TOXICOLOGICAL SCIENCES **62,** 92–102 (2001)

Spatial Glutathione and Cysteine Distribution and Chemical Modulation in the Early Organogenesis-Stage Rat Conceptus *in Utero*

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Received November 17, 2000; accepted March 14, 2001

Glutathione (GSH), cysteine, and other low-molecular-weight thiols (LMWT) play a vital role in the detoxication of xenobiotics and endogenous chemicals. Differential alterations of LMWT status in various cell types of the developing embryo may underlie cell-specific sensitivity or resistance to xenobiotics and contribute to embryotoxicity. This study describes the spatial and temporal distribution of LMWTs in rat conceptuses and alterations produced by the non-teratogenic GSH modulator, acetaminophen (APAP). Pregnant female rats were given 125, 250, or 500 mg/kg APAP (po) on gestational day 9. Conceptal LMWT was localized histochemically using mercury orange in cryosections, and GSH and cysteine concentrations were measured by HPLC analysis. Mercury orange histofluorescence revealed a non-uniform distribution of LMWT in untreated conceptal tissues, with strongest staining observed in the ectoplacental cone (EPC), visceral yolk sac (VYS), and embryonic heart. Less intense staining was observed in the neuroepithelium. Following treatment with APAP, tissue-associated LMWT decreased dramatically except in the EPC, while exocoelomic fluid LMWT, and LMWT within embryonic lumens, increased. Exposure to 250 mg/kg APAP decreased embryonic GSH after 6 and 24 h by 46% and 38%, respectively. Acetaminophen (500 mg/kg) decreased embryonic and VYS cysteine content by 54% and 83%, respectively, after 24 h. Acetaminophen alters the spatial distribution of LMWT in rat conceptuses, particularly with respect to cysteine. The mobilization of cysteine following chemical insult may influence the ability of conceptal cells to maintain normal GSH status due to reduced availability of cysteine for *de novo* GSH synthesis.

Key Words: **glutathione; cysteine; embryo; VYS; acetaminophen; mercury orange; *in utero*.**

Reduced glutathione (GSH) is the most abundant low-molecular-weight thiol (LMWT) found in nearly all adult cell types (Kosower and Kosower, 1978), although it may represent only one of a number of important thiols in developing embryos (Deneke, 2000). Reduced glutathione and glutathione disulfide (GSSG), the oxidized form of the thiol, form one of the most important redox couples in living cells. Under normal conditions, the ratio of GSH:GSSG is approximately 14 and 40 in the adult rat liver and kidney, respectively, indicating highly reducing environments (Tanimura and Shepard, 1970). Thus, changes in cellular GSH content may have profound effects on the GSH:GSSG ratio and will dramatically impact cellular redox status. Studies have shown that elevation of GSSG causes the inhibition of several metabolic enzymes, likely through the formation of protein-glutathione mixed disulfides (Benard and Balasubramanian, 1995) and oxidation of other cellular components (Benard and Balasubramanian, 1993; Meister, 1991). The redox couple is the predominant factor in establishing and maintaining cellular redox potential, which, in turn, regulates the activity of important enzymes, protein folding and conformation, membrane transport, receptor dynamics, and control of transcription activation, proliferation, and differentiation (Arrigo, 1999; Cotgreaves and Gerdes, 1998; Kosower and Kosower, 1978; Takahashi and Zeydel, 1982). Reduced glutathione also plays an important protective role in xenobiotic metabolism. Free radicals are detoxified either through direct interaction with GSH or enzymatic intervention via GSH peroxidase, producing GSSG. Glutathione can also remove reactive intermediates and electrophiles through covalent adduct formation (Ketterer *et al.*, 1983; Kosower and Kosower, 1978). Since GSH plays an important role in detoxication of reactive electrophiles, intracellular GSH content must be strictly maintained by several enzymatic processes including new GSH synthesis.

Glutathione is synthesized in a 2-step, ATP-dependent process catalyzed by γ-glutamyl cysteine synthetase (γ-GCS) and GSH synthase. Cellular GSH content is dependent on negative feedback control of γ-GCS by GSH and on the availability of cysteine. Since high intracellular cysteine concentrations are toxic to both mature and immature organisms, cysteine flux is tightly regulated (Meister, 1988; Richman and Meister, 1975). The toxicity of cystine may be related to its uptake and subsequent reduction to cysteine (Meister, 1991). Cystine was shown to enhance the toxicity of methylmercury in developing

Portions of this work were presented at the 36th annual meeting of the Society of Toxicology and the 38th Annual Meeting of the Teratology Society.

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blastocysts (Spindle and Matsumoto, 1988). The ratio of GSH: cysteine in the adult liver ranges from 30–35 (Neuschwander-Tetri and Rozin, 1996), while the *in utero* embryonic and visceral yolk sac (VYS) ratios of GSH:cysteine have not yet been determined. Studies suggest that perturbation of cysteine or cystine transport has a negative influence on GSH content and cell biochemistry. Inhibition of cystine uptake results in decreased GSH content in cultured fetal brain cells (Sagara *et al.*, 1993). Furthermore, some GSH modulators stimulate cysteine or cystine transport in favor of GSH synthesis (Bannai *et al.*, 1989; Pacitti *et al.*, 1993).

Although the effects of GSH and other LMWT depletion have been characterized in many adult tissues, the time- and dose-dependent relationships between GSH depletion and manifestation of embryotoxicity remain unclear. Slott and Hales (1987) demonstrated a dose-dependent decrease in cultured embryonic growth parameters 45 h after GSH depletion using the GSH synthesis inhibitor L-buthionine-S,R-sulfoximine (BSO). Hales and Brown (1991) showed that *in utero* GSH depletion produced a significant increase in malformations. Similarly, the embryolethality of phenytoin increased in a dose-dependent fashion after inhibition of GSSG reductase, which reduces GSSG to GSH, following administration of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on the morning of gestational day (GD) 12 (Wong and Wells, 1989). Chemical oxidation of GSH *in vitro* using diamide also resulted in embryotoxicity, affecting axial rotation and viability (Hiranruengchok and Harris, 1993; 1995). Thus, it is clear that GSH plays an important protective role in embryonic development, and disruption of cellular redox status resulting from perturbation of GSH content may play a critical role in embryotoxicity (reviewed in Wells and Winn, 1996). Furthermore, the most critical changes in GSH and other LMWT concentrations may occur selectively in a few discrete tissues or cells in the absence of major changes in the tissue environment as a whole. Adult studies indicate that GSH is spatially distributed in the tissue of some organs. For example, hepatic GSH is located primarily within hepatocytes and is largely absent from surrounding interstitial tissue and vessels (Chieco and Boor, 1983). Moreover, periportal hepatocytes have higher concentrations of GSH than do pericentral hepatocytes. Glutathione in the brain is primarily localized to the neuropil and white matter tracts (Philbert *et al.*, 1991). Clearly, whole-tissue GSH measurements would be unable to detect such heterogeneity, and since many developmental studies attempt to describe biochemical effects in whole embryonic preparations, tissue- and cell-specific alterations in GSH status may be overlooked. Therefore, this study attempts to evaluate changes in LMWT distribution in discrete embryonic cell populations and to correlate these changes with more traditional approaches to quantify GSH and cysteine concentrations.

