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Identification of apoptosis and macrophage migration events in paraquat-induced oxidative stress using a zebrafish model

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ABSTRACT

Aims: Paraquat (PQ) is a pesticide highly toxic to human beings, and a well-known trigger of oxidative stress. Although several animal models of PQ poisoning have been developed, some disadvantages limit their application in vivo. A zebrafish model was used in the present study to better define mechanisms of oxidative stress injury induced by PQ.

Main methods: The toxicity of PQ was evaluated in the AB strain of zebrafish, and apoptosis was assessed by acridine orange staining. Macrophage migration was identified using the TG (*zlyz*:*EGFP*) transgenic strain, and angiogenesis was observed using the *fli1a-EGFP casper* strain. Following the validation of gene changes by zebrafish-based in vivo quantitative real-time PCR, network analysis was performed using the Ingenuity Pathway Analysis software.

Key findings: We first established the LC_{50} of PQ in the zebrafish model, and then found that robust oxidative stress and antioxidant genes were activated after PQ exposure. Moreover, apoptosis and distinct macrophage activation and migration were identified for the first time in PQ-exposed zebrafish. Utilizing this model, both extrinsic and intrinsic pathways involved in PQ-induced apoptosis were elucidated. We further demonstrated that macrophage migration was specifically induced by PQ, and that Rho family members and JNK-MMP13 signaling participated in this process.

Significance: Zebrafish is a promising tool for investigating the mechanisms of oxidative stress injury induced by PQ, and for screening effective anti-oxidant drugs.

1. Introduction

Paraquat (PQ) is a widely used herbicide which causes high mortality in developing countries. Although progress has been made in organ replacement and life support techniques [1], the mortality rate following PQ poisoning remains at approximately 50%–90% [2]. More importantly, PQ is a well acknowledged trigger of oxidative stress [3,4], and oxidative stress based on redox cycling of PQ is generally recognized as a major cause of its toxicity [22]. However, the specific molecular pathways of PQ-induced oxidative stress have not been fully elucidated.

Disease models of PQ-induced oxidative stress injury have been reported in cell lines, *Caenorhabditis elegans* (*C. elegans*), *Drosophila* and in mammals, including mouse, rat, dog, swine and sheep [5–10]. In vitro models have not adequately simulated the actual conditions associated with the human disease and can only provide limited information. *C. elegans* and *Drosophila* have been used to evaluate PQ-induced oxidative stress and neurodegeneration like Parkinson's disease, but they may not accurately reflect disease processes in vertebrates. Rodent models for toxicological study usually require large numbers of animals, which may not be feasible or affordable. It is also time consuming when transgenic strains are needed. Moreover, in vivo observations in rodent models need 3D imaging systems, which are not widely available. Bigger mammals like dog, swine and sheep require larger feeding chambers and more demanding and costly housing requirements, and are thus seldom used.

Zebrafish is a useful vertebrate model of human disease with several unique advantages such as (1) small size and high fecundity; (2) transparent embryo and body; (3) short reproductive cycle; (4) rapid development of embryo; (5) facilitates high-throughput toxicity screening;

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and (6) many genes are functionally conserved with their human orthologues [11,12]. Here we present evidence that the zebrafish is a superior model for the study of PQ-induced oxidative stress.

2. Materials and methods

2.1. Animals

Wild-type AB strain and transgenic adult zebrafish were housed at 28 + 0.5 °C in an automatic zebrafish housing system on a 14:10 hour light: dark cycle. Adult fish were fed brine shrimp twice daily. Renewal of the water was performed daily, and water quality (pH 7.2–7.6; salinity: 0.03–0.04%) was maintained. Embryos were stored according to the literature [13]. The TG (*zlyz:EGFP*) transgenic lines were purchased from China zebrafish resource center [14]. The *fli1a-EGFP casper* transgenic line has been established as described [15,16]. These procedures have been approved by the ethics committee of Shanghai Tenth People's Hospital and are consistent with the American Veterinary Medical Association's (AVMA) Panel on Euthanasia. The zebrafish facility at Shanghai Research Center for Model Organisms is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

2.2. Toxicity and safety of PQ in the zebrafish model

AB strain zebrafish were soaked in 0.1% dimethyl sulfoxide (DMSO) as vehicle control, or in PQ (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMSO. 30 embryos in total were allocated to each condition. To determine the LC50 of PQ, zebrafish were treated from 3 days post-fertilization (dpf) to 6 dpf and mortality was recorded every 24 h. Dead zebrafish was defined as the absence of heartbeat under a dissecting stereomicroscope (Nikon SMZ645; Japan). In the initial tests, five concentrations (0.1, 1, 10, 100, and 1000 μg/mL) were used for PQ. If a LC_{50} could not be found from the initial tests, additional concentrations within the range of 0.01–3000 μg/mL were tested. Mortality curves were generated using Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA, USA).

