DIFFERENTIAL GLUTATHIONE DEPLETION BY L-BUTHIONINE-*S*,*R*-SULFOXIMINE IN RAT EMBRYO VERSUS VISCERAL YOLK SAC *IN VIVO* AND *IN VITRO*

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Abstract—L-Buthionine-*S*,*R*-sulfoximine (L-BSO), a selective inhibitor of glutathione synthesis, exhibited the capacity to deplete embryonic and visceral yolk sac glutathione (GSH) after administration *in vivo* and also after addition of L-BSO to a whole embryo culture system. Administration of L-BSO to pregnant dams at 2.5 or 18 hr prior to the explantation of day 10 conceptuses resulted in greater than 55% GSH depletion relative to control values in both the yolk sac and the embryo proper. The levels of GSH returned to normal in all embryos and in the corresponding visceral yolk sacs pretreated at 2.5 hr (but not at 18 hr) after culturing for 24 hr in L-BSO-free medium. GSH content was significantly lower in yolk sacs but not in embryos of conceptuses cultured for 24 hr in medium containing 1 mM L-BSO. This treatment, however, significantly increased the amount of protein and DNA in the embryo. The differential sensitivity of the yolk sac versus embryo and the demonstration of the ability to modulate tissue levels of GSH during organogenesis promise to provide important new tools for the study of embryonic protective mechanisms in chemical teratogenesis.

The multifunctional roles of glutathione (GSH§) include the regulation of enzymatic activities, synthesis of DNA precursors, interorgan transport of amino acid sulfur, protection of cells against free radicals and lipid peroxidation, and the metabolic processing of drugs, other foreign chemicals and some endogenous substrates [1–3]. Many xenobiotics which are bioactivated to harmful intermediates utilize GSH in their detoxication pathways [4]. It is now recognized that chemical teratogens also undergo bioactivation in both maternal and embryonic tissues [5, 6]. The nature and severity of malformations vary with both the quantity and type of metabolites produced, suggesting that embryonic target tissues may be sensitive to teratogenic insult depending on the balance of activation and inactivation in those tissues [7]. Attempts have been made to characterize the monooxygenases involved in embryonic biotransformation but, currently, virtually nothing is known concerning the capacity of embryonic tissues to protect against the toxicity of reactive intermediates [8, 9]. Enzyme activities associated with the detoxication of xenobiotics are frequently very low or undetectable until late in gestation [10]. We have, therefore, utilized sensitive methods to determine the levels of GSH in cultured embryos and their associated visceral yolk sacs, to draw comparisons with levels measurable *in utero* and to attempt to modulate such levels to determine the

potential role of GSH as a protective or regulatory influence in chemical teratogenesis. In these investigations, the modulating influence of L-buthionine-*S*,*R*-sulfoximine (L-BSO), a selective inhibitor of glutathione synthesis, has been determined. The effects of L-BSO on rat embryos during organogenesis, *in vivo* and cultured *in vitro*, are presented.

MATERIALS AND METHODS

Animals. Primagravida Sprague–Dawley rats (Wistar-derived) were obtained on day 5 or 6 of gestation from Tyler Laboratories (Bellevue, WA). Animals were allowed free access to food and water and were housed under the conditions described previously [7]. The morning following copulation was designated as day 0 of pregnancy.

Dams were anesthetized with ether at 9:00 a.m. on day 10, and blood used for preparation of serum, a component of the culture medium, was collected from the abdominal aorta. Conceptuses were prepared for culture as previously described [11, 12]. Embryos and yolk sacs used for determination of GSH and GSSG were separated using forceps, placed in 0.5 ml of 0.1 M sodium phosphate, 5 mM EDTA buffer (pH 8.0, PHOS–EDTA), and immediately frozen at −70°. Two embryos and two corresponding yolk sacs were pooled separately for each determination.

Administration of L-BSO (Sigma Chemical Co., St. Louis, MO) was via a single intraperitoneal injection, 6 mmoles/kg L-BSO in saline, at 2.5 hr or 18 hr prior to killing the animals.