Acetaminophen (APAP), a known GSH modulator, produces adult liver and renal toxicity (Jollow *et al.*, 1973; Ross *et al.*, 1989). A widely used analgesic, APAP does not cause embryotoxicity when administered *in utero* (McElhatton *et al.*, 1997; Shepard, 1989). Relatively high doses of APAP produce embryotoxicity and dysmorphogenesis in rat conceptuses in whole embryo culture, as evidenced by an increased incidence of open neural tubes (Harris *et al.*, 1989; Stark *et al.*, 1989a,b). Weeks *et al.* (1990) also demonstrated that *in vitro* exposure to 300 μM APAP results in decreased embryonic growth parameters and GSH depletion in VYSs. The difference in effects between *in utero* and *in vitro* systems was believed to result from greater direct exposure of the conceptus to APAP *in vitro*, while conceptuses *in utero* are protected by the presence of maternal biotransformation and detoxication systems. However, initial studies conducted in this laboratory have shown that *in vivo* exposure to APAP alters LMWT content in the intact conceptus without concomitant dysmorphogenesis, suggesting that APAP or one of its metabolites reaches the conceptus *in utero* in significant concentrations (Harris, unpublished observations). The lack of APAP teratogenicity suggests that GSH depletion *per se* is not embryotoxic but that toxicity may be mediated through the conceptal ability to rapidly respond and adapt to APAP-induced GSH and cysteine depletion by restoration of normal redox status.

Acetaminophen-induced GSH depletion is believed to result from different metabolic pathways in the adult and conceptus. In the adult liver, APAP can be metabolized to the reactive N-acetyl-*p*-benzoquinone imine (NAPQI) primarily via cytochromes P450 2E1 and 3A (Kostrubsky *et al.*, 1997; Lee *et al.*, 1996), although the inducible cytochrome P450 1A1 may also play a limited role in some instances. Redox cycling of NAPQI causes an initial increase in H_2O_2 resulting in GSSG formation and oxidation of NADPH (Lores-Arnaiz *et al.*, 1995). The reactive metabolite also covalently binds hepatic proteins and GSH causing a decrease in total cellular GSH content (Jollow *et al.*, 1973; Potter *et al.*, 1974). In developing conceptuses, however, APAP is metabolized to form 3-hydroxy-acetaminophen (3-OH-APAP; Harris *et al.*, 1989) by a number of peroxidases such as prostaglandin-H-synthase (Moldeus *et al.*, 1982), which has been shown to play a critical role in the bioactivation and subsequent embryotoxicity of chemicals such as phenytoin and benzo[a]pyrene (Winn and Wells, 1997). The NAPQI metabolite of APAP was shown to be more embryolethal than 3-OH-APAP in whole embryo culture. However, the incidence of open anterior neuropore was significantly decreased when embryos were exposed to NAPQI or the hepatic enzymes required to produce NAPQI than when exposed to 3-OH-APAP or the enzymes required to produce this catechol (Harris *et al.*, 1989). These data suggest that the proximate dysmorphogen in APAP toxicity is 3-OH-APAP, and that conceptuses do not metabolize APAP to form the NAPQI metabolite (Harris *et al.*, 1989; Stark *et al.*, 1990). Further oxidation of 3-OH-APAP yields an *o*-quinone that can form covalent adducts with GSH (Rashed and Nelson, 1989) or redox cycle to produce GSSG (Stark *et al.*, 1990).

Glutathione is responsible for detoxication of the reactive

o-quinone metabolite of 3-OH-APAP from embryonic tissues, and *in vitro* exposure to APAP has been shown to modulate GSH content in embryos. Therefore, APAP will be used as a tool to model spatial and temporal changes in GSH and cysteine in conceptal tissues and cells. It will then be possible to begin to draw a correlation between patterns of GSH and cysteine depletion and restoration and expected developmental outcomes. This study will also help to establish a model technique, mercury orange histofluorescence, for the evaluation of conceptal GSH and cysteine fluxes in response to chemicals, in an attempt to determine the exact role of reduced thiols and, by extension, redox status in embryonic development.

MATERIALS AND METHODS

Chemicals. Sodium methanesulfonate was obtained from Aldrich Chemical (St. Louis, MO). Monobromobimane (MBBr) was purchased from Calbiochem-Novabiochem (La Jolla, CA). HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA). Methanesulfonic acid (MSA), diethylenetriaminepentaacetic acid (DTPA), N-(2-hydroxyethyl)piperazine-N′-3-propanesulfonic acid (HEPPS), mercury orange, APAP, glutathione, and L-cysteine were obtained from Sigma Chemical (St. Louis, MO). The embedding medium OCT was purchased from Ted Pella, Inc. (Redding, CA).

Animals. Time-mated, primigravida Sprague-Dawley rats were obtained on gestational days (GD) 5–8 from the P-30 Small Animal Core Facility of the Reproductive Sciences Program at the University of Michigan. The morning following detection of a sperm-positive vaginal smear was designated GD 0. Pregnant dams were housed with a 12-h light/12-h dark cycle and allowed free access to food and water prior to and during *in utero* studies.

In utero. On the morning of GD 9 at approximately 7 A.M., dams were given an oral dose of 125, 250, or 500 mg/kg APAP suspended in 0.9% saline. Doses were selected based on initial dose-response studies, which demonstrated that 500 mg/kg APAP was the highest concentration to produce GSH depletion in conceptuses without causing overt clinical signs of maternal toxicity. Dams were anesthetized with ether and uteri removed 0, 6, 12, 24, or 48 h later. Implantation sites were dissected free of uteri and placed in warmed Hank's balanced salt solution for further manipulation. Embryonic growth parameters were measured on embryos taken at the 48-h time point. Parameters measured included crown-rump length, somite number, positive flexure/rotation, neural tube closure, and visual determination of heartbeat and yolk sac circulation.

