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Effect of Paraquat on Glutathione Activity in Japanese Quail

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In the assessment of environmental contamination, an important new approach is the use of biomarkers, generally defined as biochemical and cellular responses to xenobiotic compounds (McCarthy and Shugart 1980; Fossi 1994). The use of biomarkers to evaluate pollution hazards has noticeably increased in the past few years instead of the use of sentinel species as bioindicators because biomarkers provide an early signal of adverse effects on natural populations. At present, many biomarkers commonly are used in environmental hazard assessment. Moreover, markers with a non-specific target may be useful in monitoring pollution due to complex mixtures, for example in highly industrialized areas of developing countries (Fossi 1994).

The glutathione system is greatly involved in protecting cells against damage initiated by a variety of endogenous and exogenous compounds by acting as co-substrate for glutathione S-transferase (Kaplovitz 1980) and glutathione peroxidase (GSPx; Wendel 1980), and for these reasons it can be a promising biomarker of chemical stress. The analysis of human urinary thioethers is used to evaluate the presence of electrophilic compounds that are commonly detoxified through conjugation with glutathione (GSH) (Van Doorn et al. 1980; Mallol and Nogues 1991). Moreover, conditions of oxidative stress can lead to a depletion of intracellular GSH (Jaensche 1990).

The aim of this work was to investigate a series of parameters useful in detecting a chemical-induced stress in the blood of birds. Since in a previous work we demonstrated that GSH levels in birds can be used as a biomarker of exposition to electrophilic substances (Galvani et al. 1998), in this study we analysed the effect of paraquat, a free radical-generating agent, on Japanese quail *(Coturnix coturnix japonica)* in a dose that causes an oxidative stress without affecting the health of quail. Our attention was focused on the hematic levels of GSH and GSPx activity.

MATERIALS AND METHODS

Paraquat was purchased from Sigma Chemical Company, St. Louis, MO, USA. Adult female *Coturnix coturnix Japonica* weighting 130-150 g were obtained from a local supplier. They were housed under controlled environmental

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conditions of temperature (22 ± 1°C) and light (12 h light-12 h dark) and had free access to water and bird food. For experimentation, the birds were randomly divided into groups of 5 animals each, and paraquat was administered intraperitoneally at the dose of 1 mL/100 g body weight from a 1 mg paraquat/mL saline solution. Control animals received vehicle only (0.9% saline solution).

In order to study the short-term effects of an acute treatment, birds received a single dose of paraquat and were investigated at 0.5, 1, and 4 h post-exposition. Moreover, to investigate the effects of a prolonged expositon, the animals were treated with paraquat (10 mg/kg body weight) daily for 7 days by intraperitoneal injection, and were studied at 4 and 24 h after the last administration. At these intervals blood was collected in syringes containing sodium heparin as an anticoagulant and animals were sacrificed. Lungs were excised, immediately rinsed in ice-cold 0.9% saline solution and quickly stored at -20°C until lipid peroxidation assay. A 0.2 mL portion of whole blood was added to 0.8 mL of ice-cold 5% (w/v) metaphosphoric acid, incubated at room temperature for 15 min, and centrifuged at 14,000 x g for 10 min. The supernatant obtained was immediately frozen and stored at -80°C until GSH assay. To prepare lysate samples, 0.5 mL portions of whole blood were mixed with 0.5 mL of cold isotonic saline solution and centrifuged at 1,000 x g for 5 min. This procedure was repeated three times and the sedimented erythrocytes were diluted with 1 mL of saline. Redistilled deionized water (0.8 mL) was added to 0.2 mL of the cell suspension and the mixture was frozen and thawed three times at -20°C.

