EMBRYOPROTECTIVE ROLE OF ENDOGENOUS CATALASE

By

Julia P. Abramov

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

Your file Votre référence
ISBN: 978-0-494-78113-5

Our file Notre référence
ISBN: 978-0-494-78113-5

Embryoprotective Role of Endogenous Catalase

Doctor of Philosophy, August 2011
Julia P. Abramov
Graduate Department of Pharmaceutical Sciences
University of Toronto

ABSTRACT

Oxidative stress and reactive oxygen species (**ROS**) such as hydrogen peroxide ($\mathbf{H_2O_2}$), which is detoxified by catalase, are implicated in fetal death and birth defects, but embryonic levels of catalase are only about 5% of adult activity, and its protective role is unknown. Our approach involved the use of mice genetically modified to either: (1) express low levels of endogenous catalase (**acatalasemic, aCat**); or, (2) express human catalase resulting in elevated levels of embryonic catalase activity (**hCat**). Using these mouse models we investigated the protective importance of constitutive embryonic catalase against endogenous ROS and the ROS-initiating teratogen phenytoin in embryo culture and *in vivo*. We hypothesized that aCat mice would be more sensitive to endogenous embryonic and phenytoin-enhanced ROS production, while hCat embryos would be less sensitive. aCat and hCat embryos respectively exhibited reduced and enhanced catalase activity compared to wild-type (**WT**) controls, with conversely enhanced and reduced spontaneous and phenytoin-enhanced embryopathies and DNA oxidation. Among aCat embryos exposed to phenytoin, embryopathies increased with decreasing catalase activity, and were completely blocked by addition of exogenous catalase. The alterations in phenytoin embryopathies were not due to pharmacokinetic differences, as drug concentrations in maternal and fetal tissues were similar among all strains. However, phenytoin concentrations in fetal brain exceeded those in fetal liver or maternal tissues, which may explain the predominance of cognitive deficits over structural birth defects in children exposed *in utero* to phenytoin. Similarly in untreated aged mice (about 18 months), female aCat mice showed a substantial loss in motor coordination compared to WT controls in the rotarod test. Following *in utero* exposure to phenytoin, the effect of altered embryonic catalase activity on postnatal neurodevelopment was assessed by several pre- and post-weaning tests. Catalase deficiency (aCat), independent of drug treatment, reduced performance in surface righting, negative geotaxis tests and rotarod tests. Conversely, high catalase expression (hCat) enhanced performance in the surface righting, negative geotaxis, air righting and rotarod tests. Our results provide the first evidence that the

quantitatively minor amounts of antioxidative enzymes like catalase in the embryo and fetus provide important protection against the molecular damage and adverse fetal effects caused by developmental and drug-enhanced oxidative stress. Accordingly, interindividual variation in embryonic/fetal activities of catalase, and possibly other antioxidative enzymes, likely constitute an important determinant of risk for adverse developmental outcomes.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Peter G. Wells for his belief in me and continues support throughout the years.

Many thanks to my advisory committee members: Drs. David Riddick, Michael Wiley and Patricia Harper for their help and advice with my research.

Former lab mates: Winnie Jeng, Connie Chen, Julia Kennedy, Andrea Wong and Tom Preston. Thanks to Julia for teaching me the embryo culture technique. I'd like to thank the rest of the guys for showing me the ropes around the lab and for all your advice.

Present lab mates: Margaret Loniewska, Crystal Lee, Annmarie Ramkissoon, Lutfiya Miller, Dr. Gordon McCallum, Aaron Shapiro, Michelle Siu, Kyla Lam, Stephanie Ondovcik, and Nicole Sweeting. Words cannot describe my gratitude for my lab mates. Without you guys I would have never finished my studies and most definitely wouldn't have enjoyed this period as much as I did. Our endless conversations, lunches, shopping trips and conference travels made the years in this lab the best time of my life. You made the miserable life of a grad student to be awesome fun!

Undergraduate research project student: Audrey Tran. Dear Audrey, without you there wouldn't have been the third study. The amount and quality of work you've done for me is absolutely amazing. I am forever grateful to you.

Family: My husband Eugene, my son Nikolai, Mom, Dad and brother Dmitry. Without the support and love of my family I wouldn't have been able to do grad school and achieve all my goals. Constant support and encouragement from my loving husband was my fuel to keep me going for so many years. His patience and help with our little boy allowed me to continue to focus on my studies and I hope to make them both very proud. I love you guys!

TABLE OF CONTENTS

LIST OF TABLES

SECTION 1: INTRODUCTION

SECTION 2: STUDIES

Study 3.

LIST OF FIGURES

SECTION 1: INTRODUCTION

SECTION 2: STUDIES

Study 1

Study 2

Study 3.

LIST OF ABBREVIATIONS

aCat	acatalasemic mouse
ASA	acetylsalicyclic acid
ATM	ataxia telangiectasia mutated
CSB	Cockayne syndrome B
CYP450	cytochromes P450
EGF	epidermal growth factor
ETYA	eicosatetraynoic acid
FHS	fetal hydantoin syndrome
FOX	ferrous oxidation in xylenol orange
Fe	iron
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GD	gestational day
GPx	glutathione peroxidase
GSH	glutathione
GSH Rd	glutathione reductase
GSSG	glutathione disulfide
GST	glutathione S-transferase
hCat	human catalase expressing mouse
H_2O_2	hydrogen peroxide
HNF-1α	hepatic nuclear factor 1α
HRP	horseradish peroxidase
IQ	intelligence quotient
LPO	lipoxygenase

METH	methamphetamine
MRS	male rat serum
NADPH	nicotinamide-adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid
NER	nucleotide excision repair
NF-κB	nuclear factor kappa B
$O_2^{\bullet-}$	superoxide anion
•OH	hydroxyl radical
OGG1	oxoguanine glycosylase 1
8-oxoG	7,8-dihydro-8-oxoguanine/8-oxoguanine
PEG	polyethylene glycol
PHS	prostaglandin H synthase
PHT	phenytoin
PND	postnatal day
RBC	red blood cell
ROS	reactive oxygen species
SOD	superoxide dismutase
TPA	tetradecanoylphorbol acetate
UGT	UDP-glucuronosyltranferase
UV	ultraviolet
VEH	vehicle
WT	wild-type
XP	xeroderma pigmentosum
XPC	xeroderma pigmentosum C

LIST OF PUBLICATIONS & PRESENTATIONS ARISING FROM THIS THESIS

NOTE: Abramov JP was previously published under Perstin J

Refereed Papers

1. Abramov JP and Wells PG (2011) Embryoprotective role of endogenous catalase in acatalasemic and human catalase-expressing mouse embryos exposed in culture to developmental and phenytoin-enhanced oxidative stress. Toxicological Sciences 120(2):428-438.

2. Abramov JP and Wells PG (2011) Embryonic catalase protects against endogenous and phenytoin-enhanced DNA oxidation and embryopathies in acatalasemic and human catalase-expressing mice. FASEB Journal 25(7):2188-2200.

3. Abramov JP, Tran A, Shapiro, A and Wells PG (2011) Protective role of endogenous catalase in baseline and phenytoin-enhanced neurodevelopmental and behavioral deficits initiated *in utero* and in aged mice. (submitted)

Invited Review

1. Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJJ, Perstin J, Preston TJ, Wiley MJ, Wong AW (2009) Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits and cancer. Toxicological Sciences 108(1):4-18.

Book chapter

1. Wells PG, Lee CJJ, McCallum GP, Perstin J and Harper PA (2009) Receptor- and reactive intermediate-mediated mechanisms of teratogenesis. In: Handbook of Experimental Pharmacology, Vol. 196: Mechanisms of Adverse Drug Reactions, JP Uetrecht (ed.), pp. 131-162, Springer, Heidelberg.

Abstracts

1. Perstin J and Wells PG (2004) Embryonic catalase activity during organogenesis with endogenous and phenytoin-enhanced oxidative stress. Proceedings of the 4th annual scientific meeting of the Oxidative Stress Consortium, p.49 (No.6).

2. Perstin J and Wells PG (2004) Embryonic catalase activity during organogenesis with endogenous and phenytoin-enhanced oxidative stress. Teratology Society Meeting. (abstract accepted too late for publication).

3. Perstin J and Wells PG (2005) Endogenous embryonic catalase activity during organogenesis in embryo culture, *in vivo* and after exposure to phenytoin-enhanced oxidative stress. Toxicological Sciences (Supplement: The Toxicologist) 84(S-1):461.

4. Perstin J and Wells PG (2005) Endogenous catalase activity in conceptal tissues of wild-type and acatalasemic mice during organogenesis. Birth Defects Research Part A: Clinical and Molecular Teratology 73(5):362.

5. Perstin J and Wells PG (2005) Phenytoin embryopathies in wild-type (C3HeB/FeJ) and acatalasemic (C3Ga.Cg-*Cat*^{b}/J) mice. Society of Toxicology of Canada Meeting. *38*th *Annual Symposium "The Impact of Toxicants on Child Health"*.

6. Perstin J and Wells PG (2006) Relation of constitutive catalase activity to phenytoin embryopathies in wild-type (C3HeB/FeJ) and acatalasemic (C3Ga.Cg-*Cat*^{b}/J) mice. Birth Defects Research Part A: Clinical and Molecular Teratology 76(5):392.

7. Perstin J and Wells PG (2007) Protective role of low levels of constitutive catalase in embryonic and fetal development. Birth Defects Research Part A: Clinical and Molecular Teratology 79(5):418.

8. Perstin J and Wells PG (2009) Embryoprotective role of endogenous catalase in wild-type (C3HeB/FeJ) and acatalasemic (C3Ga.Cg-*Cat*^{b}/J) mice. Toxicological Sciences (Supplement: The Toxicologist) 108(1):355.

9. Shapiro A, Perstin J and Wells PG (2009) Gender-dependent reactive oxygen species-mediated neurodegeneration in untreated aged acatalasemic mice. Toxicological Sciences (Supplement: The Toxicologist) 108(1):454.

10. Wells PG, Jeng W, Loniewska M, McCallum GP, Perstin J, Ramkisson A and Shapiro A (2009) Oxidative stress and DNA damage in neurodevelopmental deficits in neurodegeneration. Proceedings of the 6th annual scientific meeting of the Canadian Oxidative Stress Consortium.

11. Perstin J and Wells PG (2009) Tissue drug concentrations, oxidative DNA damage and embryopathies in acatalasemic and catalase-overexpressing embryos exposed in utero to phenytoin. Journal of Pharmacy and Pharmaceutical Sciences: 12(2):87.

12. Perstin J and Wells PG (2009) Tissue drug concentrations, oxidative DNA damage and embryopathies in acatalasemic and catalase-overexpressing embryos exposed in utero to phenytoin. Birth Defects Research Part A: Clinical and Molecular Teratology 85(5):398.

13. Perstin J and Wells PG (2010) Embryoprotective role of constitutive antioxidative catalase in wild-type and acatalasemic mice *in vivo* and in embryo culture. Toxicology Letters 196S: S184.

1.1 RATIONALE AND RESEARCH OBJECTIVES

During the course of normal pregnancy, otherwise healthy women are exposed to numerous drugs and environmental chemicals, most of which are capable of crossing the placenta, and thus exposing the developing fetus as well (Bologa et al 1994). The risk of teratological outcomes resulting from these exposures depends on the biochemical and genetic makeup of the fetus, as well as environmental factors. The mechanisms underlying these teratological outcomes, which include structural and functional anomalies, are still not fully understood. It is interesting to note that there are differences with respect to the differential embryonic susceptibilities of xenobiotic-initiated teratological outcomes within exposed groups. These differences may be the result of, and reflected in, embryonic biochemical and molecular differences, which may serve as useful predictors of individual risk.

This thesis will focus on one of many potential teratogenic mechanisms; namely, oxidative stress. This mechanism is involved when proteratogens are enzymatically bioactivated to free radical reactive intermediates that generate reactive oxygen species (**ROS**) which, if not detoxified, may result in oxidative stress and damage to essential macromolecular targets, such as proteins, lipids and DNA. In the case of increased bioactivation and/or decreased detoxification or repair of macromolecular damage, changes in ROS-mediated signal transduction and/or oxidative damage may lead to teratogenesis.

My hypothesis is that teratogenesis is dependent upon embryonic and fetal imbalances between bioactivation and cytoprotective (ROS detoxification) and repair pathways. If so, then susceptibility to teratogenesis is predictable. My studies were designed to examine and elucidate the potential of endogenous embryonic catalase, an antioxidative ROS-detoxifying enzyme, to protect the developing embryo from endogenous and xenobiotic-enhanced oxidative stress. My xenobiotic model was the ROS-initiating teratogen phenytoin, a widely used antiepileptic drug taken by pregnant women.

My objectives were as follows:

1. To assess the pathogenic role of physiological and drug-enhanced ROS formation within the embryo, and the importance of endogenous catalase in protecting the embryo from developmental and drug-enhanced oxidative stress. My results provide the first direct evidence that the relatively low level of endogenous embryonic catalase provides important protection against both developmental oxidative stress and ROS-initiating

teratogens, and suggest that interindividual embryonic differences in expression of this enzyme could contribute to teratological risk.

2. To determine the mechanism underlying phenytoin-initiated birth defects by evaluating the fetal levels of DNA oxidation following exposure to endogenous and phenytoin-enhanced oxidative stress. My results show the pathogenic potential of oxidative stress, and possibly oxidatively damaged DNA, and provide the first evidence of a protective role for endogenous embryonic and fetal catalase.
3. To evaluate levels of phenytoin in maternal and fetal tissues as a potential confounding factor in the different mouse strains employed. Strain differences in phenytoin levels could not explain the observed variations in embryopathies, but higher concentrations of phenytoin discovered in fetal brain compared to fetal liver and maternal tissues may explain the high frequency of neurodevelopmental deficits compared to other fetal anomalies caused by this drug.

1.2 REACTIVE OXYGEN SPECIES

1.2.1 Introduction to reactive oxygen species

Reactive oxygen species (**ROS**) include a mixture of highly reactive forms of oxygen, like hydrogen peroxide (**H_2O_2**), and oxygen molecules with an unpaired electron in their valence shell, termed free radicals, including superoxide (**$O_2^{\bullet-}$**) and hydroxyl (**•OH**) (Halliwell & Gutteridge 1991). ROS are involved in the redox-dependent regulation of different cellular functions, including energy metabolism and the response to stress or growth signals (Valko et al 2007). H_2O_2 is formed by the dismutation reaction of $O_2^{\bullet-}$ (catalyzed by superoxide dismutases) via the hydroperoxyl radical; and •OH is mainly generated by the metal ion (iron or copper)-catalyzed decomposition of H_2O_2 (known as the Fenton reaction). Superoxide anion is formed through a 1-electron reduction of O_2 (Halliwell & Gutteridge 2007). ROS are highly unstable and will react with other molecules to gain stability. Various ROS have different properties, including reduction potential, half-life and intracellular concentrations (**Fig. 1**). Among biologically relevant ROS, H_2O_2 has the lowest reactivity, the highest stability (as measured by its half-life) and the highest intracellular concentration (Giorgio et al 2007; Halliwell & Gutteridge 2007).

1.2.2 Endogenous sources of ROS

ROS are formed via a variety of physiological and pathophysiological reactions (**Fig. 2**). A major site of endogenous ROS generation within the cell is the mitochondrial respiratory chain (Halliwell & Gutteridge 1991). During cellular metabolism electrons may leak from the electron transport chain and reduce molecular oxygen to produce ($O_2^{\bullet-}$) (Sauer et al 2001). In addition, H_2O_2 is also produced by mitochondria to control cellular growth and death (Giorgio et al 2005). Another location of electron transport is in the endoplasmic reticulum, where electrons can leak from nicotinamide-adenine dinucleotide phosphate (**NADPH**) cytochrome P450 reductase (Wang et al 2010). This enzyme is predominantly involved in the generation of ROS during the oxidation of unsaturated fatty acids.

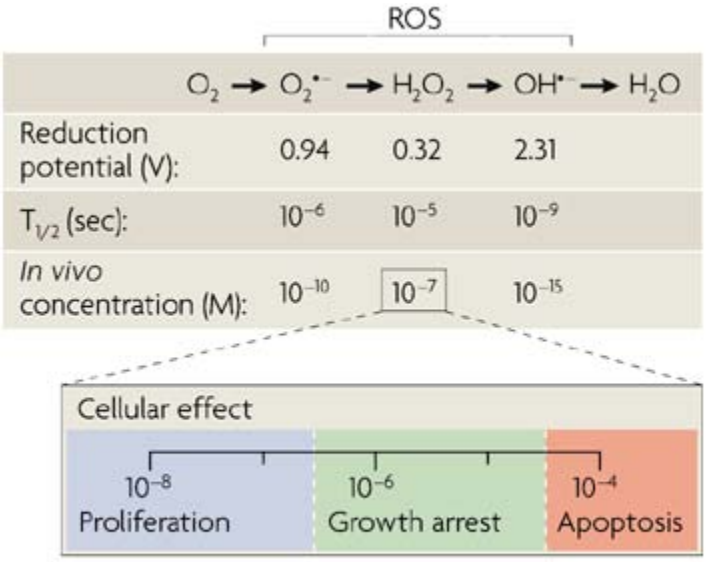


Figure 1. The O_2 reduction pathway and the family of reactive oxygen species.

From: Giorgio et al., 2007.

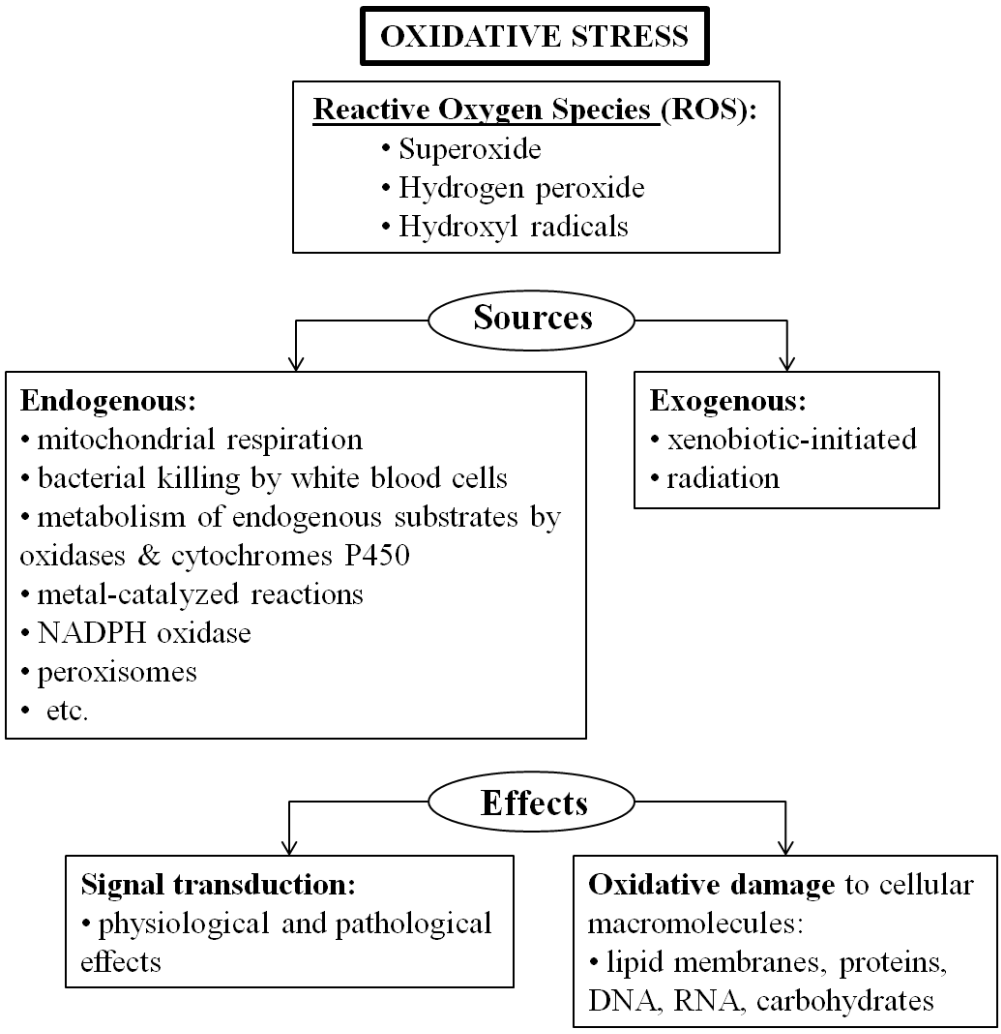


Figure 2. Sources of ROS and the general mechanisms by which oxidative stress can alter cellular function.

Modified from Wells et al, 2009.

ROS also can be produced as a by-product of a variety of enzymes such as hypoxanthine/xanthine oxidase, lipoxygenase, cyclooxygenase and gamma-glutamyl transpeptidase (Del Bello et al 1999; Dominici et al 1999; Moriscot et al 2007; Nakashima et al 2007; Radogna et al 2009; Wang et al 2010). ROS are also generated by cells of the immune system such as neutrophils and macrophages, which utilize ROS in the oxidative or respiratory burst to kill invading microorganisms (Sauer et al 2001). In addition, free radicals can be produced by the auto-oxidation of such endogenous molecules as dopamine, norepinephrine and epinephrine through a self-catalytic reaction between molecules with molecular oxygen (Graham 1978), or via bioactivation by prostaglandin H synthases (**PHSs**) to free radical intermediates (Goncalves et al 2009; Ramkissoon & Wells 2011). A quick source of ROS is through activation of membrane-bound NADPH oxidases that produce ROS for signal transduction pathways or the degradation of foreign organisms within a few minutes after cell stimulation (Gorlach et al 2000; Mohazzab et al 1994; Mohazzab & Wolin 1994). Peroxisomes, which are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen, are also known to produce H_2O_2 under physiological conditions (Valko et al 2004).

1.2.3. Exogenous sources of ROS

Intracellular ROS levels can also be increased by radiation and exposure to various xenobiotics (**Fig. 2**). Radiation results in absorption of energy by the cell water, causing oxygen-hydrogen covalent bonds to split and creating •OH (Halliwell & Gutteridge 1991). Numerous xenobiotics, such as phenytoin and thalidomide, can be bioactivated to produce ROS. Several bioactivating peroxidases, such as PHS, can catalyze oxidation of these xenobiotics to produce free radical intermediates that rapidly react with molecular oxygen to produce ROS (Kubow & Wells 1989). Some xenobiotics, such as benzo[a]pyrene and paraquat, can undergo reductive bioactivation catalyzed by NADPH cytochrome P450 reductase, followed by reoxidation by molecular oxygen, which produces ROS in a redox cycling reaction (Juchau et al 1986; Kappus 1986; Sauer et al 2000) (**Fig. 3**). Other chemicals such as hydroxyurea react directly with oxygen (nonenzymatically) to produce ROS (DeSesso et al 1994; von Sonntag 1987).

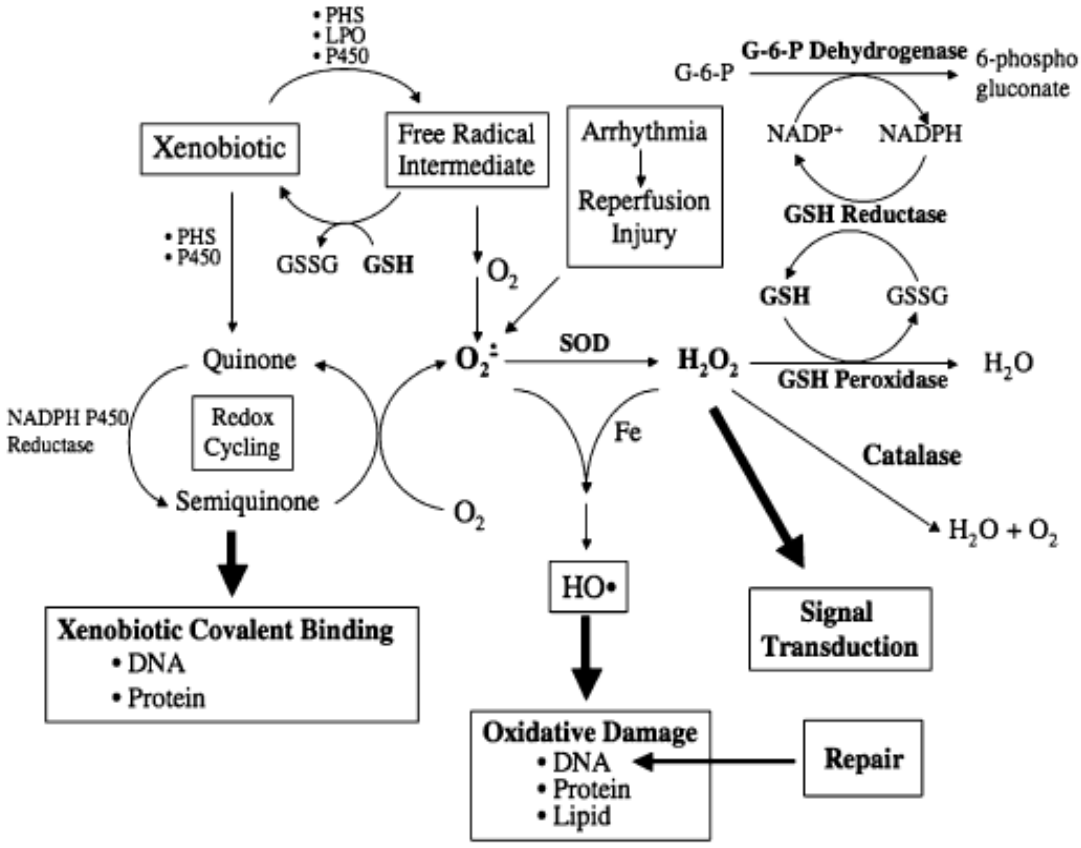


Figure 3. Biochemical pathways for the formation, detoxification and cellular effects of xenobiotic free radical intermediates and ROS.

Abbreviations: Fe, iron; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; HO•, hydroxyl radical; NADP+, nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; P450, cytochromes P450; PHS, prostaglandin H synthase; LPO, lipoxygenase; G-6-P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase.

From: Wells et al., 2009.

1.2.4 Antioxidative defense mechanisms

Numerous enzymatic and non-enzymatic mechanisms are involved in the detoxification of ROS. The developing embryo has very low levels of most antioxidative enzymes relative to adult levels, thus making it particularly susceptible to the damaging effects of ROS (Ozolins et al 1996; Wells et al 2009; Wells & Winn 1996).

1.2.4.1. Enzymatic mechanisms

There are a number of enzymes involved in the protection against ROS (**Fig. 3**). The antioxidative enzyme superoxide dismutase (**SOD**) plays a role in detoxifying ROS by converting $O_2^{\bullet-}$ to H_2O_2 and oxygen (Fridovich 1986). It is important to note that this reaction leads to the formation of H_2O_2 which, if not detoxified by either catalase or glutathione peroxidase (**GPx**), in the presence of iron can be readily and non-enzymatically converted to the highly reactive •OH. Increased activity of SOD must not exceed the capacity of downstream enzymes required to remove H_2O_2, to prevent the accumulation of this reactive and toxic product.

GPx detoxifies H_2O_2 and other peroxides in the cytosol and mitochondria (Savaskan et al 2007). This reaction requires the oxidation of glutathione (**GSH**) to glutathione disulfide (**GSSG**). GSH is regenerated from GSSG by glutathione reductase (**GSH Rd**), using NADPH as a reducing cofactor. NADPH in turn is regenerated by glucose-6-phosphate dehydrogenase (**G6PD**).

H_2O_2 is also detoxified by catalase, which is localized primarily in peroxisomes, and to a lesser extent in the cytosol. This enzyme will be discussed in greater detail below.

Other proteins that act as antioxidants are thioredoxin and glutaredoxin (Bjornstedt et al 1994). Thioredoxin is a polypeptide with 2 adjacent thiol (R–SH) groups that are converted to a disulfide (R–S–S–R) bond when thioredoxin binds to and reduces its target protein. Oxidized thioredoxin is reduced by thioredoxin reductase. Glutaredoxin is a small redox enzyme that uses GSH as a cofactor. Like thioredoxin, glutaredoxin possesses an active centre disulfide bond. Glutaredoxin catalyzes GSH-dependent disulfide reductions when coupled to GSH, NADPH and GSH Rd (Holmgren 1988).

1.2.4.2. Non-enzymatic antioxidative systems

Apart from antioxidant enzymes, all cells contain a variety of reducing substances, e.g. the vitamins A, E and C, along with lipoate, urate, ubiquinone, GSH, all of which effectively scavenge ROS (Sauer et al 2001).

Vitamin A is an effective radical-trapping antioxidant. It can act as a chain breaking antioxidant by combining with peroxyl radicals, before these radicals can propagate peroxidation in the lipid phase of the cell and generate hydroperoxides (Palace et al 1999).

Vitamin E, or otherwise known as α-tocopherol, is a lipid-soluble free radical scavenger located in the cell membrane. It prevents the propagation of lipid peroxidation by donating an electron to and thereby "trapping" toxic peroxyl radicals (Halliwell & Gutteridge 1991).

Vitamin C (ascorbic acid) is a water soluble antioxidant located in the cytosol (Frei et al 1989). It neutralizes ROS by directly donating electrons. Another function of vitamin C is to recycle vitamin E to its active reduced form.

Lipoate, or otherwise known as lipoic acid, can scavenge •OH and increase intracellular GSH (Packer et al 1995).

Urate comprises 30-65% of the peroxyl radical-scavenging capacity of blood plasma thus indicating that urate plays a considerable role in attenuating lipid auto-oxidation in plasma (Becker 1993).

Ubiquinone functions as an energy carrier and is continuously going through an oxidation-reduction cycle. As it accepts electrons, it becomes reduced. As it gives up electrons, it becomes oxidized. In its reduced form, the ubiquinone molecule holds electrons rather loosely, so this molecule will quite easily give up one or both electrons and, thus, act as an antioxidant (Solaini et al 1987). Ubiquinone inhibits lipid peroxidation by preventing the production of lipid peroxyl radicals. In addition, the reduced form of ubiquinone effectively regenerates vitamin E from the a-tocopheroxyl radical and, thereby interferes with the propagation step (Ouchi et al 2010). In contrast to other antioxidants, this compound inhibits both the initiation and the propagation of lipid and protein oxidation (Solaini et al 1987).

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as ROS (Reed 1986). In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide

(GSSG). GSH can be regenerated from GSSG by the enzyme glutathione reductase. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress (Pastore et al 2003). GSH can also function as an antioxidant through direct conjugation by forming disulfide bonds to cysteines on cytoplasmic proteins (Pompella et al 2003).

1.2.5 Effects of ROS

During normal cellular function there is a balance maintained between ROS production and detoxification. However, when this balance is disrupted, either by excess of production or a deficiency in detoxification, a number of outcomes, including macromolecular damage and/or alterations in signal transduction, may result.

1.2.5.1. Oxidative damage

Protein oxidation caused by ROS may affect the function of that protein, whereas damage to lipids that are components of cell or organelle membranes may alter the membrane permeability to ions and water. The accumulation of these substances within a cell can result in disruption of normal cell function and/or the initiation of cell death cascades. Damage to DNA can have various consequences, such as loss of genetic information, mutations and altered gene expression (Hailer-Morrison et al 2003; Khobta et al 2010; Kitsera et al 2011; Marnett 2000; Valko et al 2004; Valko et al 2006).

Hydroxyl radical, produced by the reaction of superoxide and hydrogen peroxide, has been shown to be the primary ROS that directly damages DNA (Rowley & Halliwell 1983; Valko et al 2004). ROS can target either the sugar backbone of the DNA or the DNA base (von Sonntag 1987). Damage to the sugar backbone leads to single-strand breaks, DNA-protein cross-linking, and a number of sugar lesions, whereas base damage results in abasic sites, oxidized bases and ring-opened bases (Dizdaroglu 2005). There are over 20 different types of oxidatively damaged bases, but the most prevalent and commonly studied is 8-hydroxy-2'-deoxyguanine or 8-oxoguanine (**8-oxoG**) (Dizdaroglu et al 2002) (**Fig. 4**).

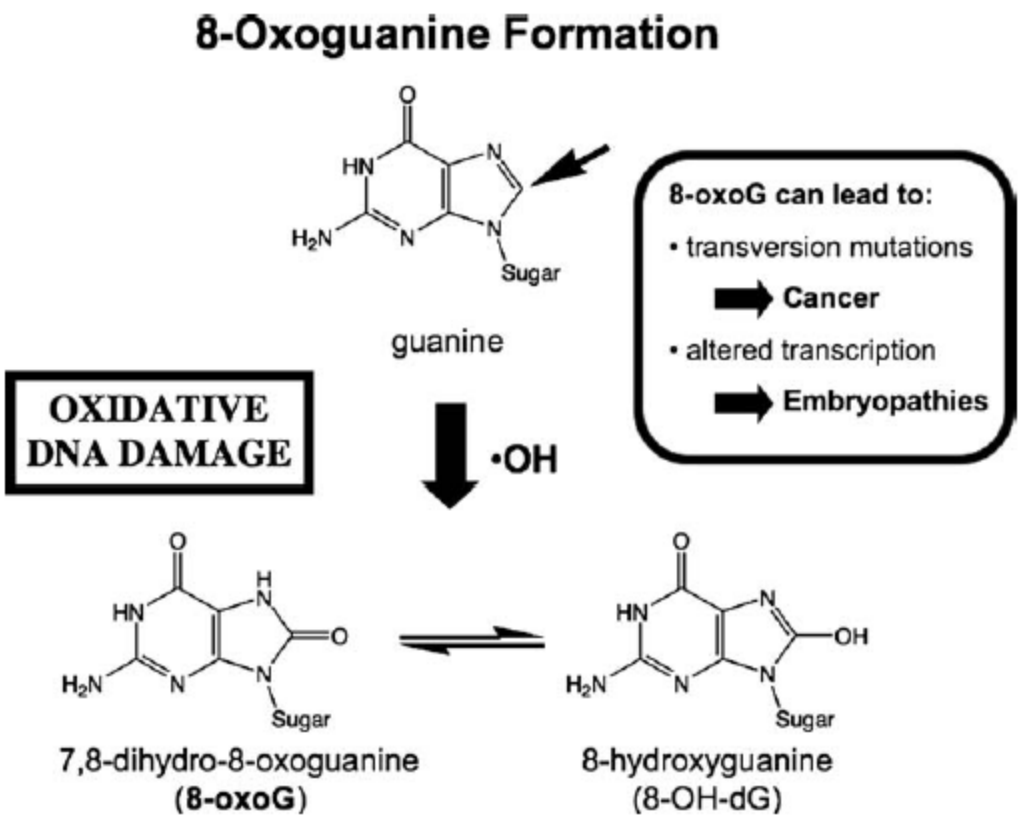


Figure 4. Reaction of hydroxyl radicals (•OH) with guanine residues of DNA.

Guanine is attacked at C8 by the hydroxyl radical to form the molecular lesion 7-8,dihydro-8-oxoguanine. This exists in equilibrium with the enol form, 8-hydroxyguanine. If not repaired, this oxidative damage can cause mutations and/or altered gene transcription, which may lead to cancer and/or embryopathies.

From: Wells et al., 2010a.

1.2.5.2. Cell death

Increased ROS formation can lead to programmed cell death, or apoptosis (Buttke & Sandstorm 1995). ROS has been implicated in both extrinsic (via induction of Fas receptors) and intrinsic (via oxidation of the mitochondria pores and subsequent cytochrome c release due to disruption of the mitochondrial membrane potential) pathways (Simon et al 2000). It has been shown that addition of antioxidants can block or delay apoptosis, and that there is efflux in antioxidants such as GSH in apoptotic cells but blockade of this efflux will delay apoptosis (van den Dobbelsteen et al 1996).

1.2.5.3 Signal transduction

Apart from causing oxidative macromolecular damage, ROS can alter signal transduction, with teratological consequences. Oxidative damage to proteins involved in signal transduction processes can alter the activities of these pathways, affecting gene expression, cell growth and cell survival (Allen & Tresini 2000). For example Ras and nuclear factor kappa B (**NF-κB**) are transduction proteins commonly involved in ROS-mediated signaling pathways, and embryonic Ras activation (Winn & Wells 2002) and NF-κB expression (Kennedy et al 2004) are both increased by phenytoin. Pretreatment with a farnesyltransferase inhibitor, which blocks Ras activation, or inhibition of NF-κB expression with antisense oligonucleotides, both block the embryopathic effects of phenytoin, suggesting that ROS-mediated signal transduction play an important role in the teratological mechanism (**Fig. 5**). Similarly ROS-mediated alterations in signaling involving GSH and the transduction proteins, NF-κB, Wnt and Akt, have been implicated in the mechanism of teratogenesis (Hansen 2006; Knobloch & Rüther 2008). ROS also stimulate the mitogen-activated protein kinase (**MAPK**) signaling pathway (Allen & Tresini 2000).

