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LINDANE EMBRYOTOXICITY AND DIFFERENTIAL ALTERATION OF CYSTEINE AND GLUTATHIONE LEVELS IN RAT EMBRYOS AND VISCERAL YOLK SACS

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Abstract — The lindane embryotoxicity and associated changes in cysteine (CYS) and glutathione (GSH) status have been investigated in the early organogenesis-stage rat conceptus utilizing whole embryo culture techniques. Direct exposure of gestational day 10 (GD 10) conceptuses to lindane (50, 100, 200, 300, and 400 μM) in the culture medium resulted in a dose- and time-dependent increase in mortality (88% at 400 μM), frequency, and severity of malformations and in decreased growth parameters. Protein and DNA contents of embryo and visceral yolk sac (VYS), likewise decreased significantly as lindane concentrations increased. Lindane exposures greater than 100 μM produced abnormal axial rotation, pooled blood on lateral cephalic surfaces, cephalic edema, and decreased VYS vasculature. Histologic sections showed a variety of abnormalities, including distended anterior cardinal veins, thinning of the neuroepithelium in forebrain and hindbrain regions, and abnormal branchial arch development. CYS and GSH levels in the VYS were not significantly affected by 100 μM lindane exposure during a 5-h incubation period on GD 10 and GD 11. In contrast, CYS and GSH levels in lindane-exposed embryos remained unchanged while control levels continued to increase with gestational age. At 5 h, treated embryos showed a significant depletion of CYS (GD 10, 22%; GD 11, 35%) and GSH (GD 10, 41%; GD 11, 24%) relative to controls. Selective lindane-induced depletion of embryonic GSH suggests involvement of the glutathione redox cycle in lindane embryotoxicity.

Key Words: lindane; embryotoxicity; cysteine; glutathione; histology; whole embryo culture; embryos; visceral yolk sac; rat.

INTRODUCTION

The organochlorine insecticide lindane (γ-hexachlorocyclohexane) is a common component in a variety of agricultural fertilizers, household insecticide sprays, human parasitic medications, and animal parasiticidal solutions (1). Although the production of lindane was banned in the United States in 1977 and technical grade lindane can no longer be purchased, it is still present in commercial products and found in significant quantities in the environment and is thus a potential hazard to human health.

In vivo studies have been conducted to ascertain the influence lindane may have on reproductive success and fetal development. A study using the mouse as a model system demonstrated that lindane had a significant effect on implantation success and early development when given orally at various times throughout pregnancy (2). When this insecticide was given early in pregnancy, prior to implantation (gestational days [GD] 1 to 4), implantation was totally inhibited in the treated females, while administration during organogenesis (GD 6 to 12) caused the complete resorption of implanted fetuses. Lindane given late in pregnancy (GD 14 to 19) caused a significant reduction in pup weight as well as subsequent neonatal death within 12 h (high dose) to 5 days (low dose) after birth. The amount of lindane given to each treated female during early and mid-gestation was 50% of the established LD_{50} (43 mg/kg body weight) for the species of mouse used in this study (Swiss; LD_{50}—86 mg/kg body weight). Lindane administered late in gestation was given at a high (50% of LD_{50}; 43 mg/kg body weight) and low (25% of LD_{50}; 21.5 mg/kg body weight) dose. Other studies, conducted in the rat, have shown that lindane exposure can cause an increase in the duration of diestrus and/or shorten the duration of estrus, lengthen the gestation period, decrease the number of fetuses born, and reduce growth in the young (3,4). In contrast, one study conducted in the rabbit and rat, using low levels of lindane (15 mg/

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kg body weight or less), provided no evidence of teratogenic effect when administered during pregnancy (5). These studies show that lindane exposure can influence reproduction and development in vivo. To date, no studies have been conducted to determine how direct lindane exposure may influence development in vitro.

It has been shown that lindane can be metabolized to excretable products by a variety of pathways in mammalian tissues. Results from in vitro studies suggest that the initial biotransformation step in mammals occurs by one of three routes: 1) γ-2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) is generated by dehydrochlorination; 2) γ-hexachlorocyclohexene (γ-HCCH) is produced by dehydrogenation; and 3) γ-tetrachlorocyclohexene (γ-TCCH) is formed by dechlorination (6–8). Further characterization of these products has been performed using in vitro methods, primarily with rat liver microsomal preparations. It was determined that the three intermediary metabolites were found simultaneously in the incubation mixture and that the enzymatic reactions resulting in dehydrochlorination (γ-PCCH) and dehydrogenation (γ-HCCH) of lindane require both oxygen and NADPH (8). In contrast, the intermediary metabolite formed from dechlorination of lindane (γ-TCCH) was produced more readily under anaerobic conditions and could have been produced by either enzymatic and/or nonenzymatic means. This metabolite (γ-TCCH) can also be derived enzymatically under aerobic incubation conditions (7,8). These lindane metabolism products appear to occur readily under in vitro culture conditions and are assumed to also be generated in vivo. Organogenesis-stage rat conceptal tissues have been shown to contain constitutive cytochrome P-450 enzymes that can biotransform xenobiotics (9,10). Therefore, lindane, as well as metabolites of lindane biotransformation, should be considered when assessing toxicity. Electrophilic metabolites produced from lindane metabolism have been shown to cause oxidative stress in livers of lindane-exposed rats, and to influence glutathione (GSH) status in hepatic tissues (11). Lindane treatment also appears to cause a decrease in the GSH/GSSG (glutathione disulfide) ratio in hepatocytes (12).

Glutathione (γ-glutamylcysteinylglycine) is the predominant low-molecular weight thiol in most cell types that provides protection against chemical insult and regulates several important cellular functions. Maintenance of cell and membrane integrity, regulation of protein and DNA synthesis, microtubule assembly, modulation of protein folding, and participation as a cofactor for various enzyme activities are a few cellular functions with which GSH has been shown to be associated (13–15). GSH provides protection to cells by removing reactive oxygen species (ROS), such as hydrogen peroxide, along with the concomitant generation of GSSG (16). In addition, GSH may interact directly with free radicals resulting in GSH depletion and GSSG formation (17). The amount of GSH available within a cell may influence GSH-mediated cellular functions and defense mechanisms essential for cell/organism viability. In addition, the availability of cysteine has been shown to be a rate-limiting factor in GSH synthesis. Lindane and the metabolite, PCCH, generated from detoxification reactions carried out in rat liver cytosol preparations, have been shown to conjugate with GSH under in vitro conditions (18). It appears that although GSH can conjugate with both parent compound and metabolite, it predominantly forms adducts with the metabolite forming dichlorophenylglutathione. In the liver, this reaction requires particle-bound transferases (such as glutathione-*S*-transferase) and/or other soluble enzymes. Using rat liver cytosol as an enzyme source, TCCH appears to be converted to 3- or 4-chlorophenylglutathione, depending upon the initial isomeric form (19). Lindane, therefore, may influence thiol status in the conceptus by forming adducts with GSH, influencing synthesis or changing redox cycling.

