An embryoprotective role for glucose-6-phosphate dehydrogenase in developmental oxidative stress and chemical teratogenesis

CHRISTOPHER J. NICOL,* JULIAN ZIELENSKI,† LAP-CHEE TSUI,†,‡ AND PETER G. WELLS*,§,1

*Department of Pharmacology, University of Toronto, Medical Sciences Building, Toronto Ontario, Canada M5S 1A8; †Department of Genetics, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8; ‡Department of Molecular and Medical Genetics, and Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada M5S 1A8; and §Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 2S2

ABSTRACT **The primary recognized health risk from common deficiencies in glucose-6-phosphate dehydrogenase (G6PD), a cytoprotective enzyme for oxidative stress, is red blood cell hemolysis. Here we show that litters from untreated pregnant mutant mice with a hereditary G6PD deficiency had increased prenatal (fetal resorptions) and postnatal death. When treated with the anticonvulsant drug phenytoin, a human teratogen that is commonly used in pregnant women and causes embryonic oxidative stress, G6PD-deficient dams had higher embryonic DNA oxidation and more fetal death and birth defects. The reported G6PD gene mutation was confirmed and used to genotype fetal resorptions, which were primarily G6PD deficient. This is the first evidence that G6PD is a developmentally critical cytoprotective enzyme for both endogenous and xenobiotic-initiated embryopathic oxidative stress and DNA damage. G6PD deficiencies accordingly may have a broader biological relevance as important determinants of infertility, *in utero* and postnatal death, and teratogenesis.—Nicol, C. J., Zielenski, J., Tsui, L.-C., Wells, P. G. An embryoprotective role for glucose-6-phosphate dehydrogenase in developmental oxidative stress and chemical teratogenesis. *FASEB J.* 14, 111–127 (2000)**

Key Words. reactive oxygen species · development · birth defects · phenytoin · human risk

GLUCOSE-6-PHOSPHATE 1-DEHYDROGENASE (G6PD) (EC 1.1.1.49) is the first and rate-limiting enzyme in the hexose monophosphate shunt (HMS) pathway, important for its role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and the production of ribose (1, 2). During cellular oxidative stress, whether endogenous in origin or initiated by drugs or environmental chemicals, collectively referred to as xenobiotics, NADPH is critical for maintaining glutathione (GSH) in its reduced form, which is essential for detoxification of reactive free radicals and lipid hydroperoxides (3–5) (Fig. 1). Another important role for NADPH is the maintenance of the catalytic activity of catalase (6–8); hence, NADPH also is important for its role in the detoxification of hydrogen peroxide. The production of ribose by the HMS is relevant to the synthesis of nucleotides used in RNA and DNA replication and, hence, cell division and possibly DNA repair (2, 9). The gene for G6PD, containing 13 exons, has been localized to the X chromosome (Xq28), and the coding sequence has been reported for a number of species including mice, rats, *Drosophila*, yeast, and humans (10, 11). Hereditary deficiencies in G6PD, first identified in the late 1950s based on studies of differential susceptibility to the hemolytic effects of primaquine (12), are the most common enzymopathy known, affecting well over 400 million people worldwide, and particularly those from the Mediterranean region and selected African and Asian countries, wherein the incidence of G6PD deficiency may approach 60% of some populations (2). The degree of G6PD deficiency varies from negligible to severe, according to both whether one or two alleles are affected and the nature of the gene mutation and protein/enzyme variant (1). To date, 100 human genetic mutations involving the 12 coding exons, and up to 400 enzyme variants, have been described (10, 13–15). Hematological problems arising in these G6PD-deficient populations from exposure to oxidizing xenobiotics have been well characterized, ranging from hemolysis of red blood cells and hereditary non-

[1] Correspondence: Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, Ontario, Canada M5S 2S2. E-mail: pg.wells@utoronto.ca

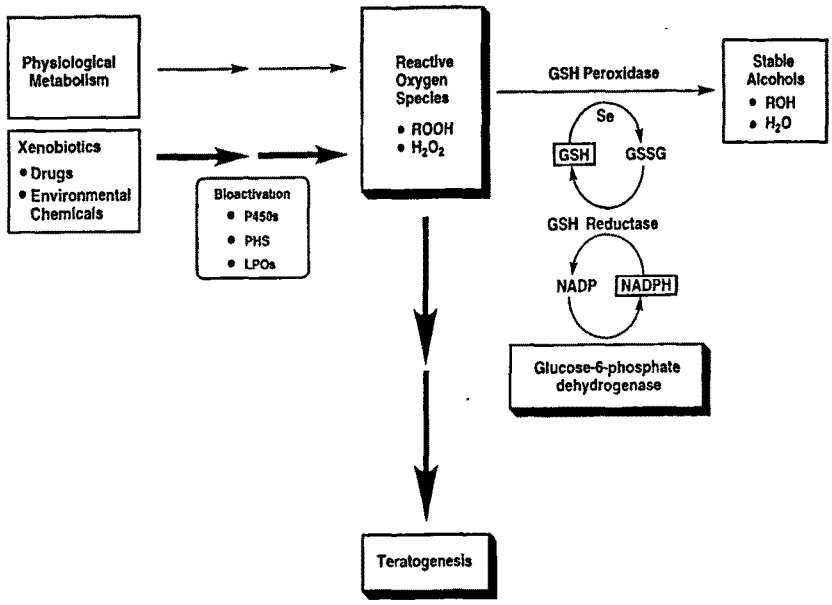

Figure 1. Postulated embryoprotective role of glucose-6-phosphate dehydrogenase (G6PD). Toxic reactive oxygen species (ROS) such as lipid hydroperoxides (LOOH) and hydrogen peroxide (H_2O_2) are formed endogenously and can be substantially enhanced by xenobiotic bioactivation catalyzed by the cytochromes P450 (P450s), peroxidases such as prostaglandin H synthase (PHS), and via reactions catalyzed by related enzymes such as lipoxygenases (LPOs) The detoxification of ROS by glutathione (GSH) peroxidase requires the cofactor GSH, which is oxidized to its disulfide, GSSG The chemical reduction of GSSG and maintenance of adequate GSH for ROS detoxification, as well as for the detoxification of free radical intermediates, ultimately is dependent on NADPH supplied by G6PD In addition, G6PD-dependent production of NADPH may be important in maintaining the activity of catalase, which also detoxifies H_2O_2 (37)

spherocytic hemolytic anemia to sepsis and life-threatening kernicterus in the newborn (9, 16).

It currently is believed that G6PD deficiencies constitute a problem only for mature red blood cells, which are non-nucleated and cannot synthesize more protective enzyme under conditions of oxidative stress (9, 13). However, embryonic tissues up to and including the critical period of organogenesis are remarkably deficient in the synthesis of many enzymes, including most of those providing cytoprotection against oxidative stress, such as GSH reductase, GSH peroxidase, superoxide dismutase, and catalase (4, 17). On the other hand, elevated G6PD activity during embryonic development corresponds to periods of both increased cellular proliferation and DNA synthesis (18, 19), suggesting that G6PD activity may be important for normal development. Accordingly, we hypothesized that G6PD-deficient embryos would be highly susceptible to normal developmental oxidative stress, and even more so to that initiated by oxidizing xenobiotics (Fig. 1). This hypothesis was tested in pregnant mutant C3H mice with heterozygous (+/−) or homozygous (−/−) deficiencies in G6PD activity, compared with congenic G6PD-normal controls (+/+). Dams either were allowed to deliver untreated or were treated during organogenesis with either the most commonly used anticonvulsant drug in North America, phenytoin (Dilantin), a human teratogen that is representative of xenobiotics known to initiate embryonic oxidative stress (20) or its vehicle. To determine the cytoprotective role of G6PD with respect to xenobiotic-initiated embryonic DNA damage, after maternal treatment with phenytoin during organogenesis, individual embryos were analyzed for both G6PD activity and DNA oxidation. The recently reported functional mutation in the mouse G6PD gene (21) was confirmed by a combination of direct sequencing and the development of a polymerase chain reaction (PCR)-based genotyping method, which was used to determine the frequency of the G6PD-deficient genotype in the remnants (resorptions) of embryos that died *in utero*. The results provide the first direct evidence of a critical embryoprotective role for G6PD in both endogenous and xenobiotic-initiated oxidative stress and DNA damage.

MATERIALS AND METHODS

Chemicals

Phenytoin (sodium salt) was purchased from Sigma Chemical (St. Louis, Mo). Carnoy's solution was made from 100% absolute ethanol, chloroform, and glacial acetic acid (6 3 1, v/v/v) (Sigma). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, Calif), and Albumin Concentrate (source: Bovine Serum) protein assay standard was purchased from Pierce (Rockford, Ill).

Animals

Breeding pairs of G6PD-mutant C3H mice were purchased from the Medical Research Council (MRC) of England (Genetics Division, MRC Radiobiology Unit, Chilton, U.K.). Same-sex animals were housed not more than three to one microisolator cage containing ground corncob bedding (Beta Chip, Northeastern Products, Warrensburg, N.Y.) and were maintained in a temperature-controlled animal facility with a 12 h light/dark cycle. Food (Laboratory Rodent Chow 5001; PMI Feeds, St. Louis, Mo.) and tap water were provided *ad libitum*. The genotype of all animals was confirmed phenotypically by analysis of RBC G6PD activity. To establish a breeding colony, three females were housed overnight with one male breeder starting at 5 00 P.M. Females were checked by 9 00 A.M. the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 1. Pregnant females were placed in their own microisolator cages and allowed to deliver spontaneously, with an average gestation of 20.5 days. The number of pups was recorded daily, and the pups were left with their mothers until weaning, 21 days after birth. The number and sex of weaned pups were recorded. Pups were ear-notched for identification and phenotyped by G6PD activity using tail vein blood.

Teratogenesis

Homozygous (+/+) G6PD-normal and heterozygous (+/−) and homozygous (−/−) G6PD-deficient females were mated with males that were hemizygous (−/y) G6PD-deficient as described above. Dams were either untreated, treated intraperitoneally at 9 00 A.M. on GDs 12 and 13 with vehicle alone or treated with a subteratogenic (20 mg/kg) or teratogenic dose (65 mg/kg) of phenytoin in saline containing 0.002N NaOH (17) and killed by cervical dislocation on GD 19. The uterus was exteriorized, implantations (fetuses and resorptions/*in utero* deaths) were noted, and fetuses and dissectable resorptions were removed. Fetuses were examined to determine sex, weight, and external anomalies. Viable fetuses were kept warm under a heat lamp (30°C) for 2 h to assess postpartum lethality. Fetuses subsequently were bled by decapitation and phenotyped for RBC G6PD activity. Resorptions and fetal tails were stored at −80°C for future study. Pinpoint resorptions were noted and left in the uterus, which was stored, similar to fetal heads and bodies, in Carnoy's solution for future analysis. Fetuses were later examined for internal anomalies.