Levels of reduced (GSH) and oxidised (GSSG) glutathione were measured using *o*-phthalaldehyde (OPT) as fluorescent reagent according to the method of Hissin and Hilf (1976). The reaction mixture contained 1.8 mL of 1 *M* sodium phosphate-5 m*M* EDTA buffer pH 8.0, 0.1 mL of diluted supernatant, and 0.1 mL of OPT solution containing 0.1 mg of OPT. After 15 min, the intensity of fluorescence at 420 nm was determined with excitation at 350 nm. For GSSG assay a portion of the supernatant was incubated at room temperature with 0.04 *M* N-ethyhnaleimide (NEM). The reaction was stopped after 30 min by the addition of cold 0.1 *N*NaOH. This mixture was taken for measurement of GSSG using the procedure for GSH assay, except that 0.1 *N* NaOH was employed as diluent rather than phosphate-EDTA buffer. Glutathione peroxidase activity was determined according to the method of Wendel (1981). The hemolysate was mixed with an equal volume of Drabkin's reagent (1.6 m*M* KCN, 1.2 m*M* $K_3Fe(CN)_6$, 23.8 m*M* $NaHCO_3$) to convert all hemoglobin to the stable cyanmethemoglobin form The reaction mixture contained 50 m*M* potassium phosphate pH 7.0 (including 1 m*M* Na_2EDTA and 1 m*M* NaN_3) 0.2 m*M* NADPH in 0.1% $NaHCO_3$ solution, 1 m*M* GSH, 0.6 U.I./mL glutathione reductase, and 0.1 mL of hemolysate-Drabkin's mixture. The reaction was started by the addition of 0.03 mL of 24 m*M* H_2O_2. NADPH oxidation was calculated at 366 nm at 37°C using 6.22 $mM^{-1}c\,m^{-1}$ as the extinction coefficient. Lipid peroxidation was measured as malondialdehyde content, using the thiobarbituric acid assay according to the method of Larson and Bull (1992). The frozen lungs were homogenised in 9 volumes of 0.15 *M* KCl.

Either the homogenate (0.25 mL) or the hemolysate (0.4 mL) were added to 3 mL of 1% H_3PO_4. One mL of 0.6% thiobarbituric acid solution (TBA) was then added and the samples were incubated at 100°C for 1 h. After cooling for 5 min, the samples were transferred to centrifuge tubes containing 4 mL, of 1-butanol, mixed vigorously, and centrifuged at 2500 x *g* for 20 min. The absorbance of the upper butanol phase was read at 534 nm against a blank that contained water instead of TBA, using an extinction coefficient of 156 $mM^{-1}cm^{-1}$ (Ernster and Nordenbrand 1967).

Hemoglobin content of lysate samples was determined by the method of van Kampen and Zijlstra (1961) using Drabkin's reagent. Protein content of homogenate samples was determined according to Lowry et al. (195 1) using bovine serum albumin as the standard.

All data (expressed as mean ± SE) were estimated by two-way analysis of variance (ANOVA) followed by a Newman-Keuls test. The difference was considered significant if the corresponding *P* value was less than 0.05.

RESULTS AND DISCUSSION

The effect of a single administration of paraquat (10 mg/kg body weight) on glutathione levels in blood is presented in Fig. 1. The administration of paraquat to quail led to a time-dependent loss of GSH content. There were no consistent differences in the amount of GSH when control and paraquat treated animals were compared at 0.5 h. Reduced (GSH) and oxidized (GSSG) glutathione levels were significantly depressed (25% of depletion) by 1 h postinjection in quail treated with paraquat.

The sensitivity of quails to paraquat is verified by the increase in lipid peroxidation, measured as malondialdehyde (MDA) content in lung and blood as shown in Fig. 2. MDA levels in lung after 0.5, 1, and 4 h increased significantly by 40, 25, and 20%, respectively. The administration caused also an increase in the erythrocyte MDA content of 10 and 20%, respectively by 0.5 and 1 h postinjection, and this effect was maintained for 4 h

The oxidative stress caused by the paraquat treatment is detectable by the GSPx activity in blood (Fig. 2). The single administration of paraquat induced a time-dependent increase in GSPx activity. Although the increase in this activity was not statistically significant after 0.5 h, it appeared noticeable (20%). A 50% increase in hemolysate GSPx activity was found 1 h after paraquat administration and the values remained at high levels for 4 h.