This study was undertaken to determine what influence lindane may exert on embryos and visceral yolk sacs of the organogenesis-stage rat conceptus in vitro. In vitro culture methods were used to evaluate the embryotoxicity of lindane on the explanted rat conceptus. Also, intracellular thiol status was analyzed to determine if lindane may influence embryogenesis through altered cellular homeostasis involving GSH redox cycling.

MATERIALS AND METHODS

Rat whole embryo culture and assessment

Time-mated, primagravida Sprague–Dawley rats were obtained on days 6 to 9 of gestation from the Reproductive Sciences Program, P-30 Animal Core Facility at the University of Michigan. Following copulation, GD 0 was determined by a sperm positive vaginal smear on the morning after mating. Pregnant dams were housed under a 14 h light/10 h dark cycle and access to food and water were ad libitum. Pregnant dams were anesthetized with ether on the morning of GD 10, blood was collected from the abdominal aorta, and uteri were removed. Collected blood was held on ice until centrifugation at 12,000 × *g* for 20 min, the serum was then decanted,

heat-inactivated at 56 °C for 30 min, and used for the embryo culture media. From dissected uteri, conceptuses were explanted into culture as previously described (16). The culture media consisted of Hanks' Balanced Salt Solution (HBSS) supplemented with 33% heat-inactivated rat serum, pH 7.4, and saturated with 20% O_2: 5% CO_2: 75% N_2 prior to addition of conceptuses. Conceptuses were cultured in sterile roller bottles containing 1 mL medium per conceptus.

A dose–response study was conducted by adding lindane (γ-hexachlorocyclohexane; Sigma Chemical Company, St. Louis, MO, 99% purity), dissolved in dimethyl sulfoxide (DMSO), directly to the culture medium containing GD 10 conceptuses. Concentrations used for the dose–response study ranged from 50 to 400 μM. These concentrations were chosen to encompass values found in vivo after human lindane exposure, 71 to 125 μM (20–22). Control conceptuses were given DMSO at concentrations equal to the amount added with the highest concentration of lindane. In all cases, the amount of DMSO was kept below 15 μL (<0.001%) to avoid possible toxicity due to vehicle alone. Conceptuses were cultured for 26 h and gassed with 95% O_2: 5% CO_2 on the morning of GD 11. Growth parameters and malformations were assessed under a dissecting microscope on the afternoon of GD 11, as previously described (16,23). Only conceptuses with a visible heartbeat and active vitelline circulation were considered viable and used for subsequent assessment and analysis. Tissue samples for protein content and DNA analysis were homogenized using ultra-sonication. Protein content was determined using the method of Bradford (24), modified for use with a microtiter plate spectrophotometer (25). Bovine plasma γ-globulins were used to prepare the standard curve. DNA was determined by the spectrofluorometric method of Labarca and Paigen (26), using bovine DNA as the standard.

Histologic preparation

Gestational day 10 conceptuses were cultured as described previously in medium containing DMSO (control), or 100 or 200 μM lindane. Concentrations of lindane used for histologic analysis were chosen because these concentrations (100 and 200 μM) caused visible affects to organogenesis-stage conceptuses while maintaining close to 50% or higher viability. After 26 h in culture, conceptuses were removed, rinsed in ice-cold HBSS, placed in ice-cold Karnovsky's fixative and quickly separated into embryo and visceral yolk sac (VYS). Tissues were fixed for at least 48 h at 0 to 5 °C. Fixed tissue samples were dehydrated in a graded ethanol bath series, embedded in glycol methacrylate, and cut into 3-μm sections. Every fifth section was saved and stained with hematoxylin and eosin. The University of Michigan Reproductive Sciences Program, Morphology Core, processed and stained fixed tissue samples for histologic analysis.

Intracellular cysteine and glutathione determinations

Levels of intracellular cysteine and reduced glutathione were determined by high-performance liquid chromatography (HPLC) as described by Fahey and Newton (27) and modified by Harris (28). The limit of detection for CYS and GSH using this procedure is 20 pmole. The conceptuses were removed from culture at the designated times and rinsed through three washes of HBSS (pH 7.4) to remove chemical and serum components. Two GD 10 or one GD 11 conceptuses were dissected to separate embryo and visceral yolk sac (VYS) in a 60- × 15-mm petri dish containing HBSS (pH 7.4). Embryos or VYSs were then placed in a 1.5 mL microcentrifuge tubes containing 200 μL of 200 mM methanesulfonic acid and promptly frozen in liquid nitrogen. Samples were stored at −75 °C until derivitized for HPLC. Thawed samples were homogenized by ultrasonic cell disruption and held on ice. To precipitate proteins, 200 μL 4 M Na methanesulfonate was added to the homogenized samples, vortexed, and centrifuged for 7 min at 13,000 × *g*. The supernatant was removed and pipetted into new microcentrifuge tubes, held on ice, to which 160 μL HEPPS buffer (1 M HEPPS, 5 mM DTPA; pH 8.5) and 20 μL monobromobimane (1.5 mg dissolved in 1 mL acetonitrile) had been added. This and the following steps were carried out in the dark. Samples were vortexed and the reaction allowed to proceed for 20 min at room temperature. To stop the reaction, 380 μL of 400 mM methanesulfonic acid was added to the tubes and vortexed. Derivitized samples were stored at −75 °C until assayed by HPLC. HPLC analysis was conducted as described by Harris (28).

