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Phenytoin-Initiated DNA Oxidation in Murine Embryo Culture, and Embryo Protection by the Antioxidative Enzymes Superoxide Dismutase and Catalase: Evidence for Reactive Oxygen Species-Mediated DNA Oxidation in the Molecular Mechanism of Phenytoin Teratogenicity

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SUMMARY

A murine embryo culture model was used to investigate phenytoin-initiated embryonic DNA oxidation and dysmorphogenesis and to determine the embryoprotective potential of superoxide dismutase and catalase, which detoxify reactive oxygen species. Gestational day 9.5 CD-1 embryos were cultured for up to 24 hr at 37° in medium containing phenytoin (20 μg/ml, 80 μM) or its vehicle (0.002 N NaOH). Embryos cultured for 24 hr were examined for embryotoxicity. After varying durations of incubation, embryonic DNA was isolated and purified, and DNA oxidation was determined from the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-2′-dG). Control embryos showed an early increase in 8-OH-2′-dG levels that was maximal between 2 and 4 hr, followed by a small but significant decrease over 24 hr, with no evidence of embryopathy. Phenytoin-treated embryos within 4 hr also demonstrated maximal 8-OH-2′-dG formation, which was substantially greater than that of controls, with a maximal 3-fold increase over controls at 24 hr ($p < 0.05$). In wash-out studies, embryos removed from the phenytoin-containing medium after 4 hr and then cultured in phenytoin-free medium for an additional 20-hr period showed no decrease in either 8-OH-2′-dG levels or embryotoxicity, compared with embryos incubated in the presence of phenytoin for 24 hr. Embryos exposed to phenytoin demonstrated substantial dysmorphogenesis, as evidenced by decreases in anterior neuropore closure, turning, yolk sac diameter, crown-rump length, and somite development ($p < 0.05$). Superoxide dismutase and catalase virtually eliminated phenytoin-initiated 8-OH-2′-dG formation and reduced or completely eliminated all phenytoin-initiated dysmorphological anomalies ($p < 0.05$). These results suggest that embryonic DNA oxidation constitutes teratologically important molecular target damage, and they provide the first direct evidence that free radical-mediated oxidative stress plays a critical role in phenytoin teratogenesis.

The anticonvulsant drug phenytoin (diphenylhydantoin, Dilantin), which is commonly used for the treatment of epilepsy, has been shown to be teratogenic in animals (reviewed in Ref. 1) and humans (2, 3). Although the teratogenic properties of phenytoin are well known, the risk associated with untreated epileptic seizures is greater than the potential risk associated with phenytoin exposure. For this reason, phenytoin therapy is generally continued throughout pregnancy. Because up to 0.5% of all pregnant women are epileptic, phenytoin-initiated teratogenesis represents a significant medical concern.

Although numerous reports suggest that phenytoin-initiated teratogenesis results from the bioactivation of phenytoin to a reactive arene oxide intermediate (reviewed in Refs. 1, 4, and 5), there are several discrepancies in this hypothesis that have led to the investigation of other enzymatic systems known to bioactivate xenobiotics (1). *In vivo* and *in vitro* studies from our laboratory indicate that phenytoin-initiated teratogenesis may involve a peroxidase-catalyzed bioactivation of phenytoin to a reactive free radical intermediate (6, 7) (Fig. 1). If this phenytoin free radical is not detoxified by cellular detoxifying mechanisms (e.g., GSH), it or a free radical-dependent arene oxide intermediate can covalently bind

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ABBREVIATIONS: GSH, glutathione; HBSS, Hanks' balanced salt solution; HPLC, high performance liquid chromatography; MRS, male rat serum; 8-OH-2′-dG, 8-hydroxy-2′-deoxyguanosine; PHS, prostaglandin H synthase; SOD, superoxide dismutase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