Signaling by ROS is mainly carried out by targeted modifications of cysteine residues in proteins (Claiborne et al 1999; Janssen-Heininger et al 2008). Under physiological conditions, some cysteine residues involved in signal transduction pathways are oxidized, which can either activate or inactivate the protein (**Fig. 6**) (Denu & Tanner 1998; Janssen-Heininger et al 2008; Lee et al 1998). This cysteine residue oxidation is reversible, and reduction provides rapid inactivation of the transduction cascade (**Fig. 7**).

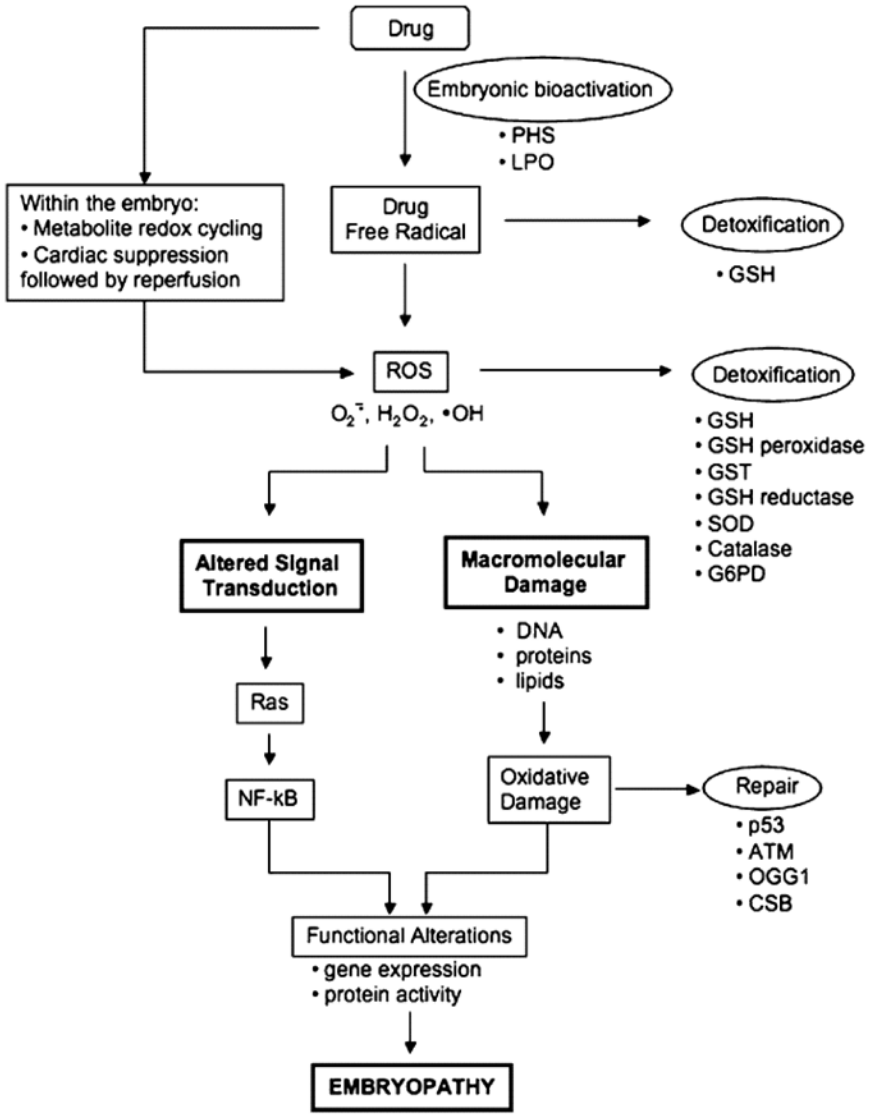


Figure 5. Potential contribution of Ras and nuclear factor-kappa B (NF-kB) proteins in signal transduction pathways initiated by drug-enhanced formation of reactive oxygen species (ROS).

Abbreviations: PHS, prostaglandin H synthase; LPO, lipoxygenase; GSH, glutathione; H_2O_2, hydrogen peroxide; HO•, hydroxyl radical; $O_2^{\bullet-}$, superoxide; GST, glutathione S-transferase; SOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase; ATM, ataxia telangiecasia mutated; OGG1, oxoguanine glycosylase 1; CSB, cockayne syndrome B.

From: Wells et al., 2009.

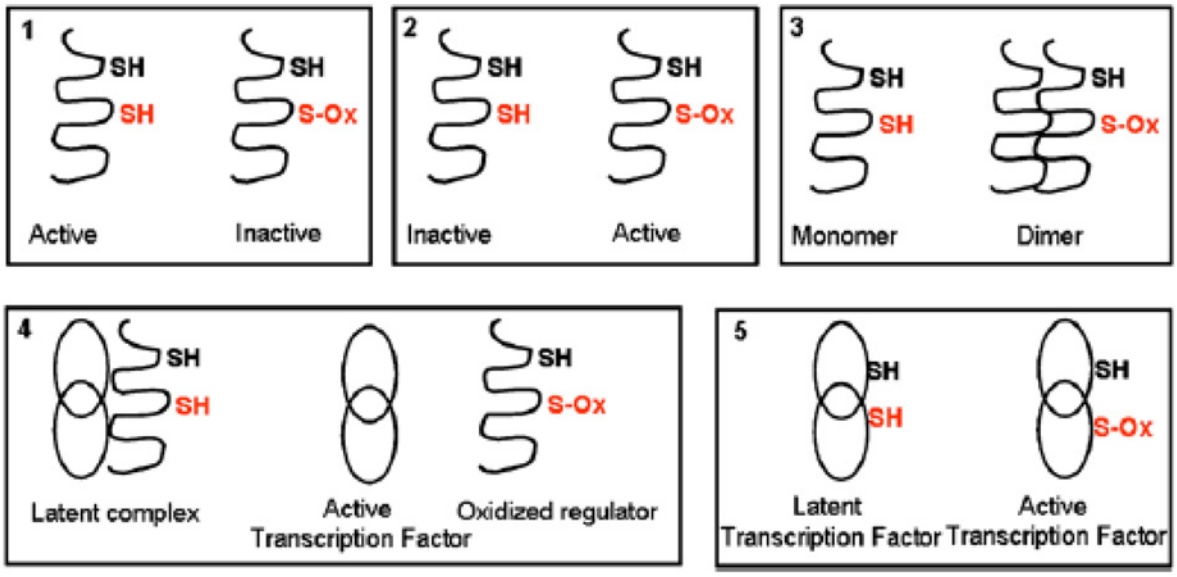


Figure 6. Schematic representation of mechanisms of redox regulation.

The scenario in which cysteine oxidation inactivates a signaling protein, such as a protein tyrosine phosphatase (box1) or caspases. Additionally, a protein with a signaling function can be directly activated by the oxidation of a critical cysteine residue (box2). Examples of this scenario are Ras, ryanodine receptor, dynamin, SERCA, and Src tyrosine kinase (Rhee et al 2005). Another mechanism whereby cysteine oxidation elicits a signal is through control of multimerization (box3), such as the dimerization of various heat shock or chaperone proteins. Box 4 visualizes the situation in where the cysteine oxidation of a regulatory protein causes its dissociation from its partner, thereby activating the function of the partner. Examples of this are the dissociation of oxidized Keap-1 from Nrf2, leading to the activation of Nrf2 as a transcription factor. Direct oxidation of transcription factors may also be sufficient to mediate their activation or inhibition through altered interactions with DNA (box5). Examples include the activation of prokaryotic OxyR, which shifts from a "dimeric" to a "tetrameric" interaction with DNA upon oxidation, and NF-κB (p50, p65) or c-Jun, whose binding with DNA is inhibited by S-oxidation.

From: Janssen-Heininger et al., 2008.

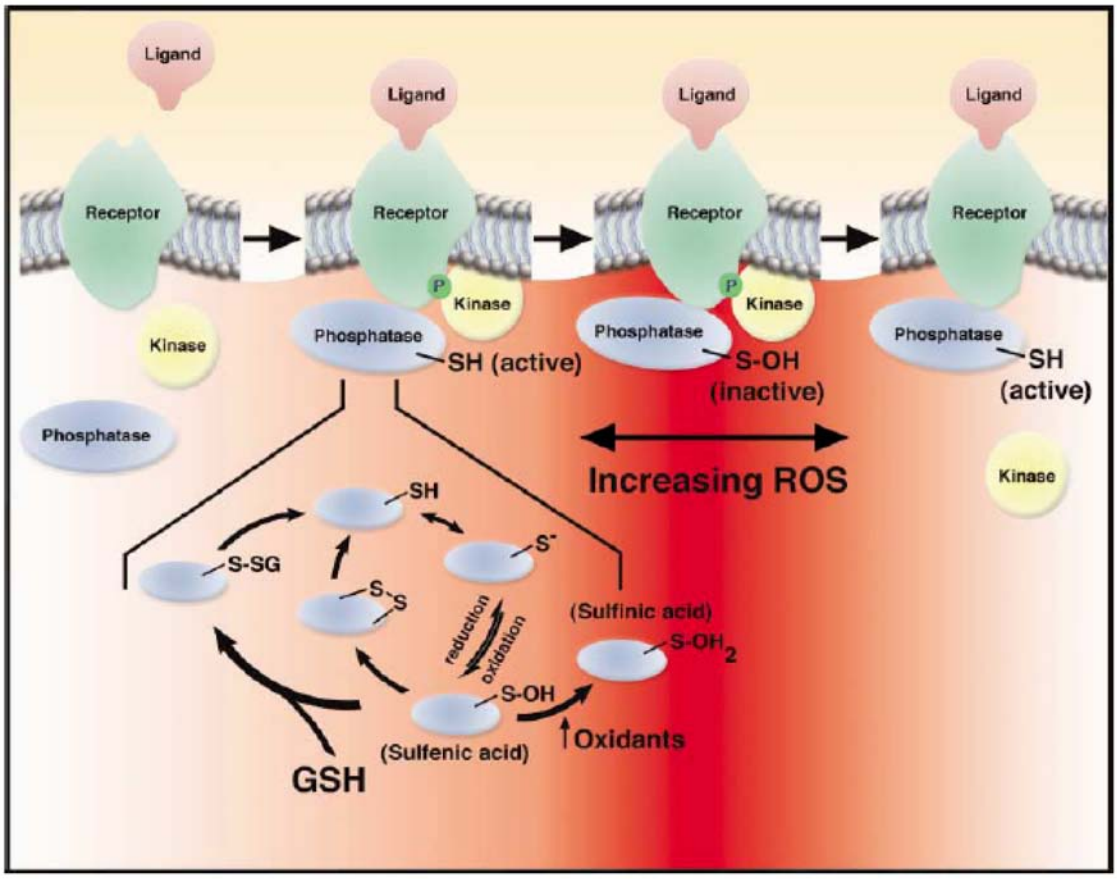


Figure 7. A model for redox regulation of protein tyrosine phosphatase (PTP) activity.

Recruitment of tyrosine phosphatases such as SHP-2 to membrane receptors is stimulated by ligand engagement. Receptor-bound, fully active PTPs could in turn destroy binding sites for other signaling molecules that have been recruited simultaneously. The observed rise in intracellular ROS can, however, transiently inactivate PTP activity, presumably through the formation of a sulfenic intermediate. The presence of either glutathione (GSH) or another intramolecular reactive cysteine allows for reversibility by providing a pathway to reduce the oxidized cysteine.

From: *Xu et al., 2002.*

With increased ROS production, multiple oxidations of the same cysteine residue and formation of sulfinic and sulfonic acids result in irreversible oxidative damage and consequently a disruption in the signaling pathways resulting in the cellular damage (**Fig. 8**) (Barrett et al 1999). There is a remarkable specificity in this process, as only certain cysteine residues can be oxidized by ROS. Unlike most cysteine residues that remain protonated at physiological pH (pKa>8.0), some cysteine residues (present in the thiolate form, RS-) within the catalytic site of particular signal transduction proteins such as the tyrosine phosphatases are extremely reactive and rapidly form a thiolate anion at physiological pH (pKa<6) (Barrett et al 1999; Xu et al 2002). The reactivity of this cysteine is in fact essential for the catalytic mechanism of tyrosine phosphatases. However, under conditions of oxidative stress, the very reactivity required for enzymatic activity also renders the catalytic cysteine vulnerable to oxidation. This target protein-dependent phenomenon attributes to the specificity of ROS-initiated alterations in signal transduction pathways through regulation of numerous enzymes such as tyrosine phosphatases, proteases (caspases), molecular adaptors (Nrf2-KEAP system), chaperones (heat shock proteins, Hsp), and transcription factors (NF-κB, p53, heat shock factor) (Janssen-Heininger et al 2008).

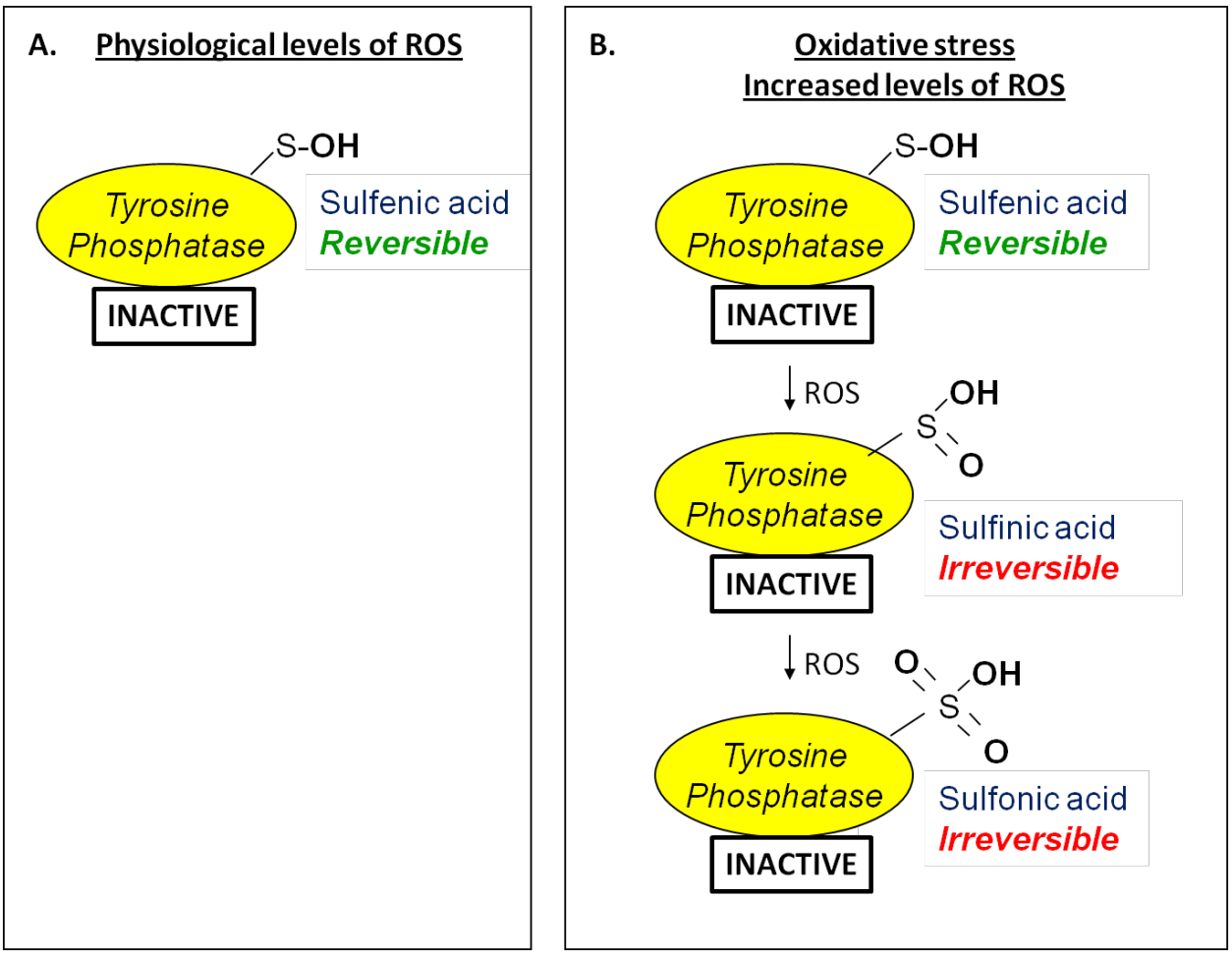


Figure 8. Proposed mechanism of excess oxidation and inactivation of signal transduction proteins.

Box A represents normal physiological conditions with oxidation of tyrosine phosphatase to a reversible sulfenic acid. Box B represents oxidative stress conditions where increased levels of ROS can irreversibly oxidize tyrosine phosphatase to sulfinic and sulfonic acids.

Modified from: Barret et al., 1999.

1.3 MECHANISMS OF CHEMICAL TERATOGENESIS

Teratogenesis refers to irreversible birth defects occurring in the developing embryo or fetus that persist after birth. These birth defects could be structural, such as shortened arms and legs and cleft palate, or functional, including cognitive, behavioral and motor deficits. **Figure 9** shows the range of potential adverse developmental consequences of *in utero* xenobiotic exposure (Wells et al 2010a). It has been estimated that over 50% of human fertilized eggs or embryos will die before birth (Roberts & Lowe 1975; Rolfe 1982), and of the children who survive to birth, at least 2-3%, and as many as 16% (Chung & Myrianthopoulos 1975) will exhibit some teratological abnormality. Susceptibility to teratological effects changes during development from the fertilization of the egg and implantation of the blastocyst, and through the embryonic and fetal periods to birth (**Fig. 10**).

Xenobiotics adversely affect development typically through either reversible binding to a specific receptor, or bioactivation to an electrophilic or free radical reactive intermediate that respectively binds covalently to cellular macromolecules or enhances ROS formation (**Fig. 11**) (Wells et al 2010a). Receptor-mediated toxicity occurs immediately following an excessive increase in the concentration of the xenobiotic, and usually resolves as the xenobiotic concentration decreases. The mechanism of receptor-mediated toxicities is usually an exaggeration of the same mechanism underlying the therapeutic effects of the drug. On the other hand, reactive intermediate-mediated toxicities are often unpredictable because they are generally unrelated to the therapeutic effects of the drug, can occur at therapeutic drug concentrations and largely depend on the balance between bioactivation and detoxification or repair of the macromolecular damage (**Fig. 12**). Thus individual susceptibility to reactive intermediate-mediated teratogenesis would depend on the biochemical balance within the developing fetus. However, it is important to note that, for some birth defects, both mechanisms could potentially contribute, thus complicating an elucidation of the mechanism of teratogenesis as well as the prediction of subjects at higher risk.

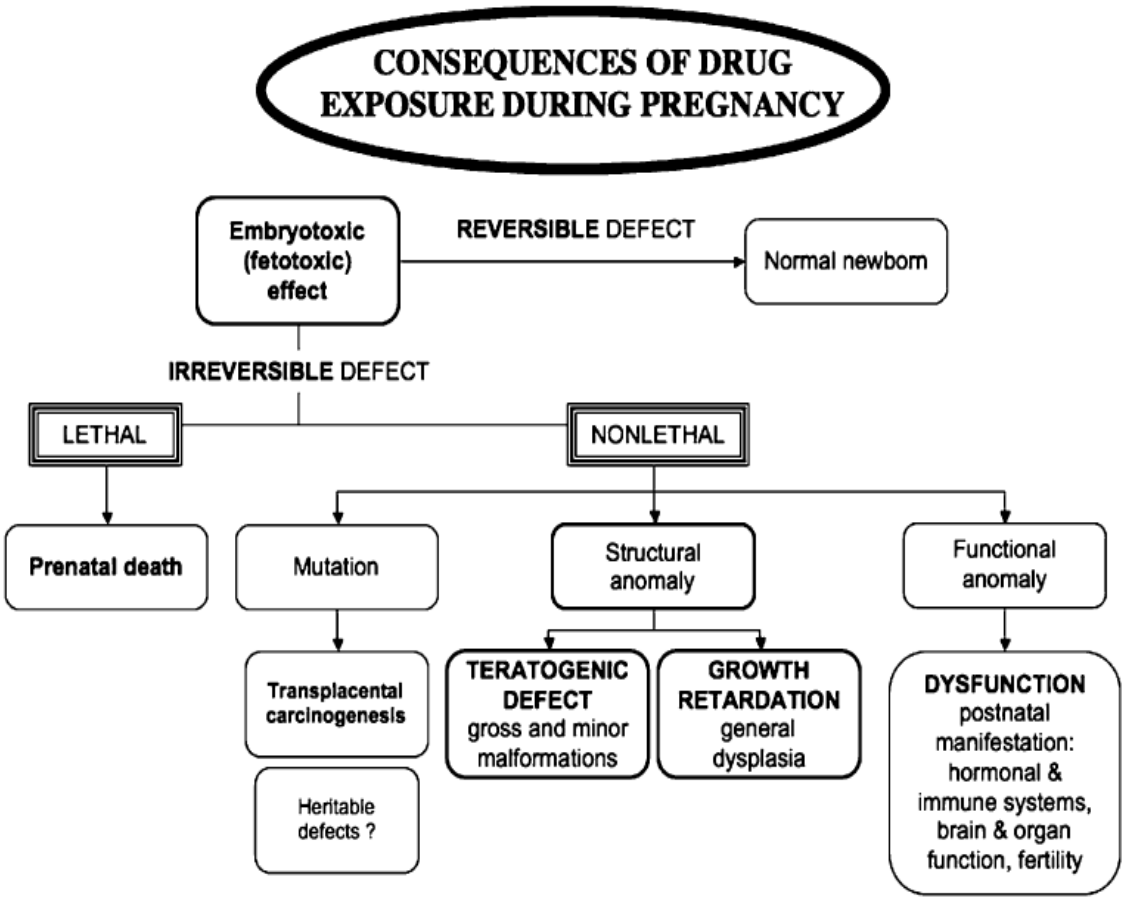


Figure 9. Consequences of drug exposure during pregnancy.

From: Wells et al., 2010a.

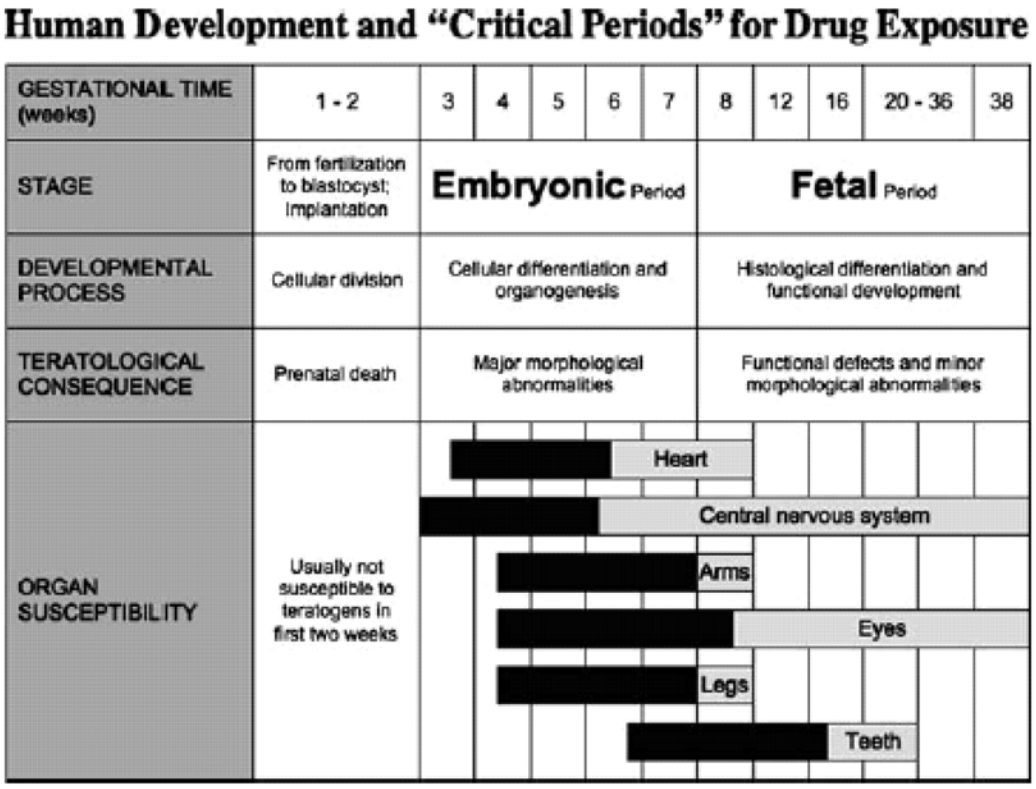


Figure 10. Human development and critical periods for drug exposure.

From: Wells et al., 2010a.

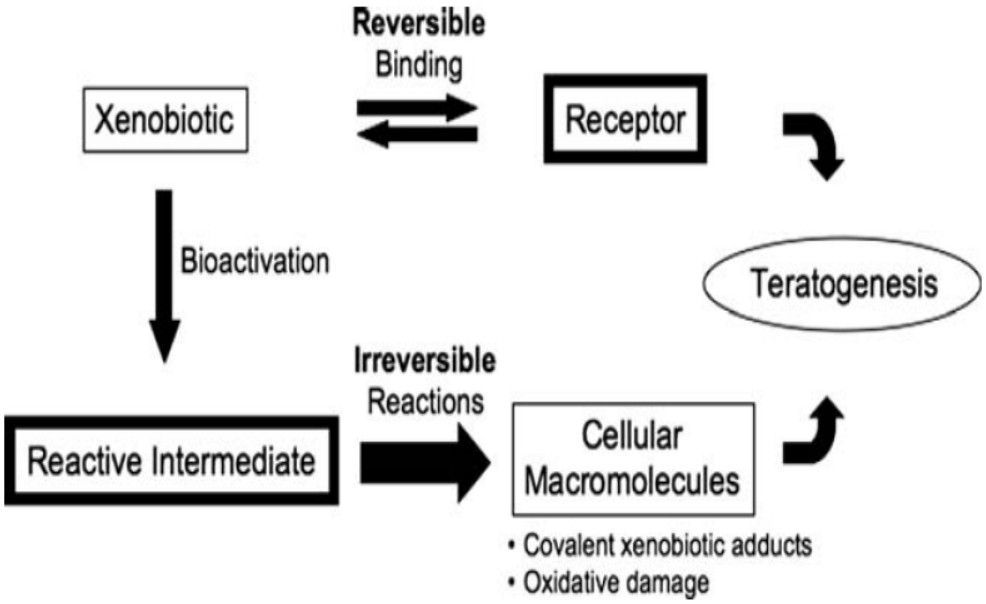


Figure 11. Receptor versus reactive intermediate-mediated mechanisms of teratogenesis.

From: Wells et al., 2010a.

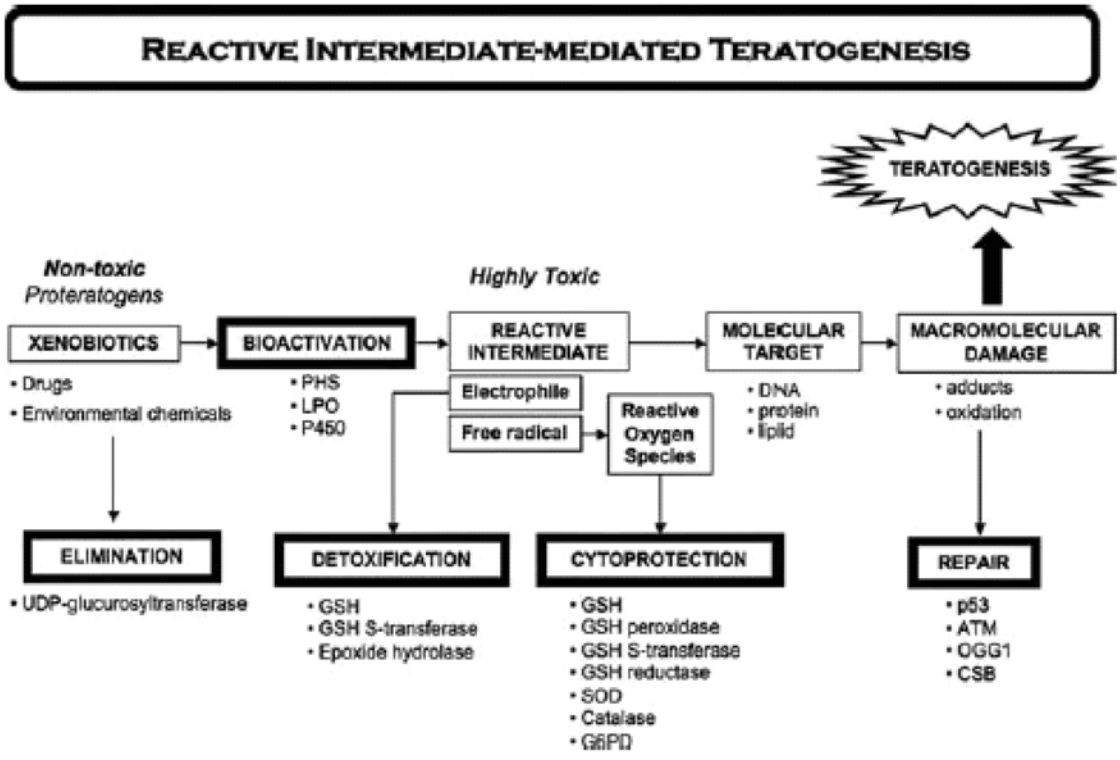


Figure 12. Enzymatic pathways involved in reactive intermediate-mediated teratogenesis.

From: Wells et al., 2010a.

Electrophiles, such as epoxides and arene oxides, contain an electron-deficient center that is highly reactive with electron-rich groups on proteins and nucleic acids, forming adducts. Bioactivation to an electrophilic reactive intermediate is primarily catalyzed by cytochromes P450 (**P450** or **CYP**) (**Fig. 13**) and PHS enzymes, and has been implicated in the mechanism of teratogenicity for a number of xenobiotics, including phenytoin and benzo[a]pyrene (Juchau et al 1986; Juchau et al 1992; Wells et al 1997; Wells et al 2009; Winn & Wells 1995a). Covalent binding of the electrophilic reactive intermediate to a developmentally important protein or gene results in the formation of a drug-macromolecular adduct that may lead to altered development or death of the embryo/fetus.

On the other hand, xenobiotics can be also oxidized by PHS and lipoxygenases (**LPO**) to a free radical intermediates (**Fig. 14**) (Wells et al 2009). Such bioactivation has been shown for drugs such phenytoin and structurally related AEDs, benzo[a]pyrene, thalidomide, methamphetamine and valproic acid (Fantel 1996; Wells et al 2009; Wells & Winn 1996). These xenobiotic free radical intermediates react directly or indirectly with molecular oxygen to initiate the formation of potentially teratogenic ROS.

The developing embryo has relatively low levels of most xenobiotic-metabolizing enzymes, including CYPs, UDP-glucuronosyltransferases (**UGTs**), glutathione S-transferases (**GSTs**), epoxide hydrolases and, most importantly for reactive intermediate-mediated teratogenesis, ROS-detoxifying enzymes (Wells et al 2009), leaving the embryo and fetus at high risk for reactive intermediate-mediated teratogenesis. Also importantly for reactive intermediate-mediated teratogenesis, the embryo and fetus have high levels of PHS and LPO, which can bioactivate many xenobiotics to reactive intermediates. The high levels PHS/LPO combined with low levels of ROS-detoxifying enzymes leaves the embryo and fetus particularly more susceptible than adults to ROS-mediated teratogenesis. Susceptibility also depends on maternal metabolism of xenobiotics. Any reduction in maternal elimination pathways can result in increased levels of the drug reaching the embryo. For example, low UGT activity in Gunn rats resulted in decreased glucuronidation of benzo[a]pyrene metabolites and a 3-fold increase in fetal resorptions (Wells et al 2004).

In the case of reactive intermediate-mediated teratogenesis, the reactive intermediate and ROS are typically too unstable to be formed maternally and cross the placenta (**Fig. 1**). Accordingly, maternal pathways do not contribute directly to this mechanism; whereas the

balance of activities of embryonic and fetal enzymes catalyzing xenobiotic bioactivation and the detoxification of xenobiotic reactive intermediates and ROS plays a key role in determining the adverse developmental consequences of xenobiotic exposure (see later section on phenytoin metabolism to a reactive intermediate).

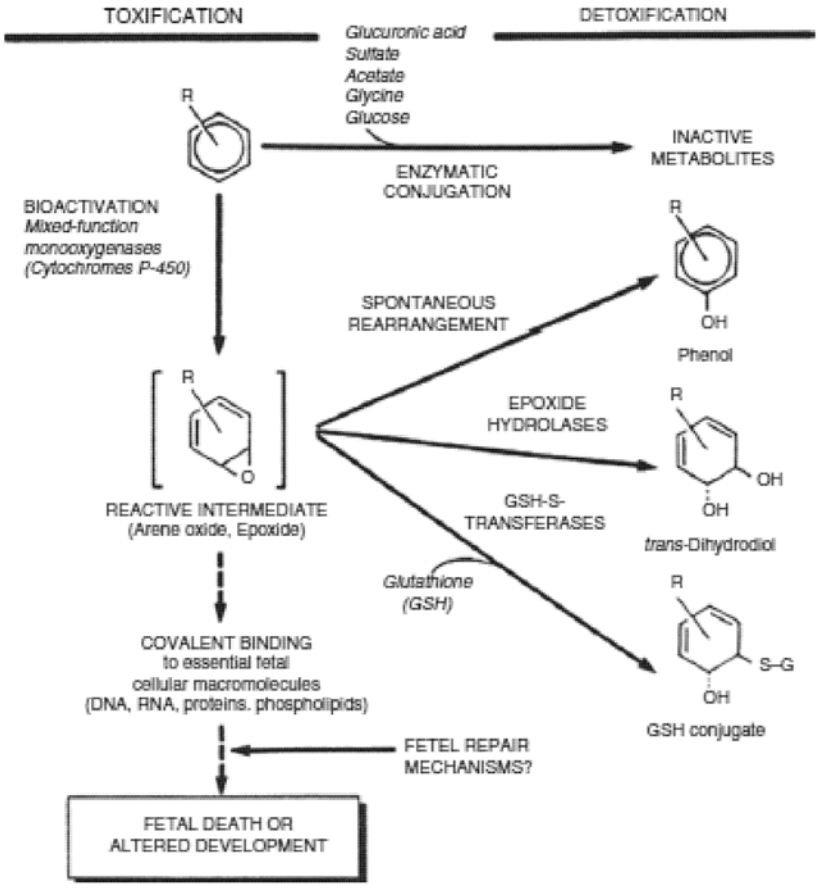


Figure 13. Formation and detoxification of an electrophilic reactive intermediate.

From: Wells et al., 2010a.

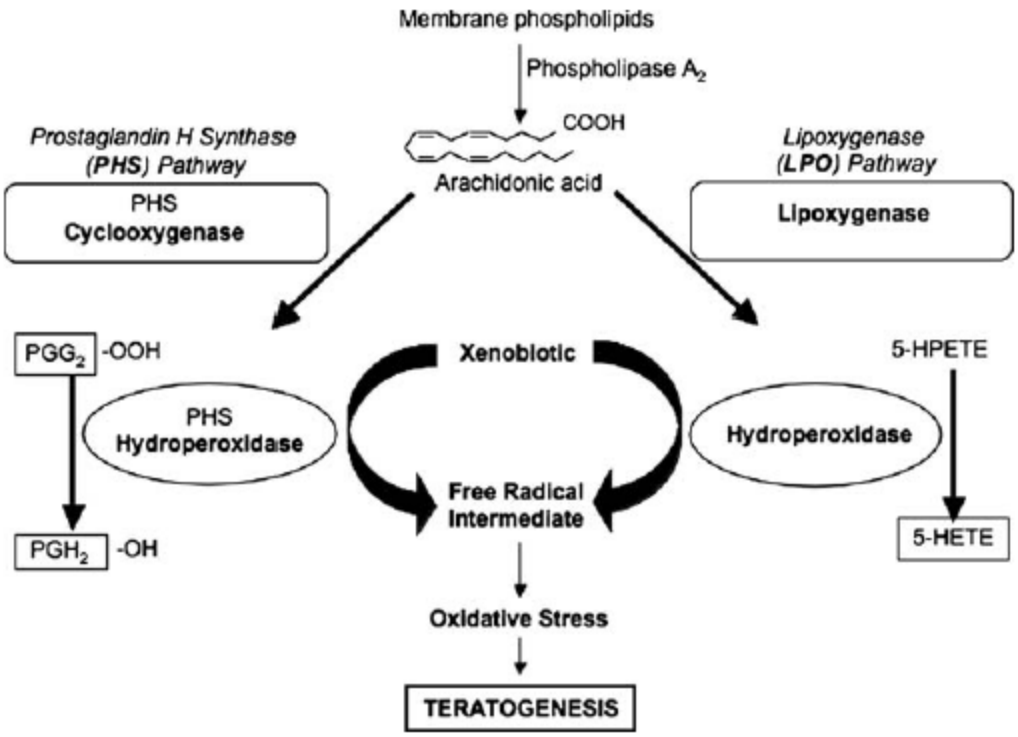


Figure 14. Bioactivation of xenobiotics via the prostaglandin H synthase (PHS) and lipoxygenase (LPO) pathways – postulated role in teratogenesis.