Glutathione modulation by conceptuses subjected to lindane

Gestational day 10 conceptuses were placed into culture media saturated with 20% O_2: 5% CO_2: 75% N_2, followed by addition of DMSO (control) or lindane (100 μM, dissolved in DMSO) to the culture bottle media. A lindane concentration of 100 μM was used because it was shown to have an effect on development, while at least 82% viability was maintained. After addition of DMSO or lindane, two

conceptuses were removed immediately (time 0 h) and at subsequent time points 0.5, 1, 2, 3, and 5 h. Conceptuses were selected at random from the culture bottle and processed for HPLC. For determinations on GD 11, conceptuses were explanted on gestational day 10 and cultured as previously described with no chemical additions for 22 to 23 h. After this incubation period, the culture media was saturated with 95% O_2 : 5% CO_2 according to standard protocols, following which DMSO vehicle (control) or lindane (100 μM) were added. The 0-h conceptus was removed immediately and samples were taken at previously stated time points (one conceptus per time point per treatment group).

Statistical analysis

Data were analyzed by the General Linear Models (GLM) procedure using the Statistical Analysis System for personal computers (29). Least square means were generated for protein, DNA, and crown–rump length values by GLM procedure and probability values calculated to find significance between treatment levels. Statistical differences between CYS and GSH levels in embryo and VYS samples were tested by means calculated in GLM procedure and analyzed using Tukey's multiple pairwise comparisons test. The main effect variable, day (replicate), was tested and found to be not significant, so it was removed from the model. For abnormal axial rotation data, Fisher's exact test (two-tailed) was conducted to find differences between control and treatment levels. A significance level of $P < 0.05$ was used for statistical testing.

RESULTS

Assessment of viability, dysmorphogenesis, and growth parameters

A dose- and time-dependent embryotoxicity was observed with respect to viability, malformations, and growth parameters in conceptuses exposed to lindane on gestational day 10 and assessed after 26 h in whole embryo culture (Table 1). A loss of viability (6%) was observed in the lowest lindane concentration investigated (50 μM). As lindane concentrations increased, a reciprocal decrease in viability was also observed. There was a high degree of correlation between these two parameters ($r = -0.99$). Lindane appeared to have a minimal effect on axial rotation at concentrations up to 200 μM (14% or less abnormal axial rotation), however, a significant increase was seen in 300 and 400 μM concentrations (81% and 100%, respectively). It should be noted that axial rotation was completely arrested in conceptuses exposed to lindane concentrations of 400 μM. Lindane exposures of 100 μM or higher also produced pooled blood on the lateral cephalic surfaces, cephalic edema, a decreased pigmentation in blood cells, and a decrease in VYS vasculature. The cephalic edema observed was unique, in that the brain region of lindane-exposed embryos appeared translucent when viewed microscopically (Figure 1C). Also, the tissues associated with this region appeared to be compressed and withdrawn from the surface ectoderm. This appearance may be due to a thin surface ectoderm in conjunction with a loss of mesoderm and a reduction in neuroepithelium (Figure 1C). The severity of these effects increased as lindane concentration increased. Growth of the conceptus, as determined by crown–rump length, was also significantly decreased by lindane exposure. Control conceptuses were found to have a significantly greater crown–rump length (3.2 ± 0.7) than all other treatment levels. Protein content was observed to decrease in the embryo and visceral yolk sac compartments as lindane concentration increased. A significant reduction in protein was evident at 200 μM concentrations and higher. Embryonic and vis-

Table 1. Influence of lindane on viability, dysmorphogenesis, and embryonic growth parameters in rat conceptuses after 26-h exposure in culture

Treatment	Viability (%)	Abnormal axial rotation (%)	Crown–rump length (mm)	Protein (μg)		DNA (μg)	
				Embryo	Visceral yolk sac	Embryo	Visceral yolk sac
Control	100 (21/21)[a]	0	3.20 ± .07[b]	303.7 ± 22.0	212.2 ± 17.5	30.5 ± 2.4	10.9 ± 0.8
Lindane							
50 μM	94 (16/17)	0	2.98 ± .06[c]	280.2 ± 15.1	178.9 ± 10.4	26.0 ± 1.8	8.4 ± 0.3[c]
100 μM	82 (18/22)	10	2.97 ± .06[c]	263.7 ± 13.5	182.5 ± 10.5	25.8 ± 1.6	9.2 ± 0.5
200 μM	48 (10/21)	14	2.74 ± .06[c]	201.7 ± 13.6[c]	163.9 ± 14.4[c]	18.9 ± 1.3[c]	7.6 ± 0.6[c]
300 μM	29 (6/21)	81[c]	2.78 ± .07[c]	184.3 ± 17.3[c]	167.3 ± 23.5	16.3 ± 1.6[c]	7.7 ± 1.3[c]
400 μM	11 (2/19)	100[c]	—	134.8 ± 14.7[c]	152.7 ± 20.8	12.0 ± 2.1[c]	6.3 ± 0.1[c]

[a]Values represent the number of viable conceptuses per total number of conceptuses cultured.
[b]Values are the mean ± SE.
[c]Statistically significant difference from control ($P < 0.05$).

