



Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Notes & Tips

Paraquat administration in *Drosophila* for use in metabolic studies of oxidative stress

T.Z. Rzezniczak, L.A. Douglas, J.H. Watterson, T.J.S. Merritt*

Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario, Canada P3E 2C6

ARTICLE INFO

Article history:

Received 14 June 2011

Received in revised form 4 August 2011

Accepted 12 August 2011

Available online xxxx

Keywords:

Paraquat

Oxidative stress

Drosophila melanogaster

ABSTRACT

Paraquat (PQ) is widely used in the laboratory to induce *in vivo* oxidative stress, particularly in the fruit fly, *Drosophila melanogaster*. PQ administration to the fly traditionally involves feeding in a 1% sucrose solution; however, a diet high in sucrose can itself be stressful. We examined a novel method of PQ administration: incorporation into the fly's standard cornmeal–sucrose–yeast diet. This method successfully delivers PQ to the fly at concentrations similar to those of the traditional method but with fewer possibly confounding complications.

© 2011 Elsevier Inc. All rights reserved.

Paraquat (PQ,¹ 1,1'-dimethyl-4,4'-bipyridinium dichloride) is commonly used in the laboratory to generate oxidative stress. PQ undergoes an *in vivo*, NADPH-dependent reduction, yielding a stable PQ radical that reacts with oxygen to generate superoxide anion, a reactive oxygen species (ROS) [1]. The accumulation of ROS, and subsequent depletion of reducing agents, creates an environment of oxidative stress (OS), where ROS can cause damage to lipids, proteins, and DNA. Oxidative damage in response to PQ exposure has been demonstrated in a wide variety of organisms [2–5].

PQ is used extensively in the *Drosophila melanogaster* model of oxidative stress [4,5]. PQ is generally administered by feeding flies a PQ/sucrose solution for 24 h [4]. Sucrose is thought to promote feeding. The ease of this administration method has led to its widespread application; however, a high-sucrose diet, even for a short term, may have a substantial metabolic effect, possibly confounding or complicating studies of OS. High-sucrose diets in *D. melanogaster* have been shown to promote OS [6] and to affect live weight, total protein, and activities of many enzymes [7,8], all of which can lead to confounding effects in studies of OS, particularly in those examining metabolism.

To simplify interpretation of PQ-driven OS effects, we suggest that PQ administration should be through incorporation into an organism's standard diet. In this study, we examined the effectiveness of administering PQ in a cornmeal–sugar–yeast (CSY) diet as a means of generating OS in *D. melanogaster*. The CSY diet is a high-quality diet that closely resembles the nutritional content of the

diet to which *D. melanogaster* is adapted in the wild [9]. We compared three concentrations of PQ in CSY medium (10, 20, and 30 mM) with the traditional method of PQ delivery (10 mM PQ in 1% sucrose [4]) by quantifying ingested PQ, measured by ultra-performance liquid chromatography (UPLC), and OS markers (mortality and superoxide dismutase [SOD] activity).

The Iso-third chromosome lines used were as described previously [10] and have different third chromosomes in otherwise identical genetic backgrounds (first and second chromosomes). Genotypes were *w*;6326; *JFL12/TM3*, *w*;6326; *MFL42/TM3*, *w*;6326; *NC25/TM3*, *w*;6326; *VA29/TM3*, and *w*;6326; *VT26/TM3*.

Adult males (3–5 days posteclosion) were fed 10 mM PQ (cat. no. 856177, Sigma–Aldrich, St. Louis, MO, USA) in (i) a simple sugar diet consisting of 1% (w/v) sucrose solution or (ii) CSY diet containing 4.52% (w/v) cornmeal, 7.05% (v/v) corn syrup, 4.52% (w/v) yeast extract, 3.84% (w/v) agar, and 0.25% (w/v) methylparaben in 95% ethanol. For the sucrose administration method, two disks of filter paper were saturated with 220 µl of 10 mM PQ in 1% sucrose solution and placed in the bottom of a standard *Drosophila* rearing vial. For the CSY method, food was allowed to cool to 35 °C and poured into standard *Drosophila* rearing vials with a PQ concentration of 10, 20, or 30 mM. A total of 15 flies were placed in each vial. Control flies were maintained on either 1% sucrose diets or CSY diets. Flies were collected following 24 h of treatment for PQ detection and SOD activity assays. Flies were maintained on treatment for 48 h to measure mortality. Ingested PQ was quantified through determination of PQ concentrations in whole fly homogenates using an Acquity UPLC system (Waters, Bedford, MA, USA) with photodiode array detection and classical standard addition as described previously [11].

Total SOD activity was measured in male flies on PQ treatments for 24 h. Extracts were prepared by homogenizing 5 flies in 500 µl

* Corresponding author. Fax: +1 705 675 4844.