The hydroperoxidase component of embryonic and fetal PHS, and hydroperoxidases associated with LPO, can oxidize xenobiotics to free radical intermediates that initiate the formation of reactive oxygen species causing oxidative stress.

From: Wells et al., 2010a.

.

1.4 PHENYTOIN TERATOGENICITY

Phenytoin (Dilantin®) (**Fig. 15**) is one of the most effective and widely used anticonvulsants in North America for the treatment of generalized tonic-clonic and simple and complex partial epileptic seizures by blocking Na^+, Ca^{2+} and K^+ channels (Rogawski & Porter 1990). However, phenytoin also has been shown to be teratogenic in a number of species (Collins et al 1990; Fort & Bantle 1990; Harbison & Becker 1972; Massey 1966; McClain & Langhoff 1980; Singh & Shah 1989) including humans (Bromley et al 2009; Buehler et al 1990; Hanson & Smith 1975; Scolnik et al 1994; Strickler et al 1985). In an animal model, it has been shown that phenytoin itself, and neither the epileptic disease state nor seizure frequency, is associated with malformations (Finnell 1980; Finnell & Chernoff 1982). In humans, the Fetal Hydantoin Syndrome describes the constellation of fetal anomalies associated with phenytoin exposure (Hanson & Smith 1975) (**Table 1**). In human studies, it has been estimated that up to 34% of exposed children will develop some characteristics of this syndrome, which includes craniofacial, limb, cardiovascular and central nervous system defects (Bromley et al 2009; Nulman et al 1997; Scolnik et al 1994; Strickler et al 1985; Van Dyke et al 1988). Nevertheless, due to the health risk of untreated seizures for both mother and fetus, phenytoin therapy is generally continued throughout pregnancy. Newer antiepileptic drugs such as levetiracetam and topiramate have been studied for their teratogenic potential and preliminary studies suggest low risk, but small sample size limit conclusions (Meador et al 2009). Also no research into the cognitive or behavioral abilities of children exposed *in utero* to these drugs has been done to date (Bromley et al 2009).

1.4.1 Postulated mechanisms of phenytoin teratogenesis

1.4.1.1 Drug-induced folate deficiency

This hypothesis states that phenytoin therapy causes depletion in folic acid, which is essential during embryonic development for DNA synthesis. There is evidence showing that patients on long-term anticonvulsant therapy develop folate deficiency, which is embryotoxic both *in vivo* and *in vitro*. The exact mechanism responsible for this phenytoin-induced deficiency is unknown, but it has been proposed to result from either impaired folate absorption or an

increase in folate catabolism. However, there is evidence to show that neither folate absorption nor catabolism are affected by phenytoin and furthermore, supplemental folic acid did not reduce all phenytoin-initiated embryotoxicity (reviewed in (Hansen 1991). In addition, newer studies did not show a decrease in folate in mouse embryos exposed to phenytoin. Overall, there is no consistent evidence supporting a role for the depletion of folic acid by phenytoin in its mechanism of teratogenesis.

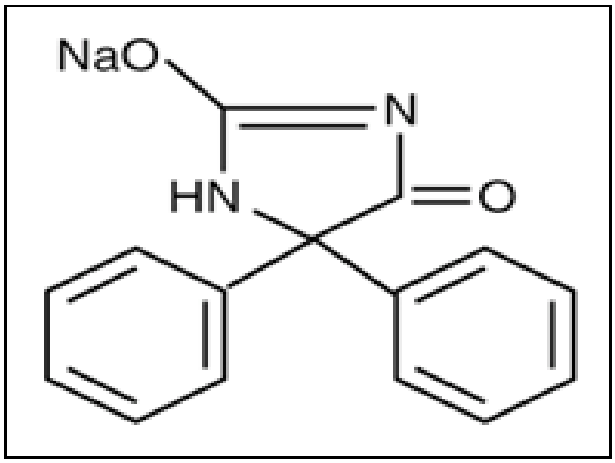


Figure 15. Chemical structure of phenytoin.

Table 1. Characteristics of the fetal hydantoin syndrome.

Adverse effects	**Reference**
Craniofacial features Microcephaly Broad & depressed nasal bridge Inner epicanthic fold Ocular hypertelorism Ridging of sutures Cleft lip/palate Low-set ears	(Hanson & Smith 1975)
Limb defects Distal phalangeal hypoplasia Nail hypoplasia Finger-like thumbs Variation in palmar creases	(Hanson & Smith 1975)
Cardiovascular anomalies Ventriculo-septal defects Tetralogy of Fallot Hypertrophic cardiomyopathy with endocardial fibroelastosis and conduction defect	(Strickler et al 1985) (Van Dyke et al 1988) (Scolnik et al 1994)
Central nervous system defects Low IQ Behavioral anomalies Developmental delays Mental retardation School/learning problems Speech/language problems	(Hanson & Smith 1975) (Hanson et al 1976) (Van Dyke et al 1988) (Scolnik et al 1994)

IQ, intelligence quotient

Modified from Hanson et al., 1976.

1.4.1.2 Receptor-mediated toxicity

It has been proposed that phenytoin exerts its teratogenic effects by binding to the glucocorticoid receptor (Katsumata et al 1982). This was based on the observation that a teratogenic dose of phenytoin increased maternal glucocorticoid levels and mimicked the effects of cortisol on programmed cell death, all of which were blocked by a glucocorticoid receptor antagonist. However, removal of maternal adrenal glands did not reduce phenytoin-initiated embryotoxicity (Hansen et al 1992), and some human studies showed that there was no correlation between maternal levels of phenytoin and phenytoin teratogenicity (Monson et al 1973). In addition, there is also evidence showing that phenytoin does not bind to the glucocorticoid receptor (Salomon & Pratt 1979). Therefore, phenytoin teratogenesis does not appear to be dependent on the glucocorticoid receptor.

1.4.1.3. Phenytoin-initiated hypoxia/ischemia

It has also been hypothesized that phenytoin exerts its teratogenic effects by delaying K^+-currents in action potentials resulting in reduced cardiac contractility and embryonic hypoxia/ischemia (Danielsson et al 1997). Severe episodes of hypoxia within the embryo are followed by reoxygenation that generates ROS ("reperfusion injury"), which causes vascular disruption and tissues necrosis. This hypothesis is supported by evidence showing phenytoin exposure decreases embryonic heart rate. However, the concentration of phenytoin needed to cause this decrease in embryonic heart rate is 10 times higher than the minimal concentration shown to initiate oxidation of embryonic DNA and protein and cause embryopathies (Shanks et al 1989; Winn & Wells 1995b; 1997). Accordingly, a hypoxia/ischemia mechanism is more likely to play a teratogenic role at higher embryonic phenytoin concentrations, and does not preclude the contribution of other mechanisms.

1.4.1.4 Metabolism to a reactive intermediate

Considerable evidence supports the enzymatic bioactivation of phenytoin to an embryotoxic reactive intermediate as a mechanism of teratogenic initiation. There are two enzymatic pathways by which this can occur: (1) CYP-catalyzed bioactivation to an electrophilic reactive intermediate (**Fig. 13**) (Shanks et al 1989); and (2) PHS- and LPO-catalyzed

bioactivation to free radical reactive intermediate (**Fig. 14**) (Kubow & Wells 1989; Miranda et al 1994; Parman et al 1998). The former covalently binds to cellular macromolecules forming a drug-macromolecular adduct, whereas the latter can initiate the formation of ROS, which can both alter signal transduction and oxidatively damage cellular macromolecules (Winn & Wells 1995a). Evidence for these two mechanisms is summarized in **Tables 2** and **3**. The formation of drug-macromolecular adducts, oxidative macromolecular damage, and ROS-mediated altered signal transduction all may adversely affect embryonic and fetal development.

Due to the discrepancies in the CYP-catalyzed bioactivation hypothesis and to growing evidence from our laboratory, we hypothesize that phenytoin-initiated teratogenicity is mediated, at least in part, through peroxidase-catalyzed bioactivation leading to the formation of a free radical and generation of ROS.

Table 2. P450-catalysed bioactivation in phenytoin teratogenesis.

Supporting evidence	Discrepancies
PHT can covalently bind to embryonic and placental protein (Harbison 1978; Harbison et al 1977; Lum & Wells 1986; Martz et al 1977) and DNA (Liu & Wells 1994a).	Rodents and humans have, respectively, minimal and low activities of most embryonic P450s (Hines 2008; Juchau et al 1992; Raucy & Carpenter 1993).
PHT-initiated *in vitro* mononuclear leukocyte toxicity required inhibition of epoxide hydrolase with trichloropropene oxide (TCPO), and cytotoxicity and covalent binding were enhanced by the P450 inducers phenobarbital or β-naphthoflavone (Riley et al 1990).	Variable susceptibility to phenytoin teratogenesis in strains of inbred mice cannot be explained by genetic differences in P450-catalysed metabolism of phenytoin (Atlas et al 1980; Hansen & Hodes 1983).
Inhibiting epoxide hydrolase with TCPO increases phenytoin teratogenesis (Blake & Martz 1980; Martz et al 1977).	Phenytoin teratogenicity *in vivo* is inhibited by pretreatment with P450 inducer phenobarbital, which should enhance bioactivation, and enhanced by pretreatment with the P450 inhibitor SKF 525A (Harbison & Becker 1970; Wells & Gesicki 1984).
Administration of the epoxide hydrolase inhibitor TCPO, and the GSH depletors dimethyl maleate, acetaminophen and buthionine sulfoxamine all enhanced phenytoin teratogenicity in mice (Harbison 1978; Lum & Wells 1986; Wong et al 1989).	The non-arene oxide-forming L-isomers of the structurally related anticonvulsants mephenytoin and nirvanol are teratogenic, while teratogenesis is not initiated by the D-isomers that do form an arene oxide intermediate (Wells et al 1982).
Children exposed *in utero* to phenytoin who developed major birth defects had lymphocytes with increased NADPH-dependent *in vitro* cytotoxicity when challenged with PHT (Strickler et al 1985).	Trimethadione and its metabolite, dimethadione, both of which lack the phenyl ring necessary for the formation of an arene oxide, are teratogenic (Wells et al 1989).
Low epoxide hydrolase activity was found in children with FHS (Buehler et al 1990), and heteropaternal twins who were discordant for FHS had correspondingly low and normal activities of epoxide hydrolase (Buehler 1984).	Enhancement of phenytoin teratogenicity by GSH depletion suggests the involvement of a reactive intermediate, but does not discriminate between electrophilic (arene oxide) and free radical reactive intermediates (Wells et al 1997; Wells & Winn 1996).
Concurrent administration of the anticonvulsant drug stiripentol to pregnant mice reduced phenytoin teratogenicity, and this protective effect was attributed to stiripentol's inhibition of P450-catalyzed bioactivation of PHT (Finnell et al 1994).	The protective effects of stiripentol in inhibiting phenytoin teratogenicity may be due to actions other than P450 inhibition, since all other P450 inhibitors evaluated have enhanced phenytoin teratogenicity (Harbison & Becker 1970; Wells & Gesicki 1984).

PHT, phenytoin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; GSH, glutathione; FHS, fetal hydantoin syndrome.

From: Winn and Wells, 1995a.

Table 3. Evidence for peroxidase-catalyzed bioactivation in chemical teratogenesis.

Parameter	Probe	Teratogen	End-points	Systems	Reference
Co-substrate-dependent	Arachidonic acid Linoleic acid	Phenytoin	Covalent binding↑ Target oxidation↑	*In vitro*	(Kubow & Wells 1989; Parman et al 1998; Yu & Wells 1995)
Purified enzymes	PHS LPO HRP	Phenytoin Mephenytoin Nirvanol Trimethadione Dimethadione Thalidomide	Covalent binding↑ Target oxidation↑	*In vitro*	(Kubow & Wells 1989; Liu & Wells 1995a; b; Parman et al 1998; Yu & Wells 1995)
Enzyme inhibitors	ASA ETYA NDGA Methimazole	Phenytoin Trimethadione Dimethadione Thalidomide	Covalent binding↓ Target oxidation↓ Teratogenicity↓	Embryo culture *In vitro* *In vivo*	(Arlen & Wells 1996; Kubow & Wells 1989; Lee et al 2011; Liu & Wells 1994b; 1995a; b; Miranda et al 1994; Parman et al 1998; Wells et al 1989; Yu & Wells 1995)
Substrate release	TPA	Phenytoin	Teratogenicity↑	*In vivo*	(Wells & Vo 1989)

PHS, prostaglandin H synthase; LPO, lipoxygenase; HRP, horseradish peroxidase; ASA, acetylsalicyclic acid (PHS inhibitor); ETYA, eicosatetraynoic acid (dual PHS/LPO inhibitor); NDGA, nordihydroguaiaretic acid (LPO inhibitor/antioxidant); Methimazole (hydroperoxidase inhibitor); TPA, tetradecanoylphorbol acetate (phospolipase A_2 activator).

From: Winn and Wells, 1995a.

1.5 ANTIOXIDANT CATALASE

1.5.1 Introduction

Catalase is a ubiquitous heme-containing protein found primarily in peroxisomes but also in the cytoplasm (Chance et al 1979). The mammalian catalase occurs as a complex of four identical subunits. Each subunit has an approximate molecular weight of 60,000 Da and contains one ferriprotoporphyrin (hematin) group. For full activity all four subunits must be assembled (Percy 1984). The crystal structure of human catalase (**Fig. 16**) indicates that each subunit contains an N-terminal threading arm that connects two subunits by hooking through a long wrapping loop around another subunit (Putnam et al 2000) (**Fig. 17**). Catalase also binds NADPH, but H_2O_2 is the source of both oxidative and reductive potential during the normal catalytic cycle. NADPH can either reduce trapped, off-pathway oxidized enzyme states and/or prevent these states from forming in the first place by providing a more attractive source of reductant (Putnam et al 2000). Catalase is the fastest enzyme characterized, capable of metabolizing 42,000 molecules/sec of H_2O_2 at 0°C (Takahara 1971). It is present in all major organs including liver, which has the highest activity, and brain, kidney, blood and bone marrow (Ogata 1991). The gene for catalase is found in humans on chromosome 11 (Wieacker et al 1980), and in mice on chromosome 2 (Dickerman et al 1968).

Catalase is responsible for removing H_2O_2 from the cells by converting it to H_2O and O_2 (**Fig. 18**). If not detoxified, H_2O_2 can interact with iron to form •OH, which can initiate a series of toxic reactions. In addition, in the presence of low concentrations of H_2O_2 ($<10^{-6}$ M), catalase can catalyze the oxidation of electron donors such as ethanol and phenol (peroxidatic activity) (Percy 1984). There are 2 distinct steps in the reaction of catalase (**Fig. 19**). In the first step, the native ferric hemoprotein (free catalase) reacts with H_2O_2 to form the primary complex, called compound I. In the second step, two electrons are transferred from an electron donor to form H_2O and an oxidized product. The electron donor can be either a second molecule of H_2O_2 (catalatic mode) or another substrate such as methanol, ethanol or formic acid (peroxidatic mode).

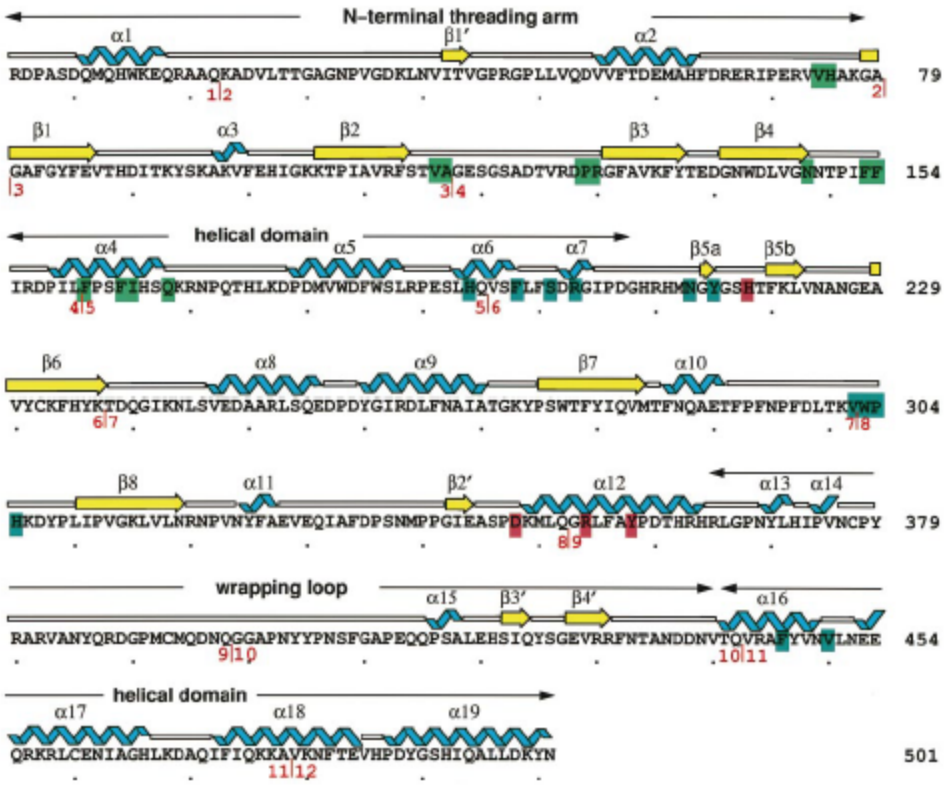


Figure 16. Secondary structure assignment for human catalase.

Blue α-helices and yellow β-sheets. Residues involved in the active site charge relay, NADPH binding and hydrogen peroxide selection are highlighted in red, blue and green, respectively. The two contiguous structural domains, the N-terminal threading arm and the wrapping loop are indicated by arrows, and exon boundaries of the human gene are illustrated in red.

From: Putnam et al., 2000.

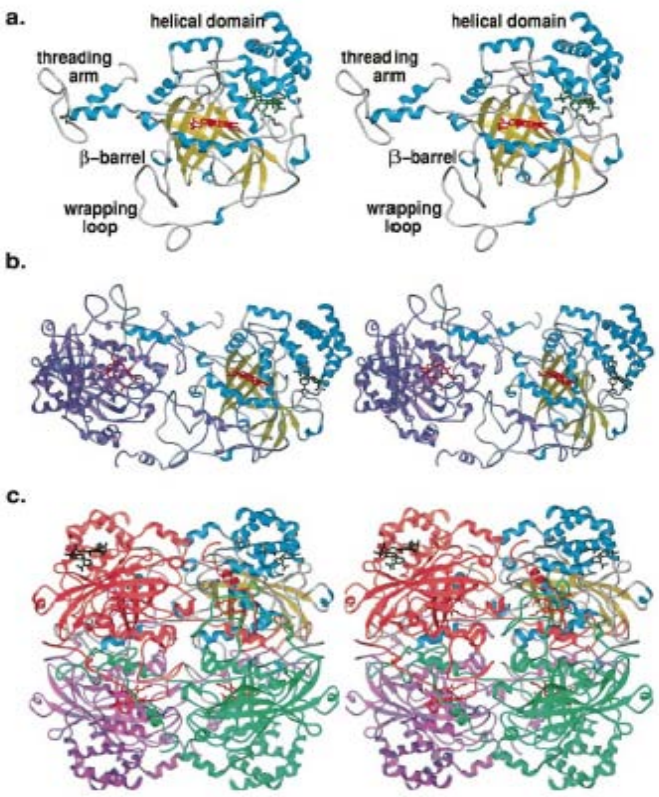


Figure 17. Structure of human catalase.

(a) Stereo view of an individual subunit of human catalase with the central β-barrel in yellow, surrounding helices in blue. The active site heme (red) is surrounded by the β-barrel, α-helices and loops with one open face that is buried upon tetramerization. The NADPH (dark green) is on the far side of the molecule in this view. (b) Stereo view of an arm-exchanged dimer with the yellow and blue subunit and a purple subunit related perpendicular to the page. The orientation is similar to (a). In this dimer, both hemes remain exposed on one face. (c) Stereo view of the catalase tetramer with the addition of a second arm-exchanged dimer (orange and green). Formation of the tetramer buries the heme active sites from solvent. The orientation of the tetramer is rotated by about 45° perpendicular to the page relative to (a) and (b).

From: Putnam et al., 2000.

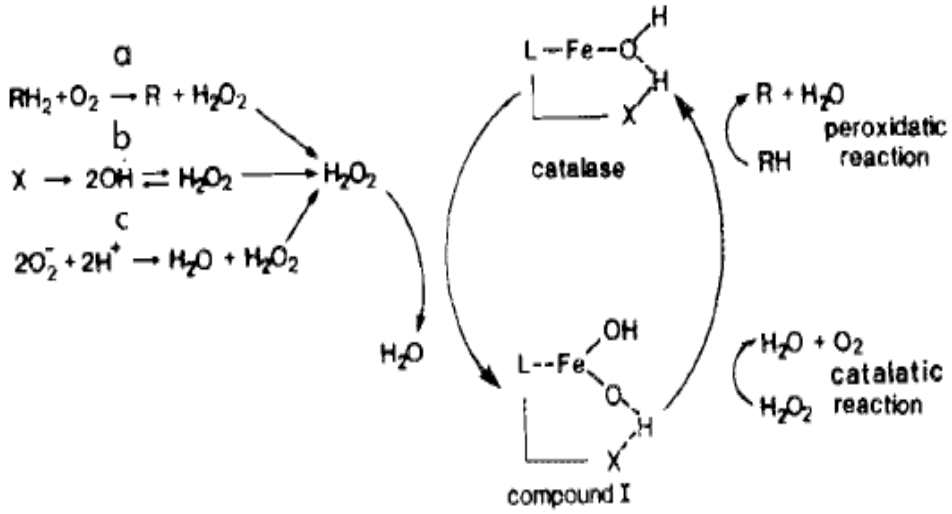


Figure 18. Cellular sources of hydrogen peroxide and its catalase mediated destruction.

(A) Flavin enzymes (uricase, D-amino acid oxidase)

(B) Autooxidation reactions (thiol compounds, ascorbate, hydrazines)

(C) Superoxide dismutase

X - represents a site on the catalase apoprotein that is postulated to undergo cyclic oxidation and reduction during the catalysis.

The iron (Fe) is located on the hematin prosthetic group represented by L.

From: Percy, 1983.

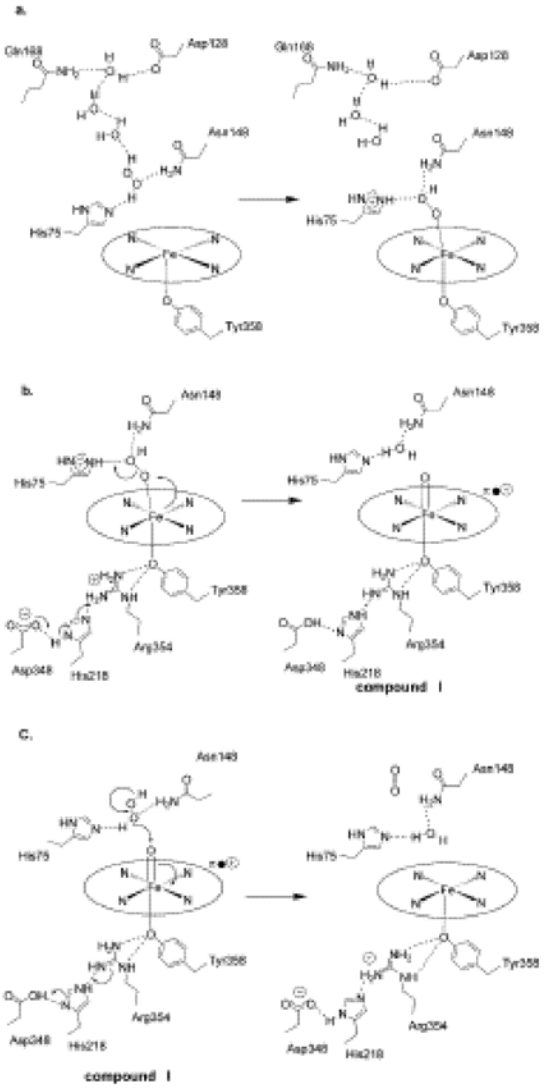


Figure 19. Proposed catalytic mechanism.

(a) Hydrogen peroxide is selected by and concentrated at the active site. (b) Heterolytic cleavage of the peroxide bond is driven through interactions with the electron-rich active site metal and the electron-withdrawing His75 and Asn148. (c) Oxidation of the second peroxide molecule likely occurs near the active site to a peroxide molecule bound by His75 and Asn148. A similar coordination of the ethanol hydroxyl group would bind the ethyl group within the hydrophobic channel. Reversal of the charge relay system after oxidation brings the molecule back to the resting state.

From: Putnam et al., 2000.

It is currently believed that both catalase and GSH peroxidase contribute to the breakdown of H_2O_2 *in vivo*, and that both enzymes have important functions, their relative contribution being dictated by their subcellular distribution, their localized concentration, and the availability of H_2O_2, hydrogen donors and GSH. In liver, the subcellular distributions of these two enzymes are strikingly different. GSH peroxidase is located mainly in the mitochondrial matrix and the cytosol, whereas catalase is highest in the peroxisomes (80%) with a smaller fraction of 20% present in the cytosol (Savaskan et al 2007).

One study showed that hepatic catalase activity could be controlled by hepatic nuclear factor 1α (**HNF-1α**) (Muppala et al 2000). Mice deficient in HNF-1α develop Laron dwarfism and non-insulin-dependent diabetes mellitus with increased oxidative stress. Oxidative stress was increased in these mice with age. Further examination of the HNF-1α-null mice showed that catalase activity was significantly reduced compared to the normal controls. Reduced catalase activity resulted from an insufficient heme pool in the liver cells caused by reduced gene expression for ferrochelatase, the enzyme that catalyzes the last step of heme synthesis. This study provides an example of a potential mechanism whereby the tissue-specific enrichment of a transcriptional regulator could play an important role in controlling localized expression of a ubiquitous factor like catalase in selected tissues.

1.5.2. Catalase mutant mouse

A mutant acatalasemic mouse (**aCat**) strain exhibiting deficient catalase expression was first described by Feinstein *et al.* (Feinstein et al 1964). This catalase mutation was identified by screening blood catalase activity levels in a group of mice following irradiation studies, and thus was considered an X-ray-induced mutation. The acatalasemic phenotype is expressed to a variable extent in different tissues, with red blood cells, kidney and liver tissue respectively exhibiting approximately 1%, 20% and 50% of normal values (Aebi et al 1968). The genetic defect that results in reduced expression of catalase in acatalasemic tissues does not mediate its effects at the level of transcription, but rather at the level of translation and/or catalase protein turnover, since similar levels of mRNA were found in all tissues (Shaffer et al 1987). It was subsequently determined that a CAG (glutamine)-to-CAT (histidine) transversion mutation in the third position of codon 11 was responsible for the deficiency (Shaffer & Preston 1990; Shaffer et

al 1990). The resulting disruption in an α-helix causes the final catalase tetramer, formed from four identical subunits, to become unstable. It is not known whether any other mutations contribute to the acatalasemia, such as in any regulatory proteins. Acatalasemic mice were shown to be more sensitive to liver injury following exposure to carbon tetrachloride (Wang et al 1996) and to low doses of radiation (Yamada et al 1997), and they developed spontaneous mammary tumors (Ishii et al 1996). For my studies, I have used C3Ga.Cg-*Cat*^{b}/J acatalasemic and C3HeB/FeJ catalase-normal wild-type mice (**Fig. 20**).

1.5.3. Human catalase-expressing mouse

The mouse expressing human catalase (**hCat**) was genetically engineered on a C57BL/6J background for studies to test oxidative stress theories in ageing. In this mouse there is an 80 kb fragment of human genomic DNA containing the 33 kb human *CAT* gene, as well as 41 kb of the 5' and 6 kb of the 3' flanking regions (**Fig. 21**). The resultant transgenic mouse model expresses human catalase in addition to endogenous mouse catalase (**Fig. 22**), resulting overall in an increase in total catalase activity of up to a 4-fold in a tissue-specific manner (Chen et al 2003). By including the introns and flanking regions of the catalase gene in the large genomic DNA construct, the regulatory elements were retained intact in the transgene, which resulted in a global pattern of catalase expression that is comparable to that for the endogenous gene.

Both hepatocytes and fibroblasts from the transgenic hCat mice were resistant to H_2O_2. Conversely, the transgenic mice exhibited a paradoxical increase in sensitivity (decreased survival) to γ-irradiation compared to wild-type mice. It was suggested that overexpression of catalase could interrupt the signaling pathways involving H_2O_2, thus leaving cells or animals that overexpress catalase more sensitive to certain stressors (Chen et al 2004), which is contrary to the hypothesis tested in this thesis.

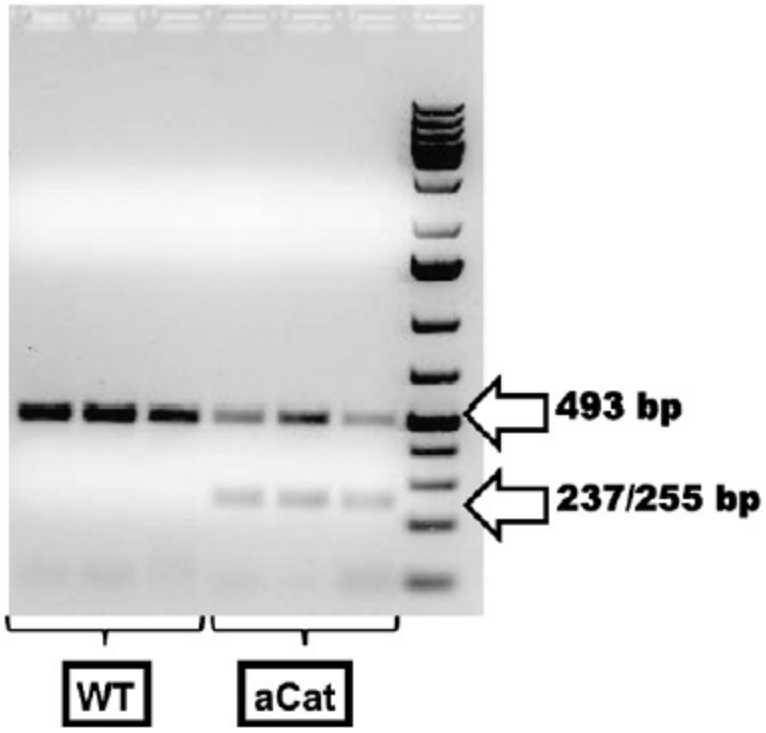


Figure 20. Representative gel for genotyping acatalasemic (aCat) and wild-type (WT) mice.

Primers target a 493 bp segment of the mouse catalase gene, and PCR products are then subject to *Nde*I enzymatic digestion resulting in the presence of a 237/255 bp fragment. The presence of two bands identifies the aCat genotype, while one band signifies WT.

From: (Miller & Wells 2011).

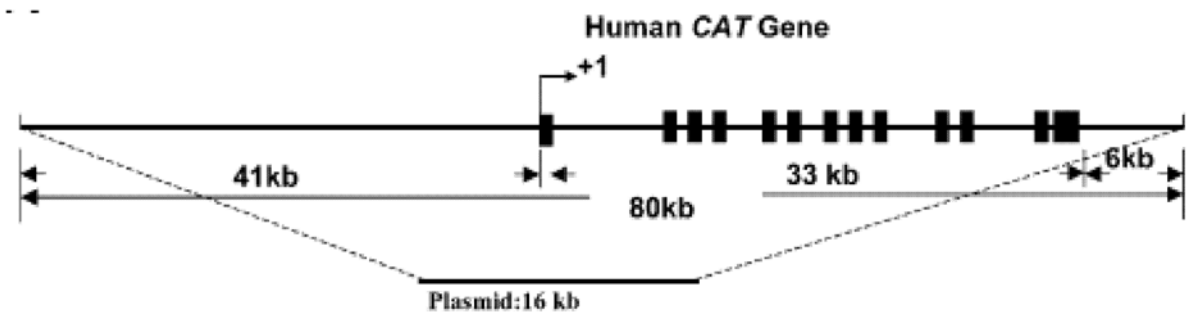


Figure 21. Map of the P1 bacteriaphage clone containing the entire human *CAT* gene.

The transcriptional start site (+1) and the 13 exons (solid boxes) of the human *CAT* gene are shown.

From: Chen et al., 2003.

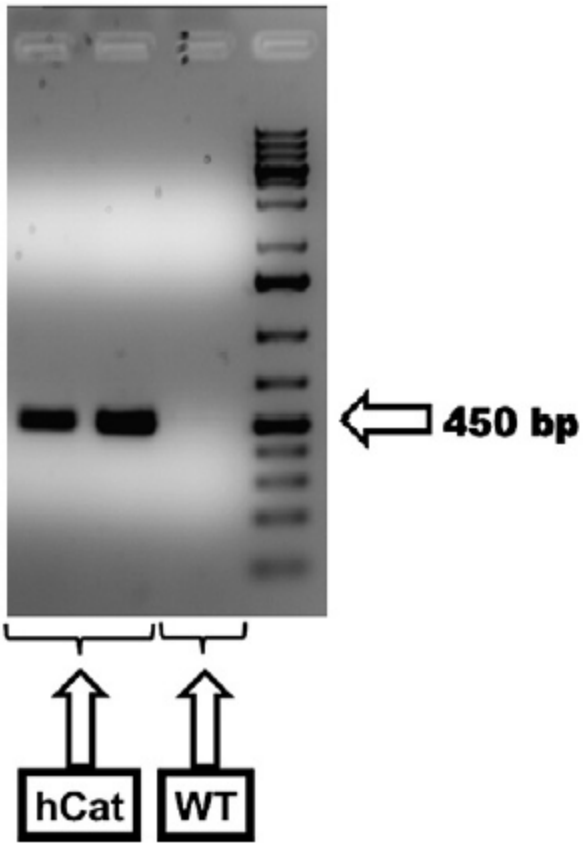


Figure 22. Representative gel for genotyping mice expressing human catalase (hCat) and their wild-type (WT) controls.

Primers targeting exon 12/13 of the human catalase gene amplify in the hCat mice a 450 bp fragment, which is absent in the WT animals. The presence of a 450 bp band identifies the hCat genotype, while no band signifies WT.

From: Miller and Wells, 2011.

1.5.4. Embryonic catalase

The catalase transcript is detectable as early as gestational day (**GD**) 8 in murine embryos. At this stage, it is primarily in the form of a 12.2 kb transcript. One additional band (2.4 kb) is also apparent at this stage although at a very low intensity. The intensity of the two bands increases with development, but only the 2.4 kb RNA band is seen at and after birth and in adults (**Fig. 23**) (El-Hage & Singh 1989). The 2.4 kb RNA is the known mature message of the catalase gene in mice. The presence of large 12.2 kb catalase-specific RNA species (seen during development *in utero* only) is interpreted as the reservoir of a primary RNA transcript, which is processed and spliced to produce 2.4 kb mature message for the catalase polypeptide. It was hypothesized that the biochemical machinery for the pre-mRNA processing, although functional, may not be highly efficient during embryogenesis. Therefore the primary transcripts accumulate and undergo delayed splicing and processing as necessary. The complete and efficient processing of this primary transcript to make catalase protein takes place only after birth. Alternatively, it is possible that the 12.2 kb transcript may also be a functional mRNA initiated from a fetal-specific promoter used only *in utero*. However, there is no corroborating evidence to date for either theory, and the relative mRNA levels are not reflective of the relative enzyme levels observed during development (**Fig. 24**) (El-Hage & Singh 1990). The relative RNA values are higher *in utero* than in the adult, but the specific activity is low at the beginning of embryonic development and continues to increase with embryonic and fetal growth. Studies of catalase activity in our laboratory showed that embryonic catalase activity is less than 10% that of maternal liver (Winn & Wells 1999).

An embryoprotective role for endogenous catalase during development has been suggested in studies of diabetic rats where decreased catalase activity was associated with increased embryonic malformations (Cederberg & Eriksson 1997; Cederberg et al 2000). In an animal model for diabetic pregnancy, offspring of the H rat strain show minor dysmorphogenesis when the mother is diabetic, whereas the offspring of diabetic rats of a U sister strain display major morphologic malformations. Embryonic catalase activity was higher in the protected H strain than in the more sensitive U strain, and maternal diabetes increased this difference in activity. Another study looked at fetal catalase activity in Sprague-Dawley rats and showed that catalase activity was significantly reduced in malformed versus non-malformed embryos (Sivan et al 1997). Embryoprotection by exogenous catalase has been shown in mice by decreases in

phenytoin-initiated DNA oxidation and embryotoxicity in embryo culture and *in vivo* (Winn & Wells 1995b; 1999), and in rat embryos by decreases in glucose-induced embryonic malformations in embryo culture (Erickson & Borg 1991).

In humans, at 7 weeks of gestation, 34% of peroxisomes showed immunoreactive catalase in fetal liver; however, earlier in the development, around week 6 of gestation, catalase activity was not detectable in the fetal liver (Espeel et al 1993). Quantitative estimates of human embryonic catalase activity do not appear to be available, and it is difficult to compare the relative embryonic and adult activities of catalase from different studies due to varying assay conditions and methods. By 8 weeks of gestation and onward, catalase activity is found in 100% of peroxisomes (Espeel et al 1997). Red blood cells (**RBC**) from premature human infants contained one-half the catalase activity of those from full-term infants; however, this may be somewhat compensated for by GSH peroxidase, since the activity of this enzyme was increased by about 50% in the RBCs of premature infants (Varga et al 1985).