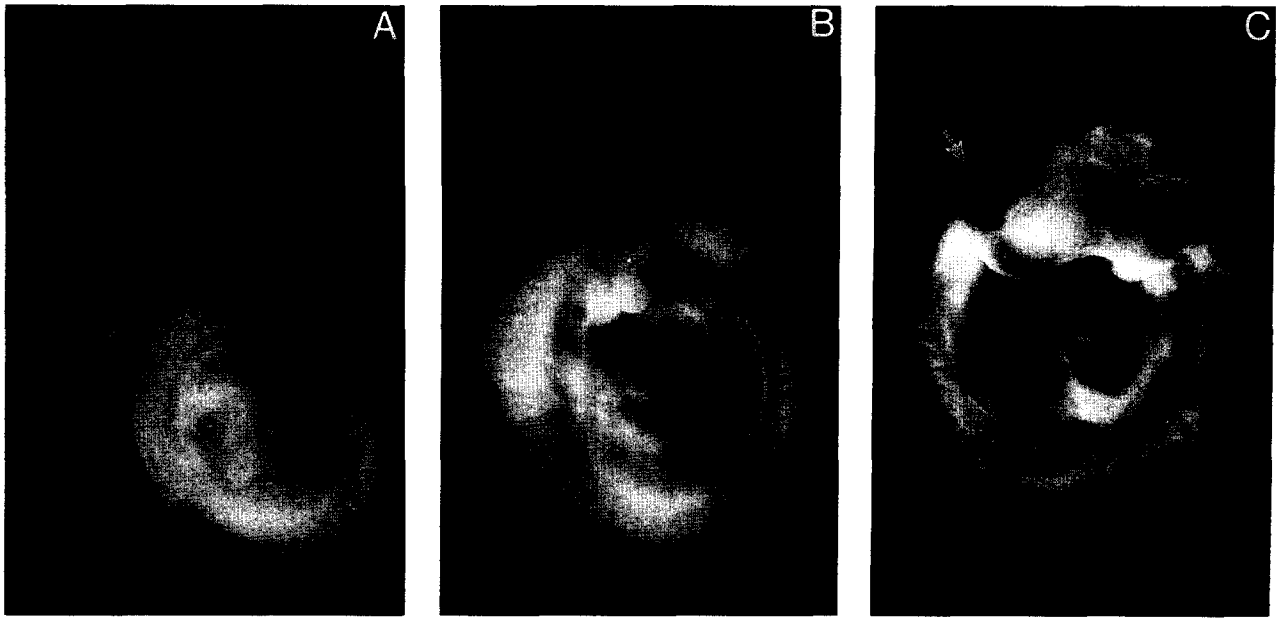

Fig. 1. Photomicrographs of representative GD 11 whole rat conceptuses cultured under in vitro conditions for 26 h in control (A), and 100 μM (B) or 200 μM (C) lindane added directly to the culture medium. Note the dose-related abnormal axial rotation and cephalic edema (arrow). The extraembryonic membranes have been removed for photography. 20×

ceral yolk sac DNA content were also seen to decrease with increasing levels of lindane. For the embryo, significantly reduced levels of DNA were observed at 200 μM or greater concentrations, whereas in the visceral yolk sac a significant loss in DNA was seen at 50-μM concentrations or greater. From these results, a treatment level of 100 μM lindane was chosen to be used for subsequent determinations of cellular thiol status.

Histologic analysis of conceptal tissue

Dysmorphic effects observed in whole embryos were reflected in histologic sections of the cephalic and branchial arch regions (Figure 2), as well as in the VYS (Figure 3). Comparable transverse sections through the cephalic region at the level of Rathke's pouch (Fig. 2A,B,C) and the first branchial arch (Figure 2D,E,F) showed quite different morphology between control and lindane-treated conceptuses. The forebrain and hindbrain areas were compressed in the lindane-treated embryos (Figure 2B,C) compared to control (Figure 2A). This may be attributed to the cephalic edema observed in whole conceptuses, which appears to be related to the abnormally distended developing blood vessels (anterior cardinal vein). Also, the neuroepithelium in both the forebrain and hindbrain regions were diminished in thickness in lindane-exposed embryos. In representative embryos exposed to 100 μM lindane (Figure 2B), the neuroepithelium exhibited some cell death, and mitotic figures were observed, suggesting that cell division was still occurring. However, at a dose of 200 μM lindane (Figure 2C), embryo neuroepithelium displayed massive cell death, blebbing of cellular material into the ventricular lumen, vacuolation, and few mitotic figures. Mesoderm cells were less densely packed in lindane-treated embryos compared to controls, and nasal placodes were absent in lindane-treated embryos. The branchial arches were abnormal in lindane-exposed embryos, and the severity increased with ascending concentrations of lindane (Figure 3). Lindane-treated embryos (Figure 3B,C) appeared to have fewer migrating neural crest cells and distended vessels in the mandibular arch (first branchial arch) compared to control embryos. Numerous apoptotic bodies were seen in regions of migrating neural crest and in the neural epithelium.

The VYS in whole conceptuses appeared to lose its vascularity as lindane concentration increased. Histologic sections support this and show an increase in the size of the vessels as well as a detachment of the mesoderm and endoderm cell layers in lindane-exposed conceptuses (Figure 3B,C) compared to control (Figure 3A).

Intracellular cysteine and glutathione levels

Gestational day 10 conceptus. Gestational day 10 conceptuses subjected to control (DMSO) or lindane (100 μM) exposures over a 5-h incubation pe-