E-mail address: tmerritt@laurentian.ca (T.J.S. Merritt).¹ Abbreviations used: PQ, paraquat; ROS, reactive oxygen species; OS, oxidative stress; CSY, cornmeal–sugar–yeast; UPLC, ultra-performance liquid chromatography; SOD, superoxide dismutase; SE, standard error.

of ice-cold homogenizing buffer (210 mM mannitol, 70 mM sucrose, and 1 mM ethylenediaminetetraacetic acid [EDTA]). Homogenates were centrifuged at 800g for 10 min at 4 °C to pellet solids. SOD activity was measured using a commercially available kit (cat. no. 706002, Cayman Chemical, Ann Arbor, MI, USA). Briefly, the kit uses hypoxanthine and xanthine oxidase to generate superoxide radical that is detected through its reaction with a tetrazolium salt to form a formazan dye that absorbs at 440 nm. SOD catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, resulting in a decrease in the amount of formazan dye detected; that is, the lower the absorbance at 440 nm, the greater the SOD activity in the sample. One unit of SOD is defined as the amount of enzyme needed to clear 50% of the superoxide. Homogenate was diluted 1:6 with homogenizing buffer and 10 μ l of homogenate was used with kit reagents, as specified by the manufacturer's protocol. SOD activity was standardized by soluble protein content (see below) and is reported as the mean and standard error (SE) of duplicate assays.

Soluble protein was measured by the bicinchoninic acid (BCA) assay using a commercially available kit (cat. no. 23225, Pierce, Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. The assays contained 10 μ l of homogenate and 100 μ l of reagent and were incubated at 37 °C for 30 min prior to measuring absorbance at 562 nm.

Sensitivity to PQ was quantified as mortality following 48 h of treatment. A total of 20 flies were maintained in vials, with five replicate vials of each genotype for each treatment. Dead flies were counted, and mortality was calculated as a percentage of the total flies per vial, reported as mean \pm SE of multiple vials.

For PQ detection and SOD activity assays, each genotype was raised in two vials and two samples of 5 flies were collected from each vial, for a total of four samples per treatment per genotype. Statistical analysis was done using the JMP software package. In analysis of PQ concentration, fly weight was used as a covariate in an analysis of covariance (ANCOVA) to standardize for differences in fly size.

We compared the traditional method of PQ delivery (10 mM PQ in 1% sucrose [4]) with a biologically simpler and less stressful delivery method (PQ in a standard fly laboratory diet [CSY medium]). We used UPLC to quantify ingested PQ in flies treated by the traditional method and in three concentrations of PQ (10, 20, and 30 mM) delivered in CSY medium. In addition, we used two biological indicators, mortality and SOD activity, to quantify OS in these same treatments.

To allow for genetic variability, we included five different lines (genotypes) of *D. melanogaster*. Fig. 1 shows the concentration of

ingested PQ in flies for each of these treatments. Flies exposed to PQ, in either CSY or sucrose, contained significant amounts of PQ and, as expected, ingested PQ varied with treatment, $F(5, 119) = 53.01$, $P < 0.0001$. The PQ concentrations from the 10- and 20-mM PQ/CSY-treated flies were not significantly different from those from the 10-mM PQ/sucrose-treated flies (Tukey's HSD, $P < 0.05$); feeding flies (10 or 20 mM PQ in CSY medium) delivers PQ at concentrations that are comparable to the traditional method of PQ delivery in sucrose. Genotype also had a significant impact on PQ concentration under both PQ administration methods, $F(4, 79) = 7.04$, $P < 0.0001$, and $F(4, 29) = 4.26$, $P < 0.0079$, for CSY and sucrose administrations, respectively. Genotypes may vary in feeding rate or PQ metabolism, but this effect is not specific to either PQ delivery method.

We quantified two biological indicators of OS, mortality and SOD activity, to verify the ability of our novel PQ delivery method to create OS in treated flies. Fig. 2A shows mortality of flies for each PQ treatment following 48 h of exposure. OS is generally associated with a reduced lifespan, likely due to the damage that ROS can cause to proteins, lipids, and DNA [12]. As expected, mortality differs with PQ treatment, $F(5, 149) = 53.36$, $P < 0.0001$, and between genotypes for both the sucrose administration method, $F(4, 49) = 35.09$, $P < 0.0001$, and the CSY administration method, $F(4, 99) = 21.41$, $P < 0.0001$. All PQ-fed flies showed increased mortality at 48 h as compared with controls (Tukey's HSD $P < 0.05$). Flies fed PQ in a sucrose solution showed a mortality of $52.9 \pm 4.6\%$ (mean \pm SE). Mortality increases with PQ concentration consistent with an increase in OS. In this assay, the 10- and 20-mM CSY flies, but not the 30-mM flies, had significantly lower mortality than the 10-mM PQ/sucrose flies (Tukey's HSD $P < 0.05$). Although flies fed 10 and 20 mM PQ in CSY medium ingested PQ concentrations that were statistically comparable to those ingested by flies fed 10 mM PQ in sucrose solution (Fig. 1), they experienced lower mortality than their sucrose-fed counterparts (Fig. 2A), consistent with the pro-oxidant effect of a high-sucrose diet observed in other studies [6]. Mortality levels after 48 h of exposure to 10 mM PQ in sucrose solution were comparable to mortality levels seen in flies exposed to 30 mM PQ in CSY medium for 48 h (Fig. 2A).