G6PD gene sequencing

RNA was isolated and purified from +/+ G6PD-normal and −/− G6PD-deficient mouse spleen samples using a Qiagen RNeasy total RNA purification kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions. Purified RNA was subsequently converted to cDNA using a Gibco BRL Superscript Preamplification System for First Strand cDNA Synthesis (Gibco BRL [Canada], Burlington, Ontario) with oligo dT primers according to the manufacturer's instructions using a Perkin Elmer GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer [Canada], Mississauga, Ontario). Oligonucleotide primers for PCR reactions were synthesized based on previously reported C57BL/6 mouse G6PD cDNA sequences (11). cDNA coding for G6PD was subsequently amplified using the PCR. Amplified cDNA templates were subsequently used for direct cycle sequencing using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham [Canada], Oakville, Ontario) according to the manufacturer's instructions, where sequencing primers for both sense and antisense strands, designed to produce overlapping sequences, were based on the published normal G6PD mouse cDNA sequence (11). Samples were run on standard 6% polyacrylamide gels and exposed overnight prior to developing film. Data for G6PD mutant mouse intronic regions were sequenced from genomic mouse DNA samples derived from tail snips.

G6PD genotyping

DNA was isolated from late-stage (dissectable) fetal resorptions by the method of Gupta (22). G6PD mouse PCR primers (sense. GGAAACTGGCTGTGCGCTAC, antisense TCAGCTCCGGCTCTCTTCTG) were made between exon 1 and intron 1, around the reported mutation site (21). PCR conditions on a Perkin Elmer 9600 thermal cycler (Perkin-Elmer [Canada]) were 94°C for 2 min, 20 s at 94°C, 20 s at 58°C, and 30 s at 72°C for a total of 35 cycles, with a 5 min extension at 72°C and kept at 4°C until ready for digestion. PCR products were digested using *Dde*I restriction enzyme (Gibco BRL) at 37°C for 1 h and run on 3% agarose gels to determine G6PD genotype.

G6PD phenotyping

G6PD activity was measured in RBCs, whole embryo homogenates, and the 9,000 *g* supernatant from homogenized maternal organs using a standard reagent kit purchased from Sigma. Activities were measured over a 5 min interval at 37°C on a UV/vis spectrophotometer (model Lambda 3, Perkin-Elmer [Canada]) using a computer-assisted kinetic program. All results were standardized with respect to total protein content and reported in International Units per gram of protein (U/g). G6PD normal control standards (Sigma) were run concurrently with samples.

DNA oxidation

Females were mated as in the teratological studies. Dams were killed 6 h after maternal treatment with phenytoin (65 mg/kg i.p.) on GD 13. The uterus was exteriorized, and embryos were removed and homogenized separately. Once G6PD activity was measured, DNA was isolated from the remainder of the individual whole embryo homogenates by the method of Gupta (22), as modified in Winn and Wells (20). Embryonic DNA oxidation was measured by the method of Shigenaga and Ames (23), using high-performance liquid chromatography with electrochemical detection of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG).

Protein concentration assay

Protein content was analyzed using the standard Bio-Rad protocol (Bio-Rad, Hercules, Calif.), as detected by spectro-

photometric absorbance at 595 nm, using bovine serum albumin concentrate as a standard.

Statistical analysis

Binomial data were analyzed using χ^2 analysis or Fisher's exact test where appropriate Continuous data were analyzed using a two-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. The level of significance was $P < 0.05$.

RESULTS

Characterization of phenotype

Figure 2 presents differences in red blood cell (RBC) G6PD activities among G6PD mutant adult progenitor mice (3 months of age, from our colony), with hemizygous (−/y), heterozygous (+/−), and homozygous (−/−) mutants having 21, 53, and 17%, respectively, of normal RBC G6PD activity. Phenotyping of congenic G6PD normal and mutant animals was based on our progenitor RBC G6PD activities (see Fig. 2) and corroborated by comparisons with expected outcomes, from both parental matings and neonatal sex, given that the G6PD mutation is inherited via the X chromosome.

Untreated mice

In untreated dams allowed to deliver spontaneously, compared with congenic +/+ G6PD-normal controls, litter sizes for −/− G6PD-deficient animals were 50% smaller at birth (**Fig. 3**, upper panel) ($P<0.05$). Subsequently, by the time of weaning, litter sizes were 90% smaller, and the incidence of preweaning offspring death was threefold higher for untreated −/−G6PD-deficient dams compared with +/+ G6PD-normal controls ($P<0.0001$) (Fig. 3, lower panel).

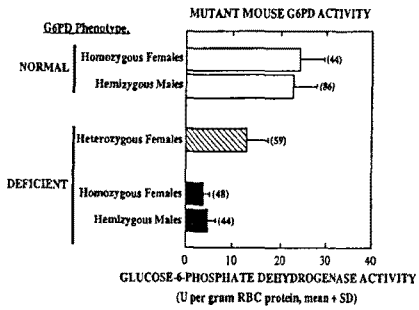

Figure 2. Characterization of G6PD phenotype Red blood cell (RBC) G6PD activities among congenic adult progenitors (3 months of age, from our colony) for homozygous (+/+) and hemizygous G6PD-normal (+/y), and heterozygous (+/−), homozygous (−/−), and hemizygous (−/y) G6PD mutant mice are presented. The total number of mice from a particular phenotype is given in parentheses Adult G6PD activities were used in subsequent studies to assess embryonic and maternal G6PD phenotype.

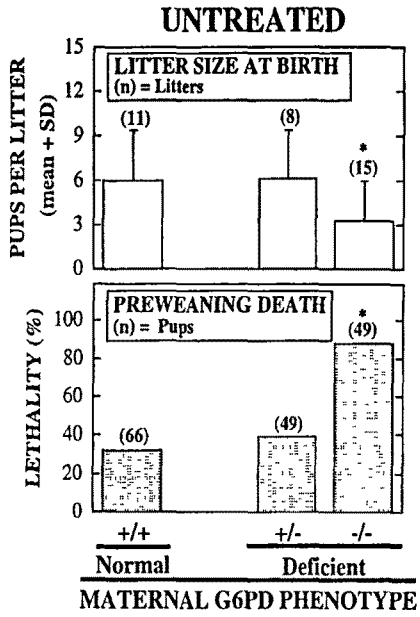

Figure 3. Spontaneous embryopathies in untreated G6PD-deficient and G6PD-normal dams Pregnant heterozygous (+/−) and homozygous (−/−) G6PD-deficient dams, and homozygous (+/+) G6PD-normals, were allowed to deliver spontaneously, and litter sizes were determined on the day of birth and 21 days later at the time of weaning *Upper panel*, litter size at birth The total number of litters from a given maternal phenotype is given in parentheses The asterisk indicates a difference from +/+ G6PD-normal controls ($P<0.05$) *Lower panel*, incidence of preweaning death of pups born live and dying before weaning The total number of viable pups for a given maternal phenotype is given in parentheses. The asterisk indicates a difference from G6PD-normal controls ($P<0.0001$)

Vehicle control mice

Homozygous G6PD-normal (+/+) and heterozygous (+/−) and homozygous (−/−) G6PD-deficient mice were injected intraperitoneally on GD 12 and 13 with the saline/NaOH vehicle for phenytoin and killed on GD 19. Compared with +/+ G6PD-normal dams, +/− and −/− G6PD-deficient dams had 6- and 7-fold increases, respectively, in fetal resorptions (*in utero* deaths) ($P<0.0001$), 6- and 11-fold increases, respectively, in postpartum lethality ($P<0.0001$), and 7 and 16% decreases, respectively, in fetal body weight ($P<0.05$) (**Fig. 4**).

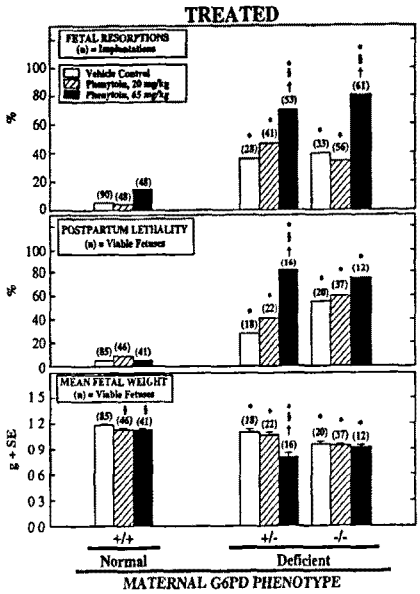

Figure 4. Embryopathies in treated normal and G6PD-deficient mice by maternal phenotype Dams were treated intraperitoneally (i p) on gestational days (GDs) 12 and 13 with either phenytoin or its vehicle and killed on GD 19. *Upper panel*, fetal resorptions (*in utero* deaths) were calculated by dividing the total number of resorptions by the total number of implantations (resorptions plus fetuses) for a particular maternal phenotype. The total number of implantations is given in parentheses. *Middle panel*, postpartum lethality was calculated by dividing the total number of fetuses born live and dying within 2 h by the total number of viable fetuses for a given maternal phenotype The total number of viable fetuses is given in parentheses. *Lower panel*, for mean fetal weights, the total number of viable fetuses for a given maternal phenotype is given in parentheses Asterisks indicate a difference from respective +/+ G6PD-normal controls (P<0 002), § indicates a difference from vehicle controls of the same phenotype (P<0.05), and † indicates a difference from respective phenytoin (20 mg/kg) groups of the same phenotype (P<0 05).

With respect to embryonic phenotype, compared with +/y G6PD-normal fetuses, independent of sex, +/− and −/− and −/y G6PD-deficient fetuses had enhanced postpartum lethality, following a gene-dose pattern with 0% in +/y G6PD-normal fetuses, 10% in +/− G6PD-deficient fetuses, and 50% in combined −/− and −/y G6PD-deficient fetuses (P<0.0001). A similar gene-dose pattern was observed with fetal body weight, which, compared with +/y G6PD-normal fetuses, was decreased by 6% in fetuses with a mutation in one G6PD allele (+/−) and by 18% in fetuses with a mutation in all alleles (−/− and −/y) (P<0.05) (**Fig. 5**) Furthermore, the mean weight of fetuses with a mutation in all G6PD alleles (−/− and −/y) was 13% lower than that of fetuses with a mutation in one allele (+/−) (P<0.05).