2.3. Oxidative stress in zebrafish

AB strain zebrafish at 3dpf were exposed to PQ (100 μg/mL, 25.72 μM) in a time-dependent manner, while zebrafish in the control group were soaked in 0.1% DMSO. At least 30 embryos for each group were collected at 24 h, 48 h and 72 h, and homogenized. Malondialdehyde (MDA) and catalase (CAT) in each group of fish were tested using assay kits according to the manufacturer's directions (A003-1 and A007-1, Jiancheng, Nanjing, China).

2.4. Acridine orange staining for apoptosis

Cell apoptosis was assessed with acridine orange (AO), which served as a nucleic acid-selective metachromatic stain [17]. 3-dpf zebrafish of AB strain were treated with PQ (100 μg/mL, 25.72 μM) for 24 h, 48 h or 72 h, and zebrafish in 0.1% DMSO served as vehicle control [18]. At the end of each time point, zebrafish were washed with fish water three times and immersed in 5 μg/mL AO (acridinium chloride hemi-[zinc chloride], Sigma-Aldrich) in fish water for 60 min. Next, zebrafish were rinsed thoroughly in fish water three times (5 min/wash) and anaesthetized with 0.016% MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO). Zebrafish were then mounted with methylcellulose in a depression slide for observation by fluorescence microscopy. Thirty zebrafish were assigned to each group. The numbers of apoptotic nuclei in the trunk were counted using the microscope [19].

2.5. In vivo macrophage migration assays in zebrafish

TG (*zlyz:EGFP*) transgenic zebrafish larvae (3 dpf) were treated with 100 μg/mL PQ for 24 h, 48 h or 72 h in 6-well plates (BD Falcon), and zebrafish soaked in 0.1% DMSO as the control. Thirty embryos were distributed in each group. At the end of each time point, zebrafish were washed with fish water three times and anaesthetized with 0.016% MS-222. Zebrafish were then mounted with methylcellulose in a depression slide for observation by fluorescence microscopy. The macrophages recruited to the body trunk were enumerated.

2.6. Angiogenesis studies in zebrafish

To determine whether PQ affects vascular development in zebrafish, 24 h post-fertilization (hpf) embryos of *fli1a-EGFP casper* strain were distributed into 6-well plates (thirty embryos per well) (BD Falcon) for a treatment period of 24 h. The test compound PQ was diluted in 0.1% DMSO. The positive control for this assay was 5 μM PTK787, a VEGFR antagonist [20], and the negative control was 0.1% DMSO. After treatment, embryos were anaesthetized with 0.016% MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO) and the numbers of complete intersegmental vessels (ISVs), such as the vessels that connect the dorsal aorta (DA) to the dorsal longitudinal anastomotic vessels (DLAV), were counted. Drug effect was calculated using the indicated formula (Fig. 4).

2.7. Quantitative real-time polymerase chain reaction

AB strain zebrafish were treated with PQ (100 μg/mL in 0.1% DMSO) or 0.1% DMSO respectively. Zebrafish at 3-dpf were exposed to triggers for 72 h for the indicated assays. Total RNA was extracted from 30 to 60 embryos per group in Trizol (Life Technologies, Lot: 87,801) according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA template using the PrimeScript reagent Kit (TaKaRa, DRR037A), followed by qRT-PCR with KAPA SYBR FAST qPCR KIT in 20 μL reaction volume for each gene. This was performed using the respective primers for 40 cycles in an ABI 7900HT Real-Time PCR System using the following protocol: 95 °C for 3 min, followed by 40 cycles of 3 s at 95 °C, 30 s at the indicated annealing temperature at 60 °C, followed by a melt of the product over 15 s at 95 °C, 15 s at 60 °C and then 15 s at 95 °C. All primers are listed in supplemental Table S1. The ΔΔCT method was used for statistical analysis to determine gene expression levels which were normalized with a reference gene, *eflα* [21].

2.8. Ingenuity Pathway Analysis (IPA) network

Network analysis was performed using Ingenuity Pathway Analysis software.

2.9. Image acquisition

Embryos and larvae were analyzed with Nikon SMZ 1500 fluorescence microscope and subsequently photographed with a digital camera. A subset of images was adjusted for levels, brightness, contrast, hue and saturation with Adobe Photoshop 7.0 software (Adobe, San Jose, California) to optimally visualize expression patterns. Quantitative image analyses were processed using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/). Inverted fluorescent images were used for processing. Positive signals were defined by particle number using Image J. Ten animals for each treatment were quantified and the total signal per animal was averaged.

2.10. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis and graphical representation of the data were performed using Graph Pad Prism