Additional experiments were performed to determine the effect of paraquat after a prolonged treatment. Fig. 3 reports the effect on glutathione levels in quail treated

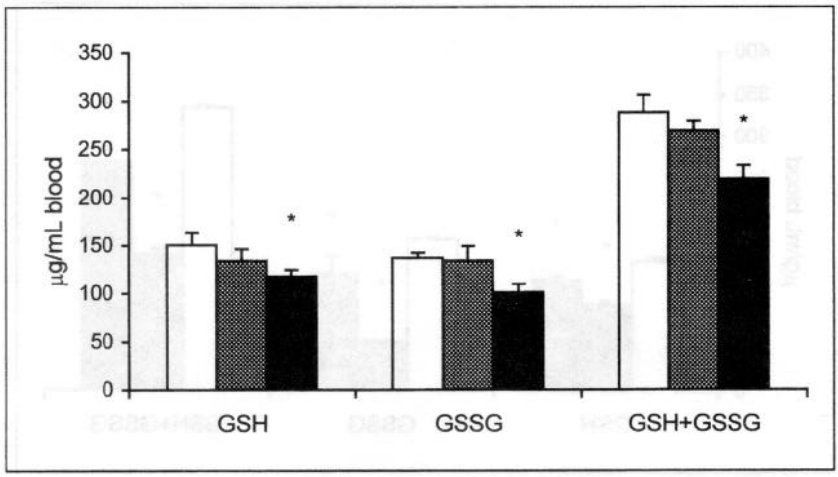

Figure 1. Effect of acute paraquat treatment on GSH, GSSG, and combined levels in whole blood. Samples were collected 0.5 (gray bar) and 1 h (black bar) after treatment; control animals are indicated by open bar. Data are mean ± SE of duplicate determinations (five animals for each group). * $p < 0.05$ vs. control (ANOVA followed by Newman-Keuls test).

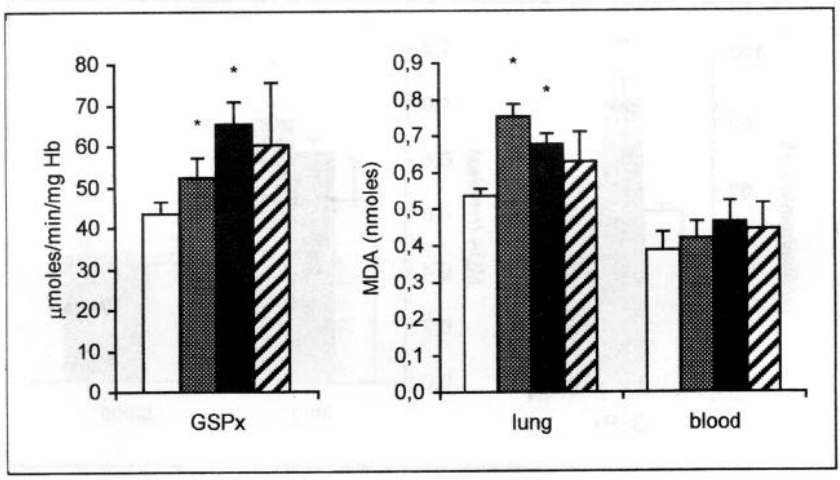

Figure 2. Effect of acute paraquat treatment on erythrocyte hemolysate GSPx activity and on MDA levels in lung (nmoles MDA/mg proteins) and blood (nmoles MDA/mg hemoglobin). Samples were collected 0.5 (gray bar), 1 (black bar), and 4 h (diagonal bar) after treatment; control animals are indicated by open bar. Data are mean ± SE of duplicate determinations (five animals for each group). * $p<0.05$ vs. control (ANOVA followed by Newman-Keuls test).

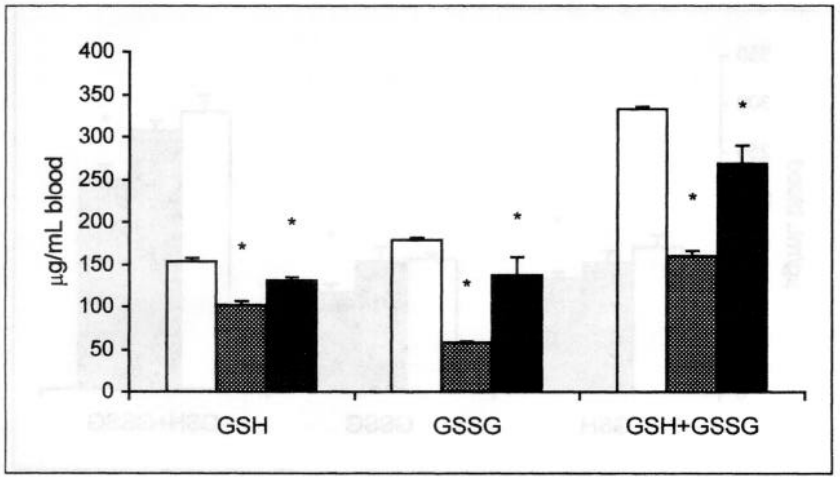

Figure 3. Effect of prolonged paraquat treatment on GSH, GSSG, and combined levels in whole blood. Samples were collected 4 h (gray bar) and 24 h (black bar) after the last injection; control animals are indicated by open bar. Data are mean ± SE of duplicate determinations (five animals for each group). * $p<0.05$ vs. control (ANOVA followed by Newrnan-Keuls test).