Phenytoin-treated mice

With respect to maternal phenotype, compared with vehicle-treated controls of the same phenotype, a standard teratogenic dose of phenytoin (65 mg/kg) enhanced fetal resorptions twofold in both +/− and −/− G6PD-deficient dams (P<0.05), and in +/− G6PD-deficient dams, enhanced postpartum lethality threefold and decreased fetal weight by 27%

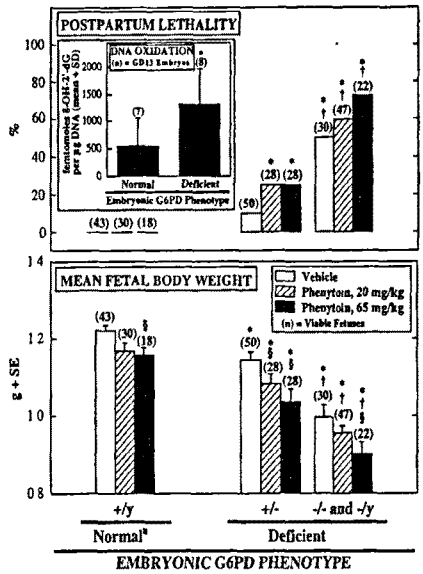

Figure 5. Embryonic DNA oxidation and embryopathies for treated G6PD-deficient mice analyzed by embryonic phenotype Fetuses from dams described in Fig. 4 were phenotyped by RBC G6PD activity. Homozygous female (−/−) and hemizygous male (−/y) G6PD-deficient fetuses with all alleles mutated were identically affected, and these data were combined in this and all subsequent analyses The total number of viable fetuses for a particular phenotype is given in parentheses. *Upper panel*, postpartum lethality was calculated as described in Fig 4. *Upper panel inset*, embryonic DNA oxidation as a reflection of oxidative stress and DNA damage in GD 13 embryos exposed 6 h before to phenytoin, 65 mg/kg i p. The total number of embryos from each group is given in parentheses, and the asterisk indicates a difference from G6PD-normal littermates (P<0 03). Normal whole embryo G6PD activity was defined as ≥25 U/g protein. *Lower panel*, mean fetal body weights. Asterisks indicate a difference from respective G6PD-normal groups (P<0 05), § indicates a difference from vehicle controls from the same phenotype (P<0 05), and † indicates a difference from respective +/− G6PD-deficient fetal groups (P<0 05). *a* indicates that +/+ G6PD-normal fetuses are not achievable with −/y male breeders.

($P<0.05$) (Fig. 4). Among phenytoin-treated dams (65 mg/kg), compared with +/+ G6PD-normal controls, in both +/− and −/− G6PD-deficient dams respectively, the incidence of fetal resorptions was enhanced 4.8- and 5.5-fold ($P<0.0003$), the incidence of postpartum lethality was enhanced 16.6- and 15.4-fold ($P<0.002$), and fetal weight was decreased by 18–29% ($P<0.05$) (Fig 4).

With a lower dose of phenytoin (20 mg/kg) that is nonteratogenic in other strains (4, 5) as well as the wild type of this G6PD mutant, all embryopathies in phenytoin-treated +/− and −/− G6PD-deficient dams were increased above the values observed in phenytoin-treated +/+ G6PD-normal controls ($P<0.05$). However, when analyzed by maternal phenotype, unlike embryonic phenotype (see below), these values were only different from vehicle controls of the same maternal phenotype among +/+ dams (Fig 4).

Embryonic phenotype

With respect to embryonic phenotype, the higher dose of phenytoin (65 mg/kg) caused a 2.4-fold increase in embryonic DNA oxidation in G6PD-deficient fetuses compared to G6PD-normal littermates ($P<0.03$) (Fig. 5, upper panel, inset), and a decrease in fetal body weight in all phenotypes, with the weight loss being progressively worse with one or two mutated G6PD alleles (Fig 5, lower panel). Thus, compared to vehicle controls of the same phenotype, the phenytoin-initiated decrease in fetal weight was 5% in +/y G6PD-normal fetuses, 9 5% in +/− G6PD-deficient fetuses, and 9% in −/− and −/y G6PD-deficient fetuses ($P<0.05$). The high dose of phenytoin also appeared to enhance postpartum lethality in all G6PD-deficient fetuses, although these differences were not statistically significant (Fig. 5, upper panel).

Among only those fetuses exposed to phenytoin (65 mg/kg), compared with +/y G6PD-normal fetuses, which had no postpartum lethality, phenytoin-initiated postpartum lethality and decreased fetal body weight were substantially worse in +/− G6PD-deficient fetuses, and even more so in −/− and −/y G6PD-deficient fetuses ($P<0$ 05) (Fig. 5). Thus, postpartum lethality initiated by phenytoin (65 mg/kg) was increased 2.9-fold in −/− and −/y fetuses compared with +/− fetuses, which in turn were substantially more affected than +/y G6PD-normal fetuses (25 vs. 0%) ($P<0.05$). Similarly, with phenytoin-exposed fetuses, the mean weight of combined −/− and −/y fetuses was decreased by 12.9% compared with +/− fetuses, which in turn had weights 10.5% lower than those in +/y G6PD-normal fetuses ($P<0.05$). There were no apparent gender differences in teratological susceptibility (data not shown).

Similar to the effects seen among +/+ dams for mean fetal body weight, when the data were analyzed by embryonic phenotype, there was evidence for an embryopathic effect of the lower dose of phenytoin (20 mg/kg). This pattern was observed for both enhanced postpartum lethality and decreased fetal body weight but was statistically significant only for the latter in +/− G6PD-deficient embryos ($P<0.05$) (Fig. 5).

Teratological syndrome

Viable fetuses from the teratological studies were examined in a blinded fashion for both external and internal anomalies (**Fig. 6**). These structural anomalies, collectively referred to here as a syndrome, included cleft palate, club foot, dilated bladder, dilated cerebral ventricles, ectopic kidney, hematoma, microcephaly, micrognathia, omphalocele, open eye, red nevus, and underdeveloped renal papilla. A gene-dose response was observed in both vehicle- and phenytoin-treated groups, and a drug-

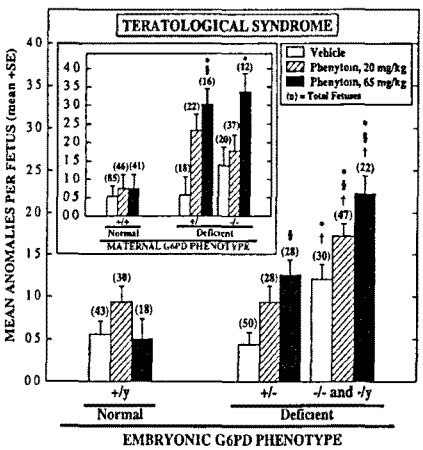

Figure 6. Teratological syndrome in treated G6PD-deficient mice by embryonic and maternal phenotype These data were obtained from the dams described in Fig 4 treated with phenytoin or its vehicle Visible anomalies, collectively referred to as a syndrome, included cleft palate, club foot, dilated bladder, dilated cerebral ventricle, ectopic kidney, hematoma, microcephaly, micrognathia, omphalocele, open eye, red nevus, and underdeveloped renal papilla The severity of the teratological syndrome was calculated as the total number of anomalies for a given phenotype divided by the total number of fetuses for that phenotype. The total number of fetuses for a particular phenotypic group is given in parentheses *Inset*, teratological syndrome by maternal phenotype Asterisks indicate a difference from respective G6PD-normal groups ($P<0$ 05), § indicates a difference from vehicle controls from the same phenotype ($P<0$ 05), and † indicates a difference from respective +/− G6PD-deficient groups ($P<0.05$)

dose response was observed in phenytoin-treated animals. This pattern was observed by both embryonic (Fig. 6) and maternal (Fig. 6, inset) phenotype, but was most clearly defined by embryonic phenotype. Data for −/− (female) and −/y (male) G6PD-deficient fetuses were analyzed independently and found to be identical, hence these two phenotypes with all alleles mutated were combined for all final analyses.

For fetuses exposed only to vehicle, anomalies in −/− and −/y G6PD-deficient fetuses were over twofold higher than in either +/− G6PD-deficient or +/y G6PD-normal fetuses ($P<0.05$) (Fig. 6). A similar twofold enhancement was observed by maternal phenotype, but was not statistically significant (Fig. 6, inset).

For fetuses exposed to the highest dose of phenytoin (65 mg/kg), compared with +/y G6PD-normal fetuses, anomalies were increased 2.5-fold in +/− G6PD-deficient fetuses and 4.5-fold ($P<0.05$) in −/− and −/y G6PD-deficient fetuses (Fig. 6). For all G6PD-deficient fetuses, this enhancement also was observed relative to vehicle controls of the same phenotype ($P<0.05$). The higher dose of phenytoin produced more anomalies than the lower dose (20 mg/kg) in −/− and−/y G6PD-deficient fetuses ($P<0.05$). With the lower phenytoin dose, compared with +/y G6PD-normal phenytoin-treated controls, anomalies were not increased in +/− fetuses, but were increased almost twofold in −/− and −/y fetuses ($P<0.05$). When compared irrespective of embryonic phenotype, the lower dose of phenytoin caused a 1.6-fold increase in anomalies compared with vehicle controls ($P<0.05$).

By maternal phenotype, a similar pattern of phenytoin-enhanced anomalies was observed, except that +/− G6PD-deficient dams were as susceptible as −/− dams, and even the lower dose of phenytoin was teratogenic. The lower phenytoin dose caused over a threefold, albeit nonsignificant, increase in anomalies in +/− G6PD-deficient dams compared with +/+ G6PD-normal phenytoin-treated dams and a significant fourfold increase when compared with pooled +/+ and +/− vehicle controls ($P<0.05$) (Fig. 6, inset). With the higher dose of phenytoin compared with respective vehicle controls, there was a fivefold increase in anomalies in +/− G6PD-deficient dams ($P<0.05$). A similar but lower twofold enhancement was observed in −/− 6PD-deficient dams because of the 2.6-fold increase in anomalies in the vehicle controls for this phenotype. When combined, +/− and −/− G6PD-deficient dams treated with the higher dose of phenytoin had over fourfold more anomalies than respective phenytoin-treated +/+ G6PD-normal dams ($P<0.05$). Similarly, combined +/− and −/− G6PD-deficient dams treated with either a high or low dose of phenytoin had three- and twofold more anomalies, respectively, compared with their combined vehicle controls ($P<0.05$). The higher dose of phenytoin was more teratogenic than the lower dose, but unlike by embryonic phenotype, the difference by maternal phenotype was statistically significant only when the data for +/− and −/− G6PD phenotypes were combined ($P<0.05$).