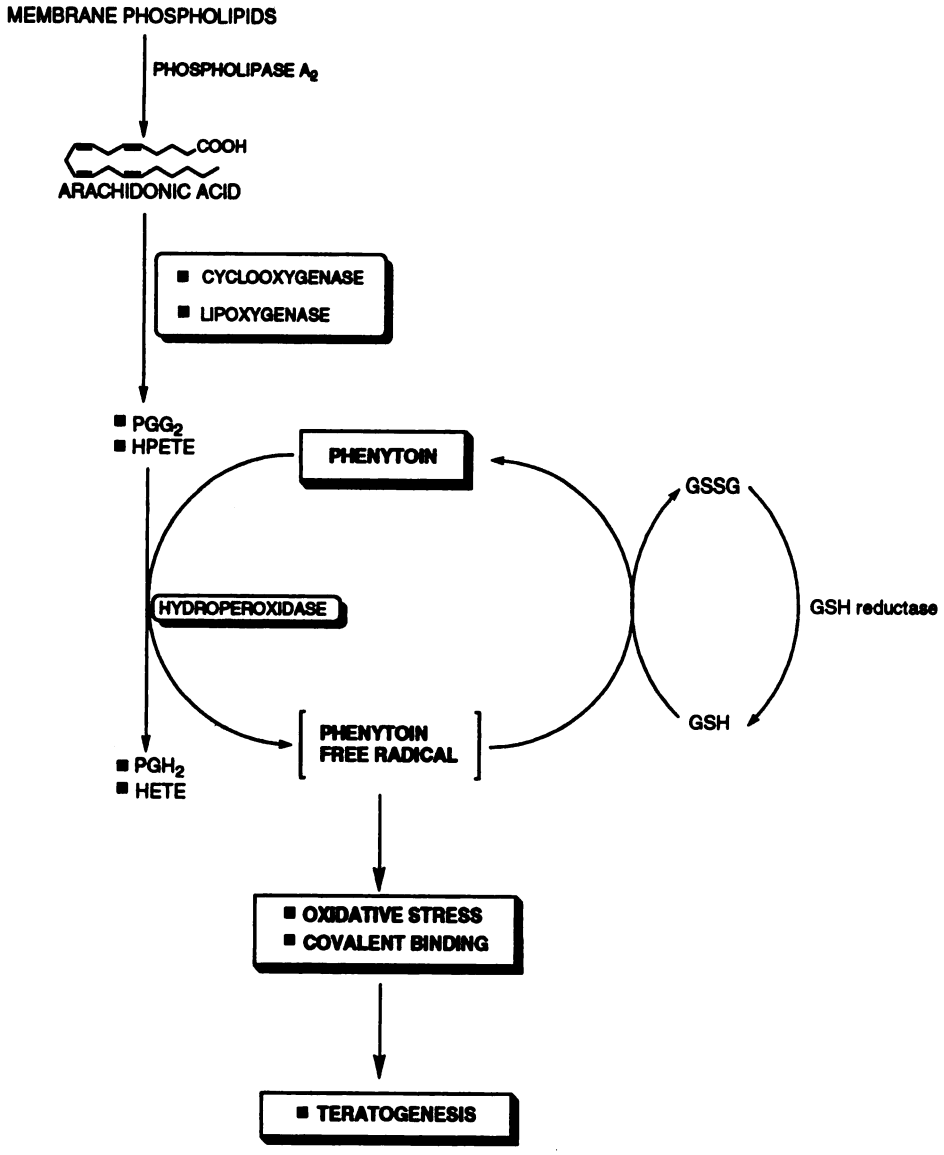

Fig. 1. Postulated mechanism of phenytoin-initiated teratogenesis. Arachidonic acid is released from membrane phospholipids by the action of phospholipase A_2. Arachidonic acid can then serve as a substrate in both the cyclooxygenase- and lipoxygenase-dependent eicosanoid pathways, generating the corresponding hydroperoxides, which are then reduced by hydroperoxidases to the corresponding alcohols. In this pathway, phenytoin may serve as a reducing co-substrate, itself being oxidized to a reactive free radical intermediate. If not detoxified, this free radical can initiate oxidative stress and/or bind covalently, thereby irreversibly damaging cellular macromolecules and initiating teratogenesis. *PGG_2*, prostaglandin G_2; *HPETE*, hydroperoxyeicosatetraenoic acid; *PGH_2*, prostaglandin H_2; *HETE*, hydroxyeicosatetraenoic acid; *GSSG*, oxidized GSH.

to embryonic macromolecules (6, 7), or it may initiate oxidative stress, leading to the oxidation of embryonic lipids, proteins, or DNA (8, 9, 10).

Due to its reducing capabilities, the phenytoin free radical may generate highly reactive oxygen species, such as hydroxyl free radical (·OH). Potentially, ·OH could be generated indirectly by the phenytoin free radical reducing Fe^{3+} complexes, initiating the Fenton reaction (11). Superoxide anions (O_2·) potentially could be formed by the phenytoin free radical reducing oxygen, ultimately producing ·OH. Although ·OH is generally thought to be a primary intermediate of *in vivo* damage (11), Wink *et al.* (12), using a simple Fe^{2+}/H_2O_2 mixture in acidic aqueous solution, have observed at least two strongly oxidizing intermediates, neither of which shows the competitive reactivities expected for ·OH. Despite the controversy over their chemical composition, these reactive oxygen species, if not detoxified by cytoprotective enzymes such as SOD and catalase, can irreversibly modify DNA (13). The damage caused by such molecular modifications has been proposed to contribute to aging, cancer, and other age-related degenerative diseases (13). Although little is known about DNA as a potential molecular target of phenytoin, *in vivo* evidence from our laboratory indicates that peroxidase-catalyzed embryonic DNA oxidation may constitute an important molecular mechanism mediating the teratogenicity of phenytoin (9), and *p53*-deficient mice lacking DNA repair are more susceptible to phenytoin teratogenicity (14), as well as to the teratogenicity of benzo[*a*]pyrene (15), another DNA-damaging xenobiotic.

The oxidized guanine analogue 8-OH-2′-dG is thought to be formed by the hydroxylation (at the C8-position) of deoxyguanosine residues in DNA by ·OH, which in turn is formed by various oxygen radical-producing agents (16). The production of 8-OH-2′-dG is thought to represent one of approximately 20 oxidative DNA modifications resulting from oxygen radical-initiated DNA damage (13). After enzymatic digestion, the 8-OH-2′-dG adduct in DNA can be readily measured among the mononucleosides by using HPLC with electrochemical detection (17). Accordingly, 8-OH-2′-dG formation can be used as a biological marker of oxidative DNA damage. *In vivo* studies have shown a correlation between the production of 8-OH-2′-dG and tumor promotion (14, 16)

or carcinogenesis (18). Liu and Wells (9) have shown *in vitro* that the formation of 8-OH-2′-dG by phenytoin and structurally related drugs is associated with their teratogenic potency. In cells, 8-OH-2′-dG-containing DNA can be repaired by nonspecific DNA repair enzymes that excise DNA adducts to release deoxynucleosides, which are enzymatically hydrolyzed to the nucleosides. Alternatively, this molecular lesion can be repaired by specific DNA repair glycosylases that release free bases (13). Both nucleosides and free bases are readily excreted from cells. We hypothesize that embryonic peroxidase-catalyzed bioactivation of phenytoin initiates free radical-mediated formation of reactive oxygen species that can oxidize guanine bases, forming 8-OH-2′-dG (Fig. 2). If not repaired, this oxidized DNA may constitute an important molecular mechanism in phenytoin teratogenesis.

To determine the significance of embryonic bioactivation and oxidation of DNA as a potential molecular mechanism mediating phenytoin teratogenicity, a murine embryo culture model was used to assess embryonic DNA oxidation, putative repair, and dysmorphogenesis resulting from exposure to a concentration of phenytoin (20 μg/ml, 80 μM) that is within the therapeutic range in maternal plasma. The embryo culture model is invaluable, in that it removes the embryo from any confounding maternal and placental effects, as well as permitting the selection of embryos at similar stages of development, which does not occur among littermates *in vivo*. To determine the potential involvement of free radical-initiated reactive oxygen species in DNA oxidation and the molecular mechanism of phenytoin teratogenicity, the potential embryoprotective effects of the antioxidative enzymes SOD and catalase were evaluated (Fig. 2). These studies provide the first direct evidence that reactive oxygen species play a critical role in phenytoin teratogenesis, and they suggest that DNA constitutes a teratologically important molecular target.

Materials and Methods

Animals. Virgin female CD-1 mice (Charles River Canada Ltd., St. Constant, Quebec, Canada) were housed in plastic cages with ground corn cob bedding (Beta Chip; Northeastern Products, Warrensburg, NY) and maintained in a temperature-controlled room with a 12-hr light/dark cycle. Food (Laboratory Rodent Chow 5001; PMI Feeds, St. Louis, MO) and tap water were provided *ad libitum*. Three females were housed with one male from 5:00 p.m. to 9:00 a.m. The presence of a vaginal plug in a female mouse was considered as gestational day 1, and these females were separated from the colony and housed together in groups of five or fewer animals/cage.