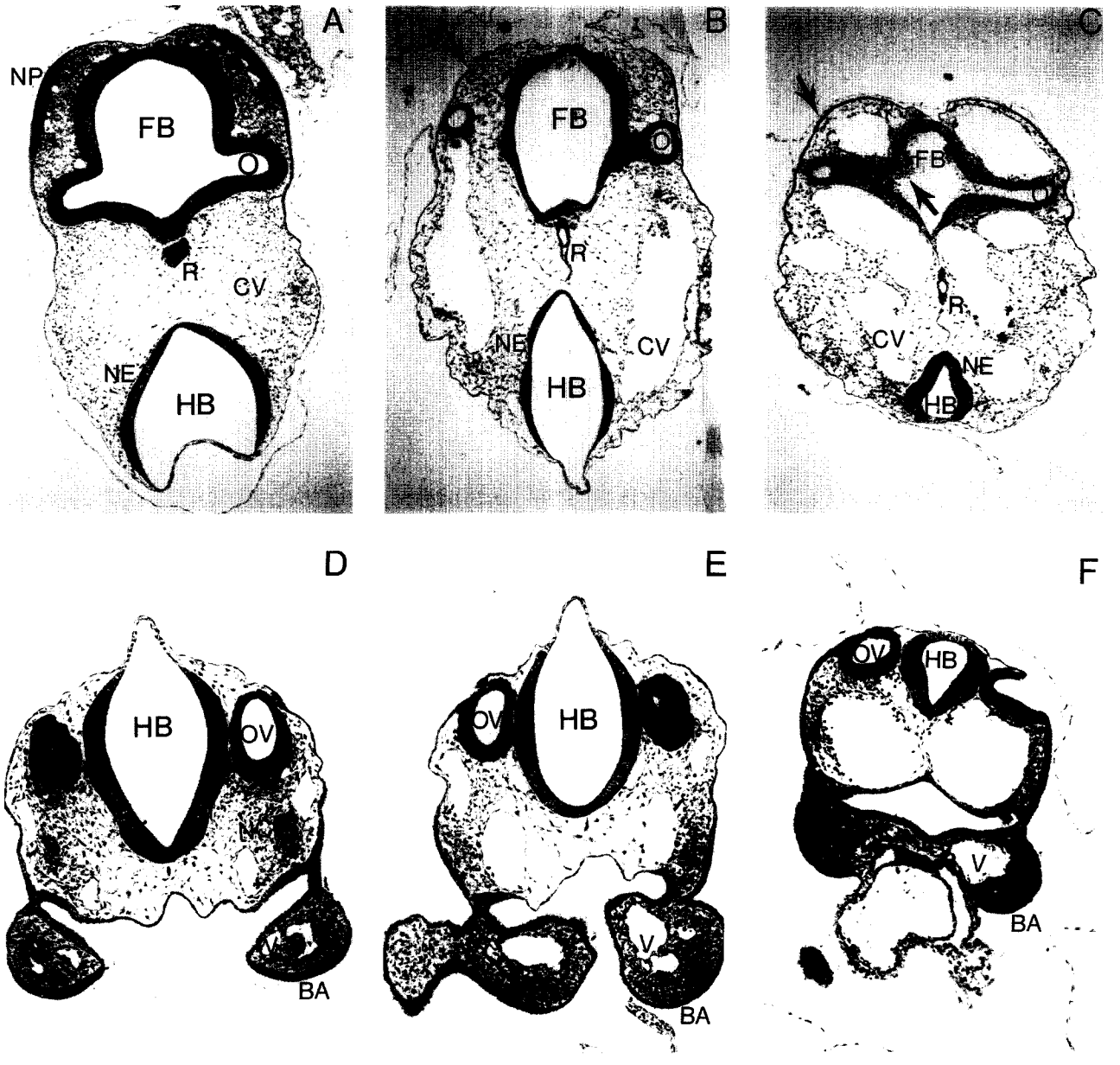

Fig. 2. Light photomicrographs of transverse histological sections of the cephalic (A–C) and branchial arch (D–F) regions showing selective morphologic defects in GD 11 embryos exposed to lindane for 26 h in vitro: control (A,D); 100 μM lindane (B,E); 200 μM lindane (C,F). Embryos were embedded in glycol methacrylate and stained with hematoxylin and eosin. Rathke's pouch (R) was used as a landmark to orient sections (A–C). Controls illustrate normally developing structures: forebrain (FB), hindbrain (HB), optic vesicles (O), nasal placodes (NP), otic vesicles (OV), anterior cardinal vein (CV), and neuroepithelium (NE). Lindane-exposed embryos display a compressed forebrain and hindbrain region, distended anterior cardinal veins, absence of nasal placodes (arrows), and less densely packed mesoderm. Note the blebbing of cellular material into the ventricular lumen in 200 μM lindane-treated embryos (arrow, C). 60×. Sections through the first branchial arch (D–F) illustrate the abnormal branchial arch (BA) development, fewer migrating neural crest cells (NC), and distended vessels (V) in the arch of lindane-exposed embryos (E,F) compared to control (D). 95×

riod responded differently with respect to changes in cysteine and glutathione (GSH) levels in the embryo or VYS. Cysteine levels appeared lower in GD 10 lindane-treated embryos when compared to control embryos although a statistically significant difference was not demonstrated (Figure 4A). There was no apparent difference in VYS cysteine levels between control and lindane-treated conceptuses (Figure 4B). A transient, but significant, intracellular cysteine peak was observed at 0.5-h incubation in

