Redox Biology 6 (2015) 648–655

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Embryonic oxidative stress results in reproductive impairment for adult zebrafish

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ARTICLE INFO

Article history:
Received 5 October 2015
Received in revised form
26 October 2015
Accepted 28 October 2015
Available online 4 November 2015

Keywords:
Oxidative stress
Reproductive toxicity
Auranofin
Zebrafish
Longitudinal cohort

ABSTRACT

Exposure to environmental stressors during embryo development can have long-term effects on the adult organism. This study used the thioredoxin reductase inhibitor auranofin to investigate the consequences of oxidative stress during zebrafish development. Auranofin at low doses triggered upregulation of the antioxidant genes *gstp1* and *prdx1*. As the dose was increased, acute developmental abnormalities, including cerebral hemorrhaging and jaw malformation, were observed. To determine whether transient disruption of redox homeostasis during development could have long-term consequences, zebrafish embryos were exposed to a low dose of auranofin from 6–24 hours post fertilization, and then raised to adulthood. The adult fish were outwardly normal in their appearance with no gross physical differences compared to the control group. However, these adult fish had reduced odds of breeding and a lower incidence of egg fertilization. This study shows that a suboptimal early life environment can reduce the chances of reproductive success in adulthood.

1. Introduction

Embryonic development can be influenced by exposure to environmental toxins and by a variety of pathologies, including oxygen deprivation, inflammation and metabolic disease [10,24]. Oxidative stress underlies many of these conditions, and excessive oxidative stress can cause direct cell damage and directly compromise the health of surviving animals. Cell culture experiments have revealed that more subtle disturbances of cellular redox homeostasis modulate signaling pathways and trigger adaptive responses [11,13]. For example, redox-dependent activation of the transcription factor Nrf2 increases expression of genes in the glutathione and thioredoxin systems, protecting the cells from subsequent stress [17,38]. However, there is little understanding of how redox disruption in the embryo affects the phenotype of the resultant organism [36].

Animal models allow the developmental consequences of environmental insults to be investigated in a way not possible in human studies [3]. The zebrafish, *Danio rerio*, is a vertebrate model of development that is increasingly used in biomedical science [25]. Advantages to using zebrafish include the well-characterized embryonic development and the ease with which toxic effects of chemicals can be studied [21,33]. In zebrafish, the chemical exposure of large numbers of developmentally synchronized embryos can be performed simply by dissolving the chemical of interest in the aquatic media [33].

Oxidative stress can be triggered by promoting oxidant generation or compromising antioxidant defences. Auranofin (AFN) is a gold-based compound (Fig. 1A) that compromises antioxidant defence by targeting selenol and thiol proteins, in particular thioredoxin reductase (TrxR), involved in maintaining the reductive capacity of a cell [5]. In cell culture systems, TrxR has been found to be completely inhibited by 1 μM AFN, with significant mitochondrial oxidative stress and apoptosis occurring as the dose is increased [12,9]. Lower doses, between 0.1–1 μM AFN, which partially inhibit TrxR activity, increase H_2O_2 levels, and activate Nrf2 [12,20,29]. In zebrafish larvae, exposure to 1 μM AFN increased transcription of the Nrf2-target gene *gstp1* in the larval gill tissue, indicating that these animals have plastic chemical defence systems capable of responding to AFN [22].

In this study we sought to model the long-term consequences of environmental oxidative stress during development by transiently exposing zebrafish embryos to AFN. While this stressor is

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http://dx.doi.org/10.1016/j.redox.2015.10.010