Embryo culture. Conceptuses were explanted and cultured using procedures described by New [11] as modified by Fantel *et al.* [12]. Details of this system

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§ Abbreviations: GSH, glutathione; L-BSO, L-buthionine-*S*,*R*-sulfoximine; GSSG, glutathione disulfide; HBSS, Hanks' balanced-salt solution; and PHOS–EDTA, buffer containing 0.1 M sodium phosphate, 5 mM ethylenediamine tetraacetic acid, pH 8.0.

have been published elsewhere [7]. Embryos had approximately 10 somites at the beginning of the culture period. For experiments *in vitro*, L-BSO dissolved in HBSS was added directly to the culture medium.

Assays and embryo assessment. Following the 24-hr culture period, conceptuses were placed in petri dishes containing HBSS and examined with a dissecting microscope. Conceptuses were regarded as nonviable in the absence of active vitelline circulation and were not evaluated further. Embryonic crown-rump length, somite count, and an index of forelimb development were recorded for each viable embryo. Embryos and yolk sacs were then placed individually in 0.5 ml PHOS–EDTA and frozen at −70°.

Thawed samples were ultrasonically disrupted and utilized immediately for the determination of GSH and GSSG. Assays were performed essentially as described by Hissn and Hilf [13]. Reaction mixtures for the determination of GSH contained 0.1 ml of tissue sample, 0.1 ml of 0.1 M formic acid, 1.0 ml PHOS–EDTA, and 0.1 ml of *O*-phthaldialdehyde (Sigma Chemical Co.) 1 mg/ml. Fluorescence was measured at 345 nm (excitation) and 425 nm (emission). Determinations of GSSG followed the same protocol except that 0.04 ml of 0.04 M *N*-ethylmaleimide (Sigma Chemical Co.) was incubated with the tissue sample for 30 min prior to the addition of other components, and 0.1 M NaOH was used in place of PHOS–EDTA. Amounts of GSH or GSSG as low as 30 pmoles could be determined accurately under these conditions.

Protein content was determined by the method of Bradford [14]. DNA content was measured fluorometrically as described by Labarca and Paigen [15].

Student's *t*-test was used to evaluate differences between sample means. Significance was based on a 95% confidence level (P < 0.05), and the numbers of determinations made in each case are indicated in the appropriate figures.

RESULTS

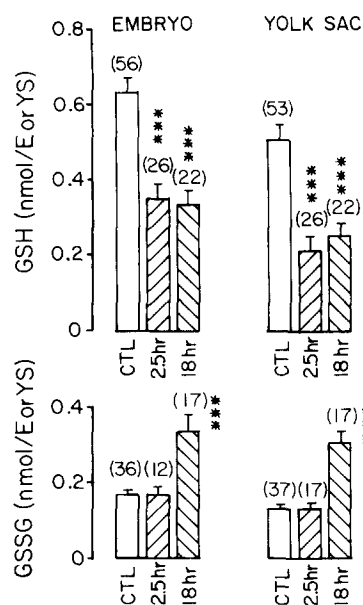Treatment of pregnant rats with L-BSO at either 2.5 or 18 hr prior to explanation resulted in significant

Fig. 1. GSH and GSSG content of day 10 rat embryos and yolk sacs from control dams (CTL) and from dams given a single i.p. dose of L-BSO (6 mmoles/kg in saline) at 2.5 hr or 18 hr prior to explantation. GSH and GSSG were determined as described in Materials and Methods. Error bars represent the mean standard error, and the number of embryos or yolk sacs included in the analysis is shown in parentheses. Comparisons significantly different from controls are designated with a triple asterisk (***), P < 0.001.

decreases in tissue levels of GSH in embryos and yolk sacs (Fig. 1) when examined at the time of explantation (morning of day 10). GSSG levels did not change in either tissue with respect to the 2.5-hr treatment but were elevated in the group treated with L-BSO 18 hr prior to explantation (Fig. 1). Protein content of embryos and yolk sacs was not altered significantly due to either treatment. DNA content, however, was increased by greater than