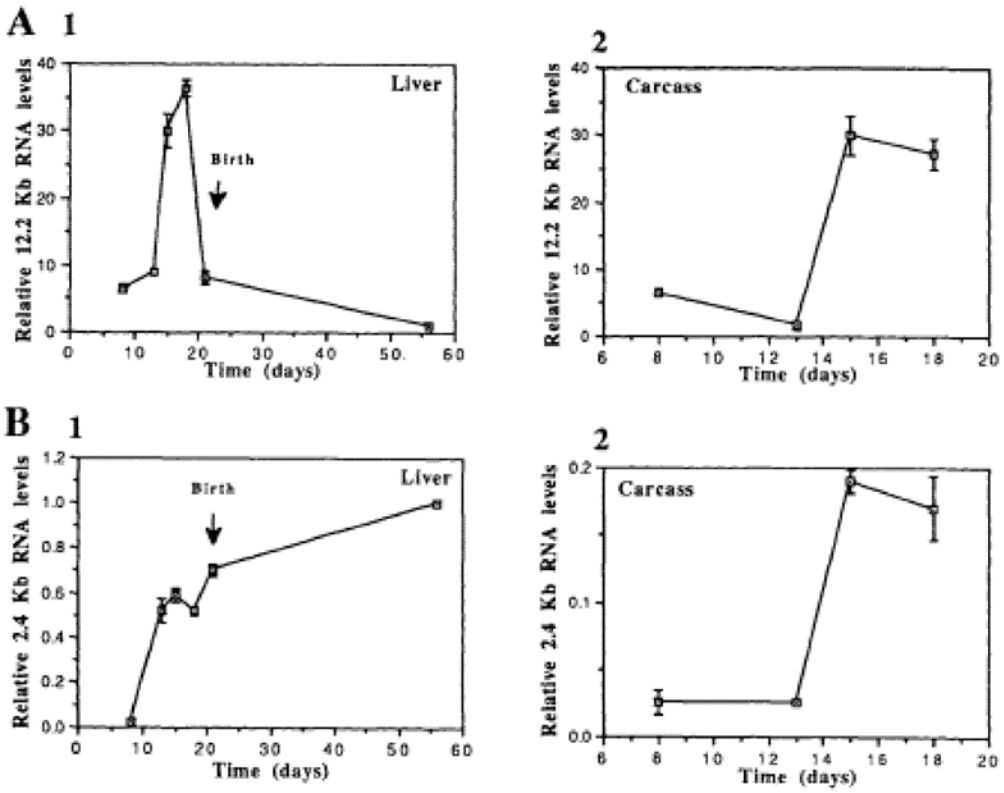


Figure 23. Quantitative analysis of the 12.2 and 2.4 kb catalase-related mRNA transcripts during development in mice.

Relative changes in RNA levels in autoradiograms were quantified by densitometry (northern blots) taking the value of the adult liver as 1. The vertical lines indicate the standard errors (n=3).
A: Quantitative changes of the 12.2 kb RNA in liver (1) and carcass (2).
B: Quantitative analysis of the 2.4 kb RNA in liver (1) and carcass (2).
Note that the time scales for the two tissues studies are independent and different.

From: El-Hage and Sing, 1989.

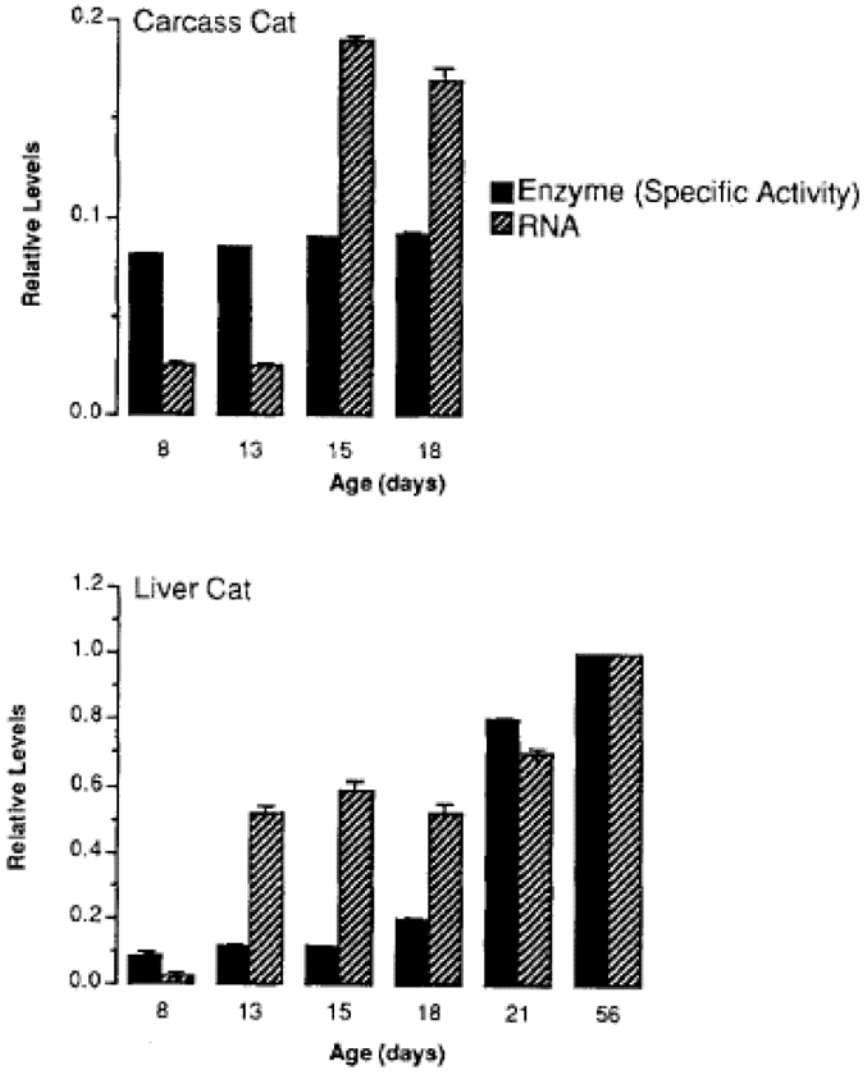


Figure 24. Developmental profile of the catalase-specific RNA and enzyme activity in the carcass and liver of mice during development.

Both plots represent values relative to the values of the adult liver.

From: El-Hage and Singh, 1990.

1.6 ACATALASEMIA IN HUMANS

1.6.1 Genetic diseases related to low catalase

Genetic defects that influence human catalase expression were first described by Takahara (Takahara 1952) in Japanese individuals who exhibited a deficiency in blood catalase activity (acatalasemia). Short-term clinical manifestations of human acatalasemia appear predominantly in the mouth. In moderate cases oral ulcerations develop, while more severe forms of this disease manifest as dental alveolar gangrene and atrophy resulting in widespread loss of teeth. It was proposed that the very low levels of catalase in blood and oral tissues would result in accumulation of H_2O_2 produced by bacteria, which in turn oxidizes hemoglobin in the circulating erythrocytes. The infected area is thus deprived of oxygen, and enzymes such as cytochromes, myoglobin and sulfhydryl-containing enzymes are oxidatively inactivated by H_2O_2. These factors are postulated to allow the multiplication of oral bacteria producing H_2O_2, thus increasing localized production of H_2O_2 and necrosis (Takahara 1971). Acatalasemia is assumed to be inherited as an incomplete autosomal recessive trait.

Another variant of acatalasemia in humans was described in the Hungarian population where both acatalasemic and hypocatalasemic (intermediate levels of catalase activity) individuals were identified (Goth 2000; 2001). An increased frequency of diabetes in catalase-deficient Hungarian patients was observed. It was postulated that severe deficiency in catalase might result in cumulative oxidative damage to pancreatic β-cells resulting in diabetes (Goth & Eaton 2000). Pancreatic β-cells are well known to be oxidant sensitive. For example, diabetic animal models are most frequently generated by the administration of oxidant drugs such as streptozotocin or alloxan that produced free radical-mediated oxidative stress (Lenzen 2008). These drugs appear to preferentially damage β-cells, with little effect on other somatic cell types. Furthermore, while β-cells are poor in catalase they also are rich in mitochondria, the major source of endogenous generation of $O_2^{\bullet-}$ and H_2O_2. Thus, one might postulate that low-level oxidative stress extending over a period of many years, combined with a deficiency of catalase, might result in the slow accumulation of oxidant damage to the β-cells and full-blown diabetes.

In the Swiss variant of acatalasemia, the catalase mRNA and protein levels in cultured fibroblasts are normal, as opposed to Japanese fibroblasts which are completely devoid of the mRNA and protein, suggesting the presence of a structural mutation in the catalase gene in Swiss

acatalasemia that results in inactivation the enzyme, as distinct from a regulatory mutation in Japanese variant in which the gene is not transcribed (Crawford et al 1988; Ogata 1991). The exact type and location of the mutation have yet to be determined. The catalase protein in the Swiss variant is rapidly (2-fold faster) degraded and has a faster electrophoretic mobility (Aebi et al 1976). No obvious pathology has been associated with this mutation.

Tables **4** and **5** summarize the clinical, biochemical and genetic observations for the different types of inherited catalase deficiencies discussed above.

1.6.2 Other conditions related to low catalase

Aniridia is an autosomal dominant disease characterized by incomplete formation of the iris, where catalase activity is decreased to 30% of normal. There is a short interstitial deletion (deletion that does not involve the terminal parts (ends) of a chromosome) of the 13p locus on chromosome 11, where the catalase gene is located (Turleau et al 1984). This deletion is associated with an increased incidence of Wilm's tumor and mental retardation, but it is not known what role if any the catalase deficiency plays in the clinical manifestations of this disease.

Xeroderma pigmentosum (**XP**) is an autosomal recessive genetic disorder of DNA repair characterized by extreme sensitivity to sunlight and a high incidence of skin cancer. The xeroderma pigmentosum C (**XPC**) protein is essential for initiating global genome nucleotide excision repair (**NER**) by recognizing the DNA lesion (cyclobutane-pyrimidine dimers) caused by ultraviolet (**UV**) light and recruiting downstream factors (Ming et al 2010). In XP patients there is a mutation in this protein that results in a reduction in or elimination of NER, leading to an increased risk of cancer. A decline in catalase activity has been found to coincide with the first symptoms of the disease and progression to tumor formation (Vuillaume et al 1983). Cultured human fibroblasts from patients with XP showed only 25% of the expected catalase activity (Vuillaume et al 1986). It was postulated that the decrease in catalase activity resulted in an increase in intracellular H_2O_2 which lead to the eventual development of the malignant process.

Table 4. Clinical and biochemical parameters in different types of inherited catalase deficiencies.

	Swiss type	Japanese type	Hungarian type
Frequency			
Acatalasemia	0.04/1000	0.8/1000	0.05/1000
Hypocatalasemia	[a]	2–4/1000	2.3/1000
Age	Mainly young	[a]	45.9 ± 19.3 years
Male/Female[a]	[a]	43/47	25/39
Takahara disease	No	~Half in 1948–1952	No
Chronic hemolysis	[a]	No	No
Diabetes mellitus (%)	[a]	[a]	12.7
Blood catalase			
Acatalasemia (%)	0.5–2.0	0–3.2	<7
Hypocatalasemia (%)	60–80	56.9	57.9
Tissue catalase <15% (%)	39–50	8–21	
Catalase protein	Changed	No change	No change
Blood			
Superoxide dismutase (%)	[a]	121	150
Glutathione peroxidase (%)	[a]	116	110
Risk factors of atherosclerosis	[a]	[a]	Increased

Note. Risk factors of atherosclerosis were cholesterol, LDL cholesterol, Apo B, Lp(a), and LDL oxidative resistance.
[a] No data are available.

From: Góth, 2001.

Table 5. Genetic parameters in different types of inherited catalase deficiencies.

Type	Mutation localization	Swiss patients	Japanese patients	Hungarian patients
		Syndrome causing mutations		
Japanese A	G to A splicing Intron 4	[a]	3 acat	Not detected
		[a]	1 hypocat	
Japanese B	T(358) deletion Exon 4	[a]	1 acat	Not detected
		[a]	4 hypocat	
Swiss	Not identified	Not identified	[a]	
Hungarian A	GA insertion Exon 2	[a]	Not detected	2 acat
		[a]	Not detected	29 hypocat
Hungarian B	G insertion	[a]	Not detected	3 hypocat
Hungarian C	T to G splicing	[a]	Not detected	7 hypocat
		Benign polymorphisms		
Patients	Mutation	Switzerland	Japan	Hungary
Japanese	Point mutations	[a]	5 forms	[a]
Hungarian	Point mutations	[a]	3 forms	6 forms

Note. acat, acatalasemic; hypocat, hypocatalasemic.
[a] No data are available.

From: Góth, 2001.

SECTION 2: STUDIES

2.1 STUDY 1: EMBRYOPROTECTIVE ROLE OF ENDOGENOUS CATALASE IN ACATALASEMIC AND HUMAN CATALASE-EXPRESSING MOUSE EMBRYOS EXPOSED IN CULTURE TO DEVELOPMENTAL AND PHENYTOIN-ENHANCED OXIDATIVE STRESS [a,b]

Running title: Protection by embryonic catalase

Julia P. Abramov[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Corresponding author:
Peter G. Wells, Pharm.D.
Faculty of Pharmacy, University of Toronto
144 College St., Toronto, Ontario, Canada M5S 3M2
E-mail: pg.wells@utoronto.ca
Tel. 416-978-3221
Fax 416-267-7797

a. Preliminary reports of this research were presented at the 2006 and 2007 annual meetings of the Teratology Society (U.S.A.) [*Birth Defects Res. Part A: Clinical and Molecular Teratology,*76(5): 392, 2006; 79(5): 418, 2007], and the 12th International Congress of Toxicology [*Toxicol. Lett.* 196S: S184, 2010]. These studies were supported by a grant from the Canadian Institutes of Health Research (CIHR).

b. Full report of this research has been published:
Abramov JP, Wells PG. 2011. Embryoprotective role of endogenous catalase in acatalasemic and human catalase-expressing mouse embryos exposed in culture to developmental and phenytoin-enhanced oxidative stress. *Toxicological Sciences* 120:428-38

2.1.1 ABSTRACT

Reactive oxygen species (**ROS**) are implicated in spontaneous and xenobiotic-enhanced embryopathies, and protein therapy with exogenous catalase suggests an embryoprotective role, although embryonic catalase activity is only about 5% of adult activity. Using mutant catalase-deficient (acatalasemic, **aCat**) mice and transgenic mice expressing human catalase (**hCat**, enhanced catalase activity) compared to a confirmed outbred CD-1 mouse model, we investigated the protective importance of constitutive embryonic catalase against endogenous ROS and the ROS-initiating teratogen phenytoin in embryo culture. Vehicle-exposed aCat and hCat embryos respectively exhibited reduced and enhanced catalase activity compared to wild-type controls, with conversely enhanced and reduced spontaneous embryopathies. Phenytoin was embryopathic in all strains without altering catalase activity, but less so in the wild-type embryos for the aCat and hCat strains, which exhibited about half the catalase activity of CD-1 embryos. Phenytoin respectively enhanced and reduced embryopathies in aCat and hCat embryos. Among aCat embryos exposed to phenytoin, embryopathies increased with decreasing catalase activity, and were completely blocked by addition of exogenous catalase, which increased embryonic catalase activity to wild-type levels. These results provide the first direct evidence that: (1) the low level of constitutive embryonic catalase protects the conceptus from developmental and xenobiotic-enhanced oxidative stress; and, (2) embryonic variations in activity of this enzyme affect development.

2.1.2 INTRODUCTION

About 20,000 children born annually in Canada exhibit a structural and/or functional birth defect (teratogenesis), including intellectual deficits (Harper 1998), but the mechanisms and determinants of risk are for the most part unknown. Reactive oxygen species (**ROS**) including hydrogen peroxide (**H_2O_2**) have been implicated in the mechanism of birth defects (Nicol et al 2000; Wells et al 2009), and the developing embryo and fetus are uniquely susceptible due to their low levels of most antioxidative enzymes. In particular, little is known about the antioxidative enzyme catalase, which detoxifies H_2O_2, with regard to its embryonic expression, inter-individual variability in catalytic activity or protective importance.

Catalase is one of the ROS-detoxifying enzymes found in all adult organs (Ogata 1991) and the embryo (Wells et al 2009). In the latter case, however, the activity of embryonic catalase, as distinct from the levels of mRNA and protein, is not well characterized and embryonic enzyme activities do not necessarily correlate well with mRNA levels (El-Hage & Singh 1990). If not detoxified by catalase, H_2O_2 can initiate signal transduction pathways, or react with iron to form highly reactive hydroxyl radicals (•OH), which can oxidatively damage cellular macromolecules (lipids, proteins, DNA, etc.), altering their function (Halliwell & Gutteridge 2007). Enhanced ROS-mediated signal transduction and/or oxidative damage to cellular macromolecules like DNA have been implicated in a spectrum of adverse structural and functional developmental consequences (Wells et al 2009). Numerous studies have demonstrated a protective role for exogenous catalase against ROS-mediated damage, including ischemic-reperfusion injury (Liu et al 1989; Oda et al 1992; Padmanabhan et al 1985), cold injury (Das et al 1991), favism (Gaetani et al 1996) and diabetic teratology (Cederberg & Eriksson 1997; Erickson & Borg 1991).

In the mouse embryo, the activity of catalase is only about 5% of that in the adult liver (El-Hage & Singh 1990; Winn & Wells 1999), which may contribute to an increased embryonic risk from developmental and xenobiotic-enhanced ROS-mediated damage. A potential protective role for catalase against teratogenesis has been demonstrated in embryo culture, where the addition of exogenous catalase enhanced embryonic antioxidative activity and protected against phenytoin-initiated DNA oxidation and embryopathy (Winn & Wells 1995b). A subsequent *in vivo* study showed that maternal administration of polyethylene glycol (**PEG**)-conjugated

catalase could increase embryonic catalase activity, decrease phenytoin-initiated protein oxidation and protect the embryo against phenytoin teratogenicity (Winn & Wells 1999).

In addition to the mechanistic insights gained by protein therapy with exogenous catalase, manipulation of catalase activity may eventually prove useful for treating acatalasemia, a homozygous hereditary condition resulting in a catalase deficiency. Acatalasemia in humans appears in several variant forms, the most widely known of which is the Japanese variant, where the deficiency is most apparent in red blood cells (Ogata 1991). Little is known about the pathogenic consequences of catalase deficiencies other than Takahara's disease, an oral gangrene attributed to H_2O_2 produced by microorganisms in the mouth, especially in cases of poor oral hygiene (Ogata 1991).

Given the pivotal role of catalase in ROS detoxification, we anticipated that a deficiency in this enzyme could be an important risk factor for adverse development mediated by either endogenous or drug-enhanced oxidative stress, as was found for another antioxidative enzyme, glucose-6-phosphate dehydrogenase (**G6PD**) (Nicol et al 2000). On the other hand, the low embryonic levels of catalase could render this enzyme relatively unimportant at least until later in the fetal period or during postnatal development. Although multiple forms of exogenous catalase therapy are cytoprotective, the role of the endogenous enzyme in protecting the embryo remains unanswered.

In the acatalasemic mouse (**aCat**), there is a G→T mutation in the catalase gene, induced by irradiation, which resulted in histidine instead of glutamine at the eleventh amino acid position (Shaffer & Preston 1990). The resulting disruption in an α-helix causes the final catalase protein, formed of four identical peptides, to become unstable (Shaffer & Preston 1990). It is not known whether any other mutations contribute to the acatalasemia, such as in any regulatory proteins. Catalase levels for this acatalasemic mouse compared to the wild-type strain were less than 10% in the blood, approximately 20% percent in the kidneys, and nearly normal levels in the liver (Shaffer & Preston 1990).

In the transgenic mouse expressing human catalase (**hCat**), there is an 80 kb human genomic DNA, containing the 33 kb human *CAT* gene as well as the 41 kb of 5' and the 6 kb of the 3' flanking regions. The resultant transgenic mouse model expresses human catalase in addition to endogenous mouse catalase, resulting overall in up to a 4-fold increase in total catalase activity in a tissue-specific manner (Chen et al 2003).

Herein, we first validated our embryo culture system by showing CD-1 mice known to be susceptible to the ROS-initiating teratogen phenytoin exhibited the same nature and extent of embryopathies previously reported. We then employed genetically modified aCat (low catalase) and hCat (high catalase) mice, including protein therapy with PEG-catalase in aCat mice, to assess the pathogenic role of physiological and drug-enhanced ROS formation within the embryo, and the importance of endogenous catalase in protecting the embryo from developmental and drug-enhanced oxidative stress. Embryos were exposed in culture to a therapeutic concentration of the antiepileptic drug phenytoin, a teratogen that is believed to alter development at least in part by enhancing ROS formation (Wells et al 2009). The results provide the first direct evidence that the relatively low level of endogenous embryonic catalase provides important protection against both developmental oxidative stress and ROS-initiating teratogens, and suggest that interindividual embryonic differences in expression of this enzyme could contribute to teratological risk.

2.1.3 MATERIALS AND METHODS

Chemicals

Phenytoin (diphenylhydantoin sodium salt), catalase, xylenol orange, ammonium iron (II) sulphate hexahydrate, potassium permanganate and polyethylene glycol-conjugated catalase (PEG-catalase) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen peroxide and sucrose were from BDH Chemicals Inc. (Toronto, Ontario, Canada), and sodium azide was from Fisher Scientific (Toronto, Ontario). Fetal bovine serum, HBSS, Waymouth's MB 752/1 medium, HEPES and penicillin-streptomycin were from Gibco Laboratories (Toronto, Ontario). All other reagents and solvents were of analytical grade.

Animals

Outbred CD-1 mice were purchased from Charles River Laboratories (St. Constant, Quebec). Breeding pairs of catalase-normal wild-type (C3HeB/FeJ) and mutant acatalasemic (catalase-deficient) (C3Ga.Cg-*Cat*^{b}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Transgenic mice expressing human catalase (hCAT) in addition to their intrinsic murine catalase (high catalase activity) (C57BL/6) were donated by Dr. Arlan Richardson's laboratory, and the wild-type (C57BL/6) mice were purchased from The Jackson Laboratory. Same-sex animals were housed in plastic cages with ground cob bedding and were maintained in a temperature-controlled animal facility with a 12 hr light/dark cycle. Food (Purina Rodent Chow, Oakville, Ontario) and tap water were provided *ad libitum*. One male was housed with two females overnight between 1700 and 0900 hr. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (**GD**) 1.

Male rat serum (**MRS**) contains undefined nutrients and factors required by murine embryos for survival and growth (Fantel 1982), and therefore was used as the medium in which the embryos were cultured. Blood was obtained from retired Sprague Dawley male rat breeders (Charles River Laboratories), which were exsanguinated under anesthesia using isofluorane. The blood was centrifuged for 5 min at 1000 x *g* at 4°C and kept on ice until blood was obtained from all animals. All blood samples were then centrifuged for 30 min at 2000 x *g* at 4°. Pooled serum was heat-inactivated for 1 hr at 58°C and gassed (5% CO_2 in air) for 30 min to evaporate

residual protein-bound isofluorane. The heat-inactivated MRS was divided into aliquots and stored at -80°C.

Embryo culture

Pregnant murine dams were sacrificed on GD 9 by cervical dislocation, and embryos were explanted according to the method of New (New 1978). Using this technique, the uterus was removed from the dam and rinsed in warm HBSS. While in HBSS, the individual implantation sites were exposed using a no. 5 watchmaker's forceps. 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°C, in a holding bottle containing pregassed holding medium (50 ml of Waymouth's MB 752/1 medium, 14 mM $NaHCO_3$, 2.5 mM HEPES, and 17 ml of MRS).

Embryos at similar stages of development (4-6 somite pairs) were used for culture. Each embryo was put in one well of a 24-well sterile plate containing 2 ml of CO_2-saturated embryo culture medium (50 ml of holding medium, 50 units/ml penicillin, and 50 mg/ml streptomycin). Plates were sealed and incubated at 37°C in an incubator on a platform rocker.

Embryos were exposed to either a therapeutic concentration of phenytoin (20 µg/ml, 80 µM) or its vehicle (0.002 N NaOH) for 24 hr. To study the role of catalase in reducing phenytoin-initiated embryotoxicity in acatalasemic mice, embryos were cultured for 24 hr in the presence of phenytoin and PEG-catalase (1 mg/ml, 1680 units/ml). After 24 hr, embryonic morphological and developmental parameters were observed using a dissecting microscope. 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).

Catalase activity

Catalase activity was measured in embryonic and adult tissues. All embryos were dissected from the surrounding membranes and catalase activity was measured in the embryo itself. Samples were homogenized in phosphate-buffered saline and catalase activity was determined using the ferrous oxidation in xylenol orange (**FOX**) assay. This method, as opposed to the traditional UV method, has a high extinction coefficient for H_2O_2 which allows the

measurement of catalase activity at very low concentrations of H_2O_2 and therefore allowed measurements to be performed in individual embryos as early as GD 9 (Gupta 1973; Jiang et al 1992; Ou & Wolff 1993; 1996). We used appropriate controls such as heat inactivation and a catalase inhibitor to confirm that only catalase activity was measured (**Fig. S1**). Briefly, 100 µl of 10 mM H_2O_2 were added to homogenates and samples were incubated at room temperature for 3 min. A 20 µl aliquot of each sample was then added to 200 µl of FOX reagent (250 µM ammonium iron (II) sulphate, 100 µM xylenol orange and 100 mM sucrose in 25 mM sulphuric acid) and samples were incubated at room temperature for 30 min. The absorbance was measured at 595 nm. Catalase activity was determined using a standard curve with bovine liver catalase as the standard.

Glutathione peroxidase activity

To evaluate the potential for compensatory increased expression of complementary antioxidative enzymes in aCat mice, glutathione peroxidase (**GPx**) activity was measured in various maternal organs. Samples were homogenized in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 mM DTT buffer and centrifuged at 10,000 x g for 15 min at 4°C. From the supernatant, a 20 µl aliquot was combined with 100 µl of assay buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA) and 50 µl of co-substrate mixture containing NADPH, glutathione and glutathione reductase. The reaction was initiated by addition of cumene hydroperoxide which served as a substrate for GPx. The absorbance was read once every minute at 340 nm.

Genotyping

DNA was isolated from tail clips by heating the sample in 300 µL of a solution containing 10 mM NaOH/0.1 mM EDTA at 95°C for 15 min. To genotype aCat mice, catalase PCR primers were, CatF: TCCTTCCAATCCCGTCCTTTCT and CatR: AAATGCCAAACTCGGAGCCATC. PCR conditions for a Perkin Elmer 9600 thermal cycler were 94°C for 5 min, 20 s at 94°C, 20 s at 65°C (-1 degree cycle), and 40 s at 72°C for a total of 10 cycles, then 20 s at 94°C, 20s at 55°C, and 40 s at 72°C for a total of 30 cycles, with a 10 min extension at 72°C and kept at 4°C until ready for digestion. PCR products were digested using NdeI restriction enzyme at 37°C for 16 hr and run on 1.5% agarose gels to determine catalase genotype. To genotype hCat mice, catalase PCR primers were, CatF:

GAGGTCCACCCTGACTACGGG and CatR: GCCTTCTCCCTTGCCGCCAAG. PCR conditions were 95°C for 5 min, 30 s at 95°C, 30 s at 60°C and 1 min at 72°C for a total of 35 cycles, with a 10 min extension at 72°C. Undigested PCR products were run on 1.5% agarose gels to determine catalase genotype.

Statistical analysis

Binomial data were analyzed using Fisher's exact test where appropriate. Continuous data were analyzed using one factor analysis of variance (ANOVA). The minimum level of significance used throughout was $p < 0.05$.

2.1.4 RESULTS

Embryopathies in CD-1 mouse embryos exposed in culture to phenytoin

Since CD-1 mice are known to be sensitive to phenytoin embryopathies *in vivo* and in embryo culture (Wells et al 2009), this strain was evaluated as a positive control for the acatalasemic strain and its wild-type controls, in which phenytoin has not been evaluated previously. CD-1 embryos demonstrated substantial dysmorphogenesis when exposed for 24 hr to phenytoin, which reduced anterior neuropore closure by 64%, turning by 45%, yolk sac diameter by 15%, crown-rump length by 11% and somite development by 19% ($p < 0.05$) (**Fig. 1**).

Endogenous catalase activity in CD-1 embryos during organogenesis and maternal livers

Embryonic catalase activity *in vivo* and in culture was about 40 U/mg of protein, constituting about 6% of maternal hepatic activity (**Fig. 2**). This activity was not affected by incubation with phenytoin for 24 hr.

Maternal catalase and glutathione peroxidase activities in aCat mice

Catalase activity in various maternal organs of the aCat mice was significantly decreased compared to the wild-type animals ($p<0.05$) (**Fig. 3,** *upper panel*). In contrast, with the exception of lungs, glutathione peroxidase activity in the aCat mice was slightly increased compared to the wild-type controls ($p<0.05$) (**Fig. 3,** *lower panel*).

Embryopathies in acatalasemic mouse embryos exposed to phenytoin

Vehicle control embryos

Compared to CD-1 embryos in the validation study (**Fig. 1**), WT embryos exposed to vehicle appeared to develop less robustly in culture, with non-significant 25% decreases in both anterior neuropore closure and turning (**Fig. 4**).

Figure 1.

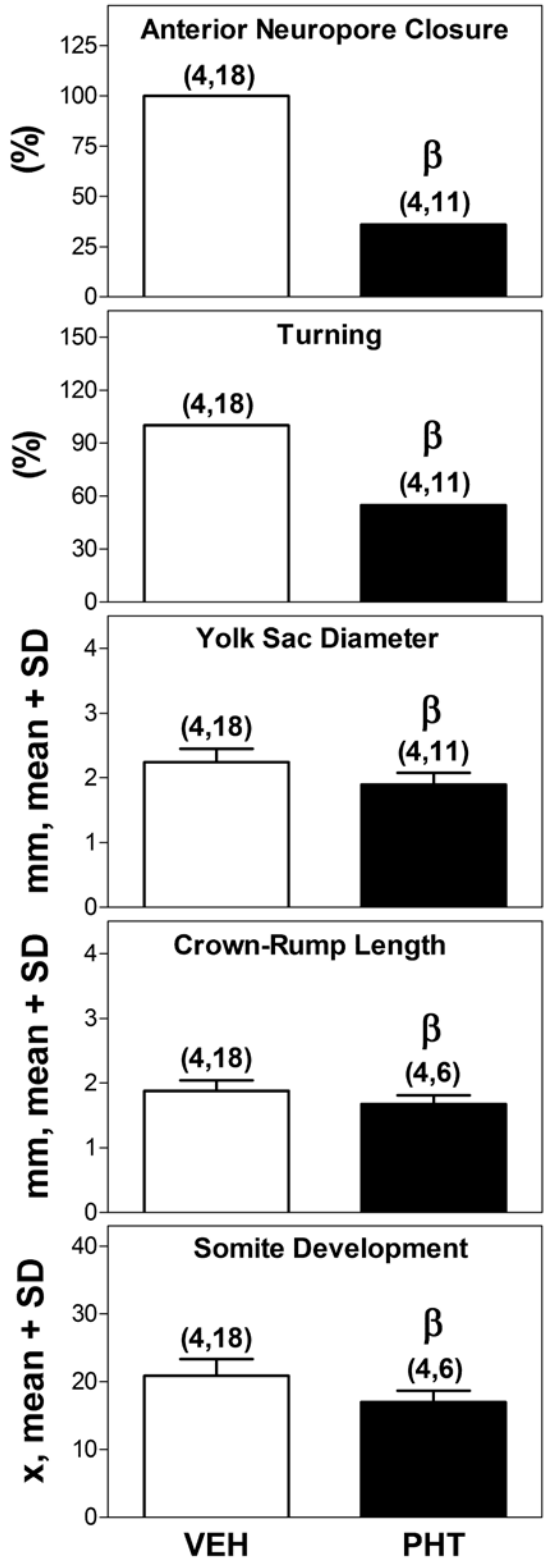

Anterior Neuropore Closure
(%)
125
100
75
50
25
0
(4,18)
β
(4,11)
Turning
(%)
150
120
90
60
30
0
(4,18)
β
(4,11)
Yolk Sac Diameter
mm, mean + SD
4
3
2
1
0
(4,18)
β
(4,11)
Crown-Rump Length
mm, mean + SD
4
3
2
1
0
(4,18)
β
(4,6)
Somite Development
x, mean + SD
40
30
20
10
0
(4,18)
β
(4,6)
VEH
PHT

Figure 1. Embryopathies in CD-1 mouse embryos exposed in culture to phenytoin or its vehicle.

Pregnant dams were sacrificed on gestational day (GD) 9 (GD 1 = vaginal plug) and embryos were explanted leaving the amnion, visceral yolk sac and ectoplacental cone intact. Embryos were staged according to the number of somite pairs, and only embryos with 4 to 6 somites were cultured with therapeutic concentration of phenytoin (20 μg/ml, 80 μM) or its vehicle (0.002 N NaOH) for 24 hours, at which point embryonic morphological and developmental parameters were assessed. Crown-rump length and somite development were evaluated only in embryos that completed turning. VEH = vehicle, PHT = phenytoin. The beta symbol indicates a difference from VEH control ($p<0.05$). (n,N) = (litters, embryos).

Figure 2.

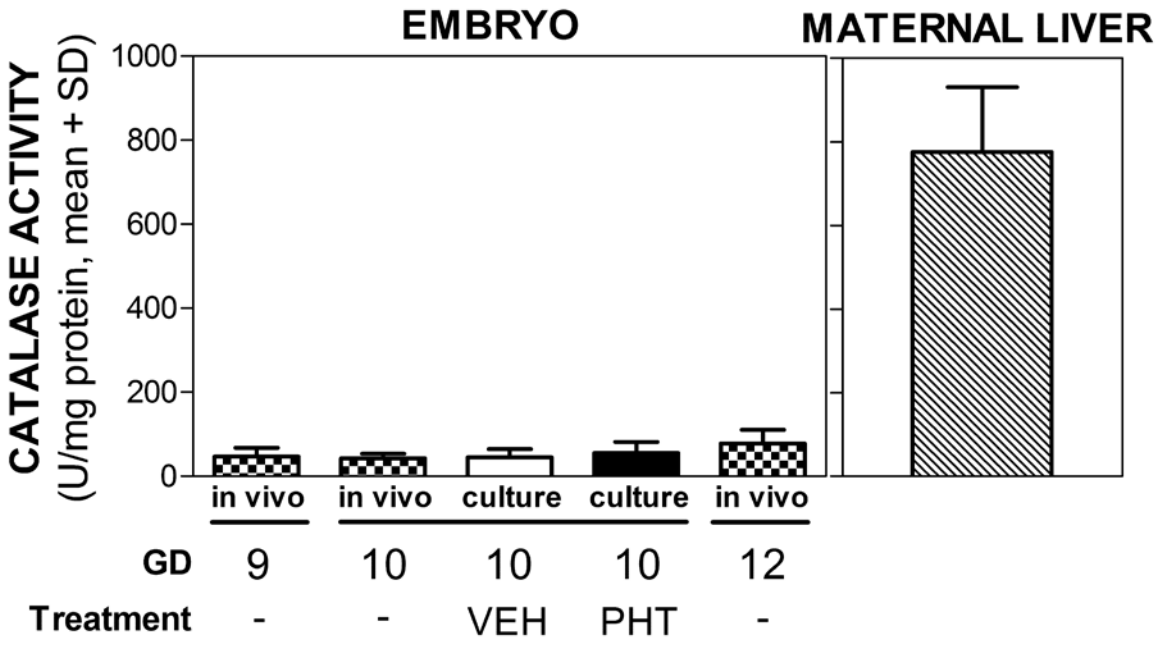
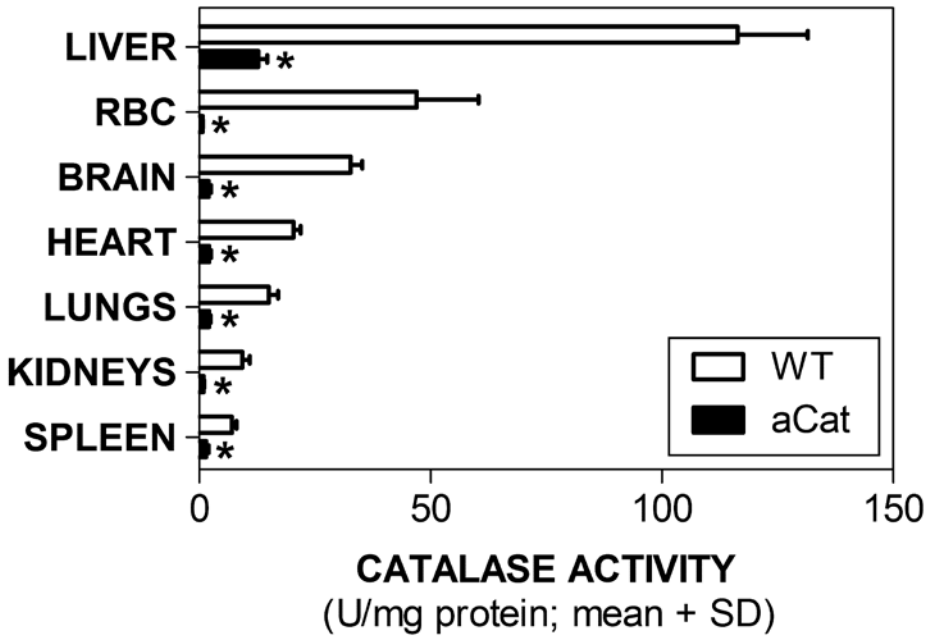

EMBRYO
MATERNAL LIVER
CATALASE ACTIVITY
(U/mg protein, mean + SD)
1000
800
600
400
200
0
in vivo
in vivo
culture
culture
in vivo
GD
9
10
10
10
12
Treatment
-
-
VEH
PHT
-

Figure 2. Endogenous catalase activity in maternal livers and CD-1 embryos during organogenesis.