Conceptuses within decidual capsules from the 125- and 500-mg/kg APAP dose groups were taken 24 or 48 h after exposure. Samples were rinsed in 20% sucrose in phosphate buffer, placed in molds containing OCT embedding medium, and snap-frozen in isopentane chilled to –70°C. Tissue blocks were stored at –75°C until sectioned.

Samples of maternal liver, decidual mass, ectoplacental cone (EPC), embryo, and VYS were placed in 200 mM MSA, which prevented artifactual oxidation of reduced thiols, for HPLC determination of GSH and cysteine content.

Mercury orange histochemistry. The regional and cellular distribution of LMWT was determined in decidual capsules with conceptuses as described by Philbert *et al.* (1991). Tissue blocks were equilibrated at –19°C for 30 min prior to sectioning. Blocks were sectioned using a Shandon Lipshaw Cryotome E Cryostat. Fresh-frozen sections (10 μm) were placed on poly-L-lysine-coated Fisher Superfrost slides and immediately immersed in an ice-cold solution of 25 μM mercury orange in toluene for 5 min. Slides were rinsed in 2 changes of ice-cold dry absolute alcohol, cleared in toluene, and coverslipped with Permount.

Slides were stored at 4°C prior to inspection on an Olympus AX70 microscope fitted with epifluorescence optics. Embryos were viewed using BG12 excitation and CB12 band-pass filters. Orange fluorescence was determined to be LMWT-induced histofluorescence, while background autofluorescence appeared green. For each time point and dose group, at least 5 conceptuses from at least 2 different litters were examined.

HPLC determination of GSH and cysteine content. Reduced glutathione and cysteine concentrations were determined using the method of Fahey and Newton (1987) as modified by Harris *et al.* (1991). Samples of maternal liver (~10 mg tissue wet weight/ml), decidual mass, EPC, embryo, and VYS were placed in microcentrifuge tubes containing 200 mM MSA. Samples were frozen in liquid nitrogen and stored at –75°C until prepared for HPLC analysis.

Thawed samples were sonicated, and aliquots were added to an equal volume of 4 M sodium methanesulfonate prior to centrifugation at 13,000 rpm for 10 min. Supernatants were buffered with 1 M HEPPS with 5 mM DTPA (pH 8.5). Samples were derivatized with 0.2 mM MBBr in the dark for 20 min. The reaction was quenched with 400 mM MSA to acidify samples prior to storage at –75°C. HPLC analysis was conducted on samples as described below.

Briefly, HPLC analyses were carried out using a Novapak® C_{18} 4-μm Radial-Pak cartridge preceded by a Novapak Guard-Pak guard column (Waters, Millipore Corporation, Milford, MA). Glutathione and cysteine were resolved with an isocratic mobile phase consisting of 14.2% methanol (v/v) and 2.5% glacial acetic acid in water (pH 3.4) at a flow rate of 1.0 ml/min. After each run, the column was washed with mobile phase consisting of 90% methanol and 2.5% glacial acetic acid in water (pH 3.4) to re-equilibrate. Detection of bimane-conjugated products was achieved using a Waters Model 470 scanning fluorescence detector (Ex, Em: 360, 455 nm) followed by peak recording and analysis using a Waters Model 746 data module. Identification and quantitation of thiol peaks was accomplished using glutathione and cysteine standards. For all GSH and cysteine measurements, values were normalized per embryo, VYS, or EPC.

Determination of protein content. Protein concentrations of pellets resuspended in 0.25 M NaOH were determined according to the method of Bradford (1976) adapted for use on a multi-well plate reader as directed by the manufacturer (Bio-Rad Laboratories, Inc., Cambridge, MA). Bovine serum albumin was used as a standard. BIORAD reagent was added to samples and standards, and protein content was determined using a V-max kinetic microplate reader and Softmax Version 2.01 software (Molecular Devices Corp., Sunnyvale, CA).

Statistical analysis. Data were analyzed using the SigmaStat package from Jandel Scientific Software. One-way ANOVA, with *post hoc* Tukey's Test, was used to determine significant differences. A level of $p < 0.05$ was considered statistically significant.

RESULTS

A single oral dose of APAP was given to dams at approximately 7 A.M. on the morning of GD 9, and implantation sites were removed from uteri 0, 6, 12, 24, or 48 h later. Viability and growth parameters were measured in embryos explanted on the morning of GD 11 (48-h time point). Viability, as measured by visual inspection of yolk-sac circulation and presence of a heartbeat, was unchanged with exposure to either 250 or 500 mg/kg APAP. All embryos showed normal axial rotation, complete closure of the neural tube, and no significant differences in crown-rump length or somite number compared to control (data not shown).

To determine the spatial distribution of LMWT within specific tissue and cellular sites, gravid rats were dosed with 125 or 500 mg/kg APAP at approximately 7 A.M. on the morning of

GD 9. Dams were killed on the morning of GD 10 (24 h) or GD 11 (48 h), and reduced thiol was localized by mercury orange staining of frozen sections. Orange histofluorescence showed the presence of LMWT, and green tissue autofluorescence indicated low thiol levels. Figure 1a shows a representative control GD 10 embryo exposed to saline *in utero*. Decidual masses exhibited strong fluorescence, with the EPC displaying the strongest intensity (Fig. 1c). The EPC is a concentration of giant trophoblast cells that migrate to the center of the antimesometrial pole of the uterine decidual mass to surround the conceptus, eventually forming the placenta (Babiarz *et al.*, 1992; Shukla *et al.*, 1991). Strong orange histofluorescence was detected in the VYS and some tissue regions of the embryo proper. Fluorescence in the neuroepithelium appeared to be more highly localized to the external limiting membrane where the nuclei of neuroepithelial cells in interphase are located (Fig. 1a). The embryonic heart displayed strong fluorescence throughout, whereas mesenchymal tissues exhibited a greater distribution of LMWT toward the cranial end of the embryo.

The pattern of mercury orange fluorescence in the GD 10 conceptus was dramatically altered by exposure to 500 mg/kg APAP on GD 9 (Fig. 1b). Decidual mass LMWT was severely depleted in all regions except the EPC, which continued to stain intensely (Fig. 1d). Embryonic and VYS tissues showed no mercury orange-detectable reduced thiol.