Table 1. Effects of L-buthionine-*S,R*-sulfoximine administered *in vivo* on levels of macromolecules, GSH and GSSG in day 10 rat embryos and yolk sacs

Treatment	Protein (μg/embryo or YS)	DNA (μg/embryo or YS)	GSH (nmoles/mg protein)	GSSG (nmoles/mg protein)
Embryo				
Control	29.6 ± 1.8 (47)*	3.0 ± 0.3 (17)	24.1 ± 1.2 (46)	5.7 ± 0.6 (33)
2.5 hr BSO†	26.1 ± 1.5 (22)	4.5 ± 0.4‡ (7)	15.7 ± 4.6‡ (21)	8.7 ± 2.9 (9)
18 hr BSO§	25.1 ± 3.4 (18)	4.3 ± 0.3‡ (17)	16.2 ± 2.2‡ (18)	24.0 ± 5.5‖ (17)
Visceral yolk sac				
Control	22.8 ± 1.4 (42)	1.9 ± 1.2 (18)	25.7 ± 1.5 (43)	6.5 ± 0.6 (31)
2.5 hr BSO	20.8 ± 1.5 (20)	3.2 ± 0.8 (7)	12.4 ± 3.2‖ (20)	7.6 ± 1.4 (18)
18 hr BSO	25.8 ± 3.9 (9)	3.1 ± 0.4¶ (17)	19.0 ± 4.3 (8)	15.4 ± 7.4‡ (5)

* Mean ± SEM (N).
† L-BSO was given to the pregnant dam as a single i.p. dose (6 mmoles/kg in saline) 2.5 hr prior to explanation of conceptuses (day 10 of gestation).
‡ P < 0.01.
§ L-BSO was given as in (†) except that dams were injected 18 hr prior to the explantation of day 10 conceptuses.
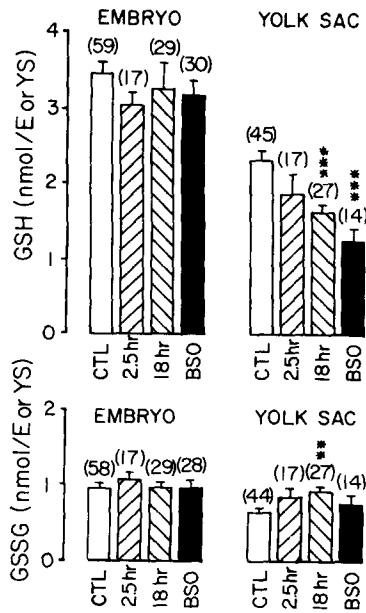‖ P < 0.001.
¶ P < 0.05.

Fig. 2. GSH and GSSG content in embryos and yolk sacs of day 11 rat conceptuses cultured for 24 hr in the whole embryo culture system. Control conceptuses (CTL) and those treated *in vivo* with L-BSO (6 mmoles/kg, i.p., in saline) for 2.5 hr or 18 hr prior to explantation were cultured for 24 hr in L-BSO-free culture medium as described in Materials and Methods. L-BSO was also added directly to the culture medium at a concentration of 1 mM (BSO) and cultured for 24 hr as indicated. Error bars represent the mean standard error, and the number of embryos or yolk sacs used in the analysis is indicated in parentheses. Comparisons significantly different from controls are designated with a double asterisk (**), $P < 0.01$ and a triple asterisk (***), $P < 0.001$.

30% in the embryos and yolk sacs of both treatment groups (Table 1). The gross morphology of both embryos and yolk sacs appeared normal for all conceptuses when examined with the dissecting micro-scope. Embryonic viability cannot be determined at this stage of development (day 10) by employing our usual criteria. The *in vivo* administration of L-BSO did not have any adverse effects on embryos, however, because cultured day 10 embryos from L-BSO-treated mothers did not differ from controls with respect to viability or other growth parameters.