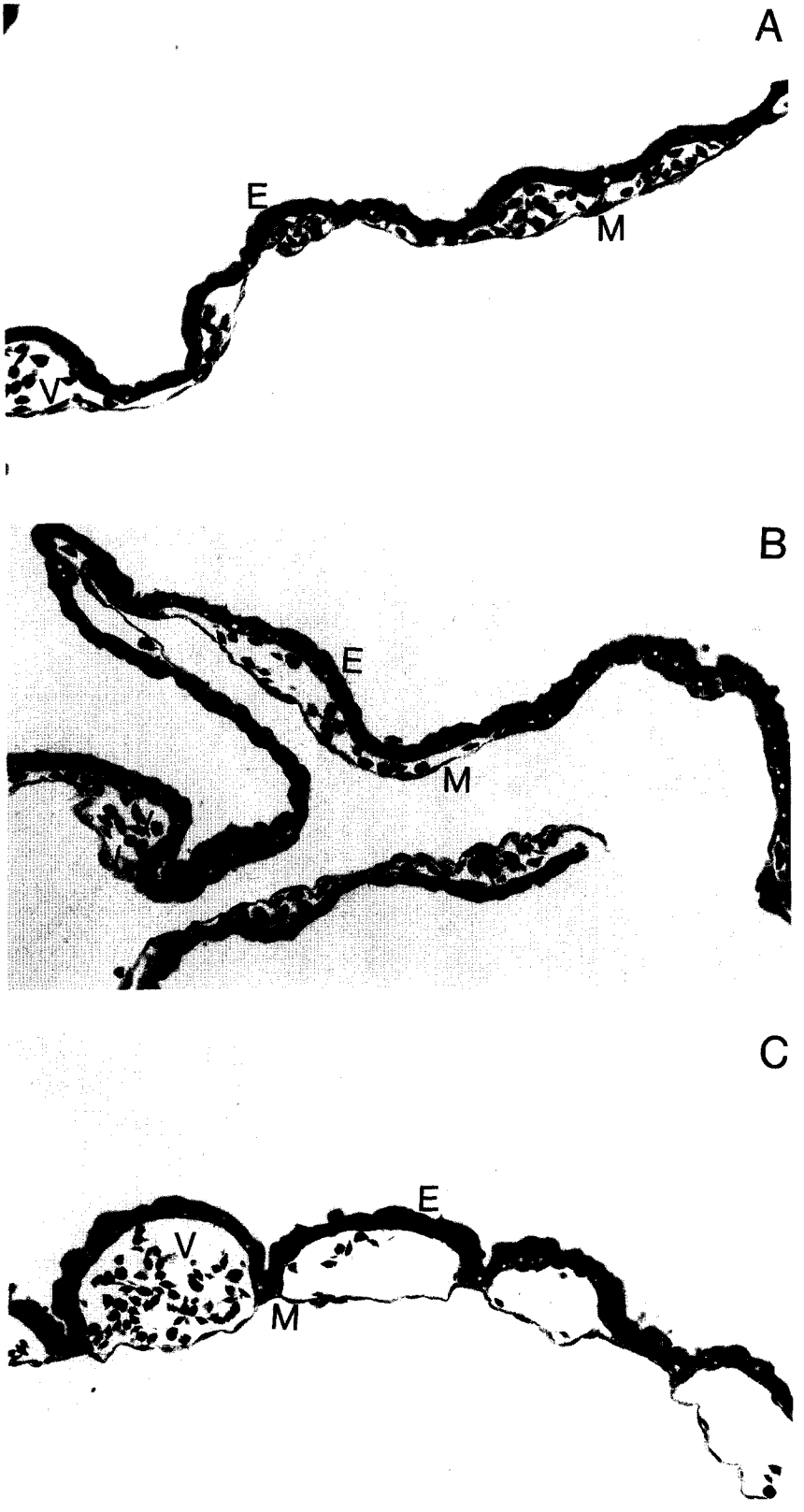

Fig. 3. Light photomicrographs of histologic sections illustrating abnormal VYS development in GD 11 conceptuses exposed to lindane for 26 h in vitro: (A) control; (B) 100 μM lindane; (C) 200 μM lindane. Conceptuses were embedded in glycol methacrylate and stained with hematoxylin and eosin. Note the increase in vessel (V) size as well as the detachment of mesoderm (M) and endoderm (E) cell layers observed with lindane exposure. 256×.

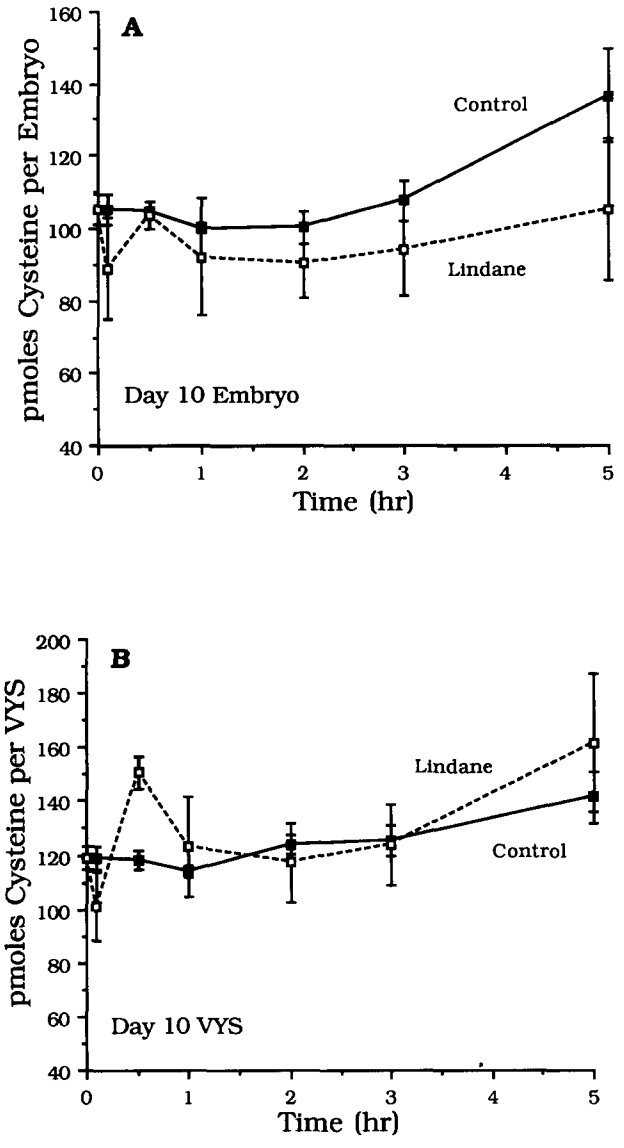

Fig. 4. The effects of lindane exposure (100 μM) on intracellular cysteine levels in GD 10 rat conceptuses subjected to in vitro whole embryo culture conditions as described in Materials and Methods. Treatment conditions were conducted over a 5-h incubation period. Values are expressed per embryo (panel A) or VYS (panel B). Each value represents the mean ± SE of three or four separate determinations derived from two conceptuses divided into embryo and VYS compartments. Initial measurements were made after approximately 5 min lindane exposure. A data point at $t = 0$ has been created to suggest the change over this initial interval.

lindane-exposed conceptuses. Embryonic GSH levels were similar in control and lindane-treated conceptuses until 3 h of incubation, following which control GSH levels continued to increase while lindane exposed embryos did not (Figure 5A). These differences in embryonic GSH were highly significant and continued until the end of the 5-h incubation period. Glutathione levels in the VYS increased by 62% over the 5-h culture period and were not affected by lindane exposure (Figure 5B).

Gestational day 11 conceptuses. Conceptuses at GD 11 responded differently to lindane exposure compared to those on GD 10. Overall, control em-