As in control GD 10 conceptuses, LMWT fluorescence showed a heterogeneous pattern of distribution throughout tissues of the control GD 11 conceptus (Fig. 1e). The decidual mass displayed strong histofluorescence, with the greatest intensity localized to the EPC. Cells of the VYS also showed intense mercury orange fluorescence. In the embryo, the branchial arches exhibited the strongest intensity of mercury orange fluorescence, while the neuroepithelium, heart, and mesenchyme displayed a lower but variable intensity of fluorescence for LMWT. Unlike control GD 10 conceptuses, reduced thiol was also observed in the exocoelomic fluid as well as the lumens of the neural tube and heart.

Forty-eight hours after exposure to 125 mg/kg APAP, tissues demonstrated a marked reduction in intracellular LMWT as shown in Figure 1f. Decidual mass LMWT was primarily localized to cells bordering the VYS, which exhibited very little reduced thiol. Neuroepithelial cells showed almost no LMWT, and the heart exhibited a dramatic decrease in histofluorescence. Mesenchymal tissues, including the branchial arches and gut epithelium, continued to display a pattern of mercury orange fluorescence similar to control. However, most remaining LMWT was observed in the exocoelomic fluid and the lumens of the neural tube, heart, and primitive gut. Forty-eight h after administration of 500 mg/kg APAP, severe depletion of LMWT was observed. The only tissue that displayed appreciable fluorescence was the EPC, which was replete with reduced thiol. Persistent orange fluorescence was primarily localized to the exocoelomic fluid.

Within a given tissue, cell-specific distribution of LMWT was observed. The cells of the basal plate in the neuroepithelium routinely stained more intensely than those of the alar plate (Fig. 1g). Within the basal plate, a heterogeneous pattern of histofluorescence was noted, wherein groups of intensely staining cells were interspersed with single cells devoid of LMWT. The opposite pattern was seen in the alar plate, where single cells with intense orange histofluorescence were seen in regions otherwise devoid of histofluorescence. Treatment with 125 mg/kg APAP eliminated most spatial differences in mercury orange fluorescence. Acetaminophen exposure resulted in a redistribution of most LMWT to extracellular spaces, yet tissues continued to display punctate foci of mercury orange fluorescence. These foci were too small to be correlated with entire cells and may instead represent an as yet unidentified subcellular pool of thiol such as that found in the mitochondria or nucleus (arrow, Fig. 1h).

Tissue concentrations of GSH were quantified using the HPLC procedure. Glutathione concentrations in control maternal liver samples fluctuated in a diurnal pattern (Fig. 2a). The concentration of GSH in controls peaked at 1:00 P.M. (6 h; 28.4 pmol/μg protein) and was lowest at 7:00 P.M. (12 h; 14.5 pmol/μg protein). No statistically significant changes in maternal liver GSH content were seen following exposure to 250 or 500 mg/kg APAP. Alternatively, GSH depletion was detected in other maternally derived tissues (decidual mass, ectoplacental cone) following exposure to APAP (Table 1). Control decidual mass GSH concentrations increased rapidly 6 h after onset of the study period but remained relatively constant thereafter. Exposure to 500 mg/kg APAP caused a significant depletion of decidual mass GSH content to 83% of control content after 6 h. After 12 h, GSH content was significantly decreased to 78% and 83% of control concentrations with exposure to 250 or 500 mg/kg APAP, respectively. Glutathione content in the decidual mass returned to control levels after 24 h. Ectoplacental cone GSH content increased 86-fold over the 48-h time period. Ectoplacental cone GSH content was significantly decreased to 67% of control levels 6 h after exposure to 250 mg/kg APAP. However, 48 h after exposure to 250 mg/kg APAP, the concentration of GSH in the EPC was significantly increased to 163% of control concentrations.

Glutathione depletion was observed in conceptal tissues following *in utero* exposure to APAP. As shown in Figures 3a and 3b, there were dramatic developmental increases in GSH content in control and conceptuses over the 48-h period. Control embryonic GSH content increased 9-fold from GD 9 to GD 10 (67 pmol and 567 pmol, respectively) and 6-fold from GD 10 to GD 11 (3255 pmol; Fig. 3a). Treatment with 250 mg/kg APAP attenuated this developmental increase, with embryonic GSH content being decreased to 54% and 62% of control content at 6 and 24 h, respectively. However, after 48 h, embryos exposed to 250 mg/kg APAP showed a rebound increase to 146% of control levels. Visceral yolk sacs also showed dramatic developmental increases over the time studied, with a 9-fold increase over the first 24-h period (60 pmol