Following a 24-hr culture period, the content of GSH and GSSG in embryos from cultured conceptuses treated *in utero* at either 2.5 or 18 hr prior to explanation was not significantly different from control values. The only significant changes in GSH and GSSG content were detected in yolk sacs from the 18-hr group in which GSH was decreased and levels of GSSG were slightly increased (Fig. 2). Protein and DNA content were identical to controls for both treatments (Table 2). Other indices of growth and development (crown-rump length, somite number and limb development index) were not affected significantly by treatment with L-BSO *in vivo* except for a slight but significant increase in the crown-rump measurement for the embryos of the group treated at 2.5 hr prior to explantation (data not shown).

When L-BSO (1 mM) was added to the medium at the onset of the culture period, significant decreases in GSH were seen after 24 hr in yolk sacs but not in embryos (Fig. 2). Levels of GSSG were not affected significantly by *in vitro* addition of L-BSO in any instance. Protein and DNA content were unchanged in the yolk sac but both increased significantly in the embryo (Table 2). Increases in protein content exceeded 20% in most instances. Viability, crown-rump length, somite number and limb development did not change significantly following additions of L-BSO to the culture medium. No measurable increases in toxicity or gross malformations were observed in cultured embryos with L-BSO concentrations as high as 5 mM. Increasing L-BSO concentrations did not, however, result in any further reductions in GSH content below the levels observed at concentrations of 1 mM.

Table 2. Effects of L-buthionine-S,R-sulfoximine on macromolecule, GSH and GSSG levels in day 11 embryos and yolk sacs cultured for 24 hr when L-BSO was administered *in vivo* or added directly to the culture medium

Treatment	Protein (μg/embryo or YS)	DNA (μg/embryo or YS)	GSH (nmoles/mg protein)	GSSG (nmoles/mg protein)
Embryo				
Control	180.0 ± 6.3 (60)*	14.9 ± 0.6 (52)	20.1 ± 0.5 (59)	5.8 ± 0.4 (58)
1 mM BSO†	229.1 ± 10.2‡ (30)	17.4 ± 0.8§ (26)	13.4 ± 0.6‡ (30)	4.6 ± 0.5 (28)
2.5 hr BSO‖	170.5 ± 9.4 (17)	14.0 ± 1.5 (17)	18.5 ± 1.5 (17)	6.5 ± 0.4 (17)
18 hr BSO‖	164.8 ± 7.7 (29)	13.7 ± 0.5 (29)	19.8 ± 1.4 (29)	6.0 ± 0.2 (29)
Visceral yolk sac				
Control	81.2 ± 3.7 (46)	5.4 ± 0.3 (39)	30.4 ± 1.4 (44)	8.3 ± 0.8 (43)
1 mM BSO	86.8 ± 6.6 (18)	5.3 ± 0.3 (14)	14.4 ± 1.6‡ (15)	8.8 ± 1.2 (14)
2.5 hr BSO	77.6 ± 3.7 (17)	5.3 ± 0.4 (17)	25.3 ± 3.6 (17)	10.8 ± 1.1 (17)
18 hr BSO	78.3 ± 2.8 (28)	4.9 ± 0.2 (27)	22.5 ± 2.5 (27)	11.0 ± 0.6 (27)

* Mean ± SEM (N).

† Embryos were cultured for 24 hr as described in Materials and Methods. BSO (1 mM) dissolved in HBSS was added to the culture medium at the start of the culture period.

‡ $P < 0.001$.

§ $P < 0.05$.

‖ Embryos were treated *in vivo* with BSO as described in Table 1 († and §). Conceptuses were then cultured for 24 hr in L-BSO-free culture medium.