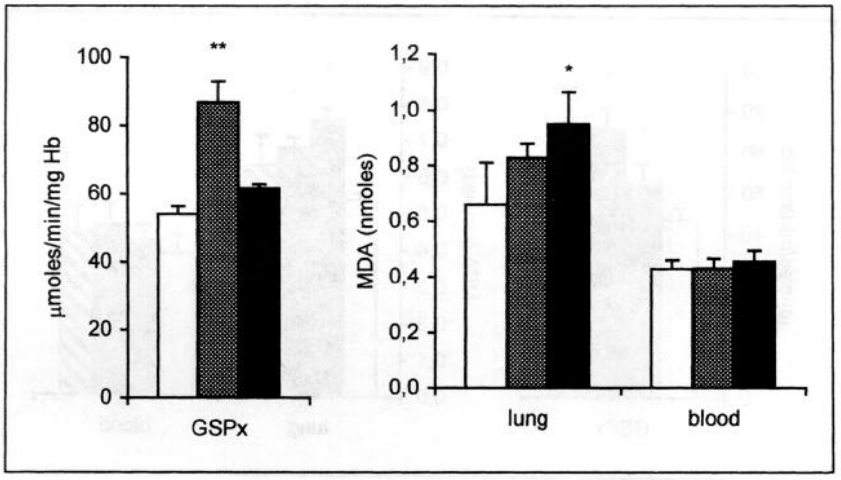

Figure 4. Effect of prolonged paraquat treatment on erythrocyte hemolysate GSPx activity and on MDA levels in lung (nmoles MDA/mg proteins) and blood (nmoles MDA/mg hemoglobin). Samples were collected 4 h (gray bar) and 24 h (black bar) after last injection; control animals are indicated by open bar. Data are mean ± SE of duplicate determinations (five animals for each group). * $p<0.05$ ** $p<0.01$ vs. control (ANOVA followed by Newman-Keuls test).

daily for 7 days by intraperitoneal injection with 10 mg/kg body weight. Paraquat induced a significant depletion of both GSH and GSSG content. In particular, a marked and significant decrease in the levels of GSH and GSSG (35 and 65%, respectively) was observed in whole blood 4 h after the last administration. Moreover, 24 h after continuous treatment a less noticeable but significant depletion of glutathione was observed.

Fig. 4 shows the effect of the prolonged paraquat treatment on GSPx activity and lipid peroxidation. As previously noticed after acute treatment, repeated paraquat administration induced a significant increase in the hemolysate GSPx activity 4 h after the injection. However, this effect is not persistent for 24 h as observed for the GSH depletion. MDA levels in lung were increased, although not significantly, 4 h after the last administration (25%), while the greatest effect was detectable after 24 h (50%). As previously shown in blood after acute treatment (Fig. 2), no significant differences in hemolysate MDA content were observed in treated animals after continuous paraquat administration.

The results show that paraquat induced a time-dependent loss of glutathione in whole blood. This depletion is evident both after a single administration and after repeated treatment. The observation that levels of glutathione in quail significantly decreased after an administration of a free radical-generating agent suggests that glutathione could be used not only to detect exposure to electrophilic compounds as previously demonstrated (Galvani et al. 1998), but also to free radical-generating agents that cause oxidative stress. In order to investigate if the depletion of glutathione is due to an oxidative stress, it is necessary to measure also the activity of the GSPx. However, the effect of paraquat on this last parameter is noticeable only within a few hours after the administration, thus the parameter detects only a recent contamination.
Our data suggest that glutathione levels, GSPx, and lipid peroxidation are easily measurable by conventional biochemical methods in whole avian blood instead of tissues, and these parameters seem promising nondestructive biomarkers in the assessment of a chemical-induced stress in natural avian populations.

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