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# The effects of glutathione depletion on reproductive success in oysters, *Crassostrea virginica*

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## Abstract

Glutathione (GSH) is a ubiquitous tripeptide that functions as a very important modulator of cellular homeostasis, including detoxification of metals and oxyradicals. Therefore, depletion of GSH may predispose organisms to pollutant stress. Reproductively active oysters (*Crassostrea virginica*) were exposed to buthionine sulfoximine in the laboratory to deplete gonadal GSH. The effects of metal exposures (Cd and Cu) on fertilization and developmental assays were evaluated using gametes from control and GSH-depleted adults. Fertilization success was not affected by GSH status, i.e. the fertilization rates of gametes derived from GSH-depleted adults were the same or slightly higher. However, GSH depletion did increase the susceptibility of developing embryos to metal toxicity, i.e. adverse effects on embryonic development were observed at lower metal concentrations with gametes derived from GSH-depleted adults. These effects may be related to diminished removal of free radicals or increased availability of metals. Whereas sperm penetration of embryonic membranes and fertilization success may be facilitated by free radicals, the persistence of free radicals during subsequent developmental periods may adversely affect differentiation and normal development. GSH probably also plays an important role in scavenging toxic metals and reducing metal interactions with essential developmental processes. These results suggest that parental depletion of GSH may increase the susceptibility of embryos to metal toxicity. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Glutathione; Oysters; Reproduction; Metals

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Glutathione (GSH) is a ubiquitous tripeptide (L- $\gamma$ -glutamyl-L-cysteinyl-glycine) that is regarded as one of the most important nonprotein thiols in biological systems. GSH functions as a very important overall modulator of cellular homeostasis, and serves numerous essential functions including detoxification of metals and oxy-radicals (Mason & Jenkins, 1995; Meister & Anderson, 1983). There is evidence that GSH depletion is associated with adverse effects of metals in marine bivalves as well as in mammals (Prozialeck & Lamar, 1995; Regoli & Principato, 1995; Ringwood, Conners, Keppler & DiNovo, 1999). Organisms may also be more susceptible to additional stressors when GSH is depleted, so GSH status has been proposed as a potential risk factor (Jones, Brown & Sternberg, 1995).

Gonadal tissues of oysters and other aquatic invertebrates typically contain very high GSH concentrations relative to other tissues, particularly during peak reproductive periods (Conners, 1998). High concentrations of GSH in associated gametes probably play important roles in ameliorating oxidative damage and metal toxicity during fertilization and development. Therefore the purpose of these studies was to determine the potential effects of gonadal GSH depletion on the susceptibility of gametes to metal toxicity. It was hypothesized that gametes and embryos derived from GSH-depleted parents would be more susceptible to metal toxicity. Cadmium (Cd) and copper (Cu) were used for the metal exposures because they have commonly been used in the kinds of fertilization and development assays used for these studies, and because they are also known to interact with glutathione (Mason & Jenkins, 1995; Ringwood, 1992).

Adult oysters were collected from a pristine site, and maintained in aerated seawater (25‰ salinity) and fed for the laboratory experiments. A subset of oysters was used to determine gonadal GSH concentrations at the start of the experiment, and the remaining oysters were randomly divided into two groups. One group (i.e. GSH-depleted parents) was exposed for 48 h to buthionine sulfoximine (BSO, 20 mg/l), a non-toxic compound that is used to experimentally deplete GSH because it is a highly specific inhibitor of  $\gamma$ -glutamylcysteine synthetase; while the control group was not exposed to BSO. The oysters were then dissected and a piece of gonadal tissue from each ripe parent was frozen for GSH analyses; and their gametes were used to conduct fertilization and development assays as described previously (Ringwood, 1992) with both GSH-depleted and non-depleted parents. Since gonadal tissues of oysters are simple follicles filled with gametes, it should be realized that gonadal GSH levels of parents actually reflect the GSH levels of gametes.

For the fertilization assays, sperm (pooled from multiple males) were exposed to a range of Cd and Cu concentrations (five replicates per treatment) for 1 h, then eggs (pooled from multiple females) were added, incubated for 2 h and then evaluated for successful fertilization (based on  $\geq 200$  eggs per replicate). Eggs that were undergoing cleavage were scored as successfully fertilized, and the results were expressed as percent fertilization. This is a standard fertilization assay that primarily reflects potential effects on sperm, but since eggs are added to the assay tubes containing metal-spiked seawater, it also reflects effects on sperm–egg interaction processes. For the development assays, the remaining sperm and eggs were mixed to allow fertili-

zation, and then exposed to the Cd or Cu treatments for 48 h (five replicates, 200 embryos per replicate). By the end of the 48-h incubation period, normal embryos have typically progressed through the embryonic period to reach the veliger larval stage characterized by a D-shaped shell; whereas abnormal embryos do not develop a shell, develop an abnormal shell, or may arrest in earlier stages.

Gonadal GSH concentrations (total glutathione including GSH and GSSG, i.e. reduced and oxidized forms combined) of individual oysters were determined by the DTNB–GSSG reductase recycling assay (Ringwood et al., 1999). Gonadal tissues were homogenized in 10 volumes 5% sulfosalicylic acid (SSA), and centrifuged (14,000 rpm, 5 min, 4°C). The supernatant was diluted 1:1 with 5% SSA and mixed with the sodium phosphate buffer containing NADPH and dithio-bis-nitrobenzoic acid (DTNB). GSSG reductase was quickly added and the rate of thio-nitrobenzoic acid (TNB) formation was monitored at 412 nm over a 90-s interval. GSH concentrations were estimated from a standard curve and reported as nM GSH/g wet weight.