Because MRS contains undefined nutrients and factors required by murine embryos for survival and growth (19), it was used as the medium in which the embryos were cultured. Blood was obtained from retired CD-1 male rat breeders (Charles River), which were exsanguinated under anesthesia using diethyl ether (J. T. Baker

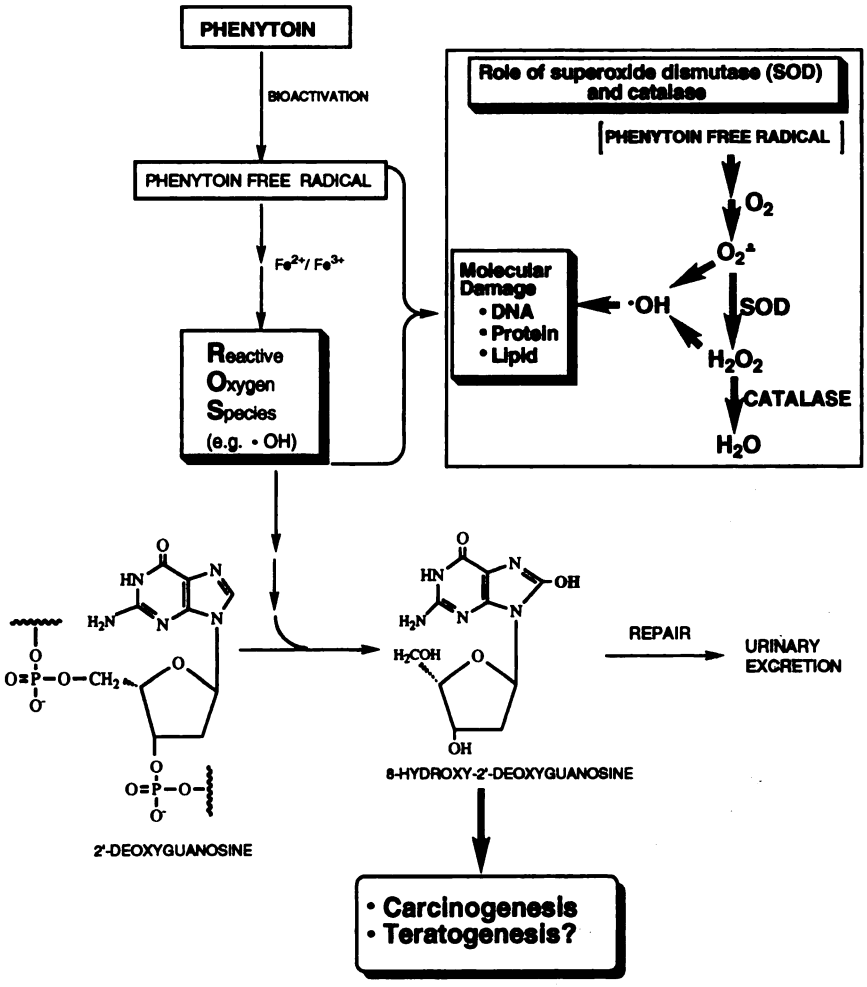

Fig. 2. Postulated formation of 8-OH-2′-dG produced from the attack of reactive oxygen species, such as hydroxyl radicals, at the C8-position of a guanine base. Reactive oxygen species may be formed in the presence of iron via peroxidase-catalyzed bioactivation of phenytoin to a free radical intermediate. *Inset*, postulated role of SOD and catalase in detoxifying reactive oxygen species generated as a result of peroxidase-catalyzed bioactivation of phenytoin to a reactive free radical intermediate.

Chemical Co., Phillipsburg, NJ). The blood was centrifuged for 5 min at 1000 × *g* at 4° (model TJ-6 centrifuge; Beckman Instruments, Toronto, Ontario, Canada) and kept on ice until blood was obtained from all animals. All blood samples were then centrifuged for 30 min at 1900 × *g* at 4° (model J2–21M centrifuge; Beckman Instruments). Pooled serum was heat-inactivated for 1 hr at 58° and gassed (5% CO_2 in air; Cannox Canada, Toronto, Ontario, Canada) for 30 min to evaporate residual protein-bound ether. The heat-inactivated MRS was divided into aliquots and stored at −80°.

Chemicals. Phenytoin (diphenylhydantoin sodium), SOD, catalase, proteinase K, ribonuclease A, ribonuclease T_1, and *Escherichia coli* alkaline phosphatase were obtained from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum, HBSS, Waymouth's MB 752/1 medium, sodium bicarbonate solution, HEPES, L-GSH, and penicillin-streptomycin were from Gibco Laboratories (Toronto, Ontario, Canada). Standard 8-OH-2′-dG was prepared as described by Kasai and Nishimura (20). All other reagents used were of analytical or HPLC grade.

Embryo culture. Pregnant murine dams were sacraficed on gestational day 9.5 by cervical dislocation, and embryos were explanted according to the method of New (21). Using this technique, the uterus was removed from the dam and rinsed in warmed HBSS. While in HBSS, the individual implantation sites were exposed using a no. 5 watchmaker's forceps (Dumont and Fils, Montignez, Switzerland). The decidua, trophoblast, parietal endoderm, and outermost membrane (Reichert's membrane) were then removed, leaving the amnion, visceral yolk sac, and ectoplacental cone intact. Explanted embryos were kept at 37°, in a holding bottle containing pregassed (5% CO_2 in air; Cannox Canada) holding medium (50 ml of Waymouth's MB 752/1 medium, 14 mM $NaHCO_3$, 2.5 mM HEPES, 1.0 mM L-glutamine, and 17 ml of MRS), until all embryos from all dams were explanted.

Embryos at similar stages of development (4–6 somite pairs) were pooled and cultured in 25-cm^2, sterile, cell culture flasks (Corning Glassworks, Corning, NY) containing 10 ml of CO_2-saturated embryo culture medium (50 ml of holding medium, 50 units/ml penicillin, and 50 mg/ml streptomycin). Flasks were incubated at 37° in a Model 3546 S/N CO_2 incubator (Forma Scientific, Toronto, Ontario, Canada) on a platform rocker (Bellco Biotechnology, Vineland, NJ).