In G6PD-normal animals, phenytoin in either dose did not increase fetal anomalies compared with vehicle controls in either +/y fetuses (Fig. 6) or +/+ dams (Fig. 6, inset).

Mutational analysis and genotyping for late *in utero* embryonic death

We were able to confirm the reported mutation (21) causing the heritable decrease in G6PD activity in our strain of mutant mice. This involved direct sequencing of not only the full-length mutant mouse cDNA, which includes the 5′ and 3′ untranslated regions and the entire coding region for the G6PD protein, but also 6 of the 12 intronic G6PD genomic DNA regions, including introns 4, 6, 7, and 10–12. Apart from the reported functional mutation in our C3H mutant mouse strain, we found a single silent A to C mutation, located at base 718 of the mouse cDNA sequence. This silent mutation differs from the reported C57 mouse strain cDNA (11) and corresponds to the human DNA sequence at base 15361 (24), maintaining the amino acid sequence code of GGC for glycine.

We also noted that the single functional point mutation, an A to T transversion in the 5′ untranslated region of the gene at the penultimate base of the 3′ end of exon 1, results in the destruction of a *DdeI* restriction enzyme site in the mutant allele. We used this information to develop a genotyping assay to characterize the functional G6PD mutation in the remnants of dissectable fetal resorptions from our teratological studies (**Fig. 7**). In short, we designed a pair of PCR primers around the mutation site, based on the known sequence of the G6PD gene, expected to produce a PCR fragment from mouse genomic DNA of 269 base pairs (bp) in length. For normal mice, subsequent digestion of PCR products with the *DdeI* restriction enzyme would produce two cleaved fragments of 214 bp and 55 bp, whereas mice with mutations in all G6PD alleles would not show a change in fragment size, and heterozygotes were expected to show all three fragments All three bands were initially observed, as expected, by polyacrylamide gel electrophoresis (PAGE) analysis, but subsequent analysis of samples was performed using agarose gel electrophoresis, in which the 55 bp fragment did not resolve from the leading xylene cyanole band of the loading buffer (Fig. 7).

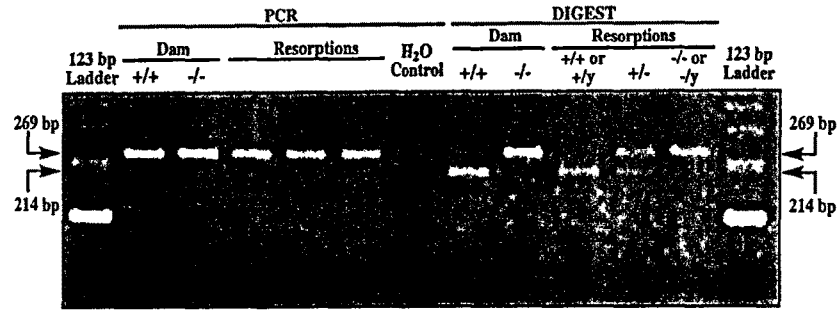

Figure 7. G6PD mutant mouse genotyping assay. PCR-based, *DdeI* digest genotyping assay of dams and late-stage (dissectable) embryonic resorptions (*in utero* deaths), illustrating wild-type (+/+ or +/y), heterozygous (+/−) and homozygous (−/−) or hemizygous (−/y) G6PD-deficient mutant mice genotypes. PCR primers were designed to generate a 269 bp product, which on digestion with *DdeI* would produce fragments of 214 bp and 55 bp in +/+ or +/y G6PD-normal mice, no change in molecular size in −/− or −/y mutant G6PD-deficient mice, and all three fragments in +/− mutant G6PD-deficient mice. Lanes 1 and 13 represent 123 bp ladder molecular weight controls. Lanes 2 to 7 represent PCR reaction products of phenotypically characterized maternal and uncharacterized embryonic resorption DNA or a water control. Lanes 8 to 12 represent *DdeI* digest products of maternal and embryonic resorption PCR products listed above. The embryonic resorptions illustrated were obtained from G6PD-dams confirmed to have a genotype different from that of the respective resorption.

Based on an approach previously established in our laboratory (25), these studies allowed us to determine the potential protective role of G6PD with respect to *in utero* fetal death (**Fig. 8**). Among the dissectable fetal resorptions reflecting *in utero* death late in gestation, compared with +/y G6PD-normal fetuses, G6PD-deficient fetuses with a mutation in either one (+/−) or all (−/− and −/y) G6PD alleles had over six- and fivefold increases, respectively, in *in utero* deaths when compared independent of treatment ($P<0.0001$) (Fig. 8). When analyzed by treatment, compared with the +/y G6PD-normal embryonic genotype, the incidence of late fetal resorptions was substantially increased to approximately the same extent in all G6PD-deficient embryonic genotypes by both vehicle and phenytoin treatments ($P<0.03$), although the difference was statistically marginal in vehicle-exposed +/− fetuses ($P<0.06$) (Fig. 8, inset).

Early *in utero* embryonic death

In dams treated with phenytoin, there also were a substantial number of pinpoint resorptions that were too small to dissect, reflecting *in utero* embryonic death early in gestation (**Fig. 9**). This early embryonic death, which was both G6PD gene dose- and phenytoin dose-related, occurred postimplantation, as the number of implantations did not differ among the maternal phenotypes. The number of implantations (mean ± SD), irrespective of treatment, was 9.79 +/− 0.85 for +/+ G6PD-normal dams vs. 8.13 ± 3.20 for ± and 8.82 ± 3.57 for −/− G6PD-deficient dams. In +/− and −/− G6PD-deficient dams, both the low dose (20 mg/kg) and the high dose (65 mg/kg) of phenytoin substantially enhanced the incidence of pinpoint resorptions to a similar extent compared with respective vehicle controls, which had no such early resorptions in any treatment group ($P<0.01$) (Fig. 9). With the high dose of phenytoin, the incidence of early embryonic death was enhanced three- and fivefold ($P<0.02$), respectively, in +/− and −/− G6PD-deficient dams compared with +/+ G6PD-normal dams, and the incidence was 1.6-fold higher in −/− dams compared with +/− dams ($P<0.03$). A remarkably identical pattern of maternal phenotypic susceptibility to, and magnitude of, early embryonic death was observed with the low dose of phenytoin ($P<0.01$), although the enhancement in +/− dams was statistically marginal ($P<0.07$).

Embryonic and maternal G6PD activities

On GD 13, within the period of organogenesis, whole embryo G6PD activity was 43% lower in G6PD-deficient embryos compared with G6PD-normal littermates (**Fig. 10**). In this case, G6PD-deficient embryos included +/−, −/y, and −/− genotypes, because these studies preceded the establishment of our genotyping technique. At the end of gestation, on day 19, G6PD activity in normal fetuses was lower than that during organogenesis and remained at this same lower level at the time of weaning (Fig. 10) and into adulthood (see Fig. 2). A similar pattern was observed in G6PD-deficient fetuses, whereby compared with GD 13 whole embryo activity, RBC activity on day 19 among combined fetuses with a mutation in one or all G6PD alleles had declined by 64%

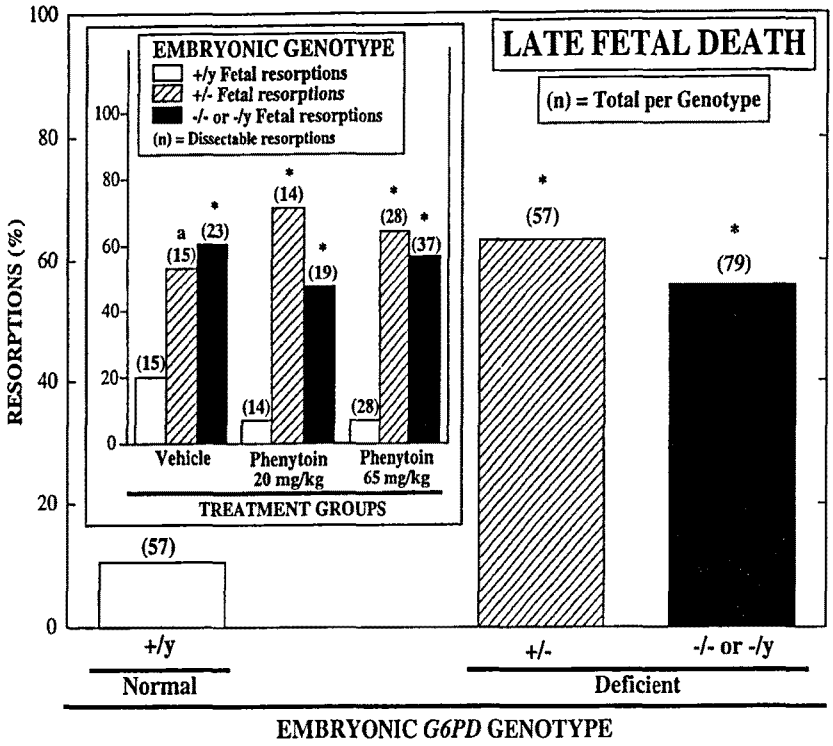

Figure 8. Effect of embryonic G6PD genotype on the incidence of late *in utero* death These data are from the animals described in Fig. 4 Late resorptions were the remnants of fetuses that died *in utero* sufficiently late in gestation that a dissectable mass remained For a particular embryonic genotype, incidence was calculated by dividing the number of typeable resorptions for a given genotype, independent of treatment, by the total number of implantations (fetuses and resorptions) for that embryonic genotype The number of implantations for a particular embryonic genotype is given in parentheses Asterisks indicate a difference from respective +/y G6PD-normal controls ($P<0.0001$). *Inset,* the individual late-stage embryonic resorption genotypes for each treatment group Incidences for a particular genotype were calculated by dividing the number of typeable resorptions for a given treatment group by the total number of dissectable resorptions for that respective treatment group The number of dissectable resorptions in each treatment group is given in parentheses Asterisks indicate a difference from respective +/y G6PD-normal controls ($P<0.05$) *a* indicates a marginal difference from respective +/y G6PD-normal controls ($P<0.06$).