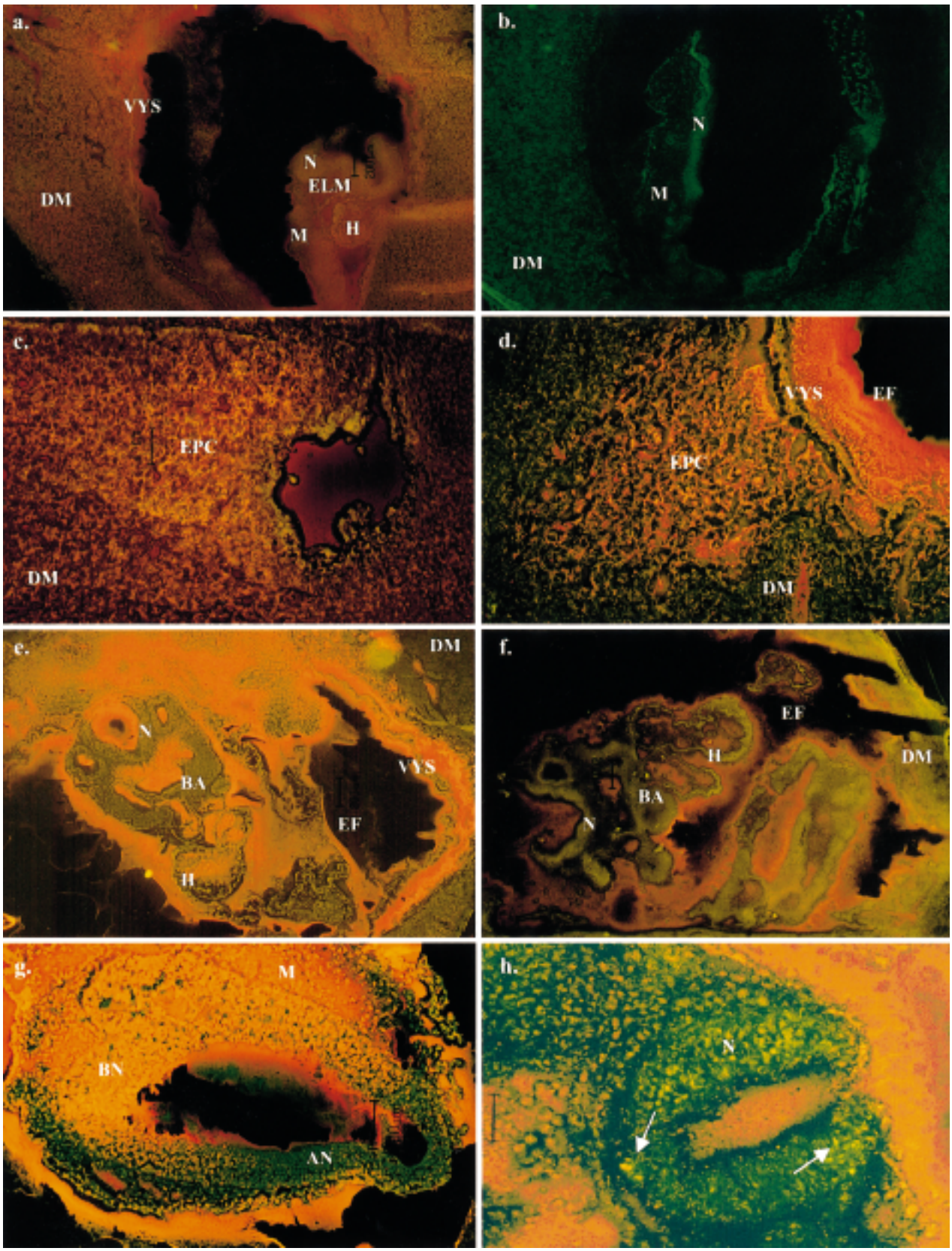

FIG. 1. Fresh-frozen sections (10 μm) of intact decidual mass with conceptus labeled with mercury orange to detect the presence of reduced LMWT. Orange histofluorescence indicates the presence of reduced thiol while green coloration was tissue autofluorescence. (a) Control GD 10 conceptus. (b) High dose GD

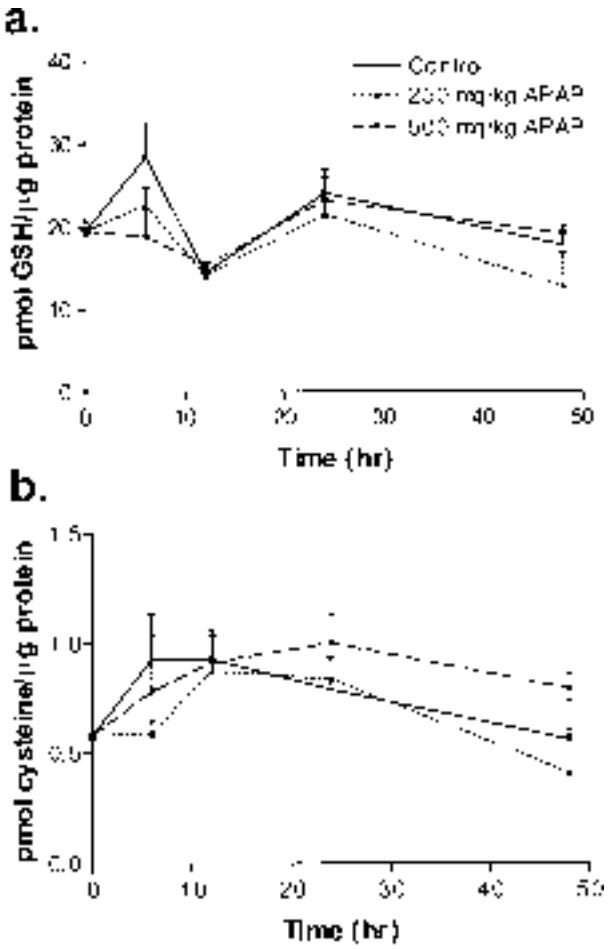

FIG. 2. Maternal liver (a) GSH and (b) cysteine concentrations following *in utero* administration of 250 or 500 mg/kg APAP on the morning of GD 9. Data are expressed as pmol GSH or cysteine/μg protein, and each point represents the mean + standard error of at least 2 samples. Asterisks indicate significant difference from equivalently timed controls ($p \leq 0.05$).

at 0 h and 561 pmol at 24 h) and a 5-fold increase over the second 24 h (2777 pmol at 48 h; Fig. 3b). Treatment with 500 mg/kg APAP caused a further increase in VYS glutathione content to 151% of control concentrations at 6 h; the increase was not significantly different from controls.

Cysteine concentrations were also measured using the HPLC procedure. Control maternal liver cysteine content initially increased at 6 h but returned to basal values at the end of 48 h (Fig. 2b). Following exposure to APAP (250 or 500 mg/kg), there were no statistically significant changes in maternal liver cysteine content at the time points chosen. Alternatively, the decidual mass and EPC showed significant decreases in cysteine content as shown in Table 1. The concentration of cysteine in the control decidual mass rose steadily through 24 h but showed a sharp decline at 48 h. Twelve h after exposure to 250 or 500 mg/kg APAP, decidual mass cysteine content was significantly depleted to 73% and 71% of control content, respectively. Acetaminophen (500 mg/kg) caused a further decrease in decidual mass cysteine concentration to 52% of control levels at 24 h, but cysteine content rebounded to 133% of control at 48 h. Control cysteine concentrations in the EPC showed a 71-fold increase over the study period. Twenty-four h after exposure to 500 mg/kg APAP, EPC cysteine content was significantly decreased to 77% of control concentrations, but was not significantly different from controls after 48 h.

As observed with GSH content, conceptuses showed profound developmental increases in cysteine content over time. Control embryonic cysteine content increased 8-fold from GD 9 to GD 10 (8 pmol and 67 pmol, respectively) and 9-fold from GD 10 to GD 11 (633 pmol; Fig. 4a). Cysteine content in the embryo was significantly decreased to 46% and 63% of control content 24 and 48 h after exposure to 500 mg/kg APAP, respectively. As shown in Figure 4b, control VYS cysteine content increased by 43% from GD 9 to GD 10 (27 pmol and 39 pmol, respectively) and 6-fold from GD 10 to GD 11 (240 pmol). Visceral yolk sac cysteine content was significantly depleted to 16% of control 24 h after exposure to 500 mg/kg APAP, but returned to control levels thereafter.