In the time course studies, embryos were exposed to either phenytoin (20 μg/ml, 80 μM) or the phenytoin vehicle (0.002 N NaOH) for various time periods. In wash-out experiments, embryos were exposed to phenytoin or the vehicle for either 4 or 24 hr. Embryos that were exposed to phenytoin for only 4 hr were removed from the culture flask, rinsed with HBSS, placed in a new culture flask containing only fresh medium and the phenytoin vehicle, and cultured for an additional 20 hr. In the SOD and catalase studies of DNA oxidation, embryos were cultured in the presence of either SOD (500 μg/ml, 1680 units/ml) or catalase (1 mg/ml, 1680 units/ml), with or without phenytoin, for 4 hr. After each experiment, embryos (with their yolk sacs intact) from the same group were pooled (three to five embryos) and homogenized using a hand homogenizer (Kontes Glass Co., Vineland, NJ), in 500 μl of DNA digestion buffer (100 ml of 1 M Tris, pH 8.0, 10 ml of 0.5 M EDTA, pH 8.0, 2 g of sodium lauryl sulfate, and 11.68 g of NaCl; volume adjusted to 1000 ml with water).

To study the role of SOD and catalase in reducing phenytoin-initiated embryotoxicity, embryos were cultured for 24 hr at 37° in the presence of phenytoin (20 μg/ml, 80 μM) and either SOD (500 μg/ml, 1680 units/ml) or catalase (1 mg/ml, 1680 units/ml). In wash-out experiments, embryos were exposed to phenytoin or the vehicle for either 4 or 24 hr. Embryos exposed to phenytoin for 4 hr were removed from the culture flask, rinsed with HBSS, placed in a new culture flask containing only fresh medium and the phenytoin vehicle, and cultured for an additional 20 hr. After 24 hr, embryonic morphological and developmental parameters were observed using a dissecting microscope (Carl Zeiss, Oberkochen, Germany), as described below. Developmental parameters included dorsal-ventral flexure (turning), anterior neuropore closure, and somite development. Morphological assessments included yolk sac diameter (in millimeters) and crown-rump length (in millimeters).

Developmental parameters. Somite development was assessed because it can be correlated with distinct developmental events and is directly related to the growth and development of the embryo (22). Somite development of individual embryos was determined as follows. At the beginning of each culture the number of somites was noted for each embryo. At the termination of the culture, this number was subtracted from the final somite count to determine somite development. The final somite count was determined by counting from the location of the anterior limb bud (13th somite) in a cranial-to-caudal direction. This technique was used because somites located cranially to the 13th somite begin to disperse in preparation for future morphological development, making accurate somite determination difficult.

Embryos were also examined for dorsal-ventral flexure, or turning. Gestational day 9.5 embryos are S-shaped, with the hindbody lying in the same plane as the head. After 24 hr of culture (day 10.5), under normal conditions the embryo turns, assuming a C-shaped position (fetal position) with the tail lying on the right side of the head.

The open cranial end of the developing neural tube, from which the central nervous system develops, is called the anterior neuropore. To ensure proper development of the nervous system and cranial tissues, sufficient neural tube growth and neuropore closure are essential. Anterior neuropore closure can be a potentially important measure of embryotoxicity, as indicated by the evidence that phenytoin can cause congenital central nervous system dysfunction in humans (2, 3) and animals (23). Therefore, each embryo was examined for anterior neuropore closure. Anterior neuropore closure occurs at the same time as the development of the 16th somite pair; therefore, embryos that had reached the 16th somite stage or greater without anterior neuropore closure were classified as having an open anterior neuropore.

Morphological parameters. The measurement of yolk sac diameter was made at the widest point perpendicular to the ectoplacental cone. Measurements were made at either 3.2× or 4.0× magnification, with an eye-piece reticle micrometer. For embryos that had turned, the crown-rump length was defined as the distance from the mesencephalon to the lumbar-sacral region. The crown-rump length was not measured for embryos that had not turned.

DNA isolation. DNA from embryo and yolk sac homogenates was isolated by a modification of the method of Gupta (24). Briefly, embryo homogenates were incubated overnight at 55° with proteinase K (500 μg/ml). After the addition of 25 μl of 1 M Tris·HCl, pH 7.4, DNA was extracted with 1 volume of chloroform/isoamyl alcohol/phenol (24:1:25) and then extracted twice with 1 volume each of chloroform/isoamyl alcohol (24:1). Each extraction step was followed by microcentrifugation at 18,000 × *g* for 1 min (model E centrifuge; Beckman Instruments). The DNA was then precipitated by addition of 500 μl of precooled (−20°) 100% ethanol and was pelleted by microcentrifugation for 1 min. The DNA was then dissolved in 500 μl of phosphate buffer, pH 7.4, and incubated at 37° in the presence of ribonuclease A (100 μg/ml) and ribonuclease T_1 (50 units/ml), to digest residual RNA. The samples were then extracted again with 1 volume of chloroform/isoamyl alcohol (24:1), followed by microcentrifugation for 1 min, and the DNA was precipitated as described above. The pellet was dissolved in 500 μl of 20 mM sodium acetate buffer, pH 4.8, and the DNA was quantified using a UV/visible spectrophotometer (Lamda 3 System; Perkin Elmer), at 260 nm, with calf thymus DNA as the standard. DNA was then digested to nucleotides by a 30-min incubation at 37° with nuclease P_1 (67 μg/ml), followed by a 60-min incubation with *Escherichia coli* alkaline phosphatase (0.37 unit/ml) at 37°. The resulting deoxynucleoside mixture was filtered (0.22-μm filter) and analyzed by HPLC with electrochemical detection.

Analysis of DNA oxidation. The formation of 8-OH-2′-dG was measured using an HPLC system (Scientific Systems) equipped with an electrochemical detector (Coulochem model 5100A; ESA, Chelmsford, MA), a reverse phase C_{18} column (Jones Chromatography, Lakewood, CO), an analytical cell (model 5020; ESA), and an integrator (Chromatopac model CR501; Shimadzu, Kyoto, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH_2PO_4 buffer, pH 5.5, and methanol (90:10, v/v), at a flow rate of 0.8 ml/min and an oxidation potential of +0.7 V (13).

Statistical analysis. Statistical significance between treatment groups in each study was determined using a standard computerized statistical program (Statview; Abacus Concepts). Groups were compared using a one-factor analysis of variance. Binomial data were examined using the χ^2 test. The minimal level of significance used throughout was $p < 0.05$.