Total SOD activity was measured for all PQ treatments (Fig. 2B). SOD catalyzes the breakdown of the superoxide anion, a harmful ROS, to hydrogen peroxide and is up-regulated under conditions of OS [13]. SOD activity differed with PQ treatment, $F(5, 119) = 2.95$, $P < 0.016$, as well as with genotype for both sucrose administration, $F(4, 39) = 3.29$, $P < 0.0237$, and CSY administration, $F(4, 79) = 5.22$, $P < 0.0011$. All PQ treatments administered through CSY medium showed significantly higher amounts of SOD activity than did controls (Tukey's HSD, $P < 0.05$), consistent with PQ-generated OS in these flies. Interestingly, there was no significant difference in SOD activity between flies fed 10 mM PQ/sucrose and control flies fed 1% sucrose. This lack of difference appears to be a function of the higher level of SOD activity in the control sucrose-fed flies and not due to a lack of OS in the flies treated with 10 mM PQ/sucrose; the 10 mM PQ/sucrose-treated flies showed an increase of 18.5% in SOD activity in comparison with CSY control flies (Tukey's HSD, $P < 0.05$). The apparent up-regulation of SOD in sucrose-fed flies may result from the pro-oxidant effect of a short-term high-sucrose diet [6]. SOD was up-regulated in PQ treated flies, although higher PQ concentrations did not result in higher SOD activity (Fig. 2B).

In conclusion, we recommend the CSY administration method for future studies of PQ-induced OS in *Drosophila* species, and the method may also be appropriate for administering other xenobiotics. Administration of 20 mM PQ/CSY results in similar levels of OS as 10 mM PQ/sucrose (Fig. 2A and B), although the total PQ concentration is higher (Fig. 1), likely reflecting the pro-oxidant nature of sucrose. Use of the CSY medium for administration will avoid the

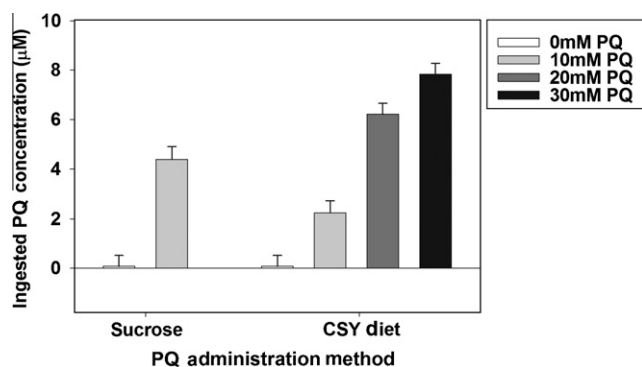


Fig. 1. Quantification of ingested PQ by UPLC following 24 h of PQ exposure. Flies were exposed to one of four treatments: 10 mM PQ in 1% sucrose or 10, 20, or 30 mM PQ in standard CSY medium. Control flies were maintained on 1% sucrose or CSY medium, respectively. Bars represent ingested PQ concentrations in micromoles per liter (mean \pm SE) across five genotypes.