($P<0.05$) and remained constant thereafter On day 19, compared with +/+ G6PD-normal littermates, RBC G6PD activity in +/− G6PD-deficient fetuses was 48% of normal ($P<0.05$) and in −/y and −/− G6PD-deficient fetuses was 18% of normal ($P<0.05$). This pattern and the respective RBC activities remained similar at weaning, 58 and 19%, respectively, of normal ($P<0.05$) (Fig. 10).

The relative distribution of G6PD activities in different organs was similar for all phenotypes, with up to an 11-fold difference between the highest (spleen) and the lowest (heart) activities (**Fig. 11**). In spleen, compared with +/+ G6PD-normal dams, G6PD activities in +/− and −/− G6PD-deficient dams were 79 and 27%, respectively, of normal ($P<0.05$), and similar patterns were evident for other organs and blood.

DISCUSSION

Toxicologic implications of G6PD deficiency

It is widely believed that serious health risks from hereditary G6PD deficiencies are limited primarily to mature red blood cells, which lack a nucleus and are unable to synthesize more protective enzyme under conditions of oxidative stress With respect to G6PD deficiency and newborns, RBC hemolysis is known to result in hyperferremia and hyperbilirubinemia

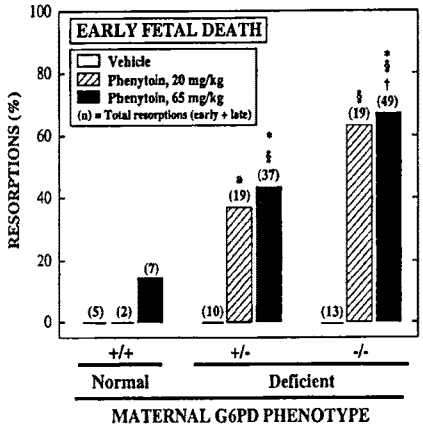

Figure 9. Effect of maternal G6PD phenotype on early *in utero* embryonic death. These data are from the animals described in Fig 4 Early resorptions were the remnants of embryos that died sufficiently early in gestation that the remaining mass of tissue was not reliably dissectable from the uterus For a particular treatment group, incidence was calculated by dividing the number of early-stage fetal resorptions by the total number of resorptions (early- plus late-stage) for the respective maternal phenotype. The total number of resorptions for a particular group is given in parentheses. Asterisks indicate a difference from respective +/+ G6PD-normal groups ($P<0.03$), § a difference from the vehicle control of the same phenotype ($P<0.01$), and † a difference from the respective +/− G6PD-deficient group ($P<0.03$) *a* indicates a marginal difference from the vehicle control of the same phenotype ($P<0.07$)

(jaundice) leading to increased sepsis and/or potentially life-threatening kernicterus, an encephalopathy caused by excessive bilirubin in the brain (9, 16, 26). However, most embryonic tissues *in utero* are known to synthesize only low levels of most enzymes involved in the detoxification of xenobiotic reactive intermediates and reactive oxygen species (ROS), particularly up to the end of organogenesis, the critical period of teratological susceptibility (4, 17). On the other hand, elevated G6PD activity during embryonic development corresponds to periods of both increased cellular proliferation and DNA synthesis (18, 19), suggesting that G6PD activity may be important for normal development. Indeed several groups have shown peak G6PD activities in various nonmurine whole embryos and their organs occur during the early stages of development and subsequently decline to adult levels near the end of gestation (18, 27–29). These results correspond to our own and are suggestive of the critical importance of G6PD during embryonic development Moreover, similar to avian species, which have nucleated RBCs (30, 31), samples from human maternal circulation found the amount of nucleated fetal RBCs per 40 ml of blood increased as gestation progressed, from 0.1% at 6 wk to 1% at term (32); the latter is comparable to the proportion of reticulocytes among all cells in normal human adult blood (33). If this increased percentage of nucleated RBCs occurs similarly within the fetus, then this may assist in providing protection for a normal developing fetus at a particular gestational age because of the ability to synthesize more G6PD enzyme under conditions of oxidative stress. However, although chickens have nucleated RBCs, they are still susceptible to phenytoin teratogenesis (34). We accordingly hypothesized that G6PD-deficient embryos would be unable to mount an adequate antioxidative response to normal developmental oxidative stress, as well as that initiated by xenobiotics, and hence be highly susceptible to ROS-mediated damage (Fig 1).

In our mutant C3H mouse model, RBC G6PD activities at weaning of heterozygous (+/−) and homozygous (−/−) G6PD-deficient females and hemizygous (−/y) G6PD-deficient males were 56, 16, and 21%, respectively, of the activity in congenic wild-type G6PD-normal female (+/+) and male (+/y) mice, similar to previously reported respective activities of 60, 15, and 20% (35). More importantly, during the period of organogenesis, while normal embryonic G6PD activity was 1.6-fold higher than RBC activity at weaning, G6PD-deficient embryos had <58% of the activity in G6PD-normal litter-

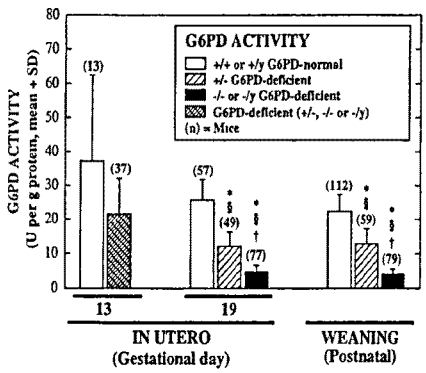

Figure 10. G6PD activity in mutant mice at various stages of development post fertilization The data for GD 13 embryos was obtained before our development of a genotyping assay, hence, for this group, the +/− G6PD-deficient embryos were not distinguishable from the −/− and −/y G6PD-deficient embryos The embryonic G6PD-deficient phenotype was based on both an activity <25 U/g protein and the expected genotype as determined from maternal and paternal matings Mice were weaned 21 days after birth Asterisks indicate a difference from GD 13 G6PD-deficient embryos ($P<0.05$), § indicates a difference from the respective G6PD-normal controls ($P<0.05$), and † indicates a difference from the respective +/− G6PD-deficient group ($P<0.05$) The number of mice is given in parentheses

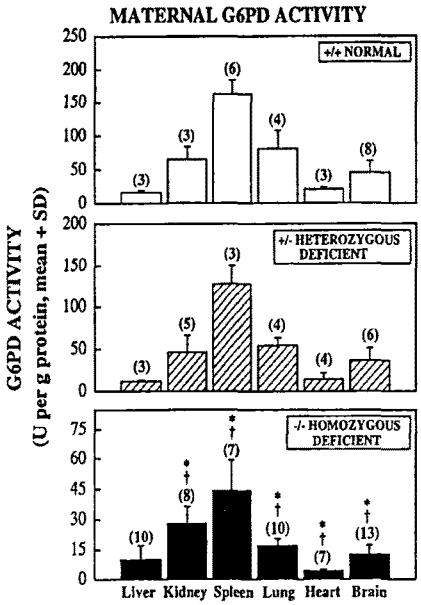

Figure 11. Maternal G6PD activity in various organs from normal and G6PD-deficient mice. Note the differences in scale of the y axes for each phenotype. Organs were obtained from dams killed on GD 19 for the teratological studies described in Fig. 4. The number of animals is given in parentheses. Asterisks indicate a difference from the same organ in +/+ G6PD-normal dams, and † indicates a difference from the same organ in +/− G6PD-deficient dams ($P<0.05$).

mates, potentially leaving the G6PD-deficient embryos more susceptible to embryopathic oxidative stress. G6PD activities likely were substantially lower in −/− and −/y than +/− G6PD-deficient embryos, but these studies were performed before the development of our genotyping assay, precluding subclassification by embryonic genotype. However, relative differences can be inferred from fetal G6PD activities on GD 19 when, compared with +/y G6PD-normal littermates, +/− G6PD-deficient fetuses had only 48% of normal fetal activity, and fetuses with all alleles mutated. (−/− and −/y) had only 18% of normal activity. The observed enhanced susceptibility of G6PD-deficient mice to the embryopathic effects of both normal developmental oxidative stress, and even more so to that initiated by the ROS-initiating anticonvulsant drug phenytoin, indicates that G6PD is a critical embryoprotective enzyme. Embryonic DNA oxidation and a broad spectrum of apparent ROS-mediated embryopathies were enhanced in G6PD-deficient animals, including fetal resorptions (*in utero* death), postpartum and preweaning lethality, decreased fetal body weight, and a syndrome of structural anomalies, including open eye, facial, renal, bladder, and cerebral ventricular defects. This C3H-derived mutant strain, similar to previous findings using inbred C3H mice (36), is resistant to one of the hallmarks of phenytoin teratogenicity, cleft palates. Although the number of fetuses affected was too small for statistical analysis, it was interesting that cleft palates were observed only in G6PD-deficient fetuses treated either with vehicle (1 out of 50 +/− fetuses and 1 out of 15 −/y fetuses) or phenytoin (65 mg/kg) (1 out of 28 +/− fetuses). The enhanced susceptibility of G6PD-deficient animals also corroborates other evidence indicating the importance of oxidative stress in the molecular mechanism of phenytoin teratogenesis (37) and further suggests that, contrary to the apparent safety of oxidizing drugs like phenytoin in adult G6PD-deficient patients (9), embryos in general, and G6PD-deficient embryos in particular, may be at serious risk.

Normal developmental oxidative stress and teratogenesis

In untreated mice allowed to deliver spontaneously in the breeding colony, the decreased litter size at birth, and increased preweaning death in homozygous (−/−) G6PD-deficient dams (Fig. 3) provides the first direct evidence that a physiological level of endogenous oxidative stress during development can be embryopathic and may contribute substantially to both apparent infertility and postnatal death. In addition, this is the first direct evidence that G6PD is a critical embryoprotective enzyme for normal development.