Embryonic and adult samples were collected on GD 9 and prepared as described in Figure 1. At the end of the culture period, embryos from the same litter were pooled and homogenized in 200 μl of PBS using a hand homogenizer. Homogenates were sonicated and catalase activity was determined using the FOX assay, standardized for protein content. Embryos were also collected from pregnant dams on GDs 9, 10 and 12 using the same method, but were immediately analyzed without culturing. The mean values for each group were determined from 12-54 embryos selected randomly from at least 3 litters. Maternal activities on GDs 9-12 were similar and the data were pooled from 5 adults. VEH = vehicle, PHT = phenytoin.

Figure 3.


LIVER
RBC
BRAIN
HEART
LUNGS
KIDNEYS
SPLEEN
WT
aCat
0
50
100
150
CATALASE ACTIVITY
(U/mg protein; mean + SD)

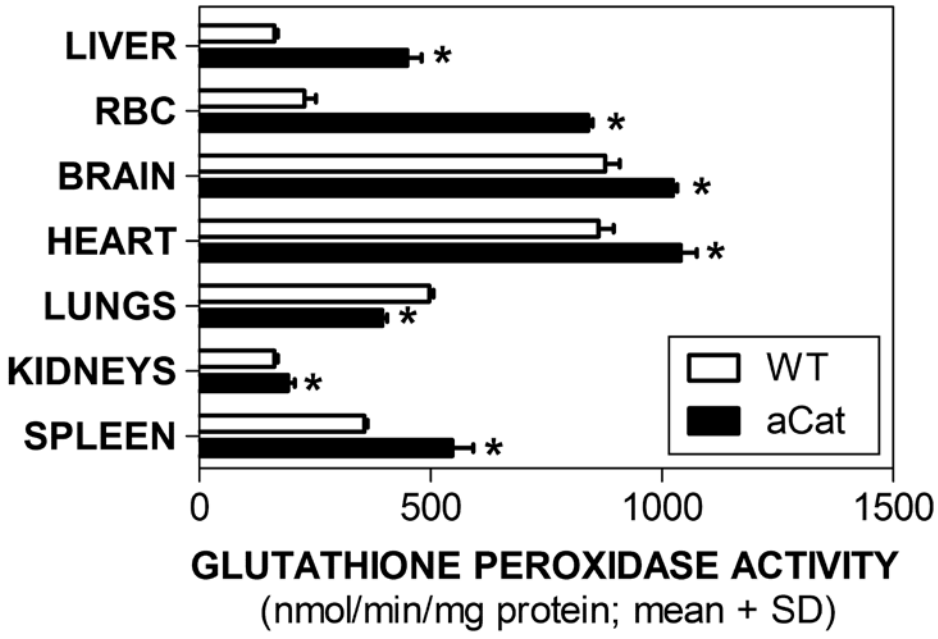

LIVER
RBC
BRAIN
HEART
LUNGS
KIDNEYS
SPLEEN
WT
aCat
0
500
1000
1500
GLUTATHIONE PEROXIDASE ACTIVITY
(nmol/min/mg protein; mean + SD)

Figure 3. Maternal tissue activities of catalase and glutathione peroxidase in aCat mice.

The activities of catalase and glutathione peroxidase in the various maternal tissues of wild-type and aCat mice were determined as described in Methods. Each group consisted of samples from 5 animals. Asterisks indicate a difference from the wild-type ($p<0.05$).

Figure 4.

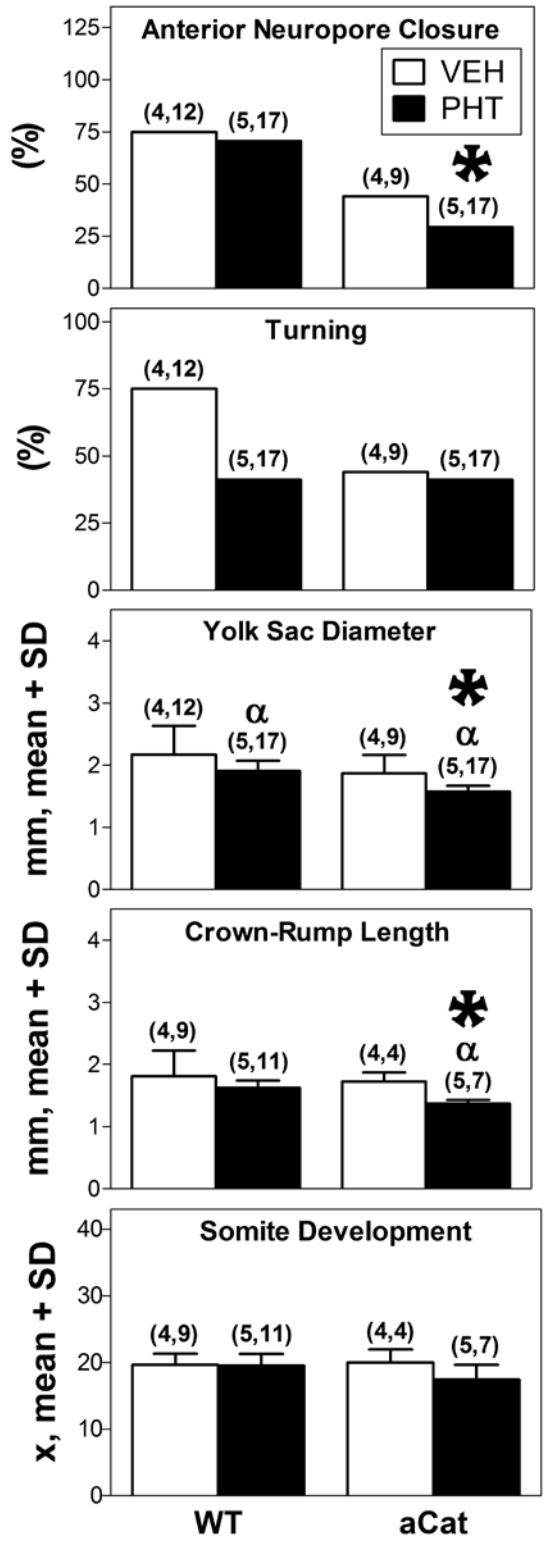

Anterior Neuropore Closure
VEH
PHT
(%)
125
100
75
50
25
0
(4,12)
(5,17)
(4,9)
(5,17)
*
Turning
(%)
100
75
50
25
0
(4,12)
(5,17)
(4,9)
(5,17)
Yolk Sac Diameter
mm, mean + SD
4
3
2
1
0
(4,12)
α
(5,17)
(4,9)
α
(5,17)
*
Crown-Rump Length
mm, mean + SD
4
3
2
1
0
(4,9)
(5,11)
(4,4)
α
(5,7)
*
Somite Development
x, mean + SD
40
30
20
10
0
(4,9)
(5,11)
(4,4)
(5,7)
WT
aCat

Figure 4. Embryopathies in wild-type (WT) and acatalasemic (aCat) mouse embryos exposed in culture to phenytoin or its vehicle.

Catalase-deficient (acatalasemic) or catalase-normal (wild-type) embryos were cultured and assessed as described in Fig. 1. VEH = vehicle, PHT = phenytoin. The alpha symbol indicates a difference from VEH-exposed embryos of the same genotype ($p<0.05$); the asterisk indicates a difference from the WT embryos exposed to the same treatment (phenytoin) ($p<0.05$). (n,N) = (litters, embryos).

Acatalasemic embryos exposed for 24 hr to vehicle alone demonstrated no significant difference in developmental and morphological parameters when compared to their WT vehicle-exposed controls, although there was a non-significant trend for increased embryopathies in acatalasemic embryos evidenced by a 31% decrease in both anterior neuropore closure and turning (**Fig. 4**).

Phenytoin-exposed embryos

WT embryos exposed to phenytoin showed a small but significant decrease in yolk sac diameter, and a non-significant 44% decrease in turning (**Fig. 4**). Overall, this C3H strain was less sensitive to phenytoin embryopathies than the CD-1 outbred strain (**Fig. 1**).

Acatalasemic embryos exposed for 24 hr to phenytoin demonstrated enhanced dysmorphogenesis when compared to their WT controls exposed to phenytoin, as evidenced by a reduction in anterior neuropore closure by 41%, yolk sac diameter by 17% and crown-rump length by 15% ($p < 0.05$) (**Fig. 4**).

Effect of embryonic catalase activity on phenytoin embryopathies in WT and aCat embryos

Independent of the genotype, catalase activity was lower in embryos that failed to close their anterior neuropore, and in embryos that failed to finish turning ($p < 0.05$) (**Fig. 5**). Similarly, embryos with an open anterior neuropore had 47% lower catalase activity when compared to embryos that had successfully closed the anterior neuropore at the end of 24 hr of culture period. Embryos that failed to complete the process of turning had 39% lower catalase activity when compared to embryos that finished turning. There was a remarkable and highly significant positive correlation of the number of somites developed with increasing catalase activity (Spearman r = 0.7; $p = 0.0015$) (**Fig. 5**).

Figure 5.

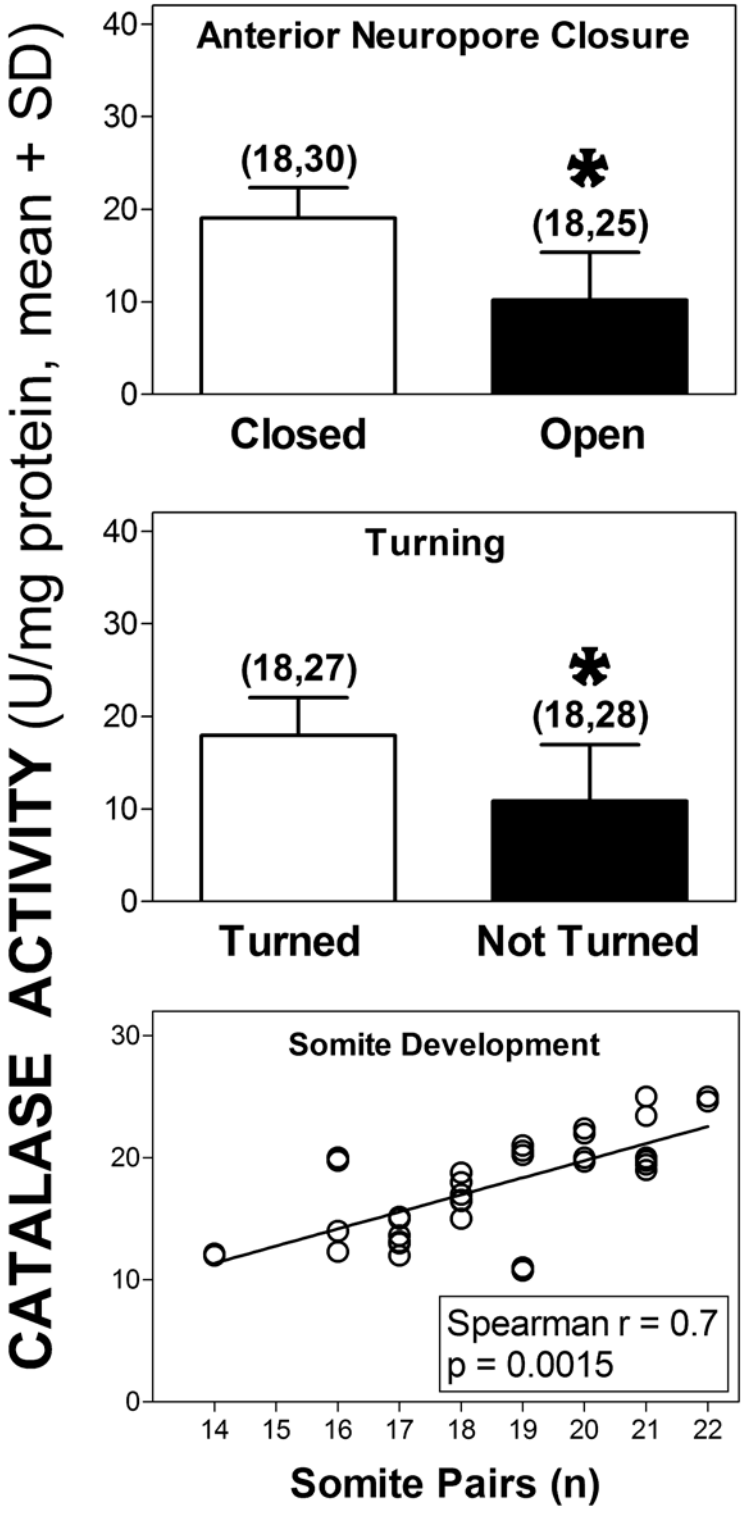

CATALASE ACTIVITY (U/mg protein, mean + SD)
Anterior Neuropore Closure
(18,30)
(18,25)
*
Closed
Open
Turning
(18,27)
(18,28)
*
Turned
Not Turned
Somite Development
Spearman r = 0.7
p = 0.0015
Somite Pairs (n)

Figure 5. Effect of embryonic catalase activity on phenytoin embryopathies in WT and aCat embryos.

Acatalasemic (aCat) and wild-type embryos from the studies shown in Fig. 4 were used to measure endogenous catalase activity in each individual embryo using the FOX assay. Embryos were evaluated by outcome rather than genotype. The asterisk indicates $p<0.05$. (n,N) = (litters, embryos). 29 embryos for WT and 26 embryos for aCat were tested.

Catalase activities and prevention of phenytoin embryopathies in acatalasemic mice in culture with catalase protein therapy

Incubation of acatalasemic embryos in the presence of catalase completely eliminated phenytoin-initiated decreases in all of the developmental and morphological parameters ($p < 0.05$) (**Fig. 6**, *panel A*). Mean catalase activity in acatalasemic embryos was only 53% of that in wild-type embryos cultured in phenytoin ($p < 0.05$) and it was doubled, reaching WT levels of about 20 U/mg protein, by adding PEG-catalase to the culture media ($p < 0.05$) (**Fig. 6**, *panel B*). In adults, catalase activity in the livers of acatalasemic mice was about 42% of that in wild-type animals ($p < 0.05$) (**Fig 6**, *panel C*). Catalase activities in WT and aCat embryos were only about 3% and 5% of the respective activities in adult WT and aCat livers (**Fig. 6**, *panel C*). Catalase activities in the WT (C3H) embryos (**Fig. 6**, *panel C*) were only about one-half of the activities observed in outbred CD-1 embryos throughout organogenesis (**Fig. 2**).

Embryopathies in embryos expressing human catalase (high activity) exposed to phenytoin

Vehicle control embryos

Compared to CD-1 embryos in the validation study (**Fig. 1**), C57BL/6 WT embryos for the hCat strain, as with the C3H WT embryos for the aCat strain, when exposed to vehicle appeared to develop less robustly in culture, with non-significant, slightly over 25% decreases in both anterior neuropore closure and turning (**Fig. 7,** *panel A*).

Compared to their wild-type vehicle controls, hCat embryos exposed for 24 hr to vehicle alone exhibited an increase in crown-rump length ($p < 0.05$), and non-significant, approximate 30% increases in anterior neuropore closure and turning (**Fig. 7**, *panel A*).

Phenytoin-exposed embryos

Phenytoin was highly embryopathic in WT embryos for hCat mice, with decreases of 58% in anterior neuropore closure, 65% in turning and 32% in somite development ($p < 0.05$) (**Fig. 7**, *panel A*). Phenytoin was also slightly embryopathic in hCat embryos compared to hCat vehicle controls, with slight but significant decreases in yolk sac diameter (11 %) and crown-rump length (15 %) ($p < 0.05$).

Compared to their phenytoin-exposed WT embryo controls, hCat embryos were substantially protected from phenytoin embryopathies, as evidenced by an increase in anterior neuropore closure by 42%, turning by 55%, and somite development by 23% ($p < 0.05$) (**Fig. 7**, *panel A*).

Catalase activities in embryos expressing human catalase (high activity)

Mean catalase activity in hCat embryos was 63% higher than that in wild-type embryos cultured in phenytoin ($p < 0.05$) (**Fig. 7**, *panel B*). In maternal liver, catalase activity in hCat mice was about 3-fold higher than that in wild-type animals ($p <0.05$) (**Fig 7**, *panel C*). Catalase activities in WT and hCat embryos were only about 13% and 7% of the respective activities in adult WT and hCat livers, with the higher percentage in WT embryos being due to the lower adult WT activity (**Fig. 7**, *panel C*) compared to outbred CD-1 mice (**Fig. 2**). Catalase activity in the C57BL/6 WT embryos for the hCat mice (**Fig. 7**, *panel C*) was identical to that in the C3H WT embryos for the aCat mice (**Fig. 6**, *panel C*), and only about one-half of the activities observed in outbred CD-1 embryos throughout organogenesis (**Fig. 2**).

Figure 6.

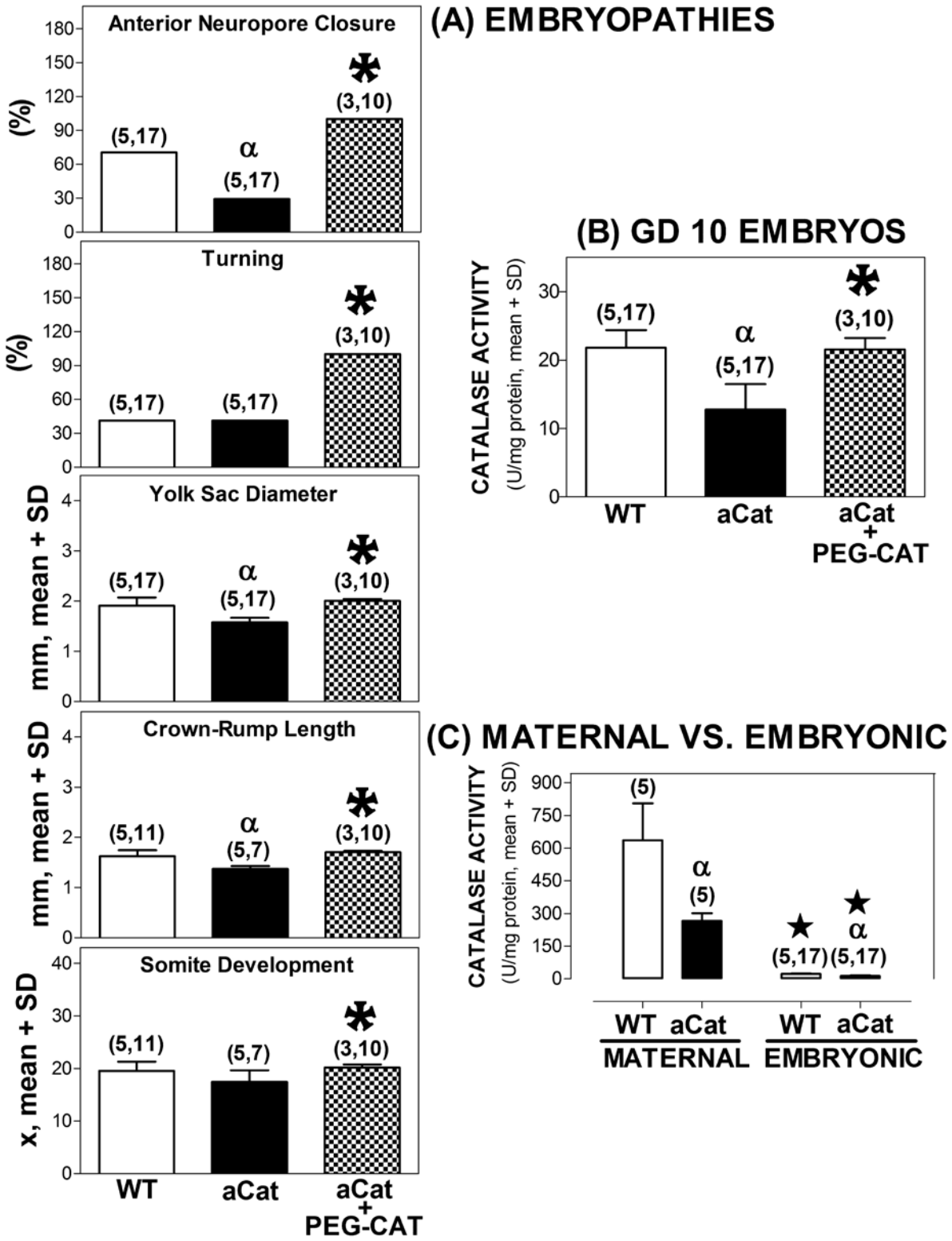

(A) EMBRYOPATHIES
Anterior Neuropore Closure
(%)
(5,17)
α
(5,17)
(3,10)
Turning
(%)
(5,17)
(5,17)
(3,10)
Yolk Sac Diameter
mm, mean + SD
(5,17)
α
(5,17)
(3,10)
Crown-Rump Length
mm, mean + SD
(5,11)
α
(5,7)
(3,10)
Somite Development
x, mean + SD
(5,11)
(5,7)
(3,10)
WT
aCat
aCat + PEG-CAT
(B) GD 10 EMBRYOS
CATALASE ACTIVITY
(U/mg protein, mean + SD)
(5,17)
α
(5,17)
(3,10)
WT
aCat
aCat + PEG-CAT
(C) MATERNAL VS. EMBRYONIC
CATALASE ACTIVITY
(U/mg protein, mean + SD)
(5)
α
(5)
(5,17)
α
(5,17)
WT aCat
MATERNAL
WT aCat
EMBRYONIC

Figure 6. Prevention of phenytoin embryopathies in aCat mice with catalase protein therapy.

Panel A: Acatalasemic and wild-type embryos were cultured with phenytoin and assessed as described in Fig. 1. **Panel B**: Embryos represented in panel A were used to measure endogenous catalase activity in each individual embryo using the FOX assay. **Panel C**: Catalase activity was measured in maternal liver samples using the FOX assay, and compared to activities in WT and aCat embryos. The alpha symbol indicates a difference from the respective WT embryonic or maternal genotype ($p<0.05$); the asterisk indicates a difference from aCat embryos exposed only to phenytoin ($p<0.05$); the star symbol indicates a difference from activity in maternal livers with the same genotype ($p<0.05$). (n,N) = (litters, embryos).

Figure 7.

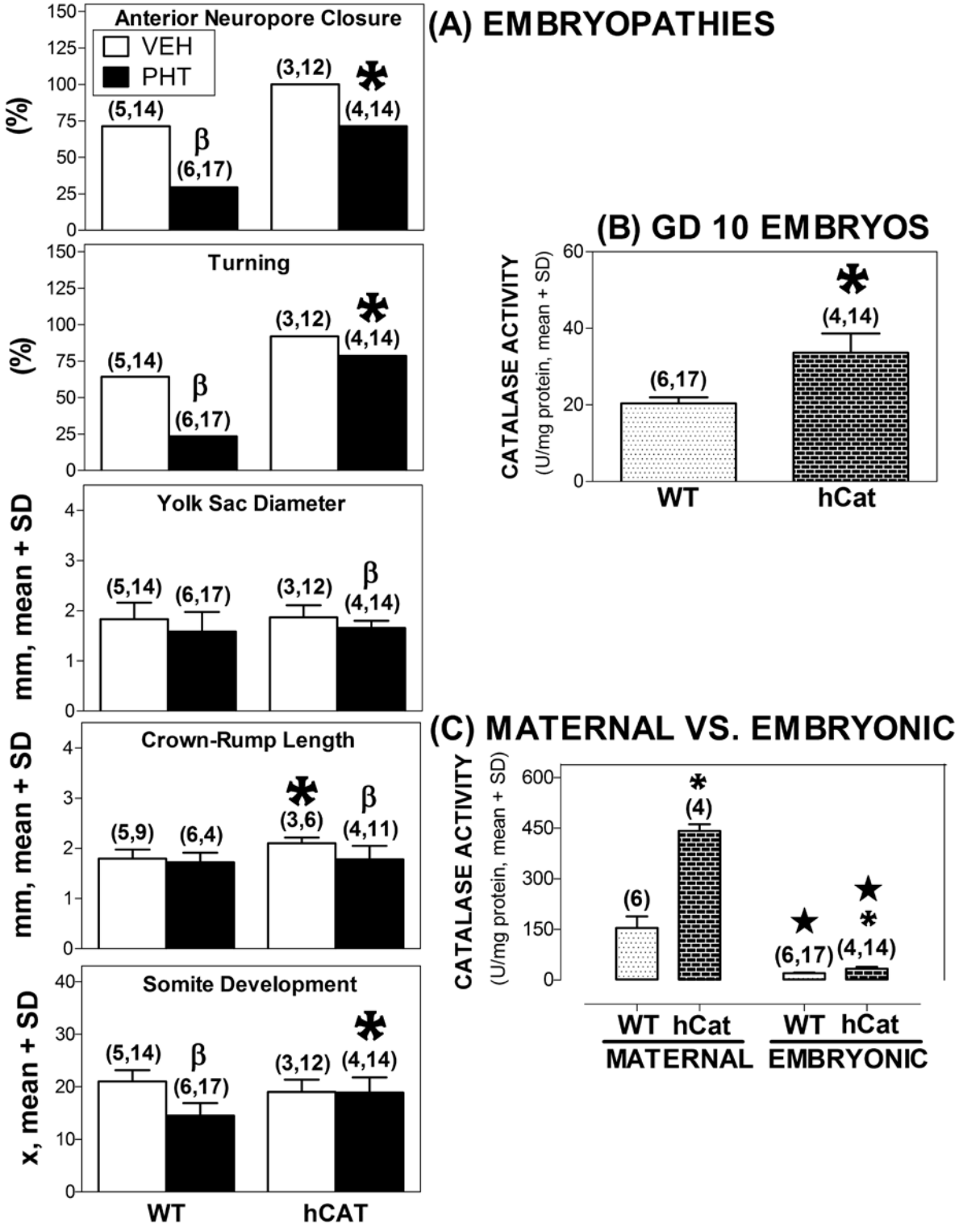

(A) EMBRYOPATHIES
Anterior Neuropore Closure
VEH
PHT
(%)
(5,14)
β
(6,17)
(3,12)
(4,14)
Turning
Yolk Sac Diameter
mm, mean + SD
Crown-Rump Length
(5,9)
(6,4)
(3,6)
(4,11)
Somite Development
x, mean + SD
WT
hCAT
(B) GD 10 EMBRYOS
CATALASE ACTIVITY
(U/mg protein, mean + SD)
(6,17)
(4,14)
WT
hCat
(C) MATERNAL VS. EMBRYONIC
(6)
(4)
(6,17)
(4,14)
WT hCat
MATERNAL
WT hCat
EMBRYONIC

Figure 7. Embryopathies in wild-type (WT) mouse embryos and embryos expressing human catalase (hCat) exposed to phenytoin or its vehicle.

Panel A. Wild-type embryos with normal catalase activity and hCat embryos expressing human catalase (high activity) were cultured and assessed as described in Fig. 1. **Panel B**. Embryos represented in panel A were used to measure endogenous catalase activity in each individual embryo using the FOX assay. **Panel C**: Catalase activity was measured in maternal liver samples using the FOX assay, and compared to activities in WT and hCat embryos. VEH = vehicle, PHT = phenytoin. The beta symbol indicates a difference from VEH-exposed embryos of the same genotype ($p<0.05$); the asterisk indicates a difference from WT embryos of the same treatment group (VEH or PHT) ($p<0.05$); the star symbol indicates a difference from activity in maternal livers with the same genotype ($p<0.05$). (n,N) = (litters, embryos).

2.1.5 DISCUSSION

The results from these embryo culture studies suggest that interindividual embryonic variations in catalase may constitute an important determinant of risk independent of maternal factors. The observed enhanced susceptibility of catalase-deficient acatalasemic (**aCat**) embryos, compared with their wild-type controls, to the embryopathic effects of both normal developmental oxidative stress, and even more so to the enhanced oxidative stress caused by the ROS-initiating anticonvulsant drug phenytoin, indicates that embryonic catalase is a critical developmentally protective enzyme. This interpretation is corroborated by the inverse decrease in embryopathies observed in embryos expressing human catalase (**hCat**, high activity) compared with their WT controls exposed to vehicle, and even more so to phenytoin. Not all developmental outcomes appeared to be modulated by catalase, at least at this gestational stage. Such outcomes may not be affected by ROS, or unaffected outcomes may reflect cell-specific or tissue-specific variations in embryonic catalase and/or other ROS-relevant protective pathways not detected by our measurements in whole-embryo homogenates. It has been shown *in vivo*, in embryo culture and *in vitro* that phenytoin and other xenobiotics can be bioactivated by embryonic enzymes such as PHS and lipoxygenases (**LPOs**) to free radical intermediates that enhance ROS formation (Kubow & Wells 1989; Miranda et al 1994; Parman & Wells 2002; Yu & Wells 1995). In contrast to low embryonic activities of alternative bioactivating enzymes like the cytochromes P450 during organogenesis (Juchau et al 1992; Wells & Winn 2010), levels and activities of PHSs and LPOs are relatively high (Datta & Kulkarni 1994; Mitchell et al 1985; Parman & Wells 2002). The formation of ROS within the embryo can alter signal transduction and cause oxidative damage to cellular macromolecules, either of which can adversely affect development (Wells et al 2009).

Embryonic catalase activity in all strains was only about 3-7% of maternal activity, but readily detectable in murine embryos during the period of organogenesis. Positive control studies with CD-1 embryos revealed that embryonic catalase activity appears to remain consistently low during the developmental period from GD 9 to GD 12 both in embryo culture and *in vivo*, and was not measurably affected by 24-hr exposure to the ROS-initiating teratogen phenytoin. This low level of embryonic activity was similar to that previously reported for this gestational age (El-Hage & Singh 1990; Winn & Wells 1999). The continuing low catalase activity on GD 12

suggests that embryos may remain susceptible to enhanced oxidative stress throughout the entire period of organogenesis.

Catalase activity was reduced in all organs of aCat mice, although the mutation affected all major organs to a varying degree. Red blood cells were most affected, exhibiting negligible catalase activity. Liver was the least affected organ, although hepatic catalase activity was decreased 9-fold. It is not known why this mutation affects each organ differently. One report speculated that the differences in tissue expression could be post-transcriptionally regulated through translational efficiency or post-translational protein stability (Reimer et al 1994).

Glutathione peroxidase catalyzes the reduction of hydroperoxides, including H_2O_2, by reducing glutathione, thereby protecting the cell from oxidative stress. In contrast to catalase which is primarily found in peroxisomes, GPx is found in the cytosol and mitochondria (Savaskan et al 2007). Constitutive peroxidase activity in embryos is only about 5% of that in maternal hepatic tissues (Ozolins et al 1996). Herein, the mutant aCat mice exhibited increased levels of this enzyme in all major organs, possibly in compensation for the reduced catalase activity. However, this increase in GPx did not measurably affect the susceptibility of this mutant mouse to the effects of endogenous and xenobiotic-enhanced oxidative stress.

The therapeutic concentration of phenytoin employed herein is generally embryopathic in mouse embryo culture (Wells et al 2009), as was confirmed in our study in CD-1 mice. On the other hand, the absence of embryopathies in wild-type C3H embryos exposed to phenytoin suggests that this genetic strain may be relatively resistant to phenytoin teratogenicity, and a similar resistance would be expected in the aCat mice, thereby reducing the potential effect of catalase deficiency on their susceptibility to phenytoin. Presumably this was reflected in the small (but significant) nature of the reductions by phenytoin in yolk sac diameter and crown-rump length in aCat embryos compared to WT controls. Nevertheless, despite the intrinsic resistance of this strain, phenytoin caused a remarkable 59% decrease in anterior neuropore closure in aCat embryos compared to WT controls. When comparing within the same genotype, aCat embryos exposed to phenytoin exhibited significantly greater decreases in yolk sac diameter and crown-rump length compared to aCat embryos exposed to vehicle, which is consistent with our hypothesis of a protective role for catalase in phenytoin embryopathies. Phenytoin caused a similar 34% decrease in aCat embryos compared to saline-exposed aCat embryos, although this difference was not statistically significant. The lack of a further effect of phenytoin on turning in

aCat embryos compared to saline-exposed aCat embryos may have been due in part to the substantial albeit nonsignificant 42% decrease in turning in saline-exposed aCat embryos compared to their saline-exposed WT controls, the latter of which is suggestive of an embryopathic role for endogenous oxidative stress. The mechanisms underlying the resistance of this strain are unknown, but could include differences in embryonic drug bioactivation, other pathways for ROS detoxification and/or repair of oxidative macromolecular damage. Despite this apparent strain resistance, the enhanced dysmorphogenesis in acatalasemic embryos exposed to phenytoin compared to phenytoin-exposed wild-type embryos provides the first direct evidence that the relatively low level of constitutive embryonic catalase provides important protection against ROS-initiating teratogens. Although there appears to be a trend toward a decrease in the parameters of anterior neuropore closure and turning in the aCat mice exposed to vehicle compared to their wild-type controls, the differences are not statistically significant. However, we believe that these trends may reflect the pathogenic potential of endogenous oxidative stress, which would require a larger study to confirm. The strong negative correlation between lower endogenous embryonic catalase activity and enhanced embryopathies seen in embryo culture suggest that interindividual embryonic differences in expression of catalase may contribute to teratological risk.

Unlike the C3H wild-type embryos for the aCat strain, the C57BL/6 WT embryos for the hCat strain showed a substantial increase in embryopathies when exposed to phenytoin, indicating that this particular strain is highly susceptible to phenytoin embryopathies. It is therefore particularly remarkable that the increased catalase activity observed in the hCat embryos was able to completely protect against phenytoin embryopathies. The phenytoin-initiated decreases in anterior neuropore closure, turning and somite development were significantly reduced in hCat mice compared to their WT controls, which is consistent with our hypothesis. Since phenytoin did not adversely affect the other two parameters (yolk sac diameter and crown-rump length), our hypothesis would not predict that these parameters would be altered in phenytoin-exposed hCat mice compared to vehicle-exposed hCat mice. A similar albeit largely non-significant trend was observed in embryos exposed only to saline vehicle, where hCat embryos exhibited almost universal anterior neuropore closure and turning, and a significant improvement in crown-rump length. This normal development was similar to outcomes in the vehicle-exposed outbred CD-1 mice, and in contrast to the vehicle-exposed

C57BL/6 WT embryos that exhibited less than a 75% incidence of anterior neuropore closure and turning. Although the protected hCat embryos exhibited a 63% increase in catalase activity over their WT controls, their activity was still only about 7% of maternal hCat activity, and about only 13% of maternal WT activity, suggesting that a relatively minor increase in embryonic catalase exerts an important protective role during development.

To confirm the embryoprotective role of catalase, as distinct from other potentially unappreciated genetic differences between the WT and acatalasemic mice, acatalasemic embryos cultured with phenytoin were pretreated with PEG-catalase. PEG-catalase added to the media crosses the membrane of the yolk-sac and enters the embryo, catalytically intact. We have found similar results previously in embryo culture (Winn & Wells 1995b), and also in the embryo *in vivo* following maternal treatment with PEG-catalase (Winn & Wells 1999). This protein therapy using exogenous catalase completely blocked phenytoin embryopathies, suggesting both a critical role for catalase in the protective mechanism, and a role for ROS in phenytoin teratogenesis. Although the protein therapy increased embryonic catalase activity in aCat embryos after only 2 hr to the level in WT embryos, the level still was only about 4% of that in maternal WT liver, and 5% of that in maternal aCat liver, showing the remarkable protective efficacy of relatively minor levels of embryonic activity.