DISCUSSION

In utero APAP exposure did not cause dysmorphogenesis in the embryo, as demonstrated previously (McElhatton *et al.*, 1997; Shepard, 1989). However, *in utero* exposure to APAP did modulate GSH and cysteine concentrations in the conceptus and extraembryonic tissues. Previous studies have suggested that GSH depletion in rodent conceptuses may be a causal factor in chemical-induced embryotoxicity (Hales and Brown, 1991; Slott and Hales, 1987). In contrast, studies in other laboratories indicate that GSH depletion does not cause teratogenicity in mouse embryos exposed to APAP *in vivo* (Lum and Wells, 1986). The data presented in this study also suggest that some chemicals elicit embryotoxic responses via more selective and direct mechanisms and that GSH depletion *per se* may play only a modulatory role in chemical-induced teratogenicity. Acetaminophen may be a useful tool for modeling the dynamics of GSH and cysteine depletion and recovery, as it is commonly used by pregnant women for analgesia

10 conceptus exposed to 500 mg/kg APAP. (c) Control GD 10 decidual mass. (d) High dose GD 10 decidual mass exposed to 500 mg/kg APAP. (e) Control GD 11 conceptus. (f) Low dose GD 11 conceptus exposed to 125 mg/kg APAP. (g) Control GD 11 neural tube. (h) Low dose GD 11 neural tube exposed to 125 mg/kg APAP. a, b, e, and f, mag 10×; c, d, g, and h, mag 20×. AN, alar plate of neuroepithelium; BA, branchial arch; BN, basal plate of neuroepithelium; DM, decidual mass; EF, exocoelomic fluid; ELM, external limiting membrane of neural tube; EPC, ectoplacental cone; H, heart; M, mesenchyme; N, neuroepithelium; Arrows, subcellular pools of thiol.

TABLE 1
Glutathione and Cysteine Concentrations Determined in Samples of Decidual Mass and EPC following *in Utero* Exposure to APAP on the Morning of GD 9

		Decidual mass		Ectoplacental	
	Hour	GSH (nmol)[a]	Cysteine (nmol)	GSH (nmol)	Cysteine (nmol)
Control	0	17.6 ± 2.1	5.2 ± 0.4	0.06 ± 0.01	0.02 ± 0.004
	6	26.0 ± 0.8	5.6 ± 0.2	0.1 ± 0.007	0.04 ± 0.006
	12	25.6 ± 0.8	8.0 ± 0.5	0.2 ± 0.03	0.1 ± 0.03
	24	31.2 ± 1.8	13.0 ± 1.1	1.1 ± 0.1	0.3 ± 0.04
	48	28.4 ± 1.2	7.0 ± 0.4	5.2 ± 0.5	1.2 ± 0.1
250 mg/kg APAP	6	23.5 ± 1.6	6.1 ± 0.5	0.08 ± 0.004*	0.02 ± 0.002
	12	20.1 ± 0.4*	5.8 ± 0.3*	0.3 ± 0.07	0.1 ± 0.008
	24	35.0 ± 2.1	16.0 ± 1.5	0.9 ± 0.05	0.2 ± 0.02
	48	28.3 ± 3.4	6.5 ± 1.0	8.5 ± 0.7*	1.5 ± 0.2
500 mg/kg APAP	6	21.6 ± 1.2*	5.5 ± 0.4	0.1 ± 0.01	0.04 ± 0.006
	12	21.2 ± 0.7*	5.7 ± 0.2*	0.3 ± 0.1	0.1 ± 0.02
	24	33.9 ± 2.0	6.7 ± 0.5*	1.0 ± 0.1	0.2 ± 0.07*
	48	26.6 ± 1.1	9.2 ± 0.9*	6.2 ± 0.5	1.0 ± 0.1

[a]Data are expressed in units of nmol GSH or cysteine as mean ± standard error of at least five determinations.
*Significant difference from equivalently timed controls ($p \leq 0.05$).

and may allow for direct comparison to human exposure. Acetaminophen is not known to be teratogenic in humans or animal models. Furthermore, use of APAP in this study may aid in understanding the role of LMWT in regulation of development in the organogenesis-stage rat conceptus.

In order to assess the regulatory and detoxication roles of GSH in the developing conceptus, it was important to localize LMWT histochemically and determine the spatial distribution of thiols within various tissues under normal physiological conditions. Mercury orange histofluorescence in control GD 10 and GD 11 conceptuses within their decidual capsules revealed a heterogeneous distribution of reduced LMWT, evident at the tissue and cellular levels. For example, the decidual mass, EPC, and embryonic heart and mesenchyme displayed the most intense histofluorescence for LMWT under normal conditions. Other embryonic tissues, including the neuroepithelium, exhibited less intense orange fluorescence. These patterns of LMWT distribution correlate with the reported relative sensitivity or resistance of embryonic cells to chemical toxicants. Heart cells on GD 10 (Fig. 1a), typically shown in this study to have greater LMWT concentrations than some other embryonic tissues, are known to be resistant to cyclophosphamide-induced toxicity. However, neuroepithelial cells on GD 10 (Fig. 1a), having relatively lower levels of LMWT than some other embryonic tissues, and these thiols being distributed in a specific pattern, are sensitive to damage by cyclophosphamide (Little and Mirkes, 1990; Mirkes, 1985). Likewise, the developing nervous system is more sensitive than other regions of the embryo to several chemicals that deplete GSH by different mechanisms including BSO, bromohydroquinone, and phenytoin (Andrews *et al.*, 1993; Hales and Brown, 1991; Miranda *et al.*, 1994). Differences in thiol and redox status may affect other embryonic cell types in a similar manner.

In utero administration of APAP (125 or 500 mg/kg) resulted in selective depletion of LMWT from maternally derived tissues of the decidual mass, such that the EPC remained unaffected by APAP while the rest of the decidual mass showed a dramatic decrease in LMWT-induced histofluorescence. The fact that the EPC did not show decreased fluorescence intensity with APAP exposure may be explained by its abundant maternal vascularization, which is responsible for inter-organ transport of GSH from the liver to other organs (Hirota *et al.*, 1989). In addition, little is known about GSH turnover in cells of the EPC. These cells may contain high gamma-glutamyl transpeptidase activity, a glutathionase responsible for degradation of GSH to its constitutive amino acids, allowing their subsequent uptake from the extracellular compartment (Meister, 1991). The EPC may also contain highly active GSH synthesis capacity, and each of these components would enable the EPC to respond rapidly to changes in LMWT content. Previous studies also have suggested that the decidual mass contains higher expression and activity of certain cytochromes P450, including P450 1A1, than the placenta and embryo, suggesting that this tissue has a greater capacity for bioactivation to the reactive NAPQI electrophile than conceptal tissues (Dey *et al.*, 1989; Schafer *et al.*, 1996).

