EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN ON EARLY DEVELOPMENT AND THE NEUROGENESIS PATHWAY IN THE ZEBRAFISH (*Danio rerio*)

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Introduction

The teratogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is known to cause a variety of effects to exposed organisms, the most sensitive vertebrates of which are fish, especially during early development[1,2]. Polychlorinated dibenzodioxin studies with a number of species including trout, zebrafish and medaka have all concentrated on signs of toxicity and characterisation of macroscopic effects[3-9]. However, effects on neuronal development have not been investigated.

Dioxins and dioxin-like PCBs have been reported to alter cognitive functions in humans[10-13], monkeys[14-15] and rats[16] so neurological effects could be expected. They are structurally similar to thyroid hormones required for orderly development, and are known to decrease or mimic their biological action in endocrine studies. Neurological abnormalities may hence occur if hormone levels are lowered, like observed in rats after developmental exposure[17-18]. Decreased levels of the neurotransmitter dopamine caused by PCBs have been documented[19] so may also contribute.

Any effects in the brain may be exerted through a ligand activated transcription factor known as the aryl hydrocarbon receptor (AhR) together with the aryl hydrocarbon nuclear translocator (ARNT). These have been found to be activated in numerous tissues after dioxin exposure, but in particular have been found in the olfactory bulb, cerebral and cerebellar cortices, and the hippocampus in exposed rats[20]. This hence may indicate that TCDD may have an effect directly on discrete neuronal populations of the brain.

These reports therefore suggest that dioxin causes more subtle effects than those previously observed macroscopically, and attention should focus on effects at the neurological level. We therefore aimed to discover effects on neurogenesis firstly via stereological assessments of retina and brain volumes, and secondly by utilising certain lines of transgenic zebrafish expressing GFP (green fluorescent protein) to examine possible disruption of the neurone developmental cascade.

Methods and Materials

Groups of newly fertilized eggs collected within 3 hours of spawning were exposed in 10mls of tank water to 10µl graded doses of TCDD (Greyhound Chromatography, UK) dissolved in acetone, tank water with 0.1% acetone (vehicle control), or just tank water (sham control), for 1 hour[8]. TCDD doses ranged from 25-200ng/l in doubling increments for length and developmental assessment, 200 and 400ng/l for GFP experiments, and 40-200ng/l in 20ng increments for all other experiments. After thorough washing the eggs were incubated in dioxin-free aerated tank water at

27.5±1^{0}C. Embryos were removed and anaesthetised in 1/7000 dilution of 3-aminobenzoic acid ethyl ester (MS222, Sigma) in embryo medium to allow assessment for gross signs of toxicity that include yolk sac and pericardial oedema, craniofacial malformations, necrosis of the brain, and mortality. Swimbladder inflation and blood flow were also assessed. Groups of fry were culled by immersion in anaesthetic and preserved in neutral buffered formalin before being embedded in resin, sectioned and stained with giemsa for stereological analysis. Transgenic fry were examined daily using a confocal scanning fluorescence microscope from 24 -144h postfertilisation. LSM500 imaging software and Kinetic's Aquistition Manager (AQM) was used to view and quantify GFP.

Results and Discussion

Egg mortality and hatching success were not affected by TCDD exposure. ED50s for macroscopic effects (Figure 1 and 2); yolk sac oedema (80ng TCDD/l (ppt)), pericardial oedema (90ppt), craniofacial malformations (54ppt), mortality (LC50, 110ppt); and a NOEC of 31.25ppt were first determined for fry at 192h postfertilisation (hpf). However, this was lowered by the retardation effects observed on length that showed a mean 9% reduction by 170hpf at lowest dose tested, 25ppt, compared to the control fry. Differences in length of all dosed fry occurred after the standard documented period of growth acceleration[21].

Figure 1: Control larva (top) and larva exposed to 100ng TCDD/l (bottom) both at 168hpf. Exposed larvae exhibit yolk sac oedema (Y), pericardial oedema (P), under-inflated swimbladder (S: healthy swimbladder), and craniofacial malformation (C: shortened snout, underdeveloped lower jaw). Note shortened length and decrease in size of the eye.