Results

Time course for phenytoin-initiated formation of 8-OH-2′-dG. Phenytoin was bioactivated in murine embryos to a reactive intermediate that initiated DNA oxidation, as measured by the formation of 8-OH-2′-dG. The formation of 8-OH-2′-dG in murine embryos occurred early and increased over the culture period in both the phenytoin-treated group and the vehicle-treated control group (Fig. 3). 8-OH-2′-dG levels were maximal within 4 hr in both groups but were 2.4-fold higher in the phenytoin-treated embryos after 4 hr, compared with controls ($p < 0.05$). A decrease in the formation of 8-OH-2′-dG was observed by 24 hr in the control group, suggesting limited embryonic repair of damaged DNA (from 331 ± 33.2 fmol/μg of DNA to 255.8 ± 28.7 fmol/μg of DNA). This decrease was not seen in the phenytoin-treated group.

Effect of removal of phenytoin from the culture medium. Embryos that were cultured in the presence of phenytoin for 4 hr and then cultured for an additional 20 hr in phenytoin-free medium did not show a significant decrease in the formation of 8-OH-2′-dG, compared with embryos cultured in the presence of phenytoin for the full 24-hr culture period (Fig. 4). Phenytoin-initiated embryotoxicity was not decreased by removal of phenytoin from the culture medium after 4 hr (Fig. 5).

Effects of SOD and catalase on phenytoin-initiated DNA oxidation. Incubation of embryos in the presence of SOD or catalase virtually eliminated the formation of 8-OH-2′-dG, compared with both the phenytoin-treated and vehicle-treated control groups (Fig. 6). SOD and catalase reduced phenytoin-initiated 8-OH-2′-dG formation by 98% and 94%, respectively, and, similarly, reduced base-line 8-OH-2′-dG formation due to culture conditions by 94% and 87%, respectively. Both enzymes reduced the level of 8-OH-2′-dG to that observed in the 0-hr embryos (Fig. 3).

Effects of SOD and catalase on phenytoin-initiated embryotoxicity. Embryos exposed for 24 hr to phenytoin demonstrated substantial dysmorphogenesis, as evidenced by decreases in anterior neuropore closure, turning, yolk sac

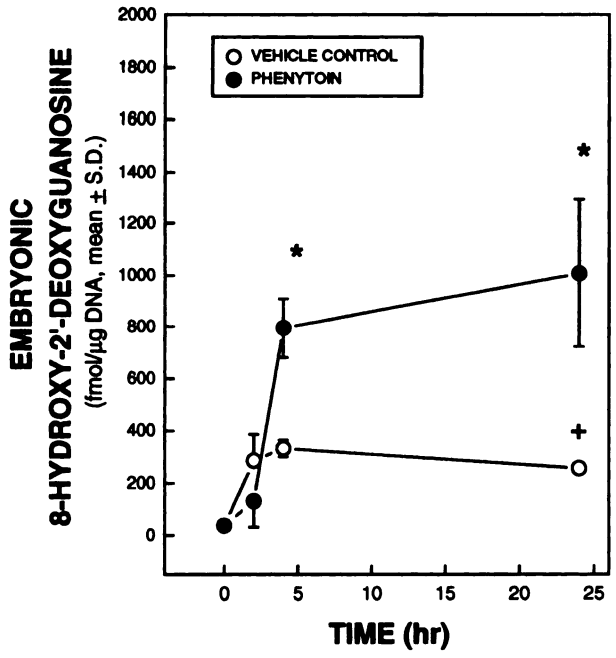

Fig. 3. Time course for phenytoin-initiated DNA oxidation in murine embryo culture. Embryos were cultured on gestational day 9.5 in the presence of either a therapeutic concentration of phenytoin (20 μg/ml, 80 μM) or the phenytoin vehicle (0.002 N NaOH). After the culture period, embryos from the same group were pooled (four or five/group) and homogenized in 500 μl of DNA digestion buffer. DNA was digested overnight with proteinase K (500 μg/ml) and then isolated according to the method of Gupta (24). DNA oxidation was quantified at various time points by determination of 8-OH-2′-dG. Each time point represents four samples of three to five pooled embryos. *, Difference from time-matched control embryos; +, difference from 4-hr control embryos ($p < 0.05$).

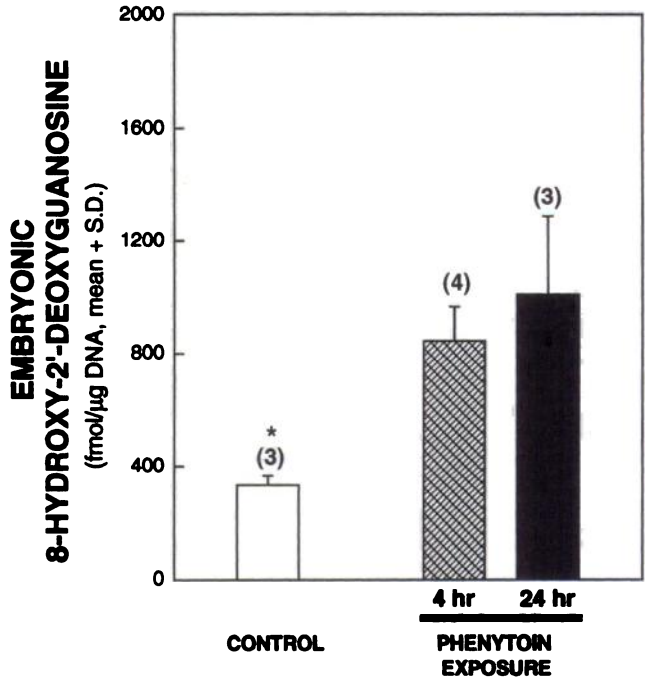

Fig. 4. Effect on DNA oxidation of removal of phenytoin from the culture medium after 4 hr of phenytoin exposure (wash-out). Embryos were removed from the culture flask, rinsed with HBSS, and placed in a new flask containing fresh medium and the phenytoin vehicle (0.002 N NaOH). Embryos were then cultured for an additional 20 hr and analyzed for DNA oxidation. Results for the vehicle-treated control group were taken from Fig. 3. Results compare control embryos with embryos from the wash-out group and embryos cultured with phenytoin for 24 hr. The number of samples analyzed is given in parentheses, and each sample represents four or five pooled embryos. *, Difference from embryos incubated in the presence of phenytoin for 24 hr ($p < 0.05$).