Strain differences in embryonic catalase activities may play a role in the relatively impaired development of WT C3H and C57BL/6 embryos, which both showed trends for reduced anterior neuropore closure and turning compared to outbred CD-1 embryos, since the latter outbred strain exhibited 2-fold higher embryonic catalase activity compared to the two inbred strains. The high susceptibility of CD-1 mouse embryos to the embryopathic effects of phenytoin in culture is consistent with their susceptibility to phenytoin teratogenicity *in vivo* (Wells et al., 2009). In addition, the CD-1 mouse is an outbred strain that we have found has a 4-fold variability in catalase activity, which may contribute to their susceptibility to phenytoin. In the case of the hCat WT mice, the C57BL/6 strain from which the hCat mice are derived are considered “hypocatalasemic” because of their relatively lower catalase activity, which may enhance the susceptibility of this strain. The mechanisms underlying the variable susceptibility of different mouse strains to phenytoin are not known, but could include strain-dependent differences in other pathways such as embryonic drug bioactivation, other pathways for ROS detoxification and/or signal transduction, and/or pathways for repair of oxidative

macromolecular damage. The protective effects of catalase observed in our studies are consistent with related *in vitro* studies showing a protective effect of catalase against ROS-initiated damage, including cell death, DNA fragmentation and single strand breaks and cell growth retardation (Abad et al 1995; Amstad et al 1991; Horwitz & Leff 1995; Kim & Kim 1991; Mann et al 1997; Noble et al 1994). More directly, the results herein are consistent with studies showing that protein therapy with exogenous catalase in CD-1 mice reduced both developmental and phenytoin-enhanced embryonic DNA oxidation and embryopathies in culture (Winn & Wells 1995b), and phenytoin teratogenicity *in vivo* (Winn & Wells 1999).

In summary, the results herein provide direct evidence that the relatively low level of endogenous embryonic catalase expressed during organogenesis provides important protection against developmental and phenytoin-enhanced oxidative stress. This is the first study to show that variations in activity of this enzyme in the embryo affect development. Further studies of endogenous embryonic catalase activity may provide new insights into the mechanisms of birth defects and the individual determinants of risk.

2.1.6 ACKNOWLEDGEMENTS

We would like to thank Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX) for generously providing a breeding pair of the transgenic hCat mice.

2.2 STUDY 2: EMBRYONIC CATALASE PROTECTS AGAINST ENDOGENOUS AND PHENYTOIN-ENHANCED DNA OXIDATION AND EMBRYOPATHIES IN ACATALASEMIC AND HUMAN CATALASE-EXPRESSING MICE [a,b]

Running title: Embryoprotective role of catalase

Julia P. Abramov[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Corresponding author:
Peter G. Wells, Pharm.D.
Faculty of Pharmacy, University of Toronto
144 College St., Toronto, Ontario, Canada M5S 3M2
E-mail: pg.wells@utoronto.ca
Tel. 416-978-3221
Fax 416-267-7797

a. Preliminary reports of this research were presented at the 2007 and 2009 annual meetings of the Teratology Society (U.S.A.) [*Birth Defects Res. Part A: Clinical and Molecular Teratology,*79(5):418; 85(5):398] and the 2009 annual meeting of the Society of Toxicology (U.S.A.) [*The Toxicologist*,108(1):355]. These studies were supported by a grant from the Canadian Institutes of Health Research.

b. Full report of this research has been published:
Abramov JP, Wells PG. 2011. Embryonic catalase protects against endogenous and phenytoin-enhanced DNA oxidation and embryopathies in acatalasemic and human catalase-expressing mice. *FASEB Journal* 25(7):2188-2200.

2.2.1 ABSTRACT

Oxidative stress and reactive oxygen species (**ROS**) such as hydrogen peroxide (**H_2O_2**), which is detoxified by catalase, are implicated in fetal death and birth defects, but embryonic levels of catalase are only about 5% of adult activity, and its protective role is not completely understood. Herein, we used mutant catalase-deficient mice (acatalasemic, **aCat**) and transgenic mice expressing human catalase (**hCat**), which respectively exhibited 40-50% reductions and 2-fold elevations in the activities of embryonic and fetal brain catalase, to show that embryonic catalase protects the embryo from both physiological oxidative stress and the ROS-initiating antiepileptic drug phenytoin. Compared to WT catalase-normal controls, both untreated and phenytoin-exposed aCat mice exhibited a 30% increase in embryonic DNA oxidation and a greater than 2-fold increase in embryopathies, both of which were completely blocked by protein therapy with exogenous catalase. Conversely, compared to WT controls, untreated and to a lesser extent phenytoin-exposed hCat mice were protected, with untreated hCat embryos exhibiting a 40% decrease in embryonic DNA oxidation and up to a 67% decrease in embryopathies. Embryonic catalase accordingly plays an important protective role, and both physiological and phenytoin-enhanced oxidative stress can be embryopathic.

2.2.2 INTRODUCTION

Catalase is an antioxidative enzyme that removes hydrogen peroxide (**H_2O_2**) from cells. It is found in all adult organs and the developing embryo (Ogata 1991), but little is known about its embryonic expression, inter-individual variability or protective importance. If not detoxified by catalase, H_2O_2 can initiate signal transduction pathways, or react with iron to form highly reactive hydroxyl radicals (•OH), which can oxidatively damage cellular macromolecules (lipids, proteins, DNA, etc.), altering their function (**Supplemental Fig. 1**) (Halliwell & Gutteridge 2007). Such oxidative stress has been implicated in the mechanism of *in utero* embryonic and fetal death, and structural and functional (neurodevelopmental) birth defects (Wells et al 2009). The developing embryo and fetus are uniquely susceptible due to their low levels of most antioxidative enzymes, together with increased rates of cell division and differentiation that must occur within narrow windows of development (Wells et al 2005).

Numerous studies have demonstrated a protective role for exogenous forms of catalase against ROS-mediated damage, including ischemic-reperfusion injury (Liu et al 1989; Oda et al 1992; Padmanabhan et al 1985), cold injury (Das et al 1991) and favism (Gaetani et al 1996). A potential protective role for embryonic catalase has been suggested by studies in murine embryo culture, where the addition of exogenous catalase enhanced embryonic antioxidative activity and protected against DNA oxidation and embryopathies initiated by the ROS-initiating antiepileptic drug phenytoin (Winn & Wells 1995b), and similarly against diabetic embryopathies (Erickson & Borg 1991). A subsequent *in vivo* study showed that maternal administration of polyethylene glycol (**PEG**)-conjugated catalase could increase embryonic catalase activity, decrease phenytoin-initiated protein oxidation and protect the embryo against phenytoin teratogenicity (Winn & Wells 1999). However, embryonic levels of catalase are only about 5% of levels in adult liver (El-Hage & Singh 1990; Winn & Wells 1999), and it is not clear whether such a quantitatively minor antioxidative enzyme in the embryo can provide biologically significant protection.

Herein we used mutant catalase-deficient mice (acatalasemic, **aCat**) and transgenic mice expressing human catalase (**hCat**), which respectively exhibited reduced and elevated levels of embryonic catalase, to determine the role of endogenous catalase in protecting the embryo *in vivo* from ROS-initiated DNA oxidation and the embryopathic effects of endogenous and drug-enhanced oxidative stress. A full description of these two mouse models is reviewed elsewhere

(Section 2.1) (Abramov & Wells 2011b). Embryos were exposed *in vivo* to either no treatment or a therapeutic concentration of the antiepileptic drug phenytoin, a teratogen that is believed to alter development at least in part by enhancing ROS formation (Wells et al 2009). Embryonic levels of oxidatively damaged DNA and developmental parameters were evaluated within the context of fetal and maternal tissue concentrations of phenytoin. Higher concentrations of phenytoin in fetal brain compared to fetal liver and maternal tissues may explain the high frequency of neurodevelopmental deficits compared to other fetal anomalies caused by this drug. Both untreated and phenytoin-exposed aCat mice exhibited increased embryonic DNA oxidation and *in utero* and postnatal death, which were blocked by protein therapy with exogenous catalase. Conversely, untreated, and to a lesser extent phenytoin-exposed, hCat mice were protected from embryonic DNA oxidation and embryopathies. These results provide the first direct *in vivo* evidence that the relatively low level of endogenous embryonic catalase provides important protection against both physiological oxidative stress and ROS-initiating teratogens, and suggest that interindividual embryonic differences in expression of this enzyme could contribute to teratological risk.

2.2.3 MATERIALS AND METHODS

Chemicals

Phenytoin (diphenylhydantoin sodium salt), mephenytoin, catalase, xylenol orange, ammonium iron (II) sulphate hexahydrate, potassium permanganate, Bouin's fixative and polyethylene glycol-conjugated catalase (**PEG-catalase**) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen peroxide and sucrose were from BDH Chemicals Inc. (Toronto, Ontario, Canada). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA.). Pure racemic (*d*/l)-methamphetamine (**METH**) was provided by the Health Environments and Consumer Safety Branch of Health Canada (Ottawa, Ontario, Canada). All other reagents and solvents were of analytical grade.

Animals

Outbred CD-1 mice were purchased from Charles River Laboratories (St. Constant, Quebec). Breeding pairs of catalase-normal wild-type (C3HeB/FeJ) and mutant acatalasemic (**aCat**) (catalase-deficient) (C3Ga.Cg-*Cat*^{b}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Transgenic C57BL/6J mice expressing human catalase (**hCat**) in addition to their intrinsic murine catalase (high catalase activity) were donated by Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX), and the wild-type (C57BL/6J) mice were purchased from The Jackson Laboratory. Same-sex animals were housed in plastic cages with ground cob bedding and were maintained in a temperature-controlled animal facility with a 12 hr light/dark cycle. Food (Purina Rodent Chow, Oakville, Ontario) and tap water were provided *ad libitum*. One male was housed with two females overnight between 17:00 and 09:00 hr. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (**GD**) 1. All animal procedures were approved by the University of Toronto animal care committee.

Teratogenesis

Homozygous aCat and hCat females and their respective wild-type controls were mated with males of the same genotype as described above. Dams were treated intraperitoneally (**i.p.**) at 10:00 a.m. on GDs 9, 12 or 13 (GDs 12 and 13, supplemental data) with vehicle alone or with a marginally teratogenic (55 mg/kg) or teratogenic dose (65 mg/kg) (Wells et al 2009) of phenytoin in saline containing 0.002 N NaOH and killed by cervical dislocation on GD 19. PEG-catalase (50 KU/kg i.p.) dissolved in 0.9% saline (pH 7.4) was administered i.p. to the pregnant dams 6 hr prior to phenytoin (65 mg/kg) administration. For females that were allowed to deliver spontaneously, the number of pups was recorded, and the pups were left with their mothers until weaning, 21 days after birth. The number and sex of weaned pups were recorded. To study the effect of physiological oxidative stress on embryopathies, pregnant females were killed by cervical dislocation on GD 13. The uterus was exteriorized and the number of implantations as well as fetal resorptions (*in utero* deaths) was recorded. For teratology studies, fetuses were examined on GD 19 to determine sex, weight and external anomalies. Viable fetuses were kept warm in a chick incubator at 37°C for 2 hr to assess postpartum lethality, and then examined for external and visceral malformations or developmental variations. In addition, fetuses were placed in Bouin's solution for subsequent examination of soft tissue abnormalities. Fetal carcasses were processed for a minimum of 3 days in Bouin's fixative. Following fixation, fetal carcasses were placed in 70% EtOH for 1 day. Fetuses were then examined for soft tissue abnormalities using Wilson's freehand razorblade-sectioning technique (Barrow & Taylor 1969).

Genotyping

DNA was isolated from tail clips by heating the sample in 300 μL of a solution containing 10 mM NaOH/0.1 mM EDTA at 95°C for 15 min. Catalase PCR primers for the aCat mice were, CatF: TCCTTCCAATCCCGTCCTTTCT and CatR: AAATGCCAAACTCGGAGCCATC. PCR conditions for a Perkin Elmer 9600 thermal cycler were 94°C for 5 min, 20 sec at 94°C, 20 sec at 65°C (-1 degree cycle), and 40 sec at 72°C for a total of 10 cycles, then 20 sec at 94°C, 20 sec at 55°C, and 40 sec at 72°C for a total of 30 cycles, with a 10 min extension at 72°C and kept at 4°C until ready for digestion. PCR products were digested using NdeI restriction enzyme at 37°C for 16 hr and run on 1.5% agarose gels to

determine catalase genotype. To genotype hCat mice, catalase PCR primers were, CatF: GAGGTCCACCCTGACTACGGG and CatR: GCCTTCTCCCTTGCCGCCAAG. PCR conditions were 95°C for 5 min, 30 s at 95°C, 30 s at 60°C and 1 min at 72°C for a total of 35 cycles, with a 10 min extension at 72°C. Undigested PCR products were run on 1.5% agarose gels to determine catalase genotype.

DNA oxidation

Females were mated as in the teratological studies. Dams were killed at times of maximal embryonic DNA oxidation, 6 hr after maternal treatment with phenytoin (65 mg/kg i.p.) (Liu & Wells 1995a), and 4 hr after maternal treatment with METH (40 mg/kg i.p.) (Jeng et al 2006), each drug dissolved in sterilized 0.9% saline and administered on GD 13 or 17. PEG-catalase (50 KU/kg i.p.) was administered i.p. 12 hr prior to drug treatment. Whole embryos from GD 13 were used, while fetal brains were isolated from GD 17 fetuses. Genomic DNA was extracted using the modified NaI extraction method (Ravanat et al 2002). Oxidative DNA damage was quantified using the 8-OHdG ELISA kit (JaICA, Japan).

Catalase activity

Catalase activity was measured in embryos on GDs 9 and 13 as well as in fetal brains from pups on GDs 17 and 19. Samples were homogenized in phosphate-buffered saline and catalase activity was determined using the ferrous oxidation in xylenol orange (**FOX**) assay (Gupta 1973; Jiang et al 1992; Ou & Wolff 1996). Catalase activity was determined using a standard curve with bovine liver catalase as the standard. Appropriate controls including heat inactivation and the use of a specific catalase inhibitor were employed to ensure that only catalase activity was measured.

Phenytoin concentration

Initial phenytoin concentrations were determined in CD-1 mice. Females were mated as described above and treated with phenytoin (65 mg/kg i.p.) on GD 17 and sacrificed 6 hr later.

Maternal and fetal liver and brain samples were isolated for determination of tissue phenytoin concentrations, and maternal blood was collected for plasma analysis. Studies in aCat and hCat mice were performed as described above employing an additional phenytoin dose of 55 mg/kg. Phenytoin was measured in 100 µl of blood or tissue homogenate. The extraction procedure and detection method using HPLC with UV detection was performed as described previously (Lum et al 1985).

Statistical analysis

Statistical significance of differences between paired data was determined by the two-tailed Student *t*-test, while multiple comparisons among groups were analyzed by one- or two-way analysis of variance (ANOVA) and the Tukey test. Binomial data were analyzed using Fisher's exact test where appropriate. The minimum level of significance used throughout was $p < 0.05$.

2.2.4 RESULTS

Spontaneous embryopathies and pup survival in untreated mice

Acatalasemic mice

Untreated aCat embryos/fetuses and pups were more susceptible than wild-type (**WT**) catalase-normal controls to both *in utero* and postnatal death, with a 2-fold greater incidence of resorptions and preweaning lethality, while exhibiting a 20% decrease in the litter size at birth ($p < 0.05$) (**Fig. 1, left panels)**.

When untreated aCat dams were killed on GD 19, resorptions were slightly but significantly increased in aCat mice, and there was a 5-fold increase in the incidence of late fetal death, although the latter was only marginally significant ($p = 0.08$) (**Supplemental Fig. 2**). There was also a non-significant trend for increased total anomalies (kinky tail, underdeveloped eye) in aCat mice on GD 19 (**Supplemental Fig. 4**).

Human catalase-expressing mice

With spontaneous delivery, untreated hCat mice had an 8% increase in the litter size at birth ($p < 0.05$) and a 3-fold decrease in the incidence of preweaning lethality compared to WT controls ($p < 0.05$) (**Fig. 1, right panels**).

When untreated hCat dams were killed on GD 19, there were no significant differences between hCat fetuses and WT controls (**Supplemental Fig. 3**).

Embryopathies in phenytoin-treated mice

Acatalasemic mice

aCat mice treated with vehicle exhibited an increased incidence (24%) of fetal resorptions (*in utero* deaths) compared to WT controls ($p < 0.05$) (**Fig. 2**). Postpartum lethality was also increased, but this was only marginally significant ($p = 0.07$). All other parameters, including late fetal death, mean fetal weight and anomalies, did not differ between the two genotypes exposed to endogenous oxidative stress.

A marginally teratogenic dose of phenytoin (55 mg/kg) produced no effect in the WT animals, but increased fetal resorptions and postpartum lethality 3- and 2.5-fold, respectively, in the aCat mice ($p<0.05$). A standard teratogenic dose of phenytoin (65 mg/kg) (Wells et al 2009) did not affect the WT animals, except for the total anomalies, which were increased by 30% ($p<0.05$). In aCat mice treated with the higher dose of phenytoin, there was a 2.5-fold increase in the incidence of fetal resorptions and a 2-fold increase in postpartum lethality, compared to the WT phenytoin-treated controls ($p<0.05$). Late fetal death was also increased in the aCat dams compared to the WT controls, but this was only marginally significant ($p = 0.08$). Compared to the vehicle-treated controls of the same respective genotype, this dose of phenytoin increased the incidence of total anomalies in aCat dams by 50% ($p<0.05$). The main malformation observed in both genotypes was exencephaly (**Supplemental Fig. 4**), with a 37% incidence in aCat mice ($p<0.05$).

Phenytoin-enhanced fetal resorptions and exencephalies observed in the aCat mice were both reduced to control levels by pretreatment with catalase protein therapy ($p<0.05$). However, the total anomalies in the aCat mice remained significantly higher than in the WT controls even after pretreatment with PEG-catalase ($p<0.05$).

In all genotypes, mean fetal weight was 11% lower in the fetuses that did not survive for 2 hr postpartum.

When dams were treated later in gestation, with 65 mg/kg phenytoin on both GDs 12 and 13, there were no major anomalies observed in either genotype, but a slight trend for an increase in anomalies was observed in both WT and aCat animals (**Supplemental Fig. 2**). Vehicle-treated aCat mice still showed a 6% increase over WT controls in fetal resorptions ($p<0.05$). With phenytoin treatment, an approximate 20% increase in fetal resorptions was observed in both WT and aCat mice compared to vehicle-treated controls, although this was significant only for the WT mice ($p<0.05$). Phenytoin increased the incidence of postpartum lethality in the aCat mice by over 2-fold compared to both the phenytoin-exposed WT controls and the aCat saline controls ($p<0.05$). There was a non-significant 2.3-fold increase in late fetal death in phenytoin-exposed aCat mice compared to phenytoin-exposed WT controls, but this increase was only 29% higher than the incidence in vehicle-exposed aCat controls.

Figure 1.

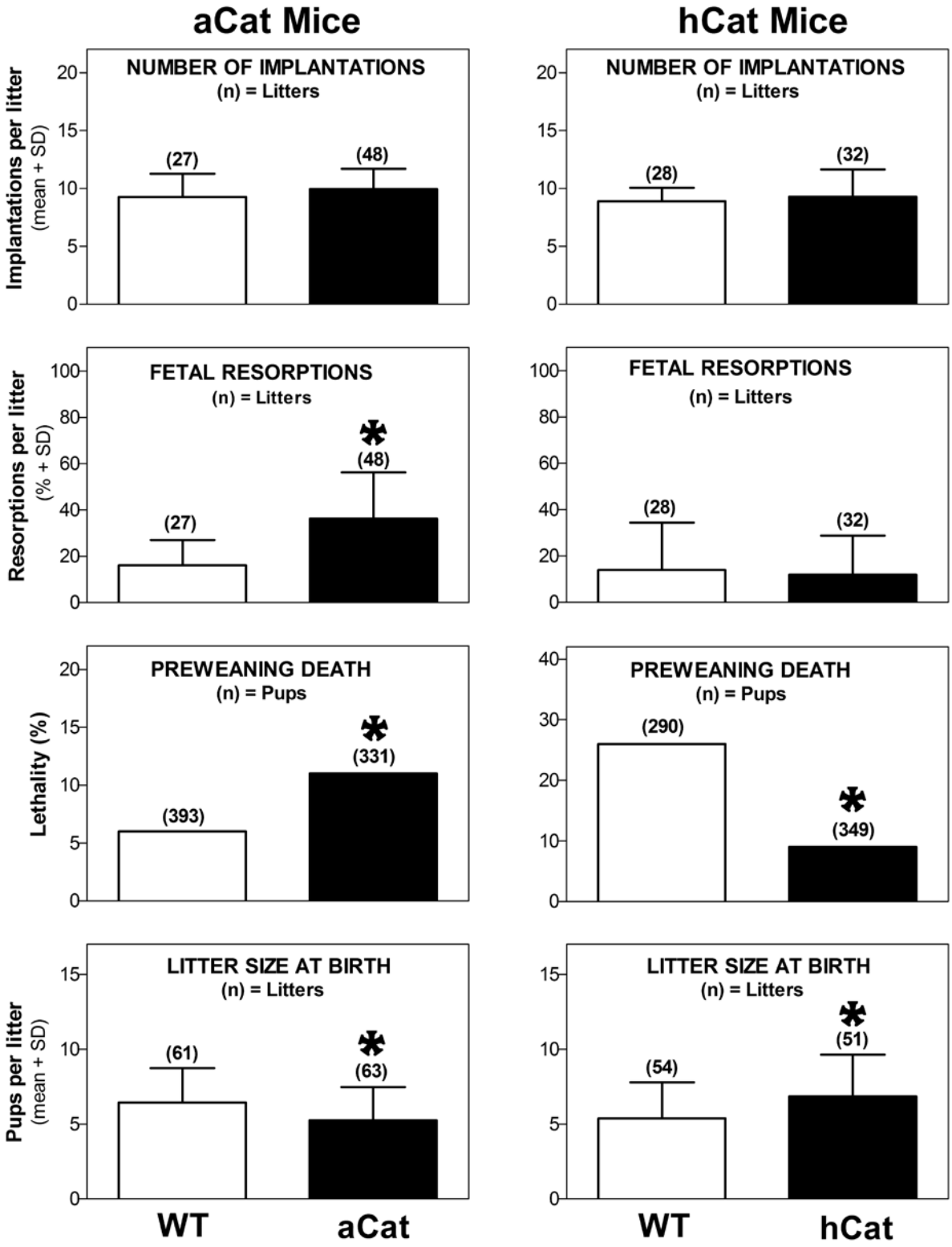

aCat Mice
hCat Mice
NUMBER OF IMPLANTATIONS
(n) = Litters
Implantations per litter
(mean + SD)
(27)
(48)
(28)
(32)
FETAL RESORPTIONS
(n) = Litters
Resorptions per litter
(% + SD)
(27)
(48)
(28)
(32)
PREWEANING DEATH
(n) = Pups
Lethality (%)
(393)
(331)
(290)
(349)
LITTER SIZE AT BIRTH
(n) = Litters
Pups per litter
(mean + SD)
(61)
(63)
(54)
(51)
WT
aCat
WT
hCat

Figure 1. Spontaneous embryopathies and pup survival in untreated mutant acatalasemic (aCat) mice and transgenic mice expressing human catalase (hCat).

Untreated pregnant mutant catalase-deficient (acatalasemic, **aCat**) and transgenic expressing human catalase (**hCat**) dams were allowed to deliver spontaneously and litter sizes were determined on the day of birth and 21 days later at the time of weaning. The number of implantations was calculated when uterine horns were exposed and embryos excised for analysis of DNA oxidation. 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 genotype. Preweaning death was calculated as the incidence of pups born live and dying before weaning. The asterisk indicates a difference from wild-type (**WT**) controls ($p<0.05$).

Figure 2.

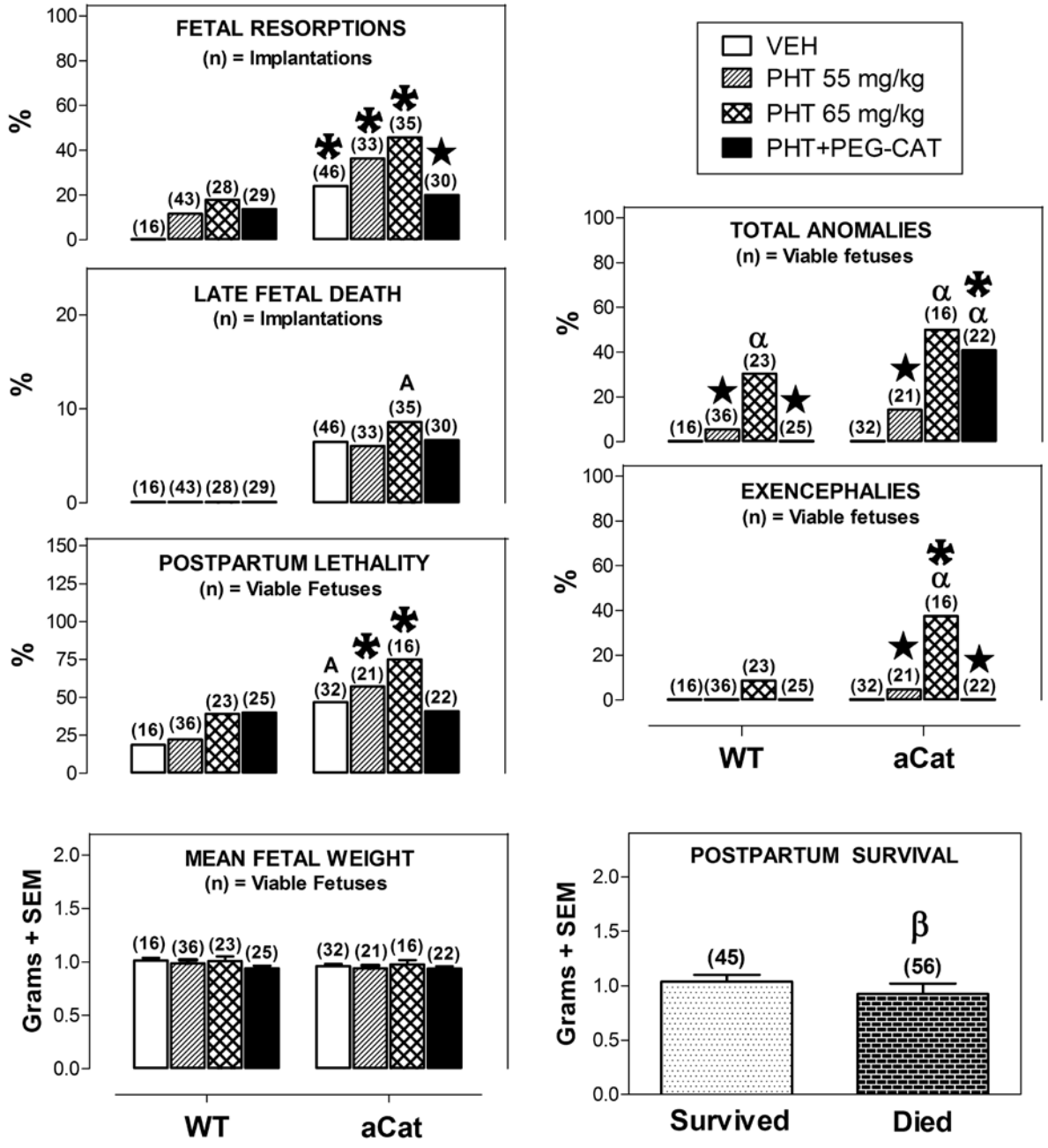

FETAL RESORPTIONS
(n) = Implantations
%
LATE FETAL DEATH
(n) = Implantations
%
POSTPARTUM LETHALITY
(n) = Viable Fetuses
%
MEAN FETAL WEIGHT
(n) = Viable Fetuses
Grams + SEM
WT
aCat
VEH
PHT 55 mg/kg
PHT 65 mg/kg
PHT+PEG-CAT
TOTAL ANOMALIES
(n) = Viable fetuses
%
EXENCEPHALIES
(n) = Viable fetuses
%
WT
aCat
POSTPARTUM SURVIVAL
Grams + SEM
Survived
Died

Figure 2. Prevention of phenytoin embryopathies in acatalasemic mice *in vivo* with catalase protein therapy.

Pregnant aCat dams were treated intraperitoneally (**i.p.**) on gestational day (**GD**) 9 with phenytoin (**PHT**), 55 or 65 mg/kg, or its vehicle (**VEH**), and sacrificed on GD 19. For the protein therapy studies, dams were pretreated with 50 KU/kg polyethylene glycol-conjugated catalase (**PEG-CAT**) 6 hr before injection with PHT, 65 mg/kg. The incidence of fetal resorptions (*in utero* deaths) was calculated as described in fig. 1. Late resorptions were the remnants of fetuses that died *in utero* sufficiently late in gestation that a dissectible mass remained. Incidence was calculated by dividing the number of typeable resorptions for a given genotype by the total number of implantations (fetuses and resorptions) for that embryonic genotype. Postpartum lethality was calculated by dividing the total number of fetuses born live and dying within 2 hr by the total number of viable fetuses. Total anomalies included exencephalies, open eye, dilated ventricles, cleft palate and hydronephrosis. The percentage was calculated by dividing the number of fetuses affected with at least one anomaly by the number of total live fetuses. For postpartum survival, mean fetal weight was calculated for all surviving and dying fetuses, independent of the genotype. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$), with a marginal difference indicated by the letter A.

Human catalase-expressing mice

hCat mice treated with vehicle exhibited a decreased incidence (66%) of postpartum lethality and increased mean fetal weight (16%) compared to the WT controls ($p < 0.05$) (**Fig. 3**). WT animals treated with phenytoin (65 mg/kg) exhibited a 20% incidence of total anomalies compared to 0% in untreated WT controls ($p = 0.11$). Phenytoin-treated hCat animals showed a trend for being protected, exhibiting an 80% reduction compared to the WT phenytoin-exposed controls ($p = 0.15$). Postpartum lethality in phenytoin-treated hCat mice was 3.5-fold higher than in vehicle-treated hCat controls ($p<0.05$), but was not different from phenytoin-exposed WT controls.

Treatment of pregnant dams later in gestation (GDs 12 and 13) with phenytoin showed no major anomalies in either genotype (**Supplemental Fig. 3**). Phenytoin-treated WT mice showed an 85% increase in late fetal death compared to vehicle-treated WT controls ($p<0.05$), whereas phenytoin-treated hCat mice were completely protected ($p<0.05$). Phenytoin treatment also increased postpartum lethality by 3.1-fold in WT mice compared to the vehicle-treated WT controls ($p<0.05$). Postpartum lethality was reduced by 49% in phenytoin-treated hCat mice compared to phenytoin-treated WT controls, but this difference was only marginally significant ($p = 0.1$).

Catalase activity in embryonic and fetal brains for teratology study

Acatalasemic mice

On GD 9, within the period of organogenesis and during the period of phenytoin treatment for the teratological study, whole embryo catalase activity was 40% lower in aCat embryos compared with WT catalase-normal embryos ($p<0.05$) (**Fig. 4, left panels**). At the end of gestation, on day 19, catalase activity in fetal brains of WT fetuses was 31% higher ($p<0.05$) than that in WT whole embryos during organogenesis (GD 9), while in aCat fetuses on GD 19, activity remained at the level for GD 9, and was 50% less than that in GD 19 WT controls ($p<0.05$). As a percentage of maternal hepatic activity, embryonic (GD 9) and fetal brain (GD 19) activities for catalase in the aCat mice were only 6% and 7% of the respective maternal activity. Maternal catalase activity, which did not vary over the GD 9-19 period, was 266 +/- 35

U/mg protein (mean +/- SD, n = 5). The WT controls exhibited similarly low embryonic and fetal brain activities, respectively constituting only 4% and 6% of maternal activity, which was 637 +/- 169 U/mg protein (n = 5).

Human catalase-expressing mice

Compared to their gestationally matched WT controls, catalase activity was increased in hCat embryos by 40% on GD 9 and 1.7-fold on GD 13, and in hCat fetal brains by 2.1-fold on GD 17 and 2.6-fold on GD 19 ($p<0.05$) (**Fig. 4, upper right panels**). GD 19 fetal brain catalase activity was increased by 12% and 1.8-fold respectively in WT and hCat fetuses when compared to the respective GD 9 whole embryo activities ($p<0.05$). GD 17 brain catalase activity in WT fetuses was decreased by 40% compared to the GD 13 whole embryo activity ($p<0.05$), whereas a lesser 24% decrease in the brain activity in GD 17 hCat fetuses compared to GD 13 whole hCat embryos was not significant. As a percentage of maternal hepatic activity, embryonic (GD 9) and fetal brain (GD 19) activities for catalase in the hCat mice were only 7% and 13% of the respective maternal activity. Maternal catalase activity was 442 +/- 20 U/mg protein (mean +/- SD, n = 5). The WT controls exhibited similarly low embryonic and fetal brain activities, respectively constituting only 13% and 15% of maternal activity, which was 154 +/- 35 U/mg protein (n = 5).

Catalase activity in embryonic and fetal brains for PEG-catalase protein therapy study

Acatalasemic mice

On GD 13, in the period of late organogenesis, whole embryo catalase activity was 52% lower in aCat embryos compared with catalase-normal embryos ($p<0.05$) (**Fig. 4, lower left panels**). Treatment with PEG-catalase resulted in a 41% increase in catalase activity in the aCat embryos 12 hr after injection ($p<0.05$).

Later in gestation, on GD 17, catalase activity in the brains of catalase-normal WT fetuses was 27% lower than activity in GD 13 WT whole embryos during organogenesis ($p<0.05$) (**Fig 4, lower left panels**). In GD 17 aCat fetuses, brain catalase activity was 48% less than activity in the GD 13 aCat whole embryo ($p<0.05$), and was only 35% of the activity in the

brains of GD 17 WT fetuses ($p<0.05$). Treatment with PEG-catalase increased catalase activity in the fetal brains of the aCat fetuses by 25%, 12 hr after the injection ($p<0.05$). A time-course study following the injection of PEG catalase in aCat GD 13 whole embryos showed that the level of catalase activity was increased 1.5-fold at 6 hr ($p<0.05$), and this increase was sustained for at least 12 hr after injection ($p<0.05$) (**Fig. 4, lower right panel**).

Endogenous and phenytoin-enhanced DNA oxidation

Acatalasemic mice

Endogenous levels of DNA oxidation in GD 13 aCat embryos did not differ from the WT controls. However, when exposed to phenytoin, aCat embryos exhibited a 37% increase in DNA oxidation compared to WT controls, which were resistant to phenytoin-enhanced DNA oxidation ($p<0.05$) (**Fig. 5, upper left panel**). Pretreatment with PEG-catalase appeared to block phenytoin-enhanced DNA oxidation in aCat mice, although this protection was only marginally significant ($p = 0.09$). In contrast to phenytoin exposure, the ROS-initiating teratogen methamphetamine (**METH**) (Jeng et al 2005; Wong et al 2008) increased the levels of DNA oxidation in both WT (58%) and aCat (40%) embryos ($p<0.05$) (**Fig. 6, upper panel insert**). aCat embryos were more susceptible than WT controls, exhibiting 20% more METH-initiated oxidative DNA damage ($p<0.05$), amounting to a 2.7-fold increase over vehicle controls, which was completely blocked by pretreatment with catalase protein therapy ($p<0.05$).

Later in gestation, on GD 17, fetal brains of aCat mice were more susceptible than WT controls to the effects of endogenous oxidative stress, as demonstrated by a 30% increase in DNA oxidation ($p<0.05$) (**Fig. 5, lower left panel**). Phenytoin enhanced DNA oxidation in both WT and aCat fetal brains by 44% and 62% respectively compared to vehicle controls ($p<0.05$), with aCat mice having a 2-fold higher level of oxidatively damaged DNA compared to the WT controls ($p<0.05$). This phenytoin-initiated increase in DNA oxidation was completely blocked by the pretreatment with PEG-catalase ($p<0.05$). Similar to phenytoin, METH treatment also increased DNA oxidation in both WT and aCat fetal brains, by 50% and 45% respectively ($p<0.05$). Pretreatment of aCat mice with PEG-catalase blocked METH-initiated DNA oxidation ($p<0.05$) (**Fig. 5, lower left panel insert**).

Figure 3.