In vehicle-treated mice killed just before the time of delivery and examined more comprehensively, a striking, broader spectrum of increased embryopathies, including fetal resorptions, postpartum lethality, decreased fetal weight, and a syndrome of teratological anomalies, was observed in G6PD-deficient animals (Figs. 4–6), suggesting an extensive embryopathic potential for developmental levels of ROS production and a broad embryoprotective role for G6PD. Given that no pinpoint resorptions reflecting early *in utero* death were observed in vehicle controls, and there were no differences in the number of implantations among all groups, it appears that the apparent infertility in G6PD-deficient animals is because of postimplantational, relatively late *in utero* death. The obligatory role of ROS in the mechanism of vehicle-initiated embryopathies is suggested by the observation that these effects were found only in G6PD-deficient dams and fetuses. By both maternal and embryonic genotype, all developmental parameters for vehicle-treated G6PD-normal animals were comparable to the normal range for extensive con-

trol groups from other murine strains studied in our laboratory (17). Previous *in vivo* studies in our laboratory also have shown that the vehicle does not measurably enhance oxidative stress and hydroxyl radical formation (38), indicating that the observed embryopathies in vehicle-treated controls are the result of normal developmental oxidative stress. For many embryopathies (fetal resorptions, postpartum lethality, decreased fetal weight), increased susceptibility was as great in +/− as in −/− G6PD-deficient dams, indicating a potentially widespread developmental relevance of G6PD deficiencies. By embryonic phenotype, the substantially increased severity of vehicle-initiated embryopathies (Fig 5), including teratologic anomalies (Fig. 6), in G6PD-deficient fetuses provided direct and proximal evidence that G6PD-deficient fetuses are exquisitely susceptible to developmental oxidative stress. While −/− and −/y G6PD-deficient fetuses were most adversely affected for several embryopathies, there was evidence of a gene-dose effect, wherein +/− fetuses also had significantly decreased body weight and appeared to be at increased risk of postpartum lethality compared with +/y G6PD-normal fetuses. The significant intermediary embryopathic risk for +/− dams and fetuses is consistent with their intermediate deficiency in G6PD activity.

Using direct sequencing in the mutant C3H model, we did not find any functional mutations in either the entire coding region or the 3′ untranslated region of the cDNA, nor in at least 6 of the 12 intronic regions of genomic DNA We did find a strain difference between our C3H mutant mouse strain and the reported C57 mouse cDNA (11) at base 718; however, this sequence alteration does not change the amino acid code for glycine at this position and as such is not expected to have any effect on G6PD activity At that time, the functional mutation was published, identifying a single point mutation, an A to T transversion, in the 5′ untranslated region of the gene at the penultimate base of the 3′ end of exon 1 (21). We confirmed this mutation and then developed a genotyping assay that allowed us to determine the G6PD genotype in the dissectable remnants (resorptions) of embryos that died *in utero* (Fig. 7), as we previously had successfully used in characterizing the role of p53 as a teratological suppressor gene (25). This approach is applicable to embryos that die later in gestation leaving a sufficient amount of tissue to be reliably dissectable without maternal tissue contamination. In vehicle-exposed embryos, the threefold greater incidence of resorptions for −/− and −/y G6PD-deficient fetuses compared with +/y G6PD-normal littermates (Fig. 8, inset) shows directly that normal developmental oxidative stress can play a major role in *in utero* death, with G6PD serving as a critical embryoprotective pathway. Remarkably, a similar threefold increase in fetal resorptions was observed even in +/− G6PD-deficient embryos, although this apparent enhancement was only marginally significant ($P<0.06$). However, the incidence of these resorptions appeared to be at a maximal level that was not further enhanced by phenytoin, and when the data were analyzed independent of treatment, heterozygous G6PD-deficient embryos were as susceptible as embryos with mutations in all G6PD alleles, demonstrating a highly significant sixfold increase over G6PD-normal littermates (Fig. 8) and indicating a potentially broad population at risk.

Embryos dying early in gestation were observed as nondissectable pinpoint resorptions, however this earlier embryopathy was not observed with vehicle-exposed embryos of any genotype, suggesting that the rate as well as the extent of embryolethality initiated by developmental oxidative stress are less than that initiated by xenobiotics. As well, the extent of this early embryopathy, especially in G6PD-deficient dams, likely contributed to the apparent lack of a xenobiotic-initiated effect among resorptions occurring later in gestation. In general, the developmental risk in G6PD-deficient heterozygotes, in some cases equivalent to that for dams and fetuses with mutations in all G6PD alleles, reveals a potentially substantial clinical risk from G6PD deficiencies under conditions of normal developmental oxidative stress

Xenobiotic-initiated oxidative stress and teratogenesis

Phenytoin and related proteratogens, including the sedative drug thalidomide, are bioactivated by embryonic prostaglandin H synthases, lipoxygenases, and related enzymes to a free radical intermediate, which causes embryonic oxidative stress, hydroxyl radical formation, and oxidative damage to DNA and other cellular macromolecules in embryonic tissues (4, 17, 37, 39, 40). Thus, the dose-dependent increase in embryopathies caused by phenytoin by both maternal and embryonic analyses, together with the enhanced susceptibility of G6PD-deficient dams and embryos compared with vehicle controls (Figs. 4–6, 8) and the enhanced phenytoin-initiated DNA oxidation in G6PD-deficient embryos (Fig 5, inset), provide the first direct evidence that G6PD is a major embryoprotective enzyme for xenobiotic-initiated oxidative stress and embryonic macromolecular target damage. The susceptibility of heterozygous (+/−) G6PD-deficient animals to phenytoin embryopathies was particularly remarkable, generally exhibiting a risk intermediate, if not equal, to that of homozygous (−/−) and hemizygous (−/y) G6PD-deficient dams and fetuses, depending on the

parameter. This pattern differed somewhat only with *in utero* death, wherein the incidence of early but not late fetal resorptions was enhanced over vehicle controls (Figs. 8, 9), although both types of *in utero* death were substantially enhanced in G6PD-deficient embryos, and the risk in heterozygotes was similar to that for homozygous and hemizygous G6PD-deficient embryos. To an extent greater than that in the vehicle controls, the embryopathic susceptibility of even heterozygous G6PD-deficient dams and embryos to phenytoin suggests that G6PD deficiencies may have broad developmental relevance for exposures to drugs and environmental chemicals like phenytoin that initiate oxidative stress

With the lower, 20 mg/kg dose of phenytoin, the enhanced embryopathies (decreased fetal body weight, early resorptions, teratological anomalies) compared with vehicle controls was remarkable, as this is <40% of a threshold teratogenic dose (55 mg/kg) in normal mice (17). For some embryopathies (e g., early resorptions, teratological anomalies), a maximal response was achieved with the lower 20 mg/kg dose in both +/− and −/− and −/y G6PD-deficient littermates. These results demonstrate that G6PD-deficient animals are exceptionally susceptible to the embryopathic effects of xenobiotic-initiated oxidative stress.

By both maternal and embryonic analysis, the dose-dependent increase in most embryopathies, including teratological anomalies, produced in G6PD-deficient animals by phenytoin compared with vehicle controls, corroborates the role of oxidative stress in the molecular mechanism of phenytoin teratogenesis. The enhanced embryonic DNA oxidation observed in phenytoin-exposed G6PD-deficient embryos further suggests that oxidative damage to embryonic cellular macromolecules may play a proximate role in embryopathic initiation. These results implicating reactive oxygen species and oxidative damage to embryonic cellular macromolecules in the mechanism of phenytoin teratogenicity are consistent with other murine studies *in vivo*, in embryo culture, and *in vitro* demonstrating phenytoin-initiated formation of reactive oxygen species, oxidative damage to embryonic cellular macromolecules, and a protective role for antioxidants (vitamin E, caffeic acid, glutathione) and other antioxidative enzymes such as glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase (37, 41). The embryopathic importance of enhanced DNA oxidation in G6PD-deficient embryos is consistent with previous studies of phenytoin and benzo-[a]pyrene, another teratogen known to initiate embryonic oxidative stress, wherein p53-deficient mice with reduced DNA repair were shown to be more susceptible to the embryopathic effects of these xenobiotics (25, 42, 43).

It has been reported that under conditions of oxidative stress, the increased expression of G6PD activity is primarily a result of an increased rate of transcription, with a minor contribution from post-transcriptional modifications (44), possibly due to posttranslational regulation of G6PD by small heat shock proteins (45). Given the above evidence for the involvement of reactive oxygen species in *in utero* death and teratogenesis, it is likely that the biochemical mechanism underlying the embryoprotective role of G6PD is its production of NADPH essential for both glutathione reductase-dependent, and possibly catalase-dependent, detoxification of lipid hydroperoxides and hydrogen peroxide, rather than G6PD-dependent pentose production. This is consistent with a recent study of diamide-initiated oxidative stress, which identified the essential protective role for G6PD as NADPH production, as distinct from its dispensable role in pentose synthesis, determined by cloning efficiency of mouse embryonic stem cells wherein the G6PD gene was selectively knocked out (46). A reduction in catalase function may contribute to the enhanced teratologic susceptibility observed with G6PD deficiencies, because NADPH is known to maintain catalase activity not only by preventing the formation of one of the inactive states of the enzyme (compound II), possibly through electron tunneling between surface-bound NADPH and the internal heme group, but also by reducing oxidized states and internal groups of catalase distinct from compound II, possibly including one of the active states of the enzyme (compound I) (6–8). This mechanism also is consistent with other studies demonstrating a protective effect of catalase therapy against phenytoin teratogenicity in embryo culture (20) and *in vivo* (41). Our results also show that G6PD, as distinct from isocitrate dehydrogenase and malic enzyme (47), provides the major supply of embryonic NADPH during organogenesis, and alternative embryonic sources are inadequate in the face of hereditary G6PD deficiencies.

Epidemiological considerations

The observed decreased litter size at birth in untreated −/− G6PD-deficient mice and, in vehicle-treated mice, the increased fetal resorptions in both +/− and −/− G6PD-deficient dams, would present in humans as an apparent decrease in fertility. Given that implantations were not decreased in G6PD-deficient dams, the decrease in viable offspring was because of postimplantation embryolethality rather than increased oocyte loss and/or increased preimplantation embryonic death. The gestational timing of susceptibility to oxidative stress may be because of, at least in part, the change from anaerobic to aerobic metabolism that begins after implantation. This change occurs as the mitochondrial

electron transport system and associated enzymes start to become functional and in rodents is complete with the establishment of the allantoic circulation. For rat embryos, this process starts between GD 10 and 11 and ends by GD 12.5, with blood circulation through the yolk sac and embryo by GD 11 (48–50). For mouse embryos, this process may run from GD 6 to 9.5, with allantoic circulation through the placenta by GD 9 (51–53). Although humans do not have a yolk sac placenta, comparisons based on stage of development and somite count between rat and human indicate that GD 11 and 12.5 in the rat are equivalent to GD 28 and 37 in the human, after which time the switch from anaerobic to aerobic metabolism should be complete (48, 54).

