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Glutathione and zebrafish: Old assays to address a current issue

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HIGHLIGHTS

- Several xenobiotic agents induces reactive oxygen species (ROS).
- Zebrafish is a common model for the study of xenobiotic toxicity.
- Glutathione a main antioxidant that neutralizes ROS.
- Glutathione peroxidase (GPx) and reductase (GR) are important antioxidant enzymes.
- Simple methods to measure glutathione, and activities of GPx and GR are outlined.

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ABSTRACT

Several xenobiotic agents (e.g. metals, polycyclic aromatic hydrocarbons, nanoparticles, etc.) commonly involve the generation of reactive oxygen species (ROS) and oxidative stress as part of their toxic mode of action. Among piscine models, the zebrafish is a popular vertebrate model to study toxicity of various xenobiotic agents. Similarly to other vertebrates, zebrafish possess an extensive antioxidant system, including the reduced form of glutathione (GSH), which is an important antioxidant that acts alone or in conjunction with enzymes, such as glutathione peroxidase (GPx). Upon interaction with ROS, GSH is oxidized, resulting in the formation of glutathione disulfide (GSSG). GSSG is recycled by an auxiliary antioxidant enzyme glutathione reductase (GR). This article outlines detailed methods to measure the concentrations of GSH and GSSG, as well as the activities of GPx and GR in zebrafish larvae as robust and economical means to assess oxidative stress. The studies that have assessed these endpoints in zebrafish and alternative methods are also discussed. We conclude that the availability of these robust and economical methods support the use of zebrafish as a model organism in studies evaluating redox biology, as well as the induction of oxidative stress following exposure to toxic agents.

1. Introduction

All aerobic organisms naturally produce reactive oxygen species (ROS). The primary contributors of cellular ROS are mitochondria, where oxygen acts as the final electron acceptor within the electron-transport chain (ETC). Approximately 0.1–0.4% of this oxygen is transformed into the oxygen radical superoxide anion ($O_2^{\bullet -}$) due to a 'leaky' ETC (Hermes-Lima, 2005). The conversion of $O_2^{\bullet -}$ into other ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), occurs within the reductive environment of the cellular milieu (Davies, 1995). An imbalance between oxidants (ROS) and antioxidants in favor of the oxidants, or oxidative stress, can disrupt redox signalling and damage cellular components (Sies and Jones, 2007). Not surprisingly, there are several defense systems against oxidative stress and damage; these include antioxidant compounds (e.g. glutathione) and enzymes (e.g. glutathione peroxidase), as well as systems to repair or replace damaged cellular components (Davies, 2000).

Among the numerous endpoints that can be assessed to estimate oxidative stress in a biological system, glutathione-associated measurements are often a primary choice. Reduced glutathione (GSH) is a tripeptide that protects against ROS and ROS-generated byproducts. It acts as an independent antioxidant or in conjunction with enzymes, including glutathione peroxidase (GPx; EC 1.11.1.9), whose action reduces hydroperoxides and lipid peroxides into water and corresponding alcohols, respectively. Upon interaction with ROS (mainly H_2O_2 and lipid peroxides), GSH is converted into its disulfide or oxidized form (GSSG) (Di Giulio and

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Meyer, 2008). GSSG is recycled back into GSH by an auxiliary antioxidant enzyme – glutathione reductase (GR; EC 1.8.1.7) (Mannervik, 1987).

A kinetic method to measure GSH and GSSG was first described in Griffith (1980). This method is based on enzymatic recycling procedure, in which GSH is sequentially oxidized by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of GR. The activity of GPx and GR can also be assessed. A kinetic method for measuring the activity of GPx was first described in Strauss et al. (1980). This two-step method involves i) neutralization of H_2O_2 by GPx in the presence of GSH and ii) recycling of resulting GSSG by GR in the presence of NADPH; consumption of NADPH is then used as a proxy for GPx activity. A kinetic method to measure GR activity was first reported in Racker (1955). The method involves the second part of the aforementioned GPx assay. Although quite old, these methods are still used today; the appearance of 96-well microplate readers and the easy access to the relatively inexpensive reagents certainly helped to simplify and increase the throughput of these assays.

Traditionally, murine models have been used to assess the potential of certain compounds to act as pro- or antioxidants, as well as to study the involvement of oxidative stress in disease. However, due to similarities across the antioxidant defense systems of mammalian and piscine organisms, piscine models are increasingly recognized for their importance in understanding oxidative stress not only as it relates to pollutant-mediated toxicity, but also as means to better understand mammalian-related phenomena (Kelly et al., 1998).