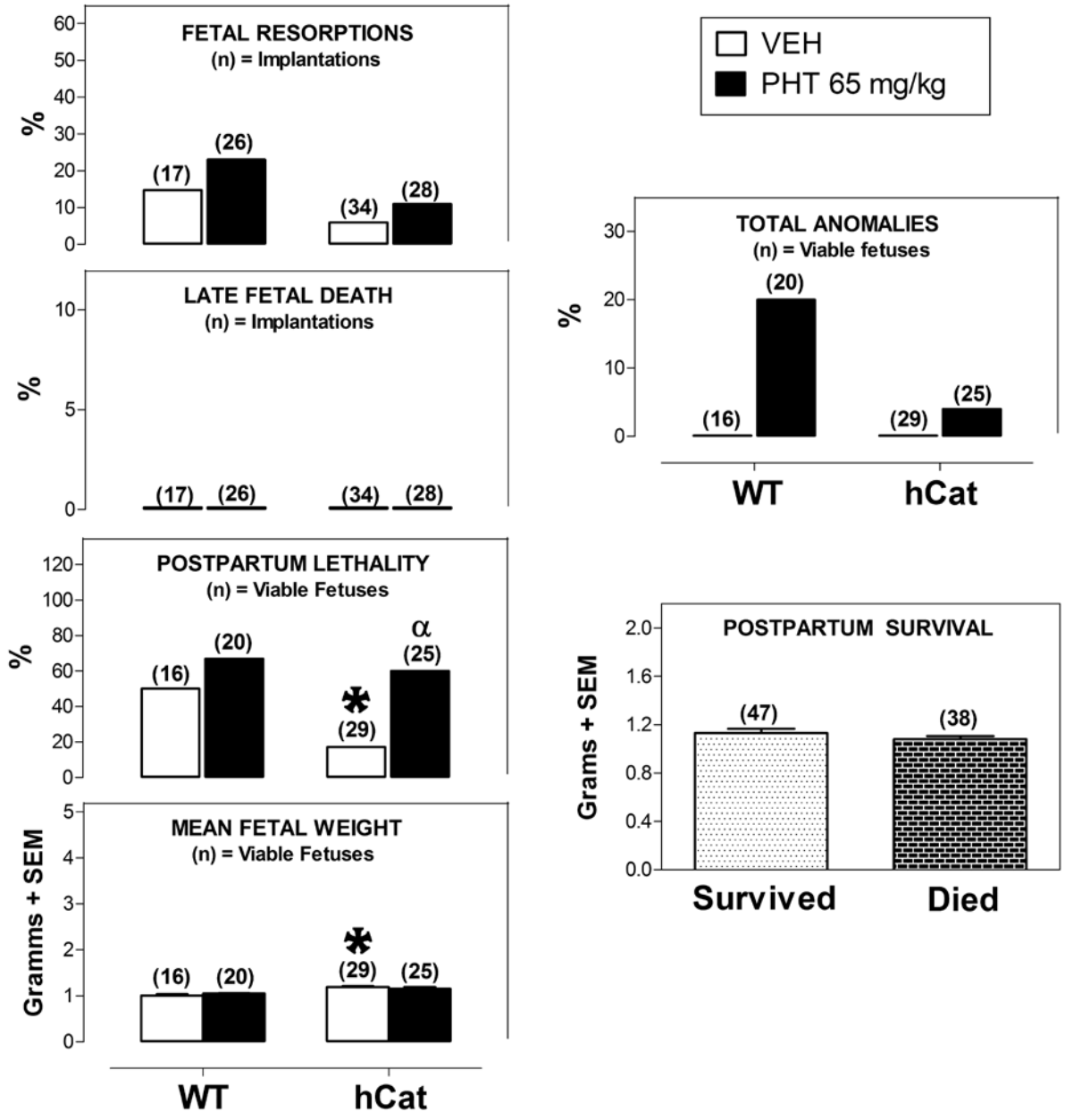
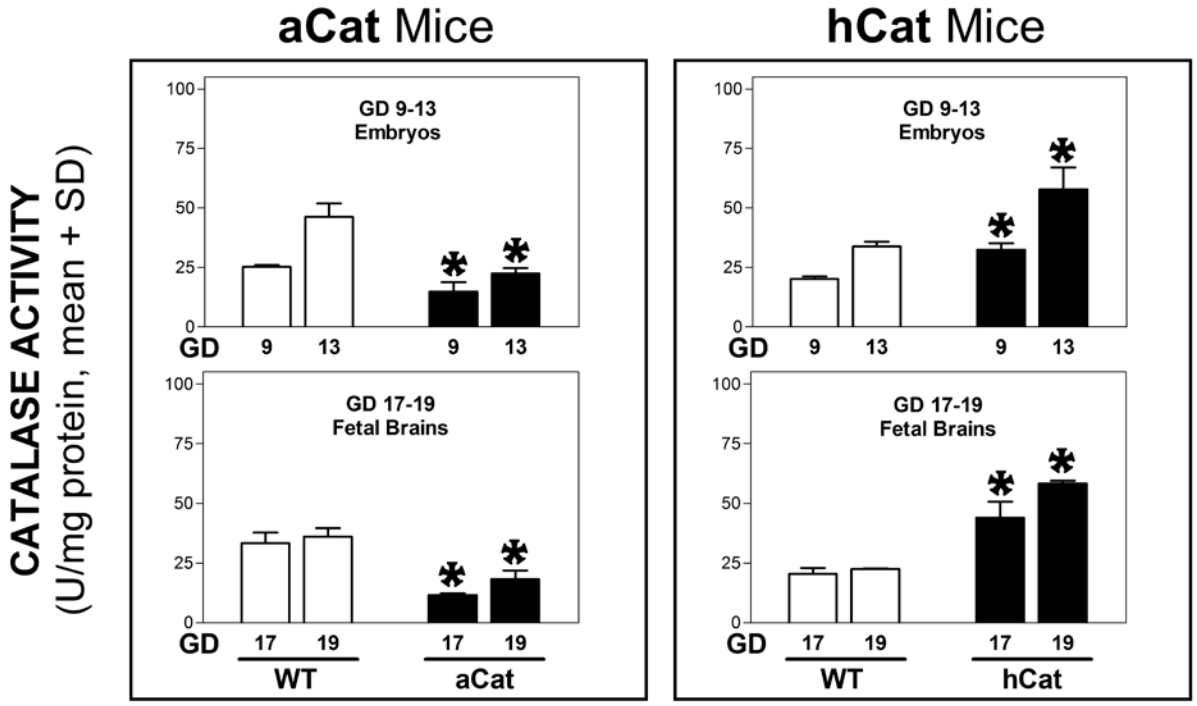

FETAL RESORPTIONS
(n) = Implantations
%
(17)
(26)
(34)
(28)
LATE FETAL DEATH
(n) = Implantations
%
(17) (26) (34) (28)
POSTPARTUM LETHALITY
(n) = Viable Fetuses
%
(16)
(20)
(29)
(25)
α
MEAN FETAL WEIGHT
(n) = Viable Fetuses
Gramms + SEM
(16) (20) (29) (25)
WT
hCat
VEH
PHT 65 mg/kg
TOTAL ANOMALIES
(n) = Viable fetuses
%
(16)
(20)
(29)
(25)
WT
hCat
POSTPARTUM SURVIVAL
Grams + SEM
(47)
(38)
Survived
Died

Figure 3. *In vivo* phenytoin embryopathies in transgenic mice expressing human catalase.

Pregnant hCat dams were treated i.p. on GD 9 with either 65 mg/kg phenytoin or its vehicle, and sacrificed on GD 19. Outcomes were analyzed as described in fig. 2. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The alpha symbol indicates a difference from VEH-treated mice of the same genotype ($p<0.05$).

Figure 4.


aCat Mice
hCat Mice
CATALASE ACTIVITY
(U/mg protein, mean + SD)
GD 9-13
Embryos
GD 17-19
Fetal Brains
GD
9
13
17
19
WT
aCat
hCat

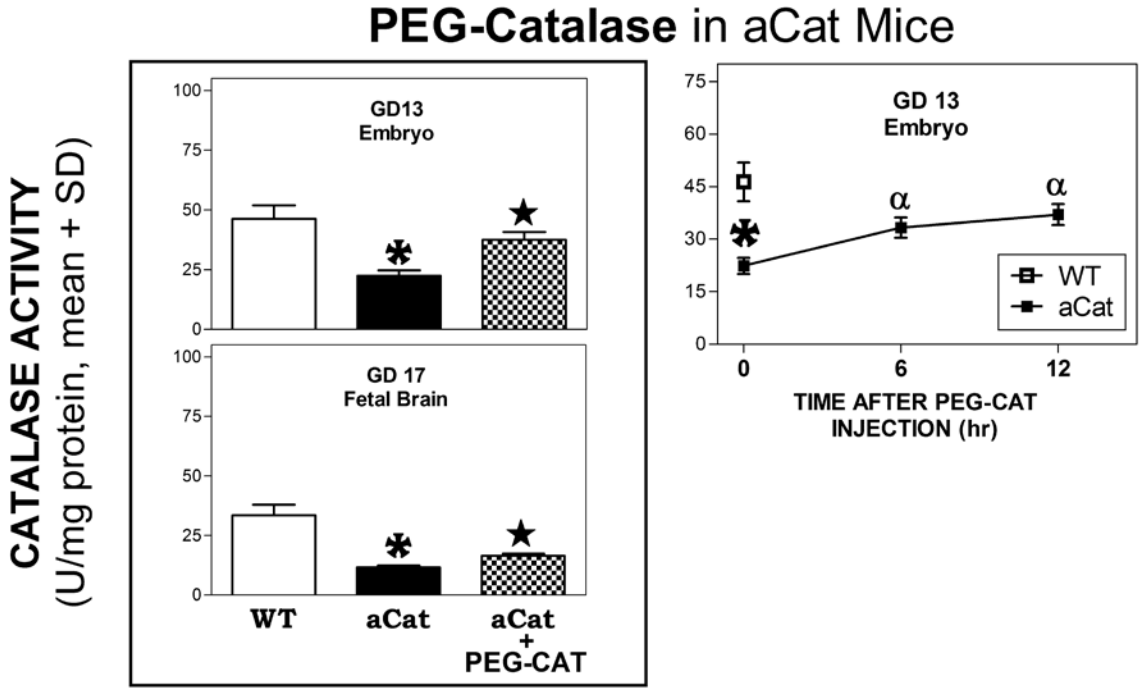

PEG-Catalase in aCat Mice
CATALASE ACTIVITY
(U/mg protein, mean + SD)
GD13
Embryo
GD 17
Fetal Brain
WT
aCat
aCat
+
PEG-CAT
GD 13
Embryo
WT
aCat
TIME AFTER PEG-CAT
INJECTION (hr)

Figure 4. Embryonic and fetal catalase activities in acatalasemic mice and mice expressing human catalase, and following maternal injection of PEG-catalase.

Embryos (GDs 9 and 13) and fetal brains (GDs 17 and 19) were analyzed for endogenous catalase activity using the FOX assay. For the PEG-CAT group, pregnant dams were administered PEG-CAT (50 KU/kg) on GD 13 and sacrificed 6 or 12 hr later. The asterisk indicates a difference from WT mice ($p<0.05$). The star symbol indicates a difference from aCat mice ($p<0.05$). The alpha symbol indicates a difference from the zero hr time point ($p<0.05$). Each group consisted of 6 samples from 3 litters.

Figure 5.

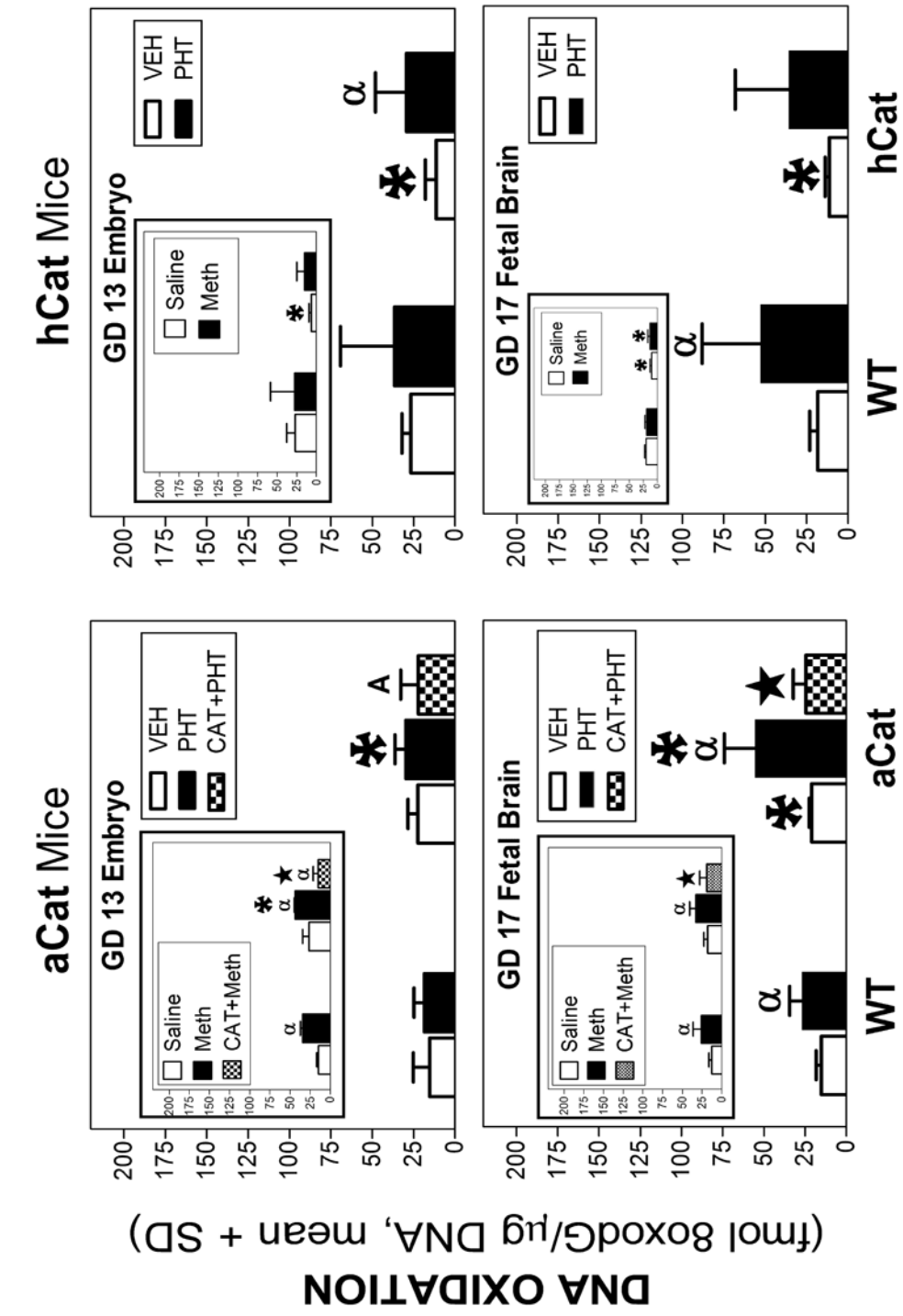

DNA OXIDATION
(fmol 8oxodG/μg DNA, mean + SD)
aCat Mice
hCat Mice
GD 13 Embryo
GD 17 Fetal Brain
VEH
PHT
CAT+PHT
Saline
Meth
CAT+Meth
WT
aCat
hCat
A
α
*
★

Figure 5. Phenytoin-initiated DNA oxidation in embryos and fetal brains from acatalasemic mice and mice expressing human catalase.

Pregnant aCat and hCat dams were treated i.p. on GDs 13 or 17 with either 65 mg/kg phenytoin or its vehicle, and sacrificed 6 hr later. As a positive control, another group of dams were treated on GD 13 with 40 mg/kg of the ROS-initiating teratogen methamphetamine (**METH**) or its saline vehicle. This group of dams was sacrificed 4 hr after injection. Embryos were explanted and analyzed for DNA oxidation using ELISA with an 8-oxodG-specific antibody. Asterisks indicate a difference from WT mice within the same treatment group ($p<0.05$). The star symbol indicates a difference from phenytoin-treated mice of the same genotype ($p<0.05$), with a marginal difference indicated by the letter A ($0.05<p<0.1$). The alpha symbol indicates a difference from vehicle-treated mice of the same genotype ($p<0.05$). Each group consisted of 10-18 embryos from 4-6 litters.

Human catalase-expressing mice

Vehicle-treated GD 13 whole embryos of hCat mice had 58% lower levels of DNA oxidation compared to WT controls ($p<0.05$). In contrast, hCat embryos were not protected from phenytoin-enhanced DNA oxidation, exhibiting levels similar to those in WT phenytoin-exposed embryos, and 2.6-fold higher than DNA oxidation in hCat embryos exposed to vehicle ($p<0.05$) (**Fig. 5, upper right panel**). With the vehicle controls for METH studies, hCat embryos exposed to saline also showed a 26% decrease in the levels of endogenous DNA oxidation compared to saline-exposed WT controls ($p<0.05$), but METH exposure did not increase DNA oxidation in either the WT or hCat embryos. (**Fig. 5, upper right panel insert**).

A similar pattern was observed later in gestation, on GD 17, with hCat fetal brains having 38% less DNA oxidation ($p<0.05$) (**Fig. 5, lower right panel**). Phenytoin enhanced DNA oxidation in WT and hCat brains by 2.9-fold and 3-fold, but only the WT increase was significant ($p<0.05$), and phenytoin-exposed hCat fetal brains were not protected compared to phenytoin-exposed WT brains. In the vehicle controls for the METH study, hCat embryos exposed to saline showed 50% less oxidatively damaged DNA ($p<0.05$) (**Fig. 5, lower right panel insert**). METH did not enhance DNA oxidation in either the WT or hCat fetal brains, but METH-exposed hCat fetal brains had 32% lower levels of oxidatively damaged DNA compared to METH-exposed WT controls ($p<0.05$).

Phenytoin concentrations in fetal and maternal tissues

Plasma phenytoin concentrations in different mouse strains and their WT controls

Plasma phenytoin concentrations following a particular drug dose were similar among all mouse strains and their WT controls when injected with either 55 or 65 mg/kg of phenytoin. There was a similar increase in the plasma phenytoin concentration in all strains when the drug dose was increased from 55 to 65 mg/kg ($p<0.05$) (**Fig. 6, lower left panel**).

Tissue phenytoin concentrations in CD-1 mice

Phenytoin concentrations in adult CD-1 liver and brain were similar at 6 hr after drug injection. The phenytoin concentration in CD-1 fetal liver was similar to that in adult liver,

whereas fetal brain tissues had a 38% increase compared to fetal liver ($p<0.05$) (**Fig. 6, upper left panel**).

Tissue phenytoin concentrations in acatalasemic mice

In both the WT and aCat adult and fetal tissues, the higher dose of phenytoin (65 mg/kg) produced a higher phenytoin concentration ($p<0.05$) (**Fig**. **6, centre panels**). In adults, phenytoin concentrations were higher in adult brain than liver with the lower 55 mg/kg dose in WT animals ($p<0.05$), and with both doses in aCat mice ($p<0.05$). In contrast, in the fetus, phenytoin concentrations were increased by the higher phenytoin dose in brain ($p<0.05$) but not significantly by the lower dose, in both WT and aCat animals. With both doses, all fetal tissues showed higher phenytoin concentrations compared to the respective adult tissues ($p<0.05$), with the exception of fetal brain following the 55 mg/kg dose in both WT and aCat animals.

Tissue phenytoin concentrations in human catalase-expressing mice

A similar pattern was observed in the WT and hCat adult and fetal tissues, with the higher drug dose (65 mg/kg) resulting in increased phenytoin concentrations ($p<0.05$) (**Fig**. **6, right panels**). In adults, phenytoin concentrations were higher in the brain than liver with the 55 mg/kg dose in WT animals ($p<0.05$) and the 65 mg/kg dose in hCat mice ($p<0.05$). In contrast, in the fetus, phenytoin concentrations in fetal brain were higher than in fetal liver with both doses of phenytoin ($p<0.05$) in the WT animals, but only with the higher dose in hCat mice ($p<0.05$). In the WT animals, phenytoin concentrations were higher in fetal brain than adult brain with both doses ($p<0.05$). With both doses, both fetal brain and liver from the hCat animals showed higher phenytoin concentrations compared to the respective adult tissues ($p<0.05$), with the exception of fetal brain following the 55 mg/kg dose.

Figure 6.

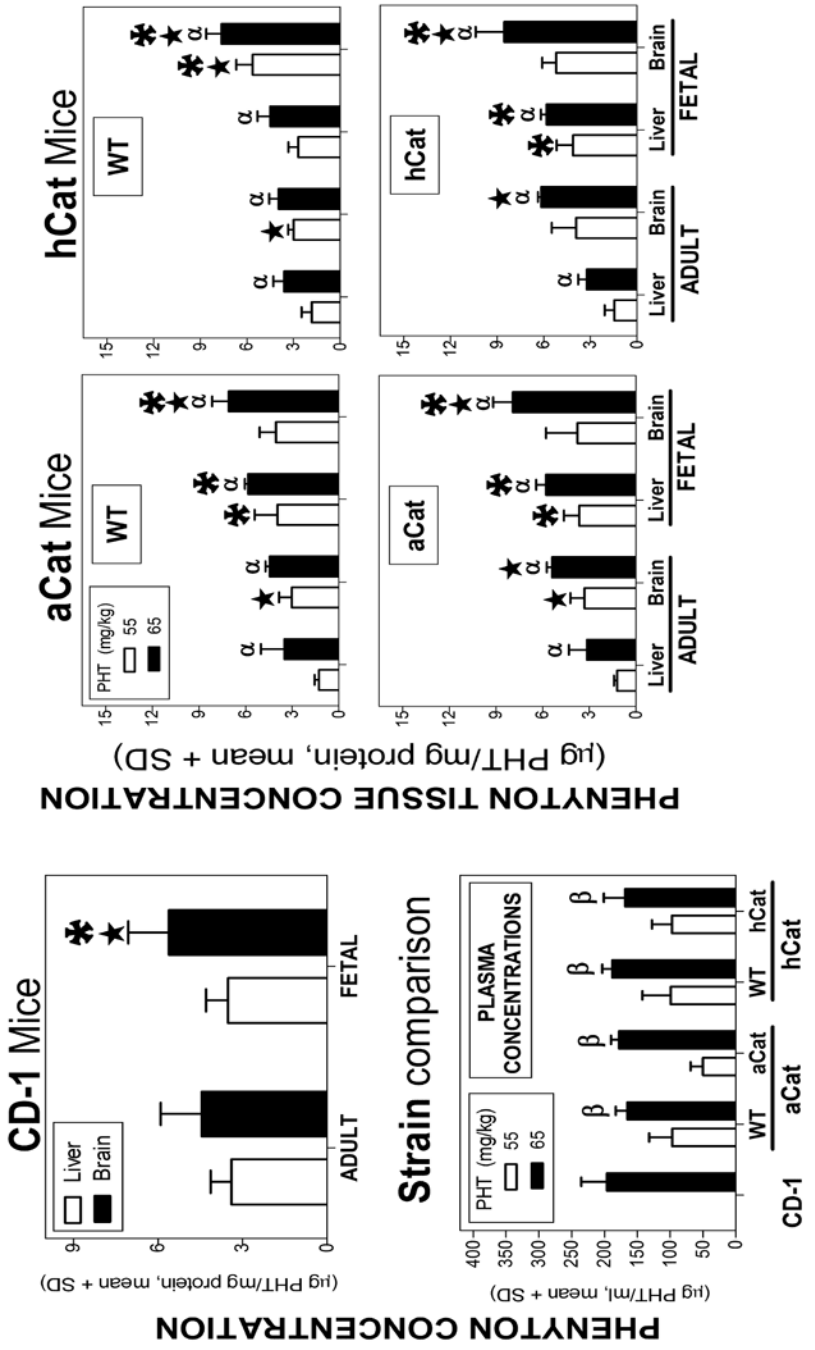

PHENYTON CONCENTRATION
(μg PHT/ml, mean + SD)
Strain comparison
PLASMA CONCENTRATIONS
PHT (mg/kg)
55
65
CD-1
WT
aCat
WT
hCat
CD-1 Mice
Liver
Brain
ADULT
FETAL
(μg PHT/mg protein, mean + SD)
PHENYTON TISSUE CONCENTRATION
(μg PHT/mg protein, mean + SD)
aCat Mice
hCat Mice
WT
aCat
hCat
Liver
Brain
ADULT
FETAL

Figure 6. Phenytoin concentrations in fetal and maternal tissues and plasma of CD-1 and acatalasemic mice and mice expressing human catalase.

Pregnant dams were treated i.p. on GD 17 with either 55 or 65 mg/kg phenytoin and sacrificed 6 hr later. Maternal and fetal brain and liver tissues as well as maternal plasma were collected and analyzed for phenytoin concentration using HPLC with a UV detector. The star indicates a difference from liver samples of the same age (adult or fetus) and treatment dose ($p<0.05$). The asterisk indicates a difference from the same organ in adults at the same dose ($p<0.05$). The beta symbol indicates a difference from the 55 mg/kg dose of phenytoin ($p<0.05$). The alpha symbol indicates a difference from the same organ and age exposed to 55 mg/kg phenytoin ($p<0.05$). Each adult group consisted of samples from 4 dams; and each fetal group consisted of samples from 4 fetuses from 3-4 litters.

2.2.5 DISCUSSION

Given the low level of embryonic and fetal catalase activity, amounting to about 5% of maternal hepatic activity, it is not known whether deficiencies in or enhanced expression of endogenous catalase activity in the developing embryo affect the developmental process. Low levels of catalase activity in humans have been linked only to adult conditions such as Takahara's disease, an oral form of gangrene (Ogata 1991). Similar susceptibilities in adult animals have been reported for acatalasemic mice, which exhibit increased susceptibility to carbon tetrachloride-initiated liver damage (Wang et al 1996), increased formation of mammary tumors (Ishii et al 1996) and increased sensitivity to hepatic tumorigenesis after exposure to ionizing radiation (Yamada et al 1997). However, during development, the embryo and fetus express relatively low levels of most enzymes involved in the detoxification of xenobiotic reactive intermediates and ROS, particularly during the period of organogenesis, the critical period of teratological susceptibility (Wells et al 2009). Although normal development has been reported for catalase knockout mice (Ho et al 2004), no detailed developmental studies were performed. To determine the developmental importance of one such quantitatively minor embryonic protein, the antioxidative enzyme catalase, we hypothesized that aCat embryos would be more susceptible to normal developmental oxidative stress, and perhaps more so to that initiated by xenobiotics, and hence would be more susceptible to ROS-mediated damage. Conversely, hCat embryos would be protected against both endogenous and xenobiotic-enhanced oxidative damage.

Among mice allowed to deliver spontaneously, in the absence of drug exposure, embryopathies were enhanced in catalase-deficient aCat animals, including fetal resorptions (*in utero* death) and preweaning lethality, showing that endogenous catalase is important in protecting the developing embryo *in vivo* against physiological oxidative stress. In aCat embryos, this physiological level of oxidative stress may contribute to both apparent low fertility and postnatal death. In contrast, human catalase-expressing mice with enhanced embryonic catalase activity were protected against physiological oxidative stress, evidenced by a lower incidence of preweaning lethality. This is the first direct *in vivo* evidence that embryonic catalase, while quantitatively minor compared to adult activity, is a critical embryoprotective enzyme for normal development. Complementary *in vitro* embryo culture studies have corroborated the efficacy of low levels of endogenous catalase in protecting the developing embryo against both normal

developmental and phenytoin-enhanced oxidative stress (Section 2.1) (Abramov & Wells 2011b). Increasing endogenous embryonic catalase activity was correlated with improved developmental parameters, and the addition of exogenous catalase was embryoprotective.

In vehicle-treated mice killed just before the time of delivery, a similar pattern of increased embryopathies was observed in the aCat mice, including an increase in fetal resorptions and postpartum lethality, although to a less significant degree than observed following spontaneous delivery. Conversely, untreated hCat mice with increased embryonic catalase activity exhibited decreased postpartum lethality and increased fetal weight. The embryoprotective effect of enhanced endogenous catalase activity complements the converse results in aCat mice in suggesting both an embryopathic potential for developmental levels of ROS production, and an important embryoprotective role for catalase. Most of the resorptions observed in both vehicle-treated mouse strains were non-dissectable pinpoint resorptions, and there were no differences in the number of implantations among all groups, indicating that the lower birth rate in aCat mice was due to post-implantational, relatively early *in utero* death.

All developmental parameters for vehicle-treated catalase-normal C3H WT and hCat animals were comparable to the normal range for extensive control groups from other murine strains studied in our laboratory (Nicol et al 2000; Winn & Wells 1995a). Previous *in vivo* studies in our laboratory also have shown that the vehicle does not measurably enhance oxidative stress (Kim & Wells 1996; Perstin & Wells 2010), indicating that the observed embryopathies in vehicle-treated controls are the result of normal developmental oxidative stress. Low catalase activity in the aCat embryos showed no effect on the mean fetal weight; however, increased activity in the hCat embryos resulted in significantly larger embryos. The data for fetal weight were pooled independent of the genotype for both C3H and C57BL/6J strains and analyzed for their relation to postpartum survival. While C57BL/6J fetuses were slightly heavier on average than C3H fetuses, there was no correlation between mean fetal weight and postpartum survival even though hCat fetuses on average had greater weight and less postpartum lethality compared to WT controls. Conversely, postpartum lethality in the vehicle-treated aCat fetuses was increased, albeit less significantly, compared to WT controls, and independent of the genotype there was a correlation between postpartum survival and mean fetal weight, with smaller fetuses being less likely to survive postpartum. Numerous reports indicate that there is strong correlation in human children born with low birth weight and impaired neuro-cognitive development

reflected by IQ scores, behavioral measures and psychiatric development (Abel et al 2010; Beyerlein et al 2010; Fattal-Valevski et al 2009; Indredavik et al 2005; Nair et al 2009; Reuner et al 2009; Roberts et al 2007). One study using neonatal rats found that lower birth weight may affect the postnatal development of serotonin projections to the frontal cortex (Himpel et al 2006).

When treated with phenytoin, a ROS-initiating teratogen (Wells et al 2009), there was a dose-dependent increase in embryopathies in aCat embryos compared with WT controls, and enhanced DNA oxidation, both of which were decreased by PEG-catalase pretreatment, indicative of a ROS-dependent embryopathic mechanism, and a protective role for catalase. Both the C3H and C57BL/6J WT strains proved to be relatively resistant to the embryopathic effects of phenytoin measured in this study, as was found in a previous *in vivo* study of the C3H strain in our laboratory (Nicol et al 2000). Similarly, preliminary *in vitro* studies in our laboratory using the same C3H and C57BL/6J mice in embryo culture also found resistance of the WT embryos to the embryopathic effects of phenytoin (Perstin & Wells 2010).

The protective role of catalase in phenytoin embryopathies observed in aCat mice was corroborated conversely in hCat mice, which were completely protected from the substantial incidence of late fetal death observed in the WT controls exposed to phenytoin on GDs 12 and 13. hCat mice also appeared to be substantially protected from total anomalies caused by phenytoin exposure on GDs 9 or 12 and 13, and to a lesser extent from fetal resorptions due to GD 9 phenytoin exposure, but the differences were not significant, possibly due in part to the relative resistance of the C57BL/6J strain to phenytoin embryopathies. The reduced phenytoin embryopathies in the hCat mice likely constitute the minimum protective potential for endogenous embryonic and fetal catalase. Although embryonic and fetal brain catalase activities were approximately doubled in the hCat mice, these activities were still very low compared to adult levels, and higher embryonic catalase activities achieved by the use of protein therapy with exogenous catalase *in vivo* and in embryo culture were highly protective (Winn & Wells 1995b; 1999). The apparent increase in postpartum lethality observed in the hCat animals treated with phenytoin compared to vehicle-treated hCat controls likely reflected the substantial protection afforded the latter compared to vehicle-treated WT controls, as the incidence of postpartum lethality was almost identical in both the WT and hCat mice exposed to phenytoin. This

suggests that the magnitude of the increase in embryonic catalase in the hCat mice is not sufficient to protect against all ROS-mediated embryopathies, particularly at higher drug doses.

Although the lower, 55 mg/kg dose of phenytoin is teratogenic in other mouse strains (Wells et al 2009), this dose did not cause typical phenytoin embryopathies in either the aCat mice or their WT controls. However, phenytoin did cause a remarkable and substantial dose-dependent increase in the incidence of exencephaly, which was not observed in the vehicle controls of any strain in this study. The cases of exencephaly were distributed across all litters, and could not be attributed to a litter effect involving a single predisposed dam. The complete mechanism for this phenytoin-initiated birth defect is not known, but it was completely blocked by pretreatment with PEG-catalase, which restored embryonic catalase activity in the aCat embryos to normal levels, suggesting that oxidative stress plays an important role. On the other hand, the total phenytoin-initiated anomalies (exencephaly, open eye, dilated ventricle, cleft palate and hydronephrosis) were somewhat but not significantly increased in the aCat fetuses compared to their WT controls, and these anomalies were slightly but not significantly reduced by PEG-catalase pretreatment, suggesting the potential involvement of ROS-independent pathways discussed elsewhere (Danielsson et al 1997; Hansen 1991).

Endogenous levels of oxidatively damaged DNA in untreated GD 13 aCat embryos did not differ from the WT controls, although there was a non-significant 25% increase. This non-significant increase in whole embryo DNA oxidation nevertheless was of the same magnitude observed in fetal brain on GD 17, which was significant, and may have contributed to the increased embryopathies observed in the untreated aCat embryos compared to their WT controls. When exposed to phenytoin, aCat embryos exhibited increased levels of DNA oxidation. Although this increase was blocked by pretreatment with PEG-catalase, the decrease was not significant. In contrast to phenytoin, methamphetamine (**METH**), another ROS-initiating teratogen (Jeng et al 2005; Wong et al 2008) that was used as a positive control for the DNA oxidation studies, increased DNA oxidation in both aCat embryos and their WT controls, and this was completed and significantly blocked by pretreatment with catalase protein therapy, confirming a ROS-dependent mechanism. Later in gestation in untreated aCat mice, the constitutive level of oxidatively damaged DNA in GD 17 fetal brains of the aCat mice was greater than that in WT controls, suggesting that normal developmental oxidative stress could be pathogenic when the fetus is deficient in even a quantitatively minor antioxidative enzyme.

DNA oxidation was increased by phenytoin on GD 17 to a much greater extent than on GD 13, and this increase was prevented by pretreatment with catalase protein therapy confirming the role of oxidative stress. With METH treatment on GD 17, a similar increase in fetal brain DNA oxidation that was blocked by PEG-catalase pretreatment was observed, but the magnitude of oxidatively damaged DNA was less than that on GD 13, unlike with phenytoin, and was not greater in aCat fetuses, suggesting that ROS-initiating teratogens would be expected to have differing effects, perhaps depending in part on their respective mechanisms of metabolism and ROS production. With phenytoin, the greater enhancement in DNA oxidation in fetal brain on GD 17 than in whole embryos on GD 13 may in part explain the higher incidence of neurodevelopmental deficits compared with structural birth defects caused by *in utero* phenytoin exposure in animals and humans (Elmazar & Sullivan 1981; Vorhees 1983; 1994).

The pathogenic potential of oxidatively damaged DNA and the protective role of embryonic and fetal catalase were corroborated in hCat embryos, which exhibited higher catalase activity and decreased levels of endogenous DNA oxidation on both GDs 13 and 17 in these embryos. Phenytoin did not increase the level of oxidatively damaged DNA in GD 13 WT embryos, consistent with the resistance of this C57BL/6J strain to structural birth defects. Although DNA oxidation in GD 13 hCat embryos exposed to phenytoin was higher than that in hCat embryos exposed vehicle, this was due to the decreased levels of DNA oxidation in the vehicle group rather than an increase in the phenytoin group, which was the same as the phenytoin-exposed WT controls. In contrast, on GD 17, phenytoin increased DNA oxidation in the C57BL/6J WT controls, suggesting that this strain might be more susceptible to neurodevelopmental deficits that are initiated during this late gestational period. There was a small and non-significant trend for a decrease in phenytoin-initiated DNA oxidation in the hCat embryos compared to the WT controls, suggesting that a higher level of catalase than was achieved in this strain might prove protective. C57BL/6J WT mice were also resistant to the effects of METH on both gestational days, similar to other studies in our laboratory with C57BL/6J mice (Jeng et al 2005; Wong et al 2008). The resistance of the C57BL/6J strain to METH-initiated DNA oxidation is consistent with the lack of structural birth defects observed in this strain (Jeng et al 2006). However, the reduction in DNA oxidation in untreated hCat embryos on GD 13 and fetal brains on GD 17, and in METH-treated fetal brains on GD 17,

corroborates the potential protective role of endogenous embryonic and fetal catalase at the macromolecular level with normal developmental oxidative stress.

The results herein implicating ROS and oxidative damage to embryonic cellular macromolecules in the mechanism of embryopathies caused by endogenous and phenytoin-enhanced oxidative stress are consistent with other murine studies *in vivo*, in embryo culture and *in vitro* demonstrating endogenous or phenytoin-enhanced formation of ROS, 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 glucose-6-phosphate dehydrogenase (Wells et al 2009; Wells et al 2010b). The embryopathic importance of respectively enhanced and reduced DNA oxidation in aCat and hCat embryos is consistent with previous studies of phenytoin and other ROS-initiating teratogens including benzo[a]pyrene, METH and ionizing radiation, for which teratogenesis or neurodevelopmental deficits were enhanced in knockout mice lacking key proteins for the detection and repair of DNA damage (p53, ATM), and particularly the repair of oxidatively damaged DNA, including 8-oxoG (OGG1, CSB) (McCallum et al 2011; Wells et al 2010b).

The level of catalase activity may be similarly important in protecting human embryos from physiological and/or drug-enhanced oxidative stress. Peroxisomes in human embryos are immunocytochemically catalase-negative at 6 weeks and only heterogeneously positive by 8 weeks (Espeel et al 1993), which represents a period in organogenesis when embryos are highly susceptible to xenobiotics.