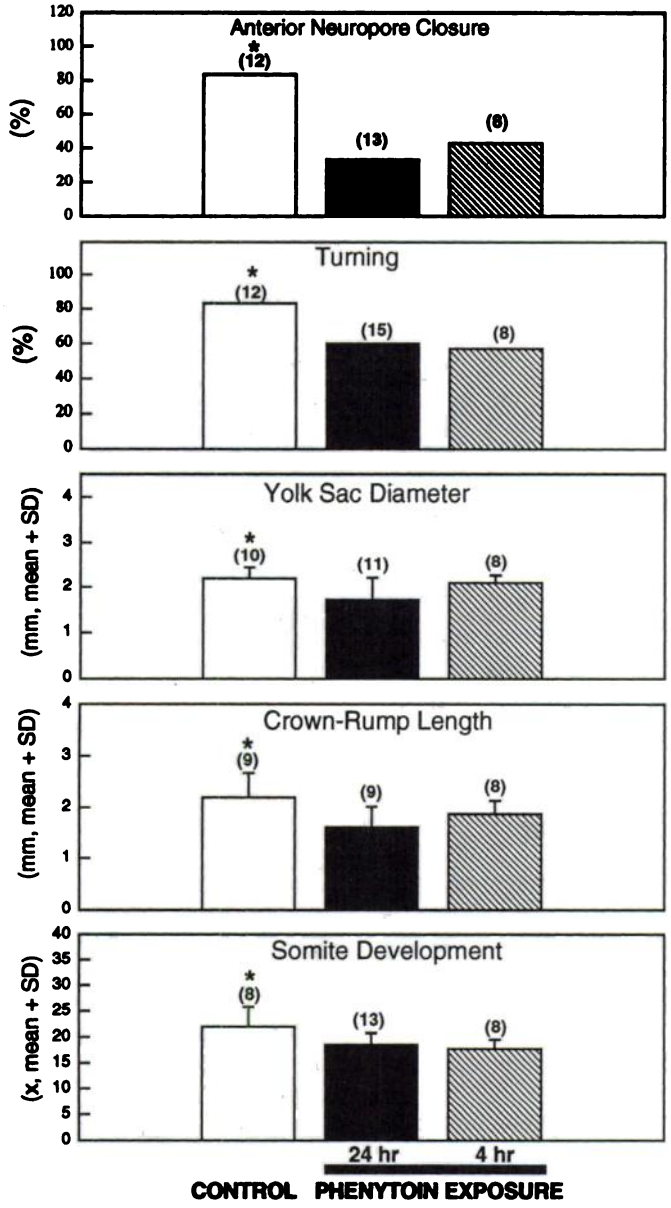

Fig. 5. Effect on phenytoin-initiated embryotoxicity of removal of phenytoin from the culture medium after 4 hr of phenytoin exposure. Incubation conditions were identical to those described for Fig. 4. Results for the vehicle-treated control group were taken from Fig. 7. Results compare embryos from the phenytoin wash-out group and embryos cultured in the presence of phenytoin for 24 hr. The number of embryos is given in parentheses. *, Difference from embryos incubated in the presence of phenytoin for 24 hr ($p < 0.05$).

diameter, crown-rump length, and somite development ($p < 0.05$) (Fig. 7). Exposure to phenytoin reduced anterior neuropore closure by 60%, turning by 28%, yolk sac diameter by 22%, crown-rump length by 27%, and somite development by 18% ($p < 0.05$). Incubation of embryos in the presence of SOD or catalase completely eliminated phenytoin-initiated decreases in all of the aforementioned developmental parameters ($p < 0.05$) (Fig. 7). Compared with vehicle-treated controls, neither SOD nor catalase improved anterior neuropore closure, turning, crown-rump length, or somite development, although there was a small but significant improvement in yolk sac diameter.

Discussion

The results from these embryo culture studies support the hypothesis that a concentration of phenytoin within the therapeutic range in maternal plasma can be embryonically bioactivated, resulting in the formation of a reactive free radical intermediate (Fig. 1) and reactive oxygen species that can oxidize DNA (Fig. 2), potentially leading to teratogenesis. It has been shown *in vivo* and *in vitro* that peroxidases such as PHS and lipoxygenases are capable of bioactivating phenytoin (6, 7, 25). This may be important, because cytochrome P450-catalyzed bioactivation may not contribute significantly to the mechanism of murine phenytoin teratogenicity. The content and activity of cytochromes P450 are very low in murine embryos at day 9.5, compared with adult levels (5). In contrast, the embryonic content and activities of peroxidases such as PHS and lipoxygenases are relatively high (26, 27) and hence may be an important determinant in phenytoin teratogenesis.

DNA damage has been the focus of several recent studies, due to its central role in information transfer between generations of somatic cells (28). *In vivo* and *in vitro* studies have found that DNA may be a molecular target mediating phenytoin teratogenesis (9), and *p53*-deficient mice lacking DNA repair are more susceptible to phenytoin teratogenicity (14). Accumulating evidence suggests that reactive oxygen species such as ·OH, which may be generated subsequent to phenytoin bioactivation (29), can produce structural alterations affecting the integrity of DNA bases (16, 17). Such alterations may occur in the form of hydroxylated residues, which can result in misreplication (30).

The formation of 8-OH-2′-dG in DNA has been widely used as a biological marker of oxidative DNA damage (18). The reported levels of 8-OH-2′-dG in various untreated tissues range from 0.006 to 0.04 pmol/μg of DNA (31). The levels reported in this study range from 0.04 pmol/μg of DNA at 0 hr to as high as 1.35 pmol/μg of DNA after a 24-hr incubation with a therapeutic concentration of phenytoin. These results are consistent with findings from a study conducted by Richter *et al.* (28), in which DNA isolated from rat liver that had been incubated with Ca^{2+} and alloxan (a prooxidant) for 15 min had levels of 8-OH-2′-dG as high as 2.70 pmol/μg of DNA. Floyd *et al.* (17) found similar levels of 8-OH-2′-dG (1.31 pmol/μg of DNA) in human granulocytes exposed to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate.

Because 8-OH-2′-dG is a potential threat to cells, due to its mutagenic activity (30), it is removed from DNA, through either general or specific repair mechanisms (13). Evidence for this repair is supported by the existence of enzymes in *E. coli* (32) and mammalian cells that are capable of selectively removing the 8-OH-2′-dG lesion from DNA as the corresponding base 8-hydroxyguanine. Although mature cells have developed various enzymatic systems to detoxify reactive oxygen species that can damage DNA (11), embryos may be deficient in these mechanisms and therefore may be more susceptible to phenytoin-initiated oxidative stress. It is known that rat and mouse embryos have low activities of GSH peroxidase, GSH reductase, SOD, and catalase (33–35).