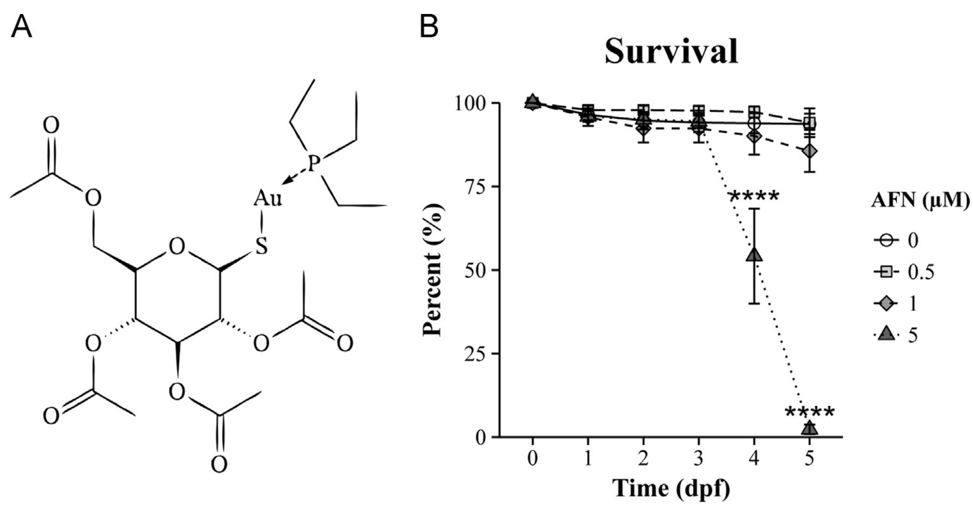

Fig. 1. Embryonic toxicity of AFN. (A) Schematic of the structural formula of $C_{20}H_{34}AuO_9PS$. The compound consists of an acetoxythioglucose group linked to triethylphosphine by a gold atom. (B) Survival following static AFN exposure beginning at 6 hpf, given as the percentage of larvae alive at each time point out of the number of embryos at 0 dpf. The values represent the mean percentage ± SEM for four biological replicates with each treatment group containing at least 30 embryos. Statistically significant differences are noted as **** $p \leq 0.0001$.

not representative of all forms of oxidative stress, AFN provides a useful pharmacological model of disrupted thiol redox homeostasis and an endogenous antioxidant stress response. First, the acute toxicity of AFN was assessed in embryos as an understanding of the developmental effects of AFN-induced oxidative stress in zebrafish is lacking. This revealed that continuous AFN exposure resulted in dose-dependent embryonic toxicity and the upregulation of antioxidant pathways. We then chose a low AFN dose to disrupt redox homeostasis during early embryogenesis, and observed reduced fertility in the resulting adults.

2. Materials and methods

2.1. Animal husbandry

Mature zebrafish were maintained under standard conditions [39]. All zebrafish research was approved by the University of Otago Animal Ethics Committee. The ABPS in-house wild type line was used for the embryonic viability experiments. To visualize blood flow, the *Tg(gata1:DsRed)* line with fluorescent red blood cells was used [37]. To visualize the craniofacial structures, the *Tg(sox10:EGFP)* line with fluorescent green neural crest cells was used [18]. For the cohort experiments, the *Tg(vas:EGFP)* line was used [23].

AFN exposed embryos (see next section) were reared using normal husbandry methods to generate three tanks of fish within each treatment arm of each cohort. As adults, the zebrafish were kept at a stocking density of approximately three fish per litre and bred as frequently as once a week. Two cohorts were produced from multiple founder pairs and raised sequentially. As such, at the time data were gathered, the first cohort was grown out to an older age than the second cohort that followed it. Cohort 1 was bred between 0.77 and 1.24 years post-fertilization (ypf). Cohort 2 was bred between 0.35 and 0.56 ypf. On average three pairs from each of three tanks were used in three spawning experiments.

Spawning was induced in the morning with a water change and the removal of the barrier between the pairs. A successful breeding event was one where at least one egg was released. As such, breeding success for each pair was classified as a binomial outcome, either successful or not. Eggs were collected with a sieve, transferred into a dish containing E3 embryo media, and incubated at 28 °C. The numbers of fertilized and unfertilized eggs were counted from each breeding pair in order to calculate the fertilization rate. The fertilization rate was classified as a Poisson count normalized by the size of the clutch produced by that pair.

2.2. Auranofin exposure

AFN (Sigma-Aldrich, cat. A6733) was made up in DMSO and kept as a 10 mM stock solution, 100 μM working stocks were made fresh in E3 media for each experiment. Final test solutions were made in a volume of 20 ml.

At 6 hours post-fertilization (hpf), embryos were randomly distributed into petri dishes. The excess media was removed and replaced with the AFN test solution or the solvent control, which contained the equivalent volume of DMSO. For embryo viability experiments there were typically 20 embryos in each dish. In the static (continuous) exposures, the test solution remained unchanged throughout the duration of the test. In the transient exposures used in the cohort experiments, the test solution was washed out with E3 media at 1 day post-fertilization (dpf). For the long-term experiment there were 50 embryos in each dish which were subsequently transferred to tanks at 5 dpf.

2.3. Scoring for developmental abnormalities

Mortality was identified in early embryos by the lack of any discernible heartbeat. Hemorrhaging was observed in the head of the larvae as a dense red region. Jaw defects were noted when the angle of the lower jaw was obviously altered in comparison to larvae exposed to the solvent control.

2.4. Measurement of transcript levels

Zebrafish embryo RNA extracts were prepared from 50 pooled embryos from each treatment group using the NucleoSpin RNA kit (Macherey-Nagel, cat. 740955.250) according to the manufacturer's instructions. RNA concentrations and purity were verified on a Nanodrop spectrophotometer. The SuperScript III First-Strand Synthesis System (Invitrogen, cat. 18080-051) was used for cDNA synthesis from 1 μg total RNA using oligo dT primers according to the manufacturer's instructions.

Primers used for quantitative real-time PCR analysis of gene expression are listed in Table S1. The primers were validated by checking the product size, sequencing the product to confirm its