DISCUSSION

Rat conceptuses were shown to contain significant amounts of GSH, with quantities on day 10 ranging from a total of 0.5 nmole per embryo or yolk sac, to as high as 3.4 and 2.3 nmoles, respectively, in embryos and yolk sacs from conceptuses that had been cultured for 24 hr (Table 2). When calculated on a per mg protein basis, GSH concentrations in embryos were comparable to those reported for the adult liver [16], with levels in the yolk sac somewhat higher. L-BSO treatment *in vivo* or *in vitro* produced no detectable adverse effects on the developing conceptus at any stage examined. Because higher concentrations of L-BSO (up to 5 mM) did not further deplete GSH in the embryo or yolk sac, we feel that the inhibition of GSH synthesis is maximal at 1 mM. The turnover of GSH is essential for its depletion following L-BSO treatment [17], and the failure of higher concentrations to further reduce levels (to less than 45% of controls) may indicate a relatively low turnover of GSH when compared to other tissues such as the adult rat kidney [17, 18]. The times chosen for *in vivo* administration of L-BSO were based on reports that show a maximal depletion of GSH after 2–3 hr in mouse liver and kidney and a recovery of enzyme activity following 18–20 hr [19, 20]. The absence of a return of GSH levels to control values in the 18-hr treatment group indicated a possible inherent inability of the embryo to resynthesize new enzyme at this stage of development. Treatment of pregnant rats with L-BSO at either 2.5 hr or 18 hr prior to explantation resulted in decreases in maternal hepatic GSH content to roughly the same levels measured in conceptuses when expressed on a per mg protein basis. It must be emphasized, however, that no attempt has yet been made to evaluate GSH depletion in different embryonic tissues. Therefore, the possibility exists that some cell types may be undergoing rapid, selective depletion of GSH. This type of selectivity may be related to the metabolism and disposition of L-BSO itself or to factors that regulate the turnover of GSH once inhibition of synthesis has occurred. The export of GSH from the cell has been implicated as a major factor in regulating the degree of GSH depletion achievable with L-BSO [21]. The yolk sac appeared much more sensitive to the depleting effects of L-BSO although it is not yet known whether this is due to a proximity effect or whether the enzymatic pathways controlling intrinsic GSH turnover and synthesis actually vary between embryonic and yolk sac tissues. To date, the role of the visceral yolk sac in chemical teratogenesis has received little attention, yet may be extremely important due to its physical vulnerability and its role in embryonic nutrition and protection.

When expressed as GSH/embryo, GSH depletion in embryonic tissues was not significant after direct additions of L-BSO to the medium, but became so when calculated on a per mg protein basis (Table 2). This was due primarily to the concomitant increase in total protein in those embryos. The reasons for significant increases in embryonic protein and DNA are not clear, but it is known that altered GSH and GSSG levels can influence rates of protein and DNA synthesis in other tissues [2].

In cultured conceptuses, GSSG levels were affected significantly only on day 10 after treatment *in utero* with L-BSO at 18 hr prior to explantation (Tables 1 and 2). No direct effects of L-BSO on the NADPH-dependent GSSG reductase (the enzyme primarily responsible for the maintenance of low GSSG levels) are known, and it has not yet been determined whether other mechanisms could contribute to this observation. While useful for the rapid measurement of large numbers of embryos and yolk sacs, the method of GSSG quantitation used in this study lacks specificity in the presence of large amounts of GSH [13, 18]. Critics of this method have pointed out that GSSG values are higher than those measured using other techniques but have also shown that these increases are not due to oxidation of GSH during sample preparation [22]. More specific and sensitive assay procedures will be necessary to determine the exact levels and functions of GSSG in the embryo and yolk sac.

The ability to regulate levels of GSH in embryos and yolk sacs *in vivo* and *in vitro* constitutes a potentially important tool in investigations into mechanisms of chemical teratogenesis. It also enables us to study the detoxication potential of GSH during development and its subsequent interactions with chemical teratogens. Advantages of this system include the ability to manipulate GSH levels in the absence of maternal influences and without apparent toxicity to other growth or regulatory pathways during the teratogen-sensitive period of organogenesis.

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