Across the various piscine models, the zebrafish (*Danio rerio*) is a common model of choice. Zebrafish have been mostly used in developmental biology and molecular genetics, but have also been proposed as a model for human disease (Lieschke and Currie, 2007) and recognized as a valuable vertebrate model for toxicology and drug discovery (Fako and Furgeson, 2009; Hill et al., 2005; Yang et al., 2009). With regards to oxidative stress, various parts of the zebrafish antioxidant system, including antioxidant enzyme activities and glutathione concentrations, were assessed previously (e.g. Massarsky et al., 2013, 2015). Given the importance of glutathione within the antioxidant defense system, this manuscript aims to outline detailed protocols to assess GSH and GSSG concentrations, as well as GPx and GR activities in zebrafish larvae. The methods described herein are specific, high-throughput, and cost-effective – putting forward zebrafish as a valuable model for the study of oxidative stress.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich.

2.2. Zebrafish processing

Typically in toxicological studies, zebrafish are exposed to various compounds up to 3–5 days post-fertilization (dpf). At the end of the exposure the larvae are collected into 1.5 mL Eppendorf tubes and rinsed with ice-cold medium. The medium is then removed and the larvae are snap-frozen in liquid nitrogen and stored at −80 °C until analyzed. This manuscript demonstrates the assays using 4 dpf zebrafish larvae (15 larvae per tube) that were not exposed to any chemicals. Note: these methods can easily be adapted for zebrafish embryos and/or larvae at other developmental stages, as well as isolated tissues or cells by adjusting the number/amount of embryos/larvae/tissue/cells used for each sample, with a target mass of ~5–10 mg.

2.3. Glutathione concentrations

2.3.1. Solution preparation

Several solutions are needed to perform the assay. Please see section 2.3.2 step 16 for a note on solution preparation.

1. Sulfosalicylic acid (SA). Prepare 5% solution by dissolving 5 g SA in double distilled water (ddH_2O) and completing to 100 mL, using a volumetric flask or a graduated cylinder. Prior to use, 5% SA should be bubbled with nitrogen (or argon) gas for 20 min, in order to replace oxygen; this step is necessary to prevent auto-oxidation of GSH in the samples. Keep 5% SA on ice until use for standard/sample preparations. When not in use, 5% SA should be kept at 4 °C.
2. Solutions A and B. Solution A is 1000 mM KH_2PO_4 (dissolve 6.12 g KH_2HPO_4 in ddH2O in a final volume of 45 mL). Solution B is 1000 mM K_2HPO_4 (dissolve 7.83 g K_2HPO_4 in ddH2O in a final volume of 45 mL). These solutions can be kept separately at room temperature and should be refreshed every several months.
3. EDTA. Prepare 134 mM EDTA (disodium dihydrate salt) solution (dissolve 1 g EDTA in ddH_2O in a final volume of 20 mL). EDTA solution can be kept at room temperature and refreshed every six months.
4. Potassium phosphate buffered solutions (KPB-100 and KPB-500). To prepare KPB-100, mix 4 mL Solution A, 6 mL Solution B, and 0.82 mL EDTA solution, complete to 100 mL with ddH_2O, and adjust to pH 7.0. To prepare KPB-500, mix 2 mL Solution A, 3 mL Solution B, and 0.074 mL EDTA solution, complete to 10 mL with ddH_2O, and adjust to pH 7.0. KPB-100 and KPB-500 can be kept at 4 °C for up to 1 week.
5. 2-vinylpyridine. Prepare a 28 μL/mL 2-vinylpyridine solution by mixing 28 μL commercially available 2-vinylpyridine stock solution with 1 mL KPB-500. 2-vinylpyridine solution should be thoroughly vortexed (white cloudy solution) prior to use.
6. NaOH. Prepare 125 mM NaOH, by mixing 100 μL 5 M NaOH with KPB-100 in a final volume of 4 mL.
7. Glutathione reductase (GR). Prepare a 2 U/mL GR solution by mixing 40 μL commercially available GR stock solution (Sigma G3664) in 4 mL KPB-100.
8. Assay solution. The Assay solution contains 0.7 mM DTNB and 0.3 mM NADPH. Combine 21 mg DTNB, 18 mg NADPH, and 80 mL KPB-100. NADPH is quite expensive; thus, it is recommended to adjust the volume of this solution according to the total number of samples (please remember that each sample or standard will be run in duplicate and that the same Assay solution will be used to assess both TGSH and GSSG concentrations).
9. GSH standards. Prepare a 10 mM GSH stock solution by adding 3 mg GSH to 1 mL 5% SA. Perform serial dilution to prepare the standard curve 100, 50, 25, 12.5, 6.25, and 0 μM.
10. GSSG standards. Prepare 10 mM GSSG stock solution by adding 6.1 mg GSSG to 1 mL 5% SA. Perform serial dilution to prepare the standard curve 10, 5, 2.5, 1.25, 0.625, and 0 μM. Ensure that there is 50 μL of each GSSG standard.

2.3.2. Sample preparation

11. Transfer the preserved samples from −80 °C onto dry ice or liquid nitrogen.
12. Add 200 μL of 5% SA to a sample and sonicate for 5–10 s. A homogenous grey suspension should be apparent at this stage (Fig. 1). Place the sample on ice and continue with the