The mean gonadal GSH concentrations of adult oysters at the start of the experiment was  $1232 \pm 93$  nM/g wet weight (mean  $\pm$  standard deviation,  $n = 4$ ). After the 48-h period, gonadal GSH concentrations of BSO-exposed adults were reduced by almost 50% (GSH concentrations of control adults were  $1397 \pm 262$ ,  $n = 10$ ; GSH levels of BSO-exposed or GSH-depleted adults were  $753 \pm 264$ ,  $n = 10$ ). When the sperm from GSH-depleted adults were exposed to Cd or Cu, fertilization rates were not significantly different from treatments in which sperm were derived from control animals and exposed to the same metal concentrations (Fig. 1A, B). In fact, fertilization rates tended to be slightly higher with sperm from the GSH-depleted adults.

However, significant effects associated with GSH depletion were observed with the developmental assays. Cd caused significant decreases in normal development in a concentration-dependent manner. When gametes from GSH-depleted adults were exposed to Cd, the effects on development were much more severe (Fig. 1A). The  $EC_{50}$ s (estimated by extrapolation from linear regression models) suggest that embryos from GSH-depleted parents would be almost five times more sensitive to Cd than embryos from parents with normal GSH levels. Embryos tend to be very sensitive to Cu, so toxicity profiles tend to decline exponentially rather than linearly as was observed with Cd (Ringwood, 1992). There was a precipitous drop in normal development from 10 to 20  $\mu\text{g/l}$  Cu (i.e. from 98 to 38%) with embryos from control parents. When embryos from GSH-depleted parents were exposed to 20  $\mu\text{g/l}$  Cu, normal development was severely reduced to only 2% (Fig. 1B).

The formation of a fertilization envelope is generally believed to be mediated by a respiratory burst that produces  $\text{H}_2\text{O}_2$ . Antioxidants such as ovothiols, GSH, and catalase are essential to the amelioration of oxyradical damage during early developmental stages (Shapiro, 1991). GSH may be particularly important when metal ions and hydrogen peroxides interact to form superoxides (Holler & Hopkins, 1990). The studies described in this paper demonstrate that oyster gametes from GSH-depleted gonadal tissues are more sensitive to metal exposures than gametes from parents containing high concentrations of GSH (approximately 1.4 mM) more

typical of normal adults. In the present study, fertilization was not impaired and tended to be slightly higher for the BSO-depleted treatment, but adverse effects on development were observed following embryonic exposures. Whereas sperm penetration and fertilization success may be facilitated by free radicals, the persistence of free radicals during subsequent developmental periods may adversely affect differentiation and normal development. Since embryos are particularly sensitive to Cu, the effects of lower GSH levels were less obvious than those observed with Cd, but still significant. These results indicate that depletion of GSH can significantly increase the sensitivity of oyster embryos to metal toxicity. Gonadal glutathione concentrations can provide valuable information regarding potential risks to reproductive success.

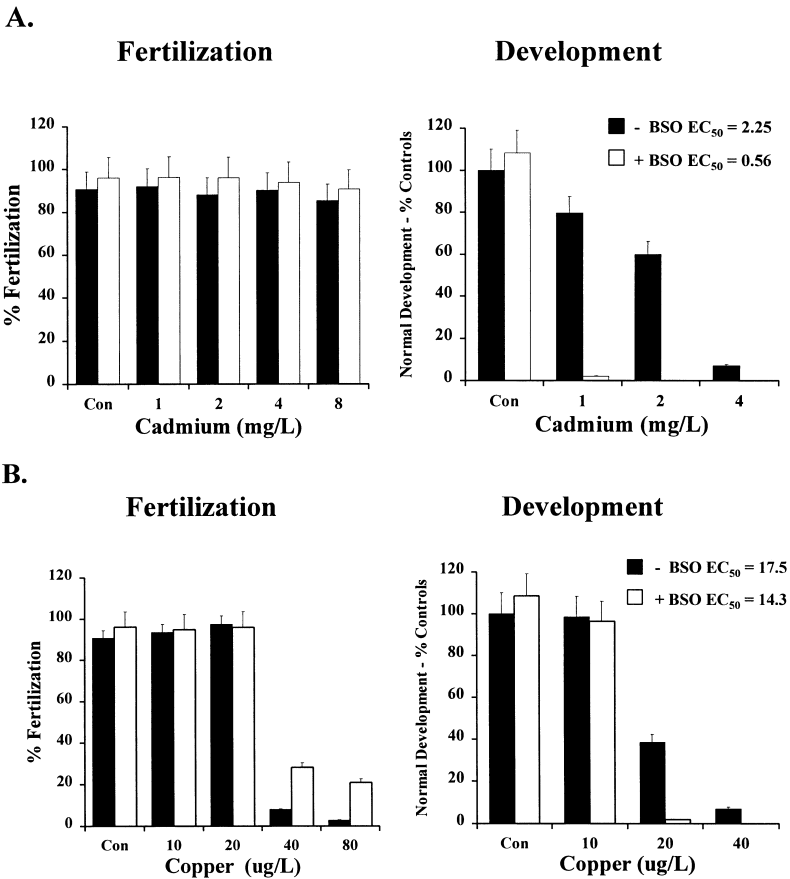


Fig. 1. The effects of cadmium (Cd) and copper (Cu) exposures on fertilization and development of gametes and embryos from glutathione (GSH)-depleted parents and non-GSH-depleted parents. Values are means + standard deviations of the five replicates. The concentrations of metals at which normal embryonic development was reduced by 50% (i.e. EC<sub>50</sub>) are also shown. Legend indicates parental treatments as follows: ■, -buthionine sulfoximine (BSO) (i.e. control, not GSH depleted); □, +BSO (i.e. GSH depleted).

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