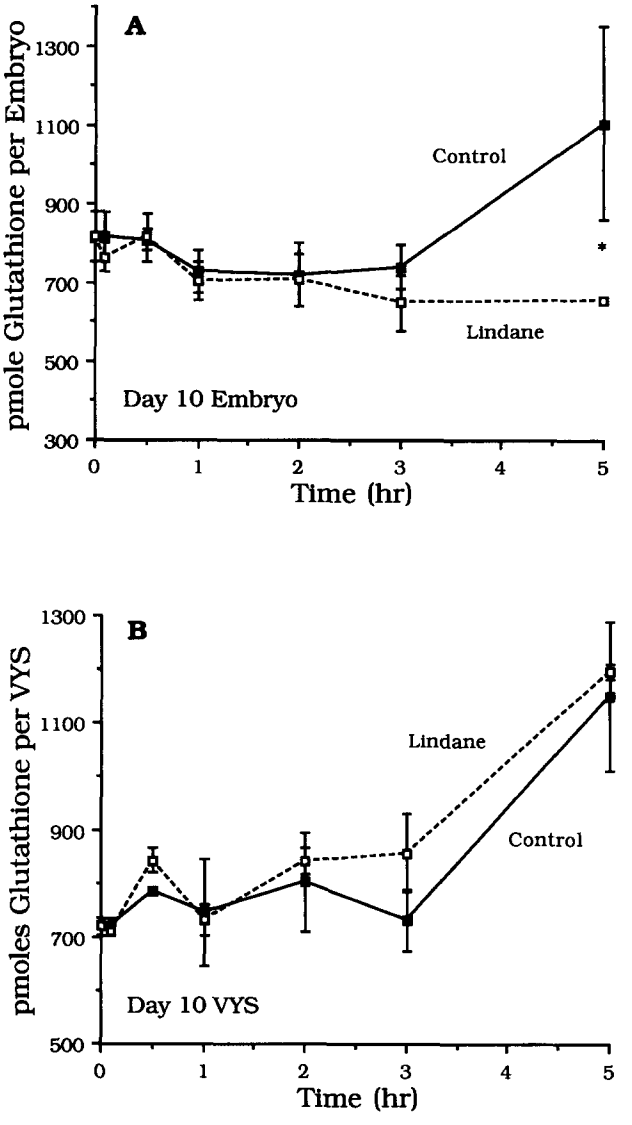

Fig. 5. Effects of lindane exposure (100 μM) on intracellular glutathione levels in GD 10 rat conceptuses subjected to in vitro whole embryo culture conditions as described in Materials and Methods. Treatment conditions were conducted over a 5-h incubation period. Values are expressed per embryo (panel A) or VYS (panel B). Each value represents the mean ± SE of three or four separate determinations derived from two conceptuses divided into embryo and VYS compartments. $^*P < 0.05$ lindane treatment vs control. A data point has been created at time 0 as explained for Figure 4.

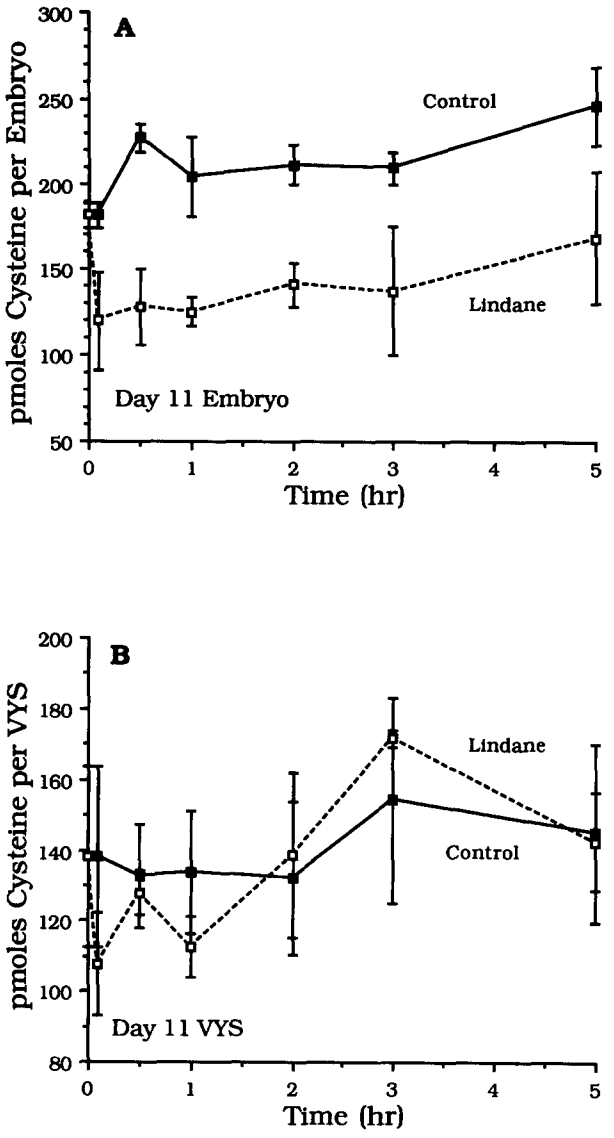

Fig. 6. The effects of lindane exposure (100 μM) on intracellular cysteine levels in GD 11 rat conceptuses subjected to in vitro whole embryo culture conditions as described in Materials and Methods. Treatment conditions were conducted over a 5-h incubation period. Values are expressed per embryo (panel A) or VYS (panel B). Each value represents the mean ± SE of three or four separate determinations derived from one conceptus divided into embryo and VYS compartments. A data point has been created at time 0 as explained for Figure 4.

bryos had a greater initial cysteine content than lindane-exposed embryos, attributable to the approximately 5-minute lindane exposure prior to protein precipitation. Both control and lindane-treated embryos exhibited an increase (19% and 13%, respectively) in cysteine levels within the first 30 min of culture, followed by a more gradual increase in cysteine for the duration of the 5-h incubation (Figure 6A). Cysteine levels in the VYS exposed to lindane were similar to controls (Figure 6B). Intracellular GSH levels did not change in control embryos until 3 h of incubation, when levels increased by 27% and remained elevated (Figure 7A). Lindane-treated embryos appeared to have decreased GSH levels at 5 h, although a statistically significant difference was not shown.

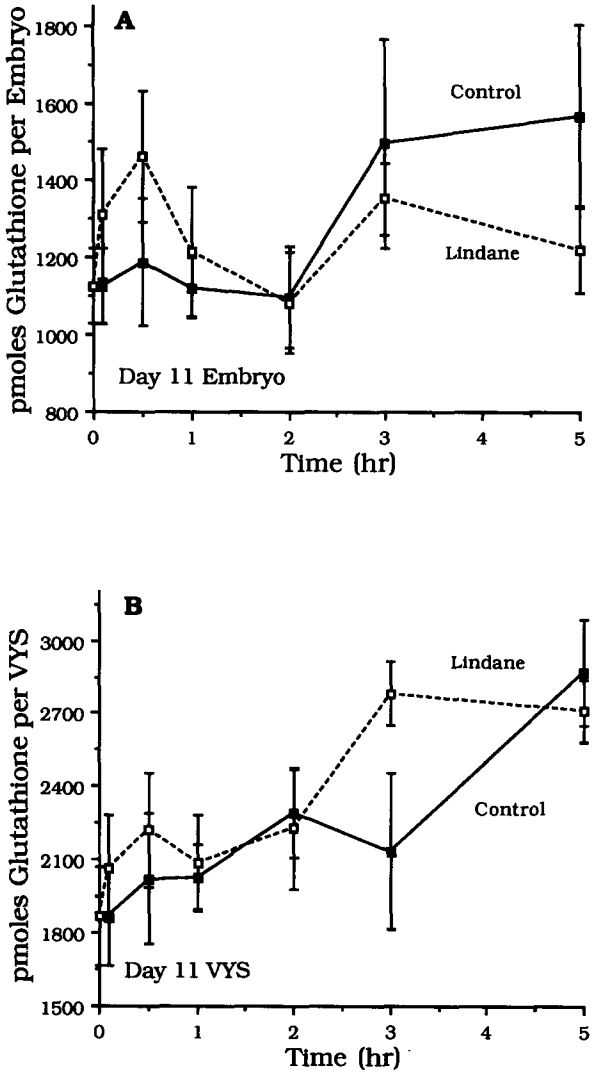

Fig. 7. Effects of lindane exposure (100 μM) on intracellular glutathione levels in GD 11 rat conceptuses subjected to in vitro whole embryo culture conditions as described in Materials and Methods. Treatment conditions were conducted over a 5-h incubation period. Values are expressed per embryo (panel A) or VYS (panel B). Each value represents the mean ± SE of three or four separate determinations derived from one conceptus divided into embryo and VYS compartments. A data point has been created for time 0 as explained for Figure 4.