Accordingly, it is not surprising that this postimplantation gestational period constitutes a critical window of susceptibility to ROS-mediated lethality for G6PD-deficient embryos Indeed, +/− G6PD-deficient embryos were as susceptible as their −/− and −/y G6PD-deficient littermates. In humans, expected incidences for G6PD-deficient −/y males and −/− females born among all G6PD-deficient groups in Europe were 0.7 and 5 1%, respectively, whereas the observed incidences of these groups were only 0 3 and 2%, respectively (2). In other words, there were 57% fewer −/y G6PD-deficient males and 61% fewer −/− G6PD-deficient females born than expected. These lower birth incidences in human G6PD-deficient populations imply a lower survival rate than expected for embryos deficient in G6PD, as was observed in our mouse model

To date, no major deletions or frameshifts within G6PD have been identified. Most variants result from point mutations or small intragenic deletions leading to a decrease of only one or two residues (14). There are few published cases of heritable mild G6PD deficiencies in erythrocytes from other species, specifically in a colony of rats (55) and in one dog out of 3,300 screened (56). In both cases, the mutation was not characterized, but difficulties in maintaining the rat colony because of increased mortality and sterility among the affected animals suggests that a homozygous knockout of G6PD would likely prove embryolethal. Similarly, there has never been a reported case of a complete human G6PD deficiency (10).

A limited number of recent human studies provide indirect evidence of other areas where G6PD deficiencies may be pathologically relevant G6PD is important for maintaining adequate concentrations of reduced glutathione, which is necessary for a number of cytoprotective antioxidative activities, including that of glutatione peroxidase (Fig. 1). Graf et al. (57) found significantly lower glutathione peroxidase activity among 37 children with the neural tube defect myelomeningocele, compared with age-matched controls. Weber and colleagues (58) reported intractable seizures, repeated infections, and intolerance to anticonvulsants in four children with glutathione peroxidase deficiencies, one of whom also was G6PD deficient. These adverse outcomes may have been because of granulocytopenia and enhanced ROS-dependent signaling pathways, respectively, both of which could result from oxidative stress and inadequate protection from antioxidative enzymes. This is consistent with results from a rat model of human chronic posttraumatic epileptic seizures, in which pretreatment with antioxidants (alpha-tocopherol and selenium) protected against a spectrum of iron-induced peroxidative injuries, including cavitation, neuronal loss, astrogliosis, and epileptiform discharges in rat isocortical regions, suggesting that deficiencies in cytoprotection against peroxidative injury may increase the risk of recurrent epileptic seizures (59). Finally, a recent study found increased levels of G6PD activity in several relevant regions of Alzheimer's brains compared with controls, possibly reflective of increased levels of cerebral oxidative challenge (60, 61). In our mouse model, normal adult brain G6PD activity was one of the lowest of all tissues examined and was further reduced by >21 and 72%, respectively, in +/− and −/− G6PD-deficient animals (Fig. 11). If oxidative stress is involved in the mechanism of neurodegenerative diseases (62, 63), then G6PD deficiencies may contribute to enhanced susceptibility. These studies are consistent with our observation of enhanced embryopathies in G6PD-deficient mice and suggest the potential for a broader range of pathological susceptibilities with G6PD deficiencies that may be further enhanced by exposure to xenobiotics like phenytoin that initiate oxidative stress.

The commonly postulated evolutionary pressure for the widespread prevalence of G6PD deficiencies is their advantage in providing resistance to malaria. This view is supported by the almost complete overlap of regions of increased incidence of G6PD deficiency with those of elevated incidence of malarial infections (2, 10) Recently, both heterozygous and hemizygous G6PD-deficient people were confirmed to be 46 and 58% more resistant, respectively, to severe malarial infection (64). Malaria-infected normal RBCs demonstrate increased lipid peroxidation (65) presumably because of parasite-generated H_2O_2 (66) and decreased antioxidant levels, with no change in G6PD activity The authors concluded this to be a possible defense mechanism by which infected host cells seek to produce and maintain unchecked oxidative stress to their advantage in a self-sacrificing attempt to limit the spread of malarial infection. As such, G6PD-deficient cells are more sensitive to this increased oxidative damage (66), which may help mediate their increased resistance to

such infections, either by predisposing the cell to premature destruction by the reticuloendothelial system (67) or macrophage-initiated lysis (68) and/or impaired growth of the parasite in G6PD-deficient erythrocytes as suggested by *in vitro* studies (69, 70), thus abolishing a suitable incubation environment for parasite development. However, this apparent selective advantage must be balanced by equally disadvantageous selective pressures, not the least of which include the developmental risks observed in our mouse study and suggested in human epidemiological studies discussed above. Developmental risks may well be increasing as a result of more frequent exposure to drugs and environmental chemicals such as phenytoin that initiate oxidative stress exquisitely toxic to G6PD-deficient embryos Such factors may be contributing to the apparently restricted increase in the prevalence of G6PD deficiencies in malaria-infected regions (64).

CONCLUSIONS

Hereditary deficiencies in G6PD are widely thought to pertain only to red blood cell hemolysis, with the most severe outcome being neonatal kernicterus (9, 10, 26). Our results provide the first evidence that G6PD is a developmentally critical enzyme, protecting the embryo from both endogenous and xenobiotic-initiated oxidative stress and DNA damage that produce a broad range of embryopathies, including teratologic anomalies. The developmental risk is potentially substantial, given both the common incidence of hereditary G6PD deficiencies and the susceptibility of even heterozygous G6PD-deficient animals.

The enhanced susceptibility of G6PD-deficient embryos to phenytoin corroborates other studies implicating embryonic oxidative stress and DNA damage in the molecular mechanism of embryopathies caused by phenytoin and related teratogens (37). G6PD deficiencies completely redefine thresholds for teratologic susceptibility to xenobiotic exposure, as evidenced by the enhanced susceptibility of even heterozygous G6PD-deficient embryos to phenytoin embryopathies at a dose that is well below the minimal teratogenic dose in G6PD-normal mice. The developmental risk from xenobiotics that initiate oxidative stress appears to be considerably greater than that observed in adults, because adult patients with G6PD deficiencies are reported to be nonsusceptible to RBC hemolysis initiated by drugs like phenytoin. FJ

Preliminary reports of this work were presented at the 35th, 36th, and 38th annual meetings of the Society of Toxicology (USA) (*Fundam. Appl. Toxicol.* Vol 30 (Suppl. 1, Part 2), p 197, 1996; *Fundam. Appl. Toxicol.* Vol 36 (Suppl. 1, Part 2), p 98, 1997, *Toxicol. Sci* 48(1-S), 17, 1999) and at the 5th International Meeting of the Society for the Study of Xenobiotics (*ISSX Proc.* Vol. 13, p. 171, 1998). We wish to thank Dr. Michael J. Wiley (Department of Anatomy and Cell Biology, University of Toronto) for consultations pertaining to teratological analyses, and Danka Markiewicz and John Tzountzouris (Department of Genetics, Hospital for Sick Children, Toronto) for their expert advice with the DNA sequencing and genotyping assays Supported by a grant to P.G W from the Medical Research Council of Canada

REFERENCES

1 Mehta, A. B (1994) Glucose-6-phosphate dehydrogenase deficiency *Postgrad. Med. J* **70**, 871–877
2 Sodeinde, O (1992) Glucose-6-phosphate dehydrogenase deficiency *Bault. Clin. Hematol.* **5**, 367–382
3 Halliwell, B , and Gutteridge, J M. C (1989) *Free Radicals in Biology and Medicine.* Clarendon, Oxford, U. K
4 Wells, P G , and Winn, L. M (1996) Biochemical toxicology of chemical teratogenesis *Crit. Rev Biochem. Mol. Biol.* **31**, 1–40
5 Wells, P. G , Kim, P. M , Nicol, C J , Parman, T , and Winn, L M (1996) Reactive intermediates In *Handbook of Experimental Pharmacology Drug Toxicity in Embryonic Development* (Kavlock, R J , and Daston, G P , eds) Vol 124, Part 1, pp 453–518, Springer-Verlag, Heidelberg, Germany
6 Kirkman, H N , and Gaetani, G F (1984) Catalase a tetrameric enzyme with four tightly bound molecules of NADPH *Proc Natl. Acad. Sci USA* **81**, 4343–4347
7 Kirkman, H N , Galiano, S , and Gaetani, G F (1987) The function of catalase-bound NADPH *J Biol. Chem.* **262**, 660–666
8 Kirkman, H N , Rolfo, M , Ferraris, A M , and Gaetani, G F (1999) Mechanisms of protection of catalase by NADPH *J Biol Chem.* **274**, 13908–13914
9 Beutler, E (1986) Drug-induced hemolytic anemia and nonspherocytic hemolytic anemia In *Glucose-6-Phosphate Dehydrogenase* (Yoshida, A , and Beutler, E , eds) pp 3–12, Academic, Orlando
10 Luzzatto, L , and Mehta, A (1995) Glucose 6-phosphate dehydrogenase deficiency In *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C R., Beaudet, A L , Sly, W S , and Valle, D , eds) Vol 7, pp 3367–3398, McGraw-Hill, Toronto
11 Zollo, M , D'Urso, M , Schlessinger, D , and Chen, E Y (1993) Sequence of mouse glucose-6-phosphate dehydrogenase cDNA *DNA Seq J DNA Seq Map* **3**, 319–322
12 Beutler, E (1959) The hemolytic effect of primaquine and related compounds a review *Blood* **14**, 103–109
13 Beutler, E (1993) Study of glucose-6-phosphate dehydrogenase history and molecular biology *Am. J Hematol.* **42**, 53–58
14 Martini, G , and Ursini, M V (1996) A new lease of life for an old enzyme *BioEssays* **18**, 631–637
15 Beutler, E , Vulliamy, T , and Luzzatto, L (1996) Hematologically important mutations glucose-6-phosphate dehydrogenase *Blood Cells Mol. Dis* **22**, 49–56
16 Abu-Osba, Y K., Mallouh, A A , and Hann, R W (1989) Incidence and causes of sepsis in glucose-6-phosphate dehydrogenase-deficient newborn infants *J Pediatr* **114**, 748–752
17 Winn, L M , and Wells, P G (1995) Free radical-mediated mechanisms of anticonvulsant teratogenicity *Eur J Neurol.* **2** (Suppl. 4), 5–29
18 Newburgh, R W , Buckingham, B , and Herrmann, H (1962) Levels of reduced TPN generating systems in chick embryos in ovo and in explants *Arch Biochem Biophys* **97**, 94–99
19 Papaconstantinou, J (1967) Metabolic control of growth and differentiation in vertebrate embryos In *The Biochemistry of Animal Development* (Weber, R , ed) Vol 2, pp 57–113, Academic, New York
20 Winn, L M , and Wells, P G (1995) 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 *Mol. Pharmacol.* **48**, 112–120