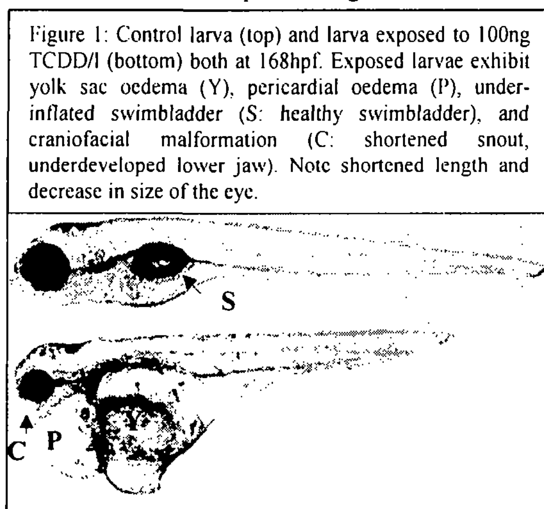
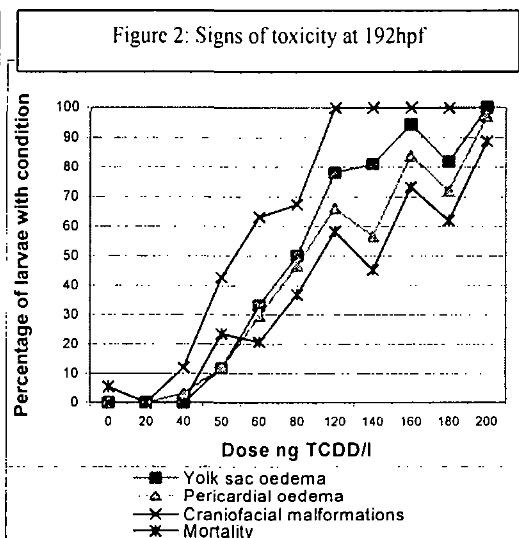


Figure 2: Signs of toxicity at 192hpf



Brain necrosis was observed in some larvae from 120hpf dosed with ≥80ppt. All occurrences coincided with cessation of blood flow. This was first seen from 96hpf (before usual rapid craniofacial growth) with doses ≥140ppt. Slowed blood flow to the trunk and pericardial oedema was not observed until 72hpf, 12 hours after healthy fry have completion of heart morphogenesis and full circulation[21], so we support the view that circulation development was not impeded in zebrafish by TCDD[10]. Cessation of blood flow was however the probable cause for the prevention of swimbladder inflation with few larvae exposed to 200ppt being successful in inflation by 192hpf, and all larvae exposed to dioxin exhibiting some delayed swimbladder inflation.

Fry at 168hpf exposed to 100ppt TCDD exhibited a statistically significant mean decrease of 16% for length (ANOVA $p<5x10^{-8}$), 11% for brain volume (ANOVA $p<0.007$) and 15% for neural retinal tissue (ANOVA $p<0.017$) compared to controls. Effects occurred in a dose dependent

manner apart from retinal volumes that had a 10-15% reduction for each dose ranging from 40 to 120ppt. A t-test showed there was no difference between vehicle and sham controls.

Zebrafish engineered to express the transgene GFP-neurogenin[22] exposed to 400ppt TCDD exhibited neurogenin down-regulation throughout the brain in fry at 48hpf (Figure 3). Mean fluorescence was 1000-1250 units for controls, 780-970 units for 200ppt dosed fry, and 125-600 fluorescence units for 400ppt dosed fry. A recovery was evident by 72hpf, possibly due to temporally different regulatory elements in the gene, like those present in sonic hedgehog[23].

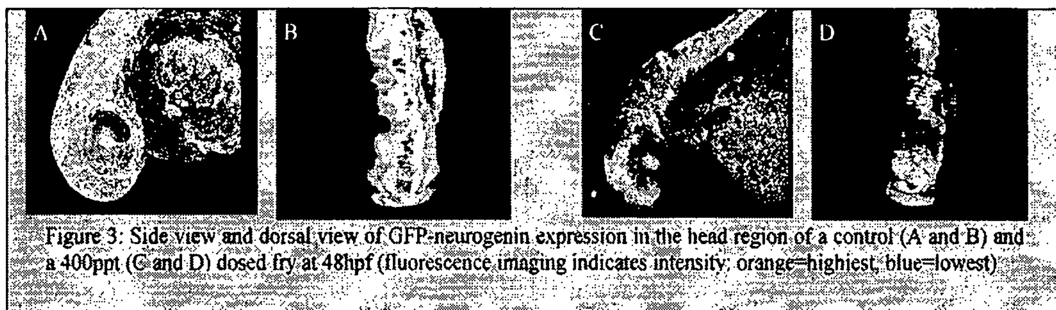


Figure 3: Side view and dorsal view of GFP-neurogenin expression in the head region of a control (A and B) and a 400ppt (C and D) dosed fry at 48hpf (fluorescence imaging indicates intensity; orange=highest, blue=lowest)

We therefore suggest that TCDD retards development of neurological tissue at levels as low as 40ppt TCDD and acts on the transcription of proneural genes either directly or somewhere upstream in the neurogenic cascade. We are currently investigating this further through the use of microarray technology.

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