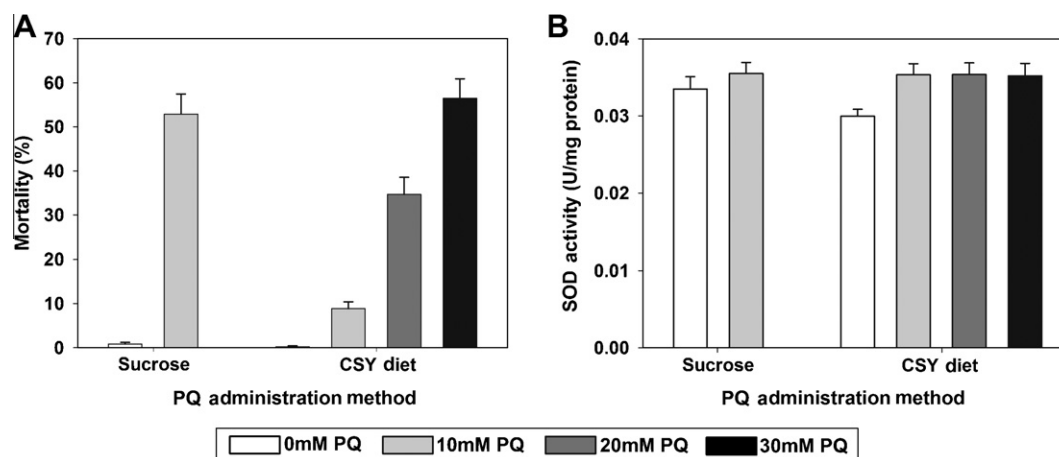


Fig.2. Biological indicators of oxidative stress compared between PQ administration methods. (A) Mortality following 48 h of PQ exposure. Mortalities are in percentage of total flies (mean \pm SE) across five genotypes. (B) Total SOD activity. SOD activities are in enzyme units per milligram soluble protein (mean \pm SE) across five genotypes.

confounding effects of a high-sucrose diet associated with the traditional method of PQ administration, improving the use of PQ in metabolic studies.

Acknowledgments

This work was supported by an NSERC Discovery grant (3414-07), a Collaborative Research and Development grant (311082-2004), a Canada Research Chair (950-215763), and a Greater Sudbury Development Fund grant.

References

- [1] J.S. Bus, J.E. Gibson, Paraquat: model for oxidant-initiated toxicity, *Environ. Health Perspect.* 55 (1984) 37–46.
- [2] T. Magwere, M. West, K. Riyahi, M.P. Murphy, R.A.J. Smith, L. Partridge, The effects of exogenous antioxidants on lifespan and oxidative stress resistance in *Drosophila melanogaster*, *Mech. Ageing Dev.* 127 (2006) 356–370.
- [3] Q. Chen, Y. Niu, R. Zhang, H. Guo, Y. Gao, Y. Li, R. Liu, The toxic influence of paraquat on hippocampus of mice: involvement of oxidative stress, *Neurotoxicology* 31 (2010) 310–316.
- [4] B.R. Strub, T.L. Parkes, S.T. Mukar, S. Bahadorani, A.B. Coulthard, N. Hall, J.P. Phillips, A.J. Hilliker, Mutations of the *withered (whd)* gene in *Drosophila melanogaster* confer hypersensitivity to oxidative and are lesions of the carnitine palmitoyltransferase I (CPTI) gene, *Genome* 51 (2008) 409–420.
- [5] S.K. Legan, I. Rebrin, R.J. Mockett, S.N. Radyuk, V.I. Klichko, R.S. Sohal, W.C. Orr, Overexpression of glucose-6-phosphate dehydrogenase extends the lifespan of *Drosophila melanogaster*, *J. Biol. Chem.* 279 (2004) 32492–32499.
- [6] J. Bussieres, E. Rock, E. Gueux, A. Mazur, P. Grolier, Y. Rayssiguier, Short-term consumption of a high-sucrose diet has a pro-oxidant effect in rats, *Br. J. Nutr.* 87 (2002) 337–342.
- [7] L. Wang, A.G. Clark, Physiological genetics of the response to a high-sucrose diet by *Drosophila melanogaster*, *Biochem. Genet.* 33 (1995) 149–165.
- [8] I. Zinke, C.S. Schutz, J.D. Katzenberger, M. Bauer, M.J. Pankratz, Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response, *EMBO J.* 21 (2002) 6162–6173.
- [9] J. Ye, X. Cui, A. Loraine, K. Bynum, N.C. Kim, G. White, M. De Luca, M.D. Garfinkel, X. Lu, D.M. Ruden, Methods for nutrigenomics and longevity studies in *Drosophila*: effects of diets high in sucrose, palmitic acid, soy, or beef, *Methods Mol. Biol.* 371 (2007) 111–141.
- [10] T.J.S. Merritt, K. Kuczyński, E. Sezgin, C-T. Zhu, S. Kumagai, W.F. Eanes, Quantifying interactions within the NADP(H) network in *Drosophila melanogaster*, *Genetics* 182 (2009) 564–574.
- [11] T.J.S. Merritt, L. Douglas, T.Z. Rzezniczak, J. Watterson, Rapid and simple analysis of paraquat in tissue homogenate by ultra-performance liquid chromatography, *Anal. Methods* 3 (2011) 1428–1432.
- [12] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress, and the biology of ageing, *Nature* 408 (2000) 239–247.
- [13] J.M. McCord, I. Fridovich, Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein), *J. Biol. Chem.* 244 (1969) 6049–6055.