Furthermore, previous studies using APAP show that it is unlikely that GSH is being conjugated to an electrophilic intermediate in the embryo or its membranes (Stark *et al.*, 1990). Rather, the proposed metabolism of APAP to 3-OH-APAP could result in GSH depletion as a result of its oxidation to GSSG. Tiboni *et al.* (1997) showed that the EPC maintains higher GSSG reductase activity than the VYS and embryo

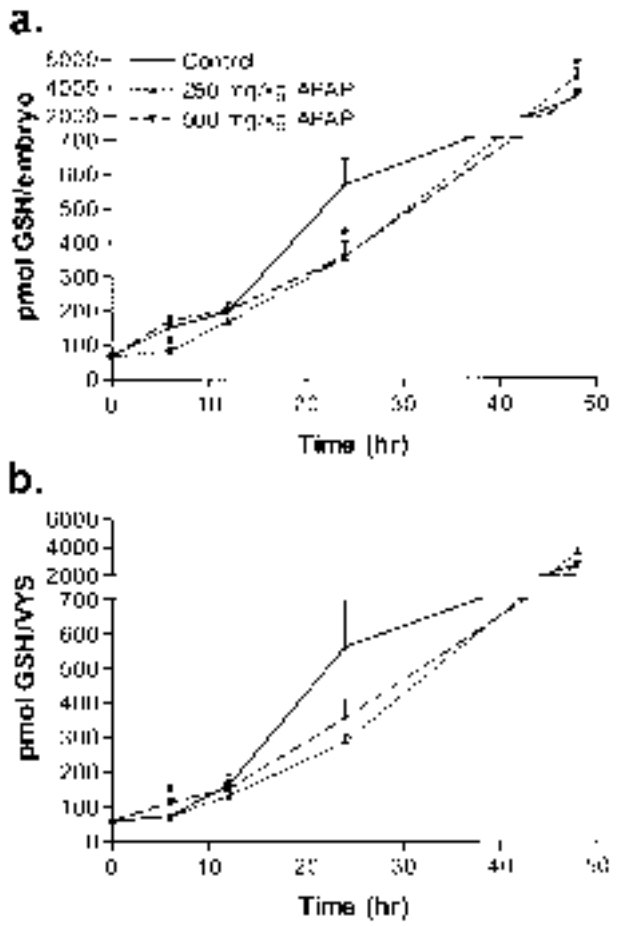

FIG. 3. Glutathione concentration in (a) embryos and (b) VYSs following *in utero* administration of 250 or 500 mg/kg APAP on the morning of GD 9. Data are expressed as pmol GSH/embryo or VYS, and each point represents the mean + standard error of at least 5 samples. Asterisks represent significant difference from equivalently timed controls ($p \leq 0.05$).

proper, so it would be better equipped to recover from GSH oxidation. These studies are in agreement with the data provided in this study showing dramatic loss of LMWT from the decidual mass but not the EPC. It should be noted that the high dose used in this study (500 mg/kg) is greater than that required to produce adult liver toxicity (300 mg/kg) in several studies using mice, which are more sensitive to APAP toxicity (Schnellmann *et al.*, 1999; Hinson *et al.*, 2000). However, the doses employed here are substantially lower than doses used in a preimplantation mouse study, which showed lethality of 25% of dams exposed to 1430 mg/kg APAP, but which did not show changes in embryonic development (Laub *et al.*, 2000).

In contrast to the selective LMWT depletion observed in maternal tissues, embryos exposed *in utero* to APAP (125 or 500 mg/kg) showed a uniform depletion of LMWT from tissues; it is not known whether lower doses of APAP would result in a selective tissue depletion in the embryo. Patterns of histofluorescence after APAP exposure showed that LMWT was redistributed to extracellular spaces, including the exocoelomic and amniotic fluids and the lumens of the embryonic neural tube, heart, and primitive gut. The presence of reduced thiol in the extracellular spaces suggests that GSSG, proposed to be formed during APAP metabolism in the embryo, may be transported out of the cells and reduced in the extracellular environment. The mechanisms for regulation of GSH and GSSG efflux from conceptal tissues have not been established, nor has the exact identity of the reduced LMWT responsible for the pattern of extracellular histofluorescence been confirmed. However, studies in adults suggest that, under normal and stress conditions, reduced and oxidized thiols are transported across the plasma membrane, and GSH conjugates are also removed from the cell in a similar manner (Khanna *et al.*, 1994; Meister, 1991; Wallin *et al.*, 1999).

Quantitation of GSH and cysteine by HPLC analysis, and changes in LMWT distribution detected using the mercury orange technique, did not correlate exactly. Exposure to APAP caused GSH depletion, as detected by HPLC analysis, within 6 h in most intrauterine tissues, but was returned to control

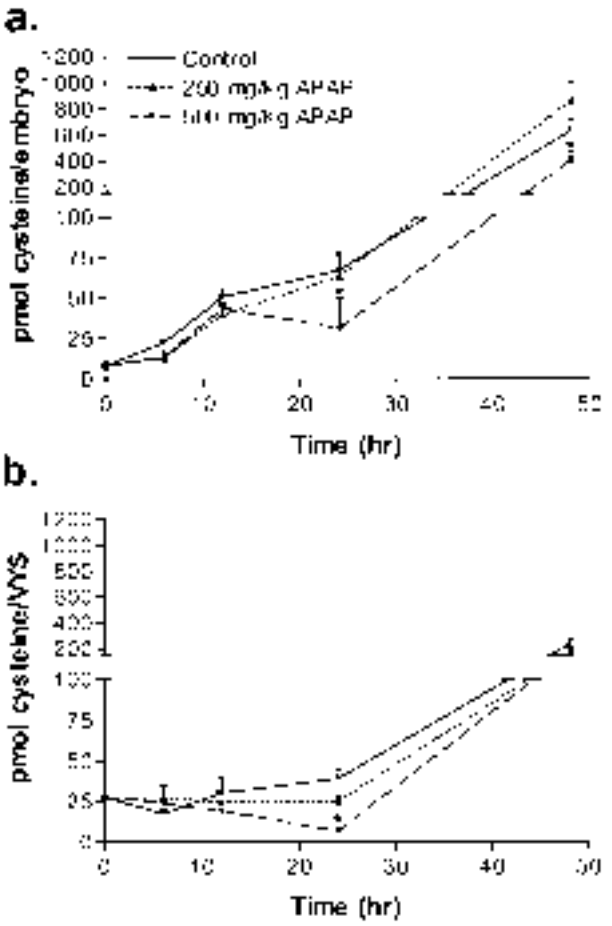

FIG. 4. Cysteine concentration in (a) embryos and (b) VYSs following *in utero* administration of 250 or 500 mg/kg APAP on the morning of GD 9. Data are expressed as pmol cysteine/embryo or VYS, and each point represents the mean + standard error of at least 5 samples. Asterisks indicate significant difference from equivalently timed controls ($p \leq 0.05$).