In these studies, control embryos showed evidence of minimal DNA repair between 4 and 24 hr, as seen in a small but significant decrease in the levels of 8-OH-2′-dG originally produced endogenously, presumably due to enhanced oxida-

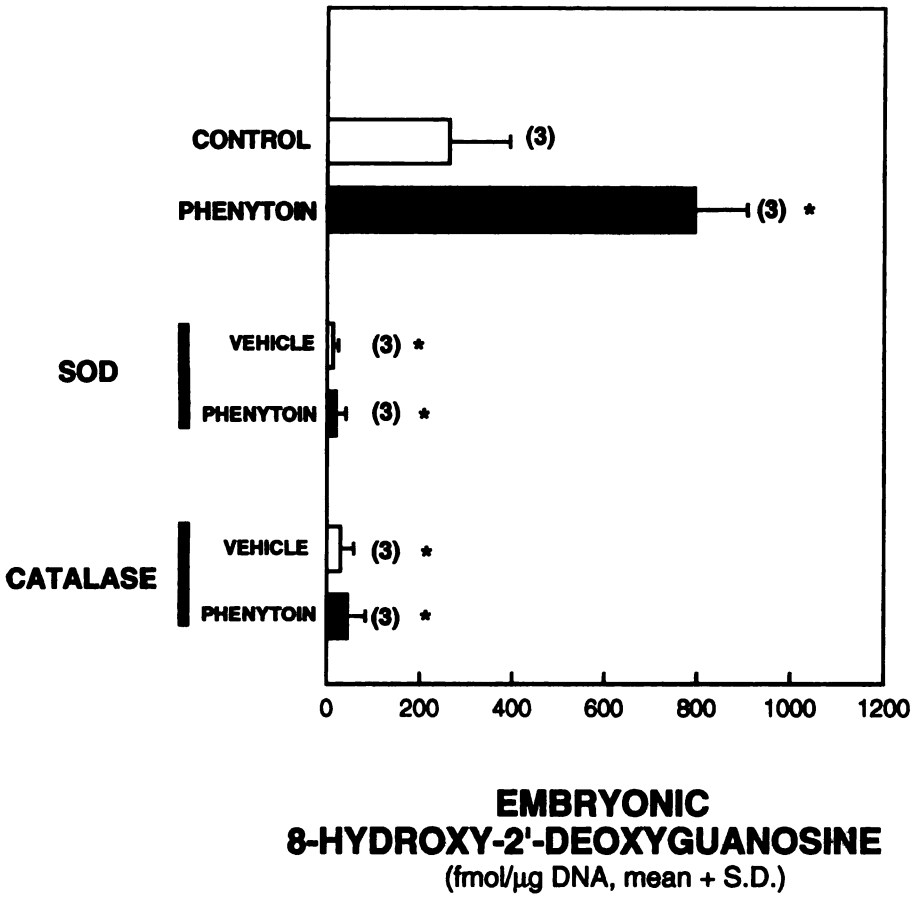

Fig. 6. Effect of SOD or catalase on phenytoin-initiated DNA oxidation in murine embryo culture. Treatment groups were as follows: vehicle-treated control (0.002 N NaOH); phenytoin (20 μg/ml, 80 μM); SOD (500 μg/ml, 1680 units/ml); SOD (500 μg/ml, 1680 units/ml) plus phenytoin (20 μg/ml, 80 μM); catalase (1000 μg/ml, 1680 units/ml); and catalase (1000 μg/ml, 1680 units/ml) plus phenytoin (20 μg/ml, 80 μM). Embryos were cultured for 4 hr. The number of samples analyzed is given in parentheses, and each sample represents four or five pooled embryos. *, Difference from control embryos ($p < 0.05$).

tive stress during embryo culture (Fig. 3). In recent *in vivo* studies, phenytoin initiated maximal maternal and embryonic DNA oxidation within 6 hr, but virtually all 8-OH-2′-dG was removed, indicating substantial DNA repair, from both maternal and embryonic tissues within 24 hr.[1] Conversely, in this *in vitro* study, embryos incubated with phenytoin for 24 hr showed no evidence of repair mechanisms (Fig. 3). The observed base-line embryonic oxidative stress in culture, evidenced by enhanced 8-OH-2′-dG formation in control embryos, may overwhelm the DNA repair capacity and/or directly inhibit repair processes, precluding the repair of phenytoin-initiated DNA oxidation.

Even when phenytoin was washed out after 4 hr, neither the level of 8-OH-2′-dG nor embryotoxicity 20 hr later was reduced (Figs. 4 and 5), which raises several interesting possibilities. Perhaps the most interesting is that the irreversible molecular damage initiated by phenytoin occurs very early and continued exposure is teratologically irrelevant. The fact that removal of phenytoin from the culture medium after 4 hr did not reduce phenytoin-initiated embryotoxicity supports the teratological importance of early, irreversible, DNA damage by reactive intermediates. This concept deserves further study, both to elucidate critical molecular mechanisms of teratogenesis and to provide the potential for more definitive therapeutic interventions. Alternatively, it is known that a teratogenic dose of phenytoin inhibits DNA synthesis in murine embryos (33), which may be related to phenytoin-initiated DNA oxidation. It is possible that phenytoin may damage the DNA that codes for repair enzymes, which would compromise embryonic DNA repair. If so, then this effect is not evident *in vivo*, where embryonic DNA repair in the continued presence of phenytoin was not measurably affected (9). If DNA repair is inhibited directly or indirectly by phenytoin, it is possible that embryos incubated for a period longer than the additional 20 hr, or embryos at a later gestational age, may have increased activities of DNA repair enzymes and thus decreased levels of 8-OH-2′-dG. Finally, although the persistent levels of 8-OH-2′-dG after phenytoin wash-out may have been due to residual phenytoin within the embryos, this concentration would be relatively low after an initial concentration equivalent to a concentration in the maternal therapeutic range.