21 Sanders, S, Smith, D. P., Thomas, G A, and Williams, E. D. (1997) A glucose-6-phosphate dehydrogenase (G6PD) splice site consensus sequence mutation associated with G6PD enzyme deficiency *Mutat. Res* **374**, 79–87

22 Gupta, R. C. (1984) Nonrandom binding of the carcinogen N-hydroxy-2-acetylfluorene to repetitive sequences of rat liver DNA in vivo *Proc Natl. Acad. Sci USA* **81**, 6943–6947

23. Shigenaga, M. K., and Ames, B. N. (1991) Assay for 8-hydroxy-2'-deoxyguanosine a biomarker of in vivo oxidative damage *Free Rad. Biol. Med* **10**, 211–216

24 Chen, E Y, Cheng, A, Lee, A., Kuang, W J, Hillier, L, Green, P, Schlessinger, D., Ciccodicola, A, and D'Urso, M (1991) Sequence of human glucose-6-phosphate dehydrogenase cloned in plasmids and a yeast artificial chromosome *Genomics* **10**, 792–800

25 Nicol, C J, Harrison, M L, Laposa, R. R., Gimelshtein, I L, and Wells, P G (1995) A teratologic suppressor role for p53 in benzo[a]pyrene-treated transgenic p53-deficient mice *Nat Genet* **10**, 181–187

26 Washington, E C, Ector, W, Abboud, M, Ohning, B, and Holden, K. (1995) Hemolytic jaundice due to G6PD deficiency causing kernicterus in a female newborn *South. Med J* **88**, 776–779

27 Burt, A. M, and Wenger, B S (1961) Glucose-6-phosphate dehydrogenase activity in the brain of the developing chick *Dev Biol.* **3**, 84–95

28 Jolley, R L, Cheldelin, V H, and Newburgh, R. W (1958) Glucose catabolism in fetal and adult heart *J Biol. Chem* **233**, 1289–1294

29 Jolley, R. L, Cheldelin, V H, and Newburgh, R W (1959) The catabolism of glucose in soluble adult and fetal-heart preparations *Biochim Biophys Acta* **33**, 64–68

30. Lucas, A M, and Jamroz, C (eds) (1961) *Atlas of Avian Hematology*, pp 17–30, United States Department of Agriculture, Washington, D C.

31. Sinclair, G D, and Brasch, K. (1975) The nucleated erythrocyte a model of cell differentiation *Revue Can Biol* **34**, 287–303

32 Ganshirt, D, Smeets, F. W, Dohr, A, Walde, C, Steen, I, Lapucci, C, Falcinelli, C, Sant, R, Velasco, M, Garritsen, H S, and Holzgreve, W (1998) Enrichment of fetal nucleated red blood cells from the maternal circulation for prenatal diagnosis experience with triple density gradient and MACS based on more than 600 cases *Fetal Diagnostic Ther* **13**, 276–86

33 Guyton, A. C. (ed) (1987) Red blood cells, white blood cells and resistance of the body to infection In *Human Physiology and Mechanisms of Disease*, 4th Ed, p 195, W B Saunders, Toronto

34 Singh, M, and Shah, G L. (1989) Teratogenic effects of phenytoin on chick embryos *Teratology* **40**, 453–458

35 Pretsch, W, Charles, D J, and Merkle, S (1988) X-linked glucose-6-phosphate dehydrogenase (G6PD) deficiency in Mus musculus *Biochem Genet* **26**, 89–103

36 Finnell, R H, and Chernoff, G F (1984) Variable patterns of malformation in the mouse fetal hydantoin syndrome *Am. J Med Genet* **19**, 463–471

37 Wells, P G, Kim, P. M, Laposa, R. R, Nicol, C J, Parman, T, and Winn, L. M (1997) Oxidative damage in chemical teratogenesis *Mutat Res* **396**, 65–78

38 Kim, P M, and Wells, P G (1996) Phenytoin-initiated hydroxyl radical formation characterisation by enhanced salicylate hydroxylation *Mol. Pharmacol.* **49**, 172–181

39. Parman, T, Chen, G, and Wells, P G (1998) Free radical intermediates of phenytoin and related teratogens prostaglandin H synthase-catalyzed bioactivation, electron paramagnetic resonance spectrometry and photochemical product analysis *J Biol. Chem* **273**, 25079–25088

40 Parman, T, Wiley, M J, and Wells, P G (1999) Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity *Nature Med.* **5**, 582–585

41 Winn, L. M, and Wells, P G (1999) Maternal administration of superoxide dismutase and catalase in phenytoin teratogenicity *Free Rad Biol. Med* **26**, 266–274

42 Laposa, R R, and Wells, P G (1995) Preliminary evaluation of phenytoin teratogenicity in transgenic mice deficient in the p53 tumor suppressor gene *Fundam. Appl. Toxicol.* **15**, 161

43 Laposa, R R, Chan, K L, Wiley, M J, and Wells, P G (1996) Enhanced phenytoin embryopathy in p53-deficient mice characterisation of embryonic p53 genotype and the p53, p21 and Bax DNA damage response proteins *Fundam. Appl. Toxicol.* **30(Suppl 1, part 2)**, 195

44 Ursini, M V, Parrella, A, Rosa, G, Salzano, S, and Martini, G. (1997) *Biochem. J* **323 (Pt. 3)**, 801–806

45 Preville, X, Salvemini, F, Giraud, S, Chaufour, S, Paul, C., Stepien, G, Ursini, M V, and Arrigo, A-P. (1999) Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery *Exp Cell Res* **247**, 61–78

46 Pandolfi, P P, Sonati, F, Rivi, R, Mason, P, Grosveld, F, and Luzzatto, L (1995) Targeted disruption of the housekeeping gene encoding glucose-6-phosphate dehydrogenase (G6PD) G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress *EMBO J* **14**, 5209–5215

47 Mayes, P A (1996) Biosynthesis of fatty acids In *Harper's Biochemistry* (Murray, R K., Granner, D K., Mayes, P. A, and Rodwell, V W, eds) 24th Ed, pp 216–223, Appleton, and Lange, Stamford, CA

48 Robkin, M A (1997) Carbon monoxide and the embryo *Int. J. Dev Biol.* **41**, 283–289

49 Tanimura, T, and Shepard, T H (1970) Glucose metabolism by rat embryos in vitro *Proc Soc Exp Biol Med* **135**, 51–53

50 Aksu, O., Mackler, B, Shepard, T H, and Lemire, R. J. (1968) Studies of the development of congenital anomalies in embryos of riboflavin-deficient, galactoflavin fed rats *Teratology* **1**, 93–102

51 Clough, J R, and Whittingham, D G (1983) Metabolism of [^{14}C]glucose by postimplantation mouse embryos in vitro *J Embryol. Exp Morph* **74**, 133–142

52 Edwards, R G, and Fowler, R. E (1959) Fetal mortality in adult mice after superovulation with gonadotrophins *J Exp Zool.* **141**, 299–322

53 West, J D (1993) A genetically defined animal model of anembryonic pregnancy *Hum. Reprod* **8**, 1316–1323

54 Altman, P L., and Dittmer, D S (1972) *Biology Data Book*, pp 176–180, Federation of American Societies for Experimental Biology, Bethesda, MD

55 Werth, G, and Muller, G (1967) Inheritable glucose-6-phosphate dehydrogenase deficiency in erythrocytes of rats *Klin Wochenschr* **45**, 265–269

56 Smith, J E, Ryer, K, and Wallace, L (1976) Glucose-6-phosphate dehydrogenase deficiency in a dog *Enzyme* **21**, 379–382

57. Graf, W D, Oleinik, O E, Pippenger, C E, Eder, D N, Glauser, T A, and Shurtleff, D B (1995) Comparison of erythrocyte antioxidant enzyme activities and embryologic level of neural tube defects *Eur J Ped Surgery* **5 (Suppl. 1)**, 8–11

58 Weber, G F, Maertens, P, Meng, X, and Pippenger, C E (1991) Glutathione peroxidase deficiency and childhood seizures *Lancet* **337**, 1443–1444

59 Willmore, L J, and Rubin, J J (1981) Antiperoxidant pretreatment and iron-induced epileptiform discharges in the rat EEG and histopathologic studies *Neurology* **31**, 63–69

60 Balazs, L., and Leon, M (1994) Evidence of an oxidative challenge in the Alzheimer's Brain *Neurochem. Res.* **19**, 1131–1137

61 Martins, R N, Harper, C G, Stokes, G B, and Masters, C L (1986) Increased cerebral glucose-6-phosphate dehydrogenase activity in Alzheimer's disease may reflect oxidative stress *J Neurochem.* **46**, 1042–1045

62 Cooper, A J, and Kristal, B S (1997) Multiple roles of glutathione in the central nervous system *J Biol. Chem.* 378, 793–802

63 Jenner, P (1996) Oxidative stress in Parkinson's disease and other neurodegenerative disorders *Pathologie Biologie* **44**, 57–64

64 Ruwende, C, Khoo, S C, Snow, R W, Yates, S N. R., Kwiatkowski, D, Gupta, S, Warn, P, Allsopp, C E M, Gilbert, S C, Peschu, N, Newbold, C I, Greenwood, B M, Marsh, K., and Hill, A V S (1995) Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature (London)* **376**, 246–249

65 Erel, O, Kocyigit, A, Avci, S, Aktepe, N, and Bulut, V. (1997) Oxidative stress and antioxidative status of plasma

and erythrocytes in patients with vivax malaria. *Clin Biochem* **30**, 631–639
66. Friedman, M. J (1979) Oxidant damage mediates variant red cell resistance to malaria. *Nature (London)* **280**, 245–247
67. Eckman, J. R., and Eaton, J. W. (1979) Dependence of plasmodial glutathione metabolism on the host cell *Nature (London)* **278**, 754–756
68. Yuthavong, Y., Bunyaratvej, A., and Kamchonwongpaisan, S (1990) Increased susceptibility of malaria-infected variant erythrocytes to the mononuclear phagocyte system *Blood Cells* **16**, 591–597
69. Roth, E F., Jr., Raventos-Suarez, C., Rinaldi, A., and Nagel, R. L. (1983) Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of plasmodium falciparum. *Proc. Natl. Acad. Sci. USA* **80**, 298–299
70. Kosower, N S., and Kosower, E. M. (1970) Molecular basis for selective advantage of glucose-6-phosphate dehydrogenase-deficient individuals exposed to malaria *Lancet* **2**, 1343–1344

Received for publication May 14, 1999.
Revised for publication July 25, 1999