppressed locomotor act., muscle contraction, Visual impairment

**Adults**

Suppressed locomotor activity and cognitive function

**MIE**

AChE Inhibition >95%

**Cells**

Decreased axonal growth and neuronal cell signaling disruption

**Organ**

Nervous system disruption, Hyperexcitation/convulsions

**Larvae**

Suppressed locomotor activity, muscle fibers impairment, Visual impairment

**MIE**

Total AChE Inhibition

**Cells**

Decreased axonal growth and neuronal cell signaling disruption

**Organ**

Nervous system disruption

**Larvae**

Suppressed locomotor activity, muscle fibers impairment, Visual impairment

**MIE**

NMDA activation

**Cells**

Calcium accumulation

**Organ**

Neurodegeneration, necrosis, edema, Visual degeneration

**Larvae**

Death

**MIE**

Oxidative stress

**Cells**

Mitochondrial damage
Conclusions

• **Chemical models** of mild, moderate and severe OPP can be **easily generated in zebrafish by exposing larvae from 7 to 8 dpf to** different concentrations of the prototypic OP compound **chlorpyrifos-oxon**

• Zebrafish models of OPP mimic most of the pathophysiological mechanisms behind human OPP, including AChE inhibition, NMDA-receptor activation, Ca\(^{2+}\) dysregulation as well as inflammatory and immune response.

• Zebrafish models of OPP can be classified as “partial models”

• Developed zebrafish models of OPP can be used for the identification of new antidotes or combinations of antidotes to fight against this toxidrome.
THIS WORK WAS SUPPORTED IN PART BY:
RNAseq Data Results

- 4 samples/phenotype
- Average 45M reads/sample
- Very good quality data (QS>30)