To investigate the role of phenytoin-initiated formation of reactive oxygen species (Fig. 2) and their involvement in the subsequent oxidation of DNA, forming 8-OH-2′-dG, the protective effects of the antioxidative enzymes SOD and catalase were evaluated. In these studies, both SOD and catalase dramatically decreased the formation of 8-OH-2′-dG to base-line levels observed in 0-hr embryos (Fig. 6). SOD, catalase, and GSH peroxidase are present in murine embryos at day 9.5, which is within the period of organogenesis; however, the activities of these enzymes are substantially lower than those observed later, near the end of gestation (days 18–19) (34). Addition of SOD and catalase also significantly reduced phenytoin-initiated embryotoxicity (Fig. 7), supporting a role for reactive oxygen species in phenytoin teratogenesis. Although *in vivo* studies show that protection by SOD depends on the encapsulation of SOD by liposomes, free SOD does prevent acetaminophen-induced cell death in cultured hepatocytes (37). Likewise, it has been shown that free SOD does pene-

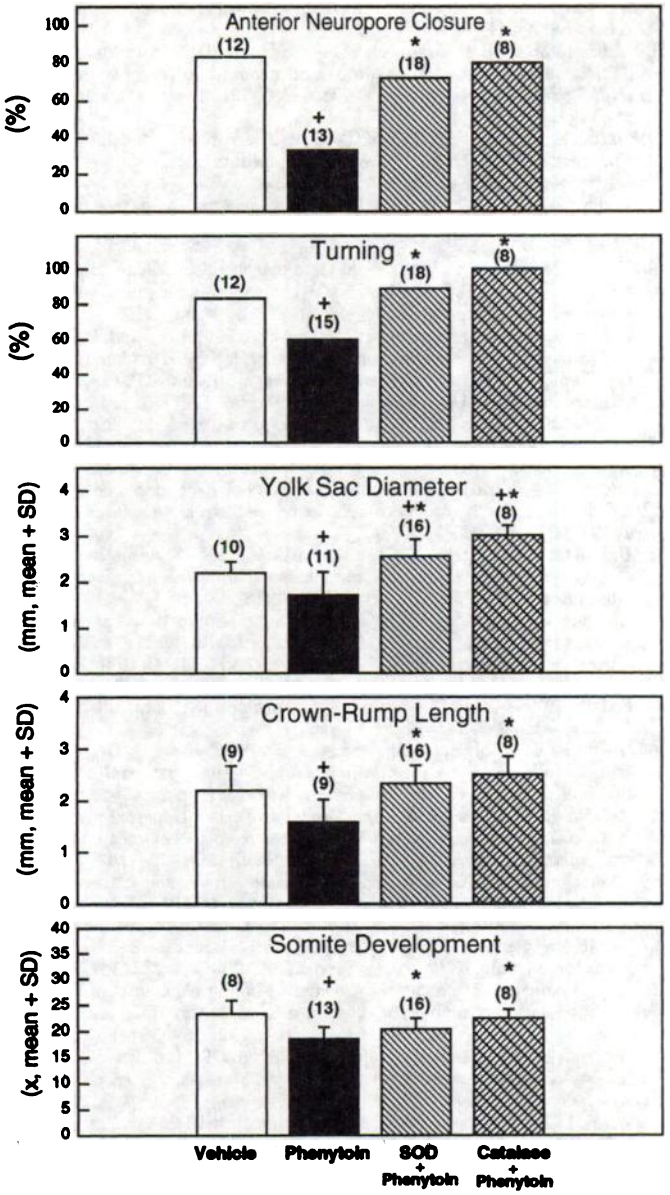

Fig. 7. Effect of SOD or catalase on phenytoin-initiated embryotoxicity. Incubation conditions were identical to those described for Fig. 5, except that embryos were cultured for 24 hr. The number of embryos is given in parentheses. *, Difference from phenytoin-treated embryos; +, difference from vehicle-treated controls ($p < 0.05$).

trate cells, although at a slower rate than liposomally encapsulated SOD (38). The cytoprotection afforded by antioxidative enzymes in embryo culture provides strong evidence that reactive oxygen species play a major role in phenytoin-initiated DNA oxidation and teratogenesis.

Agents that inhibit phenytoin teratogenicity *in vivo* have been shown to inhibit phenytoin-initiated DNA oxidation *in vivo* and *in vitro* (9). These agents include the PHS inhibitor acetylsalicylic acid and the free radical spin-trapping agent α-phenyl-*N*-*t*-butylnitrone. Furthermore, the abilities of phenytoin and structurally related teratogens to oxidize DNA in an *in vitro*, peroxidase-dependent, bioactivating system correlate well with their potencies as *in vivo* murine teratogens (9). Finally, *p53*-deficient mice, which have low DNA repair capabilities, are more susceptible to phenytoin teratogenicity (14). However, although these observations have led to our recent focus upon DNA as a teratologically critical molecular target, both arylation and oxidation of proteins (8, 10, 39, 40) and lipids (8, 10) also have been demonstrated with phenytoin, and the teratological relevance of these molecular lesions has yet to be established.

The virtual elimination by SOD and catalase of 8-OH-2′-dG formation in control embryos also provides some insight into the biochemical events occurring during dissection, staging, and culturing of untreated embryos (Fig. 5). The significant base-line elevation in embryonic 8-OH-2′-dG levels occurs within 2 hr, indicating early endogenous oxidative stress from reactive oxygen species, as evidenced by the inhibition by antioxidative enzymes. This level of oxidative stress and DNA oxidation in untreated cultured embryos may be within physiologically tolerable limits, because in our experiments SOD and catalase produced no improvement in anterior neuropore closure, turning, crown-rump length, or somite development. Although yolk sac diameter appeared to be slightly reduced by oxidative stress, as evidenced by a small but significant improvement with the addition of SOD or catalase, this did not appear to adversely affect the embryo itself. Other studies also have found that embryos in culture generally develop comparably to *in vivo* controls (19, 25), although Goto *et al.* (41) observed an increase in *in vitro* embryonic development when mouse embryos were cultured in medium containing SOD. These results demonstrating cytoprotection with SOD and catalase, although the first in embryo culture, are similar to those obtained in other biological systems, such as the decrease in 12-*O*-tetradecanoylphorbol-13-acetate-initiated 8-OH-2′-dG formation in intact granulocytes after exogenous addition of SOD and catalase (17). Given their remarkable efficacy in preventing DNA oxidation in embryo culture, the potential use of exogenous antioxidative enzymes as a therapeutic intervention in animal models of chemical teratogenesis merits further study.

In summary, these studies provide the first direct evidence that a concentration of phenytoin within the therapeutic range in maternal plasma can be embryonically bioactivated in sufficient amounts to initiate substantial DNA oxidation. The protection afforded by SOD and catalase supports the involvement of a reactive free radical intermediate and reactive oxygen species in the molecular mechanism of phenytoin teratogenesis. The role of reactive oxygen species in chemical teratogenesis and the potential therapeutic implications of antioxidative enzymes merit further study.

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