DISCUSSION

No studies have yet been conducted to ascertain what effects lindane may exert directly on the developing rat conceptus. Previous studies have shown how lindane given to females at various stages of pregnancy may influence reproductive success and fetal development. Lindane administration causes a variety of effects in pregnant females resulting in prolonged gestation, fetal loss, reduced pup weight and growth, and/or neonatal death. The present study clearly shows that lindane, even at low concentrations (50 μM), can have significant effects on the organogenesis-stage rat conceptus manifested as a significant decline in crown–rump lengths. This result is not surprising because lindane administered to pregnant females at doses that did not cause fetal loss in utero but permitted dams to bear live young produced significantly reduced body weights. In addition, loss in DNA content, whether due to a decrease in cell growth, cell death, or damage to DNA strands, may influence physiologic functions.

The embryotoxicity produced by lindane exposure was characterized by abnormal axial rotation, pooled blood on lateral cephalic surfaces, cephalic edema, and decreased visceral yolk sac vasculature. These malformations were consistently present in lindane-exposed conceptuses and increased in severity as lindane concentrations increased. Cephalic edema is a malformation observed following exposure to a number of different chemicals. The pattern and severity, however, can be very different between chemicals. The cephalic edema resulting from lindane exposure appears to be caused by abnormally large, developing vessels (anterior cardinal vein), as observed from histologic preparations. The embryotoxicity found in lindane-treated conceptuses was similar, in some aspects, to that seen in conceptuses subjected to retinoic and valproic acid (30) and salicylic acid (31) treatments. Both retinoic and valproic acids appear to cause their respective malformations by intracellular fluid accumulation, while salicylic acid exerts a cytotoxic effect on selective cell membranes, resulting in cell disruption and blebbing. Although quantitation of histologic preparations was not performed in this study, either of the above-mentioned consequences of toxic exposure may lead to the lindane-induced malformations observed.

Availability of intracellular GSH and the ability of the conceptus to restore GSH stores via de novo synthesis or redox cycling is an important mechanism for protection of the conceptus from chemical insult. Results from this study illustrate that lindane influences thiol levels in the organogenesis-stage rat conceptus and appears to differentially effect the GD 10 embryo compared to the VYS. However, the possibility exists that embryonic effects may be due to indirect influences by lindane exposure on the VYS. In this study, the VYS on conceptuses continued to increase GSH concentrations with time (5-h incubation), irrespective of whether they were cultured under control conditions or exposed to lindane (100 μM). The embryo, however, appeared unable to maintain cysteine levels. Embryonic GSH levels in conceptuses (GD 10) exposed to lindane gradually decrease over time, while GSH content in controls increased. These results suggest that lindane exposure depletes embryonic cysteine and GSH, but does not affect thiol status in the VYS in the GD 10 conceptus. A similar deficit in embryonic cysteine and GSH was suggested when lindane was first administered on GD 11 (Figures 6 and 7).

Lindane and its metabolites have been shown to form adducts with GSH and may be responsible for depleting GSH pools in the GD 10 embryo. Low cysteine in the embryo may contribute to the inability to maintain GSH levels, but does not appear to be a limiting factor in the VYS. The inability of the embryo to restore GSH may be due to the embryo's reliance on the VYS for supply of free amino acids necessary for GSH synthesis, as has been suggested previously (28). At this stage of organogenesis, the embryo may also have minimal synthetic capacity and may be unable to rapidly restore cysteine and GSH levels depleted as a result of detoxification activity.

Another contributing factor in the preferential depletion of embryonic GSH may be related to the embryo being in a relatively anaerobic environment at this developmental stage. Under these conditions lindane may be selectively converted to an intermediary metabolite, such as γ-TCCH, which may be more toxic than lindane and more reactive toward GSH (19). Lindane, when subjected to anaerobic conditions has been shown to be converted, via nonenzymatic and enzymatic means, to the intermediary metabolite γ-TCCH. The physical structure of γ-TCCH has electrophilic properties favorable for nonenzymatic GSH adduct formation and thus may be affecting GSH levels in the embryo by conjugating with GSH or possibly influencing GSH redox cycling. The VYS, in a relatively more aerobic environment, would be less likely to produce the electrophilic intermediate that results in GSH depletion.

Lindane exposure did not have such a significant effect on thiol status in the GD 11 conceptus. Cysteine levels in lindane-exposed embryos and

VYS were found to be close to control values over the 5-h incubation period. However, GSH levels, although relatively unchanged in the lindane-treated VYS over the 5-h time course, in the embryo appeared to steadily decrease over time. These results again suggest, as observed in the GD 10 conceptus, that lindane appears to preferentially deplete GSH stores in the embryo. This may be due to the embryo's not being able to synthesize or restore GSH as readily as in the VYS. Also, these data suggest that if the embryo is reliant on the VYS for some of its GSH stores, then under unfavorable conditions, such as chemical insult, the VYS may not be able to provide sufficient additional GSH to the embryo, thus causing defective cellular function leading to impaired development (28).

In general, results from this study suggest that lindane may have significant effects on the developing conceptus. This study demonstrates that even low concentrations of this insecticide can influence cellular function and possibly lead to impaired development. This chemical is of special interest since it appears to target the embryo of the developing rat conceptus. Although actual environmental exposures may be somewhat lower than those shown to elicit observed effects in vitro, changes in thiol status may influence the toxicity of other agents. This has, in fact, been shown for lindane with respect to the embryotoxicity of cadmium (32). Conclusions from this study warrant further investigation into the mechanisms of lindane embryotoxicity.

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