levels after 48 h, an effect not observed in mercury orange-processed tissues. We assume that the HPLC quantitation measures both intracellular and extracellular GSH within the conceptus, so subtle changes in LMWT distribution would be undetectable by this technique. On the other hand, the mercury orange histofluorescent technique is limited by the requirement of a threshold concentration of LMWT for optimal staining conditions (Larrauri *et al.*, 1987). It is possible that the lack of APAP dose-dependency is a result of GSH depletion by both doses of APAP to below this threshold concentration. Furthermore, the use of mercury orange histofluorescence to localize GSH in adult tissues assumes that GSH is the predominant LMWT. Mercury orange histofluorescence is unable to distinguish between GSH and cysteine, so a decrease in fluorescence in the conceptus may result from a loss of either GSH or cysteine. Indeed, the pattern of fluorescence observed after APAP exposure did more closely correlate with dramatic decreases in embryonic and VYS cysteine, as demonstrated by HPLC detection. Acetaminophen exposure decreased cysteine concentrations after 24 h in the EPC, embryo, and VYS, with embryonic cysteine remaining depleted after 48 h. These data differ from studies suggesting that the mercury orange technique is highly selective for GSH (Chieco and Boor, 1983; Philbert *et al.*, 1991). However, it must be noted that adult liver GSH:cysteine ratios are 30–35 (Neuschwander-Tetri and Rozin, 1996), while ratios from conceptal tissues obtained in this study were 2–14, depending on the tissue and time point chosen. These *in utero* data are in contrast to previously reported GSH:cysteine ratios of ~20 in cultured embryos and VYSs; the difference is due to higher GSH and slightly lower cysteine concentrations measured *in vitro* (Harris, 1993). The difference between *in utero* and *in vitro* data likely results from upregulation of GSH synthesis in cultured conceptuses, a possible consequence of increased production of reactive oxygen species (Ozolins and Hales, 1997). Moreover, this laboratory has detected a 2-fold increase in embryonic GSH content after culture (Akella *et al.*, 2000). Taken together, these data indicate that a decrease in either GSH or cysteine content will result in dramatic loss of mercury orange histofluorescence in the conceptus.

These data have important implications for maintenance of GSH content following chemical exposure. Cysteine availability has been shown to be rate-limiting for GSH synthesis, suggesting that tissues unable to maintain an adequate supply of cysteine will be more sensitive to GSH modulators. Furthermore, in times of decreased visceral yolk sac GSH content, amino acid pumps normally shuttling cysteine or cystine from the VYS to the embryo may be reversed to replenish depleted visceral yolk sac GSH. Glutathione depletion has been shown to stimulate cystine uptake and reduction to cysteine (Bannai, 1984; Bannai *et al.*, 1989). Thus, the embryo, shown here to have a substantially higher absolute cysteine concentration than the VYS, may act as a cysteine reservoir, providing cysteine for GSH synthesis in the VYS. Consequently, the depleted cysteine concentrations noted in the embryo by HPLC detection, and the reduced LMWT that was observed in the exocoelomic fluid by mercury orange histofluorescence, might not be a result of toxicity. Rather, these data may represent an adaptive response whereby embryonic cysteine is transported to the VYS. In cases where cysteine cannot be redirected to the VYS, embryotoxicity may occur.

Although most LMWT was redistributed to extracellular spaces after APAP exposure, Figure 1h shows that specific cells continued to display punctate fluorescence too small to be correlated with entire cells. These punctate foci may represent a subcellular pool of LMWT, suggesting that APAP causes sustained depletion of cytosolic LMWT without similar effects on certain subcellular pools of LMWT, such as the mitochondria or the nucleus, although future studies will be conducted to verify this hypothesis. Evidence exists to show that depletion of mitochondrial GSH, rather than cytosolic GSH depletion, is required to produce cytotoxicity in adult tissues (Olafsdottir and Reed, 1988; Shan *et al.*, 1993). The absence of an effect on particular subcellular pools of LMWT after exposure to APAP, as suggested by the present study, may explain why APAP is not embryotoxic when other GSH modulators such as ethanol, which selectively depletes mitochondrial GSH (Beck *et al.*, 2000), are embryotoxic. Other proposed mechanisms of ethanol include impaired protein synthesis, impaired nutrition, hypoxemia, and oxidative stress, and have been reviewed by Smith *et al.* (1991) and Guerri *et al.* (1994).

This study evaluates spatial and temporal differences in conceptal GSH and cysteine concentrations using the mercury orange histofluorescent technique. The study provides relevant data regarding the normal redox environment of the rat conceptus during early organogenesis, as well as demonstrating the dynamic redistribution of thiol after exposure to a nonteratogenic GSH modulator. Therefore, these results can be used as a baseline to which GSH and cysteine depletion by known teratogens may be compared. Several pertinent questions have been raised by these results, including the role played by specific subcellular pools of LMWT in response to chemical modulators of GSH. The study also suggests a critical role of cysteine as a potential modulator of embryotoxicity and teratogenicity. Future studies will be aimed at confirming and quantifying the data obtained in this study, using more sensitive fluorescent probes, as well as identifying the mechanism(s) involved in regulation of subcellular GSH content using confocal microscopy and intracellular cysteine content as a means for protection from embryotoxicity.

ACKNOWLEDGMENTS

The authors would like to thank Surekha Akella, Nick Choe, Malia Dixon, Jenny Warner, and Shannon Yancy for their technical assistance. This work was supported by PHS NIH grants ES 08846, ES 05235, and ES 07062.

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