To ensure that pharmacokinetic differences did not confound our interpretation of the effects of genetic modulation of endogenous catalase activities on the embryopathic effects of phenytoin, we measured the concentrations of phenytoin in maternal plasma and in maternal and fetal liver and brain tissues. There were no strain differences in plasma or tissue phenytoin concentrations, indicating that pharmacokinetic factors did not play a role in the differences in phenytoin embryopathies observed in aCat and hCat mice and their respective WT controls. Phenytoin freely crossed the placenta and achieved similar concentrations in fetal and adult liver. Remarkably, phenytoin concentrations were higher in fetal brain compared to fetal liver, and in most cases to adult brain. Since fetal and adult liver concentrations were similar, this suggests a role for fetal brain transporters, as distinct from placental transporters. Phenytoin is reported to

be a substrate for the placental P-glycoprotein efflux transporter (Fromm 2002; Potschka & Loscher 2001), which may actively pump phenytoin from the placenta back to the maternal circulation and thus protect the embryo (Ceckova-Novotna et al 2006). However, the ontogeny of this and other potential phenytoin transporters in fetal brain remains to be determined. The blood-brain barrier (BBB) is composed of specialized endothelial tight junction cells, particular patterns of enzymatic activity, a distinct electrochemical gradient, and specific BBB transporters (Ballabh et al 2004). In rodents, the BBB is not fully functional until after birth (Watson et al 2006); however, the P-glycoprotein efflux transporters, which are located in the capillary endothelium and astrocytes (Tishler et al 1995), were expressed in the brain of the developing fetus, but only at about 10% of adult levels (Tsai et al 2002). In contrast, expression of this efflux transporter in the liver of the developing fetus is near adult levels (Mahmood et al 2001). Therefore, the resultant increased concentration of phenytoin in the fetal brain is most likely due to the inefficient activity of the efflux transporters in the developing brain, although the possible contribution of an unknown fetal influx transporter cannot be excluded. The increased concentration of phenytoin in fetal brain compared to fetal liver and maternal brain may explain the high frequency of neurodevelopmental deficits compared to other anomalies caused by this drug, and raises interesting questions as to the mechanisms underlying this selectivity.

In summary, the results herein provide direct evidence that the relatively low levels of endogenous embryonic and fetal catalase provide important protection against physiological and drug-enhanced oxidative stress, but may be insufficient for protection against at least some developmental effects of ROS-initiating teratogens, particularly at higher doses. These studies suggest that interindividual differences in the expression of embryonic and fetal catalase likely constitute an important determinant of risk for ROS-mediated developmental pathologies.

2.2.6 ACKNOWLEDGEMENTS

We would like to thank Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX) for generously providing a breeding pair of the transgenic hCat mice.

2.3 STUDY3: PROTECTIVE ROLE OF ENDOGENOUS CATALASE IN BASELINE AND PHENYTOIN-ENHANCED NEURODEVELOPMENTAL AND BEHAVIORAL DEFICITS INITIATED *IN UTERO* AND IN AGED MICE[a]

Running title: Neuroprotective role of endogenous catalase

Julia P. Abramov[1], Audrey Tran[2], Aaron M. Shapiro[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Corresponding author:
Peter G. Wells, Pharm. D.
Faculty of Pharmacy, University of Toronto
144 College St., Toronto, Ontario, Canada M5S 3M2
E-mail: pg.wells@utoronto.ca
Tel. 416-978-3221
Fax 416-267-7797

a. A preliminary report of this research was presented at the 2009 annual meeting of the Society of Toxicology (U.S.A.) [*The Toxicologist*,108(1):454]. These studies were supported by a grant from the Canadian Institutes of Health Research.

Shapiro, A.M. – performed rotarod testing on some of the aged mice, sacrificed all of the aged mice and collected their brain tissues.

Tran A. – performed all behavioural testing on the young pups and analyzed the data. I trained her to conduct the studies, and supervised her in carrying out the studies and analyzing the data.

2.3.1 ABSTRACT

We used mutant catalase-deficient mice (acatalasemic, **aCat**) and transgenic mice expressing human catalase (**hCat**) to determine the neuroprotective role of catalase *in utero* and in aged animals treated with vehicle or the reactive oxygen species (ROS)-initiating drug phenytoin. Phenytoin-initiated postnatal death was enhanced in aCat mice and reduced in hCat mice. Catalase deficiency reduced postnatal surface righting, negative geotaxis and rotarod performances independent of drug treatment, and enhanced phenytoin-initiated negative geotaxis and rotarod deficits in aCat females. Untreated aged female but not male aCat mice exhibited reduced motor coordination. Conversely, hCat offspring showed treatment-independent increased surface righting, negative geotaxis, air righting and, in females, improved phenytoin-impaired rotarod performance. Gender dependencies were consistent with higher brain catalase activities in male than female neonatal and aged animals. Endogenous catalase plays an important gender-dependent neuroprotective role *in utero* and in aged mice, and reduces neurodevelopmental effects of phenytoin.

2.3.2 INTRODUCTION

Continuation of the antiepileptic drug phenytoin (5,5-diphenylhydantoin, Dilantin™) during pregnancy until recently has been a common practice among physicians due to the high risks to the mother and fetus associated with uncontrolled maternal seizures, as well as the proven efficacy of phenytoin in controlling seizures (Harden & Sethi 2008). Evidence that *in utero* exposure to phenytoin may cause structural and behavioral teratogenesis was first reported by Hanson and Smith, who coined the term "fetal hydantoin syndrome" (**FHS**) (Adams et al 1990). Children diagnosed with FHS presented with a variety of symptoms, including craniofacial anomalies, growth deficiencies and cognitive impairment, shown by reduced IQ scores (Adams et al 1990; Bromley et al 2009; Scolnik et al 1994; Vanoverloop et al 1992; Wide et al 2002)**.**

Numerous studies in pregnant rat or mouse models have demonstrated both the structural and developmental abnormalities associated with human *in utero* phenytoin exposure. Structural congenital abnormalities, including orofacial anomalies, abnormalities of the brain and kidney, skeletal defects and cardiac arrhythmia were shown to occur in a dose-dependent manner that was independent of maternal seizure disorder (Finnell et al 1989; Harbison & Becker 1969). Similarly, some functional neurodevelopmental anomalies, such as air righting, have been shown to be dose-dependent (Vorhees 1987). Delayed air righting as well as an impaired rotarod performance were first observed in the offspring of pregnant rats exposed to 100 mg/kg of phenytoin in the drinking water or by gastric intubation during days 7-19 of pregnancy (Elmazar & Sullivan 1981). Subsequent studies in pregnant rats using phenytoin doses of 50-200 mg/kg by gavage also observed a delay in the development of the air righting reflex (McCartney et al 1999; Minck et al 1991; Vorhees 1983; 1987). Numerous other neurodevelopmental deficiencies, such as impaired startle reflex, impaired maze performance and abnormal circling tendency, have been described in rat offspring exposed *in utero* to phenytoin (McCartney et al 1999; Minck et al 1991; Pizzi & Jersey 1992; Vorhees 1983; 1987). Gender may exert a modulatory influence, as phenytoin exposure caused a greater delay in air righting and acceleration in the negative geotaxis test in females, while a greater acceleration in olfactory orientation was seen in males (McCartney et al 1999). Despite the wealth of functional data in the literature, the mechanisms underlying phenytoin-initiated neurodevelopmental deficits and the modulatory influences of gender have not been determined.

Several mechanisms have been implicated in the teratogenicity of phenytoin (Hansen 1991), one of which involves embryonic oxidative stress caused by reactive oxygen species (**ROS**), which include superoxide anion ($\mathbf{O_2^-}$), hydrogen peroxide ($\mathbf{H_2O_2}$) and hydroxyl radical (**•OH**) (Wells et al 2010a). Teratogens can enhance embryonic ROS formation via several mechanisms, including their direct bioactivation to a free radical intermediate by embryonic enzymes like prostaglandin H synthases (**PHS**) and lipoxygenases (**LPO**) (Wells et al 2010a; Wells et al 2009), and indirect ROS formation secondary to reperfusion following cardiac suppression (Azarbayjani & Danielsson 1998). If not cleared by embryonic antioxidative enzymes, such as superoxide dismutase (**SOD**) and catalase, ROS can oxidatively damage cellular macromolecules such as DNA, RNA, protein and lipids, and/or interfere with signaling pathways, adversely affecting developmental processes (Wells et al 2010a; Wells et al 2010b).

The embryo and fetus are particularly susceptible to ROS, as the embryonic activity of antioxidative enzymes like catalase, which detoxifies H_2O_2, may amount to only about 5% of maternal activity (Sections 2.1 and 2.2) (Abramov & Wells 2011a; b; El-Hage & Singh 1990; Winn & Wells 1999). The embryopathic importance of ROS and the potential protective role of catalase have been implicated by studies in embryo culture and *in vivo*, where the administration of exogenous enzyme reduced phenytoin-initiated DNA oxidation and embryopathies (Winn & Wells 1995b; 1999). Similarly, a role for endogenous catalase in protecting the embryo from structural embryopathies caused by endogenous and drug-enhanced oxidative stress in culture and *in vivo* was found in genetically altered mice with deficient or enhanced levels of embryonic catalase (Sections 2.1 and 2.2) (Abramov & Wells 2011a; b). Oxidative stress also has been implicated in neurodegeneration in the aging brain (Jeng et al 2011; Lee et al 2000; Simpson et al 2010), where catalase activity is similarly low (Chen et al 2004; Yang & Lin 2002). However, it is not clear whether the low level of endogenous catalase in the embryo and adult brain are sufficient to protect against the respective neurodevelopmental and aging effects of baseline and drug-enhanced ROS formation in brain.

This study investigated the role that endogenous catalase in the developing and aging brain may play in detoxifying ROS generated by normal biological processes and phenytoin bioactivation. Offspring of mutant acatalasemic mice (**aCat**) (low catalase) and transgenic mice expressing human catalase (**hCat**) (high catalase) were exposed *in utero* to a single dose of phenytoin, or to its vehicle alone as a reflection of normal developmental oxidative stress. Pups

were tested postnatally using a battery of tests to determine the presence of neurodevelopmental deficits. A similar study of motor coordination was conducted in untreated aged aCat and hCat mice. These studies provide the first evidence of a protective role for endogenous catalase against neurodevelopmental deficits caused by both developmental and phenytoin-enhanced ROS formation, and a related role in the modulatory effect of gender. A similar gender-dependent protective role for endogenous catalase against ROS-related neurodegeneration was observed in untreated aged mice.

2.3.3 MATERIALS AND METHODS

Chemicals

Phenytoin (diphenylhydantoin sodium salt) (CAS # 630-93-3), catalase (CAS # 9001-05-2), xylenol orange (CAS # 1611-35-4), ammonium iron (II) sulphate hexahydrate (CAS # 7783-85-9), potassium permanganate (CAS # 7722-64-7) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen peroxide (CAS # 7722-84-1) and sucrose (CAS # 57-50-1) were from BDH Chemicals Inc. (Toronto, Ontario, Canada). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA.). All other reagents and solvents were of analytical grade.

Animals

Breeding pairs of catalase-normal wild-type (C3HeB/FeJ) and mutant acatalasemic (aCat) (catalase-deficient) (C3Ga.Cg-*Cat*^{b}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Transgenic mice expressing human catalase (hCat) in addition to their intrinsic murine catalase (high catalase activity) (C57BL/6J) were donated by Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX) (Chen et al 2003), and the wild-type (C57BL/6J) mice were purchased from The Jackson Laboratory. Same-sex animals were housed in plastic cages with ground cob bedding and were maintained in a temperature-controlled animal facility with a 12-hr light/dark cycle. Food (Purina Rodent Chow, Oakville, Ontario) and tap water were provided *ad libitum*. One male was housed with two females overnight between 17:00 and 09:00 hr. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (GD) 1.

For aging studies, adult aCat and hCat mice were housed 4 animals per cage in the same conditions as above, and tested at the age of 18-20 months.

Treatment

Homozygous aCat and hCat females and their respective wild-type (**WT**) controls were mated with males of the same genotype as described above. Dams were treated intraperitoneally

(**i.p.**) at 10:00 a.m. on GD 17 with vehicle alone or with phenytoin in saline containing 0.002 N NaOH, and allowed to deliver spontaneously. The doses of phenytoin were based upon previous studies of structural teratogenesis in mice, including a marginally teratogenic (55 mg/kg) or teratogenic dose (65 mg/kg) (Wells et al 2009). Gestational length was determined by checking the pregnant females every day starting on GD 19. Cages were inspected every morning, and the presence of a litter was designated as postnatal day (**PND**) 1. On that day the numbers of viable and dead pups were recorded, and the pups were left with their mothers until weaning, 21 days after birth.

For the aging studies, no treatments were administered to the mice.

Tests

For neonatal studies, all pups were subjected to the following battery of functional tests (Elmazar & Sullivan 1981; McCartney et al 1999):

Surface righting. Beginning on PND 4, each pup was placed on its dorsal surface and the time required for the pup to right on to all four feet was recorded, to a maximum of 30 sec. The test was conducted three times daily until the pup was successful on all three trials.

Negative geotaxis. Beginning on PND 6, each pup was placed facing downwards on an incline of 25° and the time required for the pup to turn 180 ± 45° (to face upwards), to a maximum of one minute, was measured. The test was conducted three times daily or until the pup was successful on all three trials.

Olfactory orientation. Beginning on PND 9, each pup was placed inside a 30 cm plastic tube at the halfway point between a dish of home bedding, and a dish of clean bedding. The time required for the pup to walk towards and recognize the home bedding, to a maximum of two minutes, was measured. This test was conducted once daily, or until the pup was successful.

Air righting. Beginning on PND 13, each pup was placed upside down, so that all four paws touched a plastic surface 30 cm above a padded surface. The pup was released and observed for the ability to land on all four feet. Each pup was scored as follows: 0, for landing on its back; 1, for an incomplete landing on its side or on a few feet; and 2, for landing on all four feet. Performance was calculated as the percent per group that received a score of 2 on their final trial on each day. This test was conducted three times daily until PND 22.

Post-weaning Testing

Rotarod. Beginning on the 6th postnatal week, offspring were tested on a rotating rod. Mice were placed on the stationary rod for 90 seconds, and then allowed to run for 90 seconds at 5 rpm. This procedure was repeated following a 15-minute break and subsequently, the speed was slowly increased to 20 rpm. Latency to fall was recorded. This test was repeated during weeks 8, 10 and 12.

For the aging studies, the same rotarod test was performed on the aged adult mice. The test was repeated one week later.

Catalase activity

Catalase activity was measured in brains of pups on PND 4,9,13, 17 and 31, as well as in aged adults older than 18 months. Samples were homogenized in phosphate-buffered saline and catalase activity was determined using the ferrous oxidation in xylenol orange (**FOX**) assay (Gupta 1973; Jiang et al 1992; Ou & Wolff 1996). Catalase activity was determined using a standard curve with bovine liver catalase as the standard.

Genotyping

DNA was isolated from tail clips by heating the sample in 300 μL of a solution containing 10 mM NaOH/0.1 mM EDTA at 95°C for 15 min. To determine the genotype of the acatalasemic mice and their WT controls, catalase was amplified by PCR using the following primers, CatF: TCCTTCCAATCCCGTCCTTTCT and CatR: AAATGCCAAACTCGGAGCCATC. PCR conditions for a Perkin Elmer 9600 thermal cycler were 94°C for 5 min, 20 sec at 94°C, 20 sec at 65°C (-1 degree cycle), and 40 sec at 72°C for a total of 10 cycles, then 20 sec at 94°C, 20 sec at 55°C, and 40 sec at 72°C for a total of 30 cycles, with a 10 min extension at 72°C and kept at 4°C. Acatalasemic mice contain a point mutation, which produces an NdeI restriction site in the region amplified by the abovementioned primers. Following digestion at 37°C for 16 hr, acatalasemic samples contain two bands at approximately 250 bp while the wild-type (undigested) products appear at 493 bp as determined on a 1.5%

agarose gel. To genotype hCat mice, catalase PCR primers were, CatF: GAGGTCCACCCTGACTACGGG and CatR: GCCTTCTCCCTTGCCGCCAAG. PCR conditions were 95°C for 5 min, 30 s at 95°C, 30 s at 60°C and 1 min at 72°C for a total of 35 cycles, with a 10 min extension at 72°C. Undigested PCR products were run on 1.5% agarose gels to determine catalase genotype. There was a visible band at 450 bp for the hCat genotype and no band for the WT genotype.

Statistical analysis

Statistical differences in continuous data (rotarod) were evaluated using a two-way repeated measures analysis of variance (ANOVA) for genotype and drug effects with a Bonferroni post-hoc test. Binomial data were analyzed using Fisher's exact test where appropriate. The minimum level of significance used throughout was $p < 0.05$.

2.3.4 RESULTS

Table 1 summarizes the effects of phenytoin on the pregnant females and reproductive performance. Compared to vehicle controls, exposure to the lower dose of phenytoin in aCat mice increased gestational length by one day ($p<0.05$), and resulted in 50% decrease in the number of pups born ($p<0.05$).

Postnatal death

1. aCat mice

In utero exposure to phenytoin caused a dose-dependent increase in litter death at birth in both aCat mice and their WT controls ($p<0.05$) (**Fig. 1, top panel**). aCat offspring were substantially more susceptible than their WT controls to phenytoin-initiated prenatal fetal death. At the lower dose of phenytoin (55 mg/kg), aCat mice exhibited a 2.4-fold higher incidence of newborn deaths compared with WT controls ($p<0.05$). A similar but non-significant 53% increase was observed in the aCat vs. WT offspring exposed to the higher dose of phenytoin (65 mg/kg).

A maximal increase in postnatal death of aCat pups born viable but dying prior to weaning was observed with the lower dose of phenytoin ($p<0.05$), and this was not increased by the higher dose, which caused a somewhat lesser and non-significant increase (**Fig. 1, bottom panel**). aCat offspring were substantially more susceptible to phenytoin-initiated postnatal death. The lower dose of phenytoin caused a greater than 3-fold increase in postnatal death in aCat pups compared with both their WT controls exposed to the same dose of phenytoin, and the aCat saline controls ($p<0.05$). The higher dose of phenytoin caused a similar but non-significant 3-fold increase in postnatal death in aCat pups compared with both their WT phenytoin-exposed controls and the aCat saline controls.

Table 1. Dose-dependent effects of phenytoin (PHT) on reproductive outcomes.

PHT Dose (mg/kg)	0				55				65			
	WT	**aCat**	**WT**	**hCat**	**WT**	**aCat**	**WT**	**hCat**	**WT**	**aCat**	**WT**	**hCat**
Number of litters	3	3	4	3	3	4	5	4	3	3	3	3
Maternal weight gain (g)	15±8	10±4	14±1	15±2	6±2	7±4	15±1	14±4	11±1	6±4	12±3	15±3
Gestational length (days)	20±0	20±0	20±0.5	20±0	21±1	21±1[b]	20±2	20±0.5	20±1	21±0	20±1	20±1
Live pups, PND 1	7±2	7±1	8±1	9±1	4±1	2±1[b]	2±3	6±5	3±4	3±1	3±4	4±5
Viability index §	96±6	83±7[a]	64±36	93±11	80±5	27±32[c]	33±47	70±48	43±61	58±8	25±43	27±47
Weaning index †	100±0	92±7	56±41	93±11	100±0	50±58	31±46	70±48	50±71	100±0	29±.50	30±52

Mean of litters ± SD

[a]$0.05<p<0.1$, compared to WT

[b]$p<0.05$ compared to PHT 0 mg/kg

[c]$p<0.05$ compared to WT

§ Live pups PND 4 / live pups PND 1 x 100

† Live pups PND 21 / live pups PND 4 x 100

Abbreviations: WT, wild-type; aCat, acatalasemic (low catalase); hCat, human catalase overexpressing (high catalase); GD, gestational day; PND, postnatal day

Figure 1.

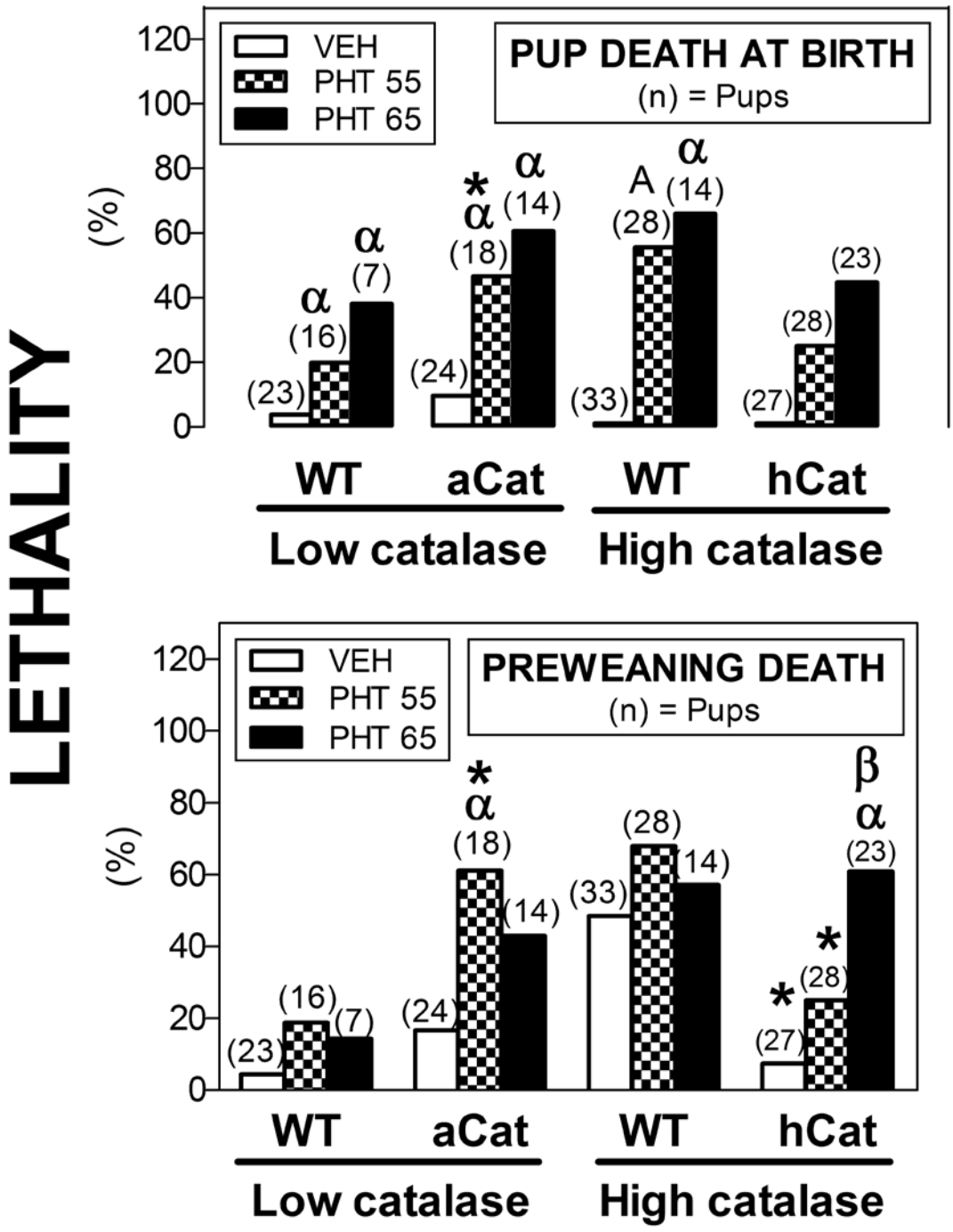

LETHALITY
PUP DEATH AT BIRTH
(n) = Pups
VEH
PHT 55
PHT 65
(%)
120
100
80
60
40
20
0
(23)
(16)
(7)
(24)
(18)
(14)
(33)
(28)
(14)
(27)
(28)
(23)
α
*
A
WT
aCat
WT
hCat
Low catalase
High catalase
PREWEANING DEATH
(n) = Pups
VEH
PHT 55
PHT 65
(%)
120
100
80
60
40
20
0
(23)
(16)
(7)
(24)
(18)
(14)
(33)
(28)
14)
(27)
(28)
(23)
α
β
*
WT
aCat
WT
hCat
Low catalase
High catalase

Figure 1. Effect of endogenous catalase expression on baseline and phenytoin-initiated prenatal pup lethality and postnatal death of offspring prior to weaning.

Phenytoin, 55 or 65 mg/kg i.p., or its vehicle was administered on gestational day (**GD**) 17 to dams with deficient (aCat) or enhanced (hCat) catalase activity. The dams delivered their litters spontaneously, stillborn pups were recorded, postnatal pup deaths prior to weaning on postnatal day (**PND**) 22 were recorded, and surviving offspring were subsequently tested for neurodevelopmental deficits. **Upper panel:** Pup death at birth was calculated from all the litters. Each treatment group for each genotype consisted of 3-4 litters. **Lower panel:** Preweaning death was calculated for the pups that survived immediately after birth and died any time between birth and the weaning day. The number of surviving pups is given in parentheses. Each treatment group for each genotype consisted of 3-4 litters. The asterisk indicates a difference from the respective WT mice receiving the same treatment ($p<0.05$). The alpha symbol indicates a difference from the vehicle group of the same genotype ($p<0.05$), with a marginal difference indicated by the letter A ($0.05<p<0.1$). The beta symbol indicates a difference from PHT 55 mg/kg-treated mice of the same genotype ($p<0.05$).

2. hCat mice

In the hCat mice prenatal death was similarly enhanced by both doses of phenytoin in the WT animals. This enhancement was significant for the higher dose of phenytoin ($p<0.05$), and marginally so for the lower dose ($0.05<p<0.1$) (**Fig. 1, top panel**). hCat offspring appeared to be protected from this effect of phenytoin, as: (1) the incidence of prenatal death with either dose was not significantly different from vehicle controls; and, (2) the incidence of prenatal death following exposure to the lower dose of phenytoin was reduced by 54% in hCat offspring compared with their WT controls, although this reduction was not significant.

In contrast to prenatal death in hCat offspring, and to postnatal death in aCat offspring, phenytoin caused a dose-dependent increase in postnatal (preweaning) death of hCat offspring (**Fig. 1, bottom panel**). The incidence of spontaneous postnatal death was relatively high in the saline-exposed WT offspring for this strain (49%), and this was not further enhanced by phenytoin. hCat offspring were protected from both spontaneous and phenytoin-initiated postnatal death. In saline-exposed offspring, postnatal death was 85% lower in hCat mice compared with WT controls ($p<0.05$). Similarly, in offspring exposed to the lower dose of phenytoin, the incidence of postnatal death was 63% lower in hCat mice compared with WT controls ($p<0.05$). hCat offspring were not protected from the higher dose of phenytoin.

Due to the substantial 61% incidence of postnatal death for hCat offspring and their WT controls exposed *in utero* to the higher dose of phenytoin, all the functional tests were performed on pups that were exposed to only the lower dose (55 mg/kg) of phenytoin or its vehicle.

Preweaning functional tests

Surface Righting

1. aCat mice

Compared to vehicle, phenytoin exposure did not affect the performance of surface righting in either WT or aCat mice from postnatal days (**PND**) 4 to 9 (**Supplementary Fig. S-1, left panels**). Compared to WT, aCat mice treated with vehicle showed initial 57% reduction in the percent of pups righting ($p<0.05$), although eventually all pups were able to complete this task (**Fig. 2, left panels**). When treated with phenytoin, aCat mice similarly showed an initial

45.5% decrease in the percent of pups righting compared to WT ($p<0.05$). Altered performances returned to normal by PND 9.

2. hCat mice

In the WT offspring, *in utero* phenytoin exposure appeared to increase development of the surface righting reflex as approximately 2-fold more pups were able to complete this task on PND 6 and 7 compared to the vehicle-exposed pups, but this effect was not significant (**Supplementary Fig. S-1, right panels**). This trend was not seen in the hCat mice. However, when exposed to vehicle, compared to WT, hCat pups exhibited a 2.5-fold increase in the completion of this task ($p>0.05$) (**Fig. 2, right panels**). There was no difference in the performance of hCat and WT pups when exposed to phenytoin. Altered performances returned to normal by PND 9.

Negative Geotaxis

1. aCat mice

WT animals did not show any difference in performance when exposed to phenytoin compared to vehicle exposure on PND 6 to 8 (**Supplementary Fig. S-2, left panels**). On the other hand, aCat pups showed 3-fold decrease in performance with phenytoin exposure on the first day of testing compared to the vehicle ($p<0.05$). Similarly, compared to WT, aCat mice exposed to phenytoin exhibited a 62% decrease in performance ($p<0.05$) (**Fig. 3, left panels**). No difference in performance was seen between aCat and WT pups with vehicle exposure. Altered performances returned to normal by PND 8.

2. hCat mice

WT animals performed showed a 3.5-fold increase in performance when exposed to phenytoin, compared to vehicle exposure ($p<0.05$) (**Supplementary Fig. S-2, right panels**). hCat animals appeared to be protected from this affect of phenytoin, as they did not exhibit any difference in performance from the hCat offspring exposed to vehicle. However, no difference in performance was observed between phenytoin-exposed hCat and WT offspring. Compared to

WT, hCat pups performed 4-fold better when exposed to vehicle ($p<0.05$) (**Fig. 3, right panels**). Altered performances returned to normal by PND 8.

Olfactory Orientation

1. aCat mice

Phenytoin treatment did not affect the performance of WT animals compared to vehicle-exposed offspring on PND 9 to15 (**Supplementary Fig. S-3, left panels**). However, aCat mice exposed to phenytoin showed a 68% decrease in initial performance compared with vehicle-exposed but this was not statistically significant. No significant difference in performance was observed between aCat and WT pups exposed to either vehicle or phenytoin (**Fig. 4, left panels**). Altered performances returned to normal by PND 14.

2. hCat mice

Phenytoin exposure in hCat pups resulted in 2-fold increase in performance compared to vehicle ($p<0.05$), but this effect was not observed in WT offspring (**Supplementary Fig. S-3, right panels**). When exposed to vehicle, there was no observed difference in performance between hCat and WT pups (**Fig. 4, right panels**). On the other hand, when exposed to phenytoin, there was an initial 2-fold increase in performance in hCat pups compared with WT but this was not statistically significant. Altered performances returned to normal by PND 14.

Air Righting

1. aCat mice

Throughout the period of testing both WT and aCat pups performed similarly when exposed to both vehicle and phenytoin on PND 13 to 22 (**Supplementary Fig. S-4, left panels**). Only on PND 17, aCat pups exposed to phenytoin showed 4-fold increase in performance compared with vehicle-exposed ($p<0.05$). There were also no significant differences in performance between aCat and WT pups with either vehicle or phenytoin exposures (**Fig. 5, left panels**).

Figure 2.

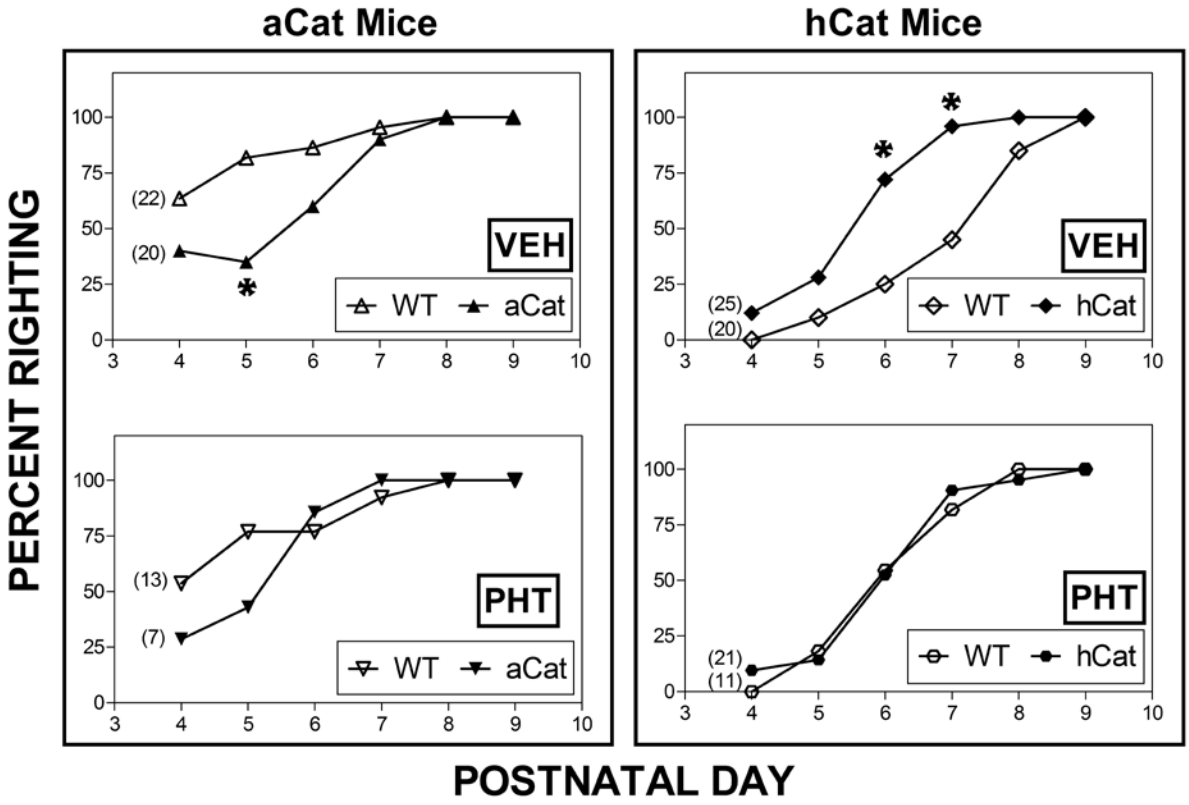

aCat Mice
hCat Mice
PERCENT RIGHTING
POSTNATAL DAY
VEH
PHT
WT
aCat
hCat
(22)
(20)
(13)
(7)
(25)
(20)
(21)
(11)
*

Figure 2. Effect of endogenous catalase expression on baseline and phenytoin-initiated changes in offspring surface righting.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and the percent of animals in each group successfully righting themselves was recorded. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parentheses.

Figure 3.

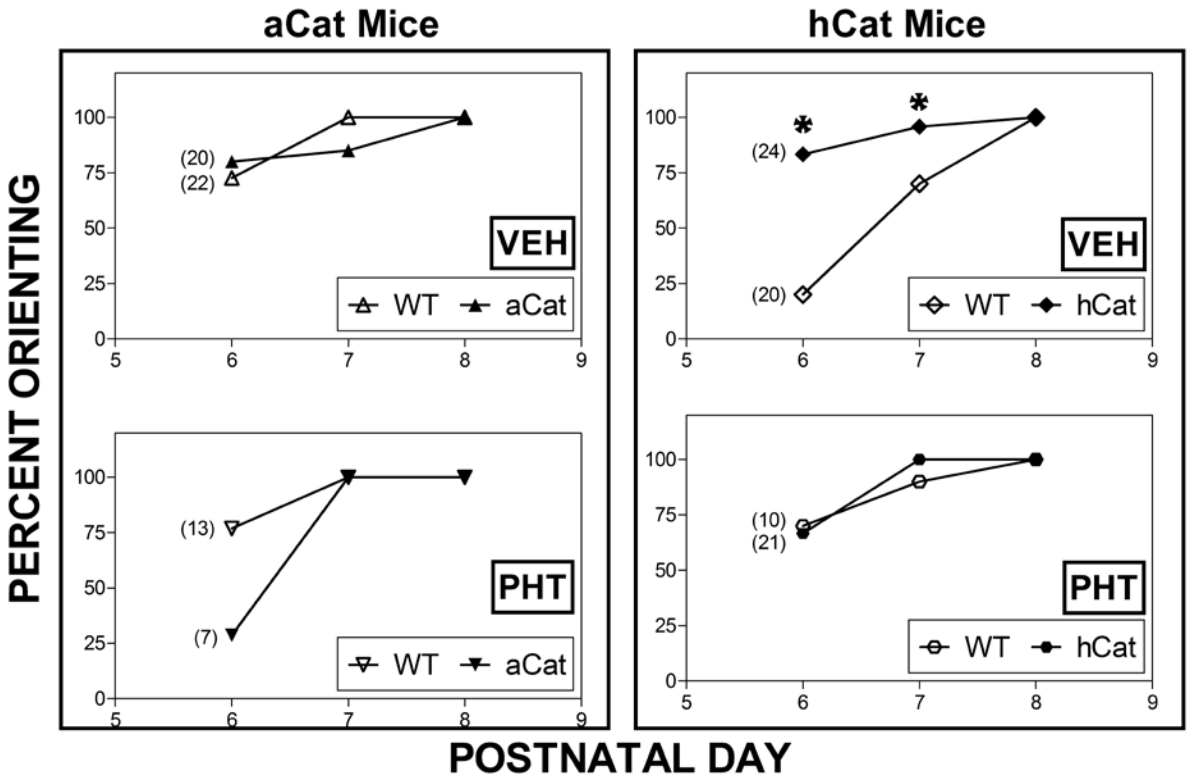

aCat Mice
hCat Mice
PERCENT ORIENTING
POSTNATAL DAY
VEH
PHT
WT
aCat
hCat
(20)
(22)
(24)
(13)
(7)
(10)
(21)
*
*
0
25
50
75
100
5
6
7
8
9

Figure 3. Effect of endogenous catalase expression on baseline and phenytoin-initiated changes in offspring negative geotaxis.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and the percent of animals in each group successfully orienting themselves upward on the incline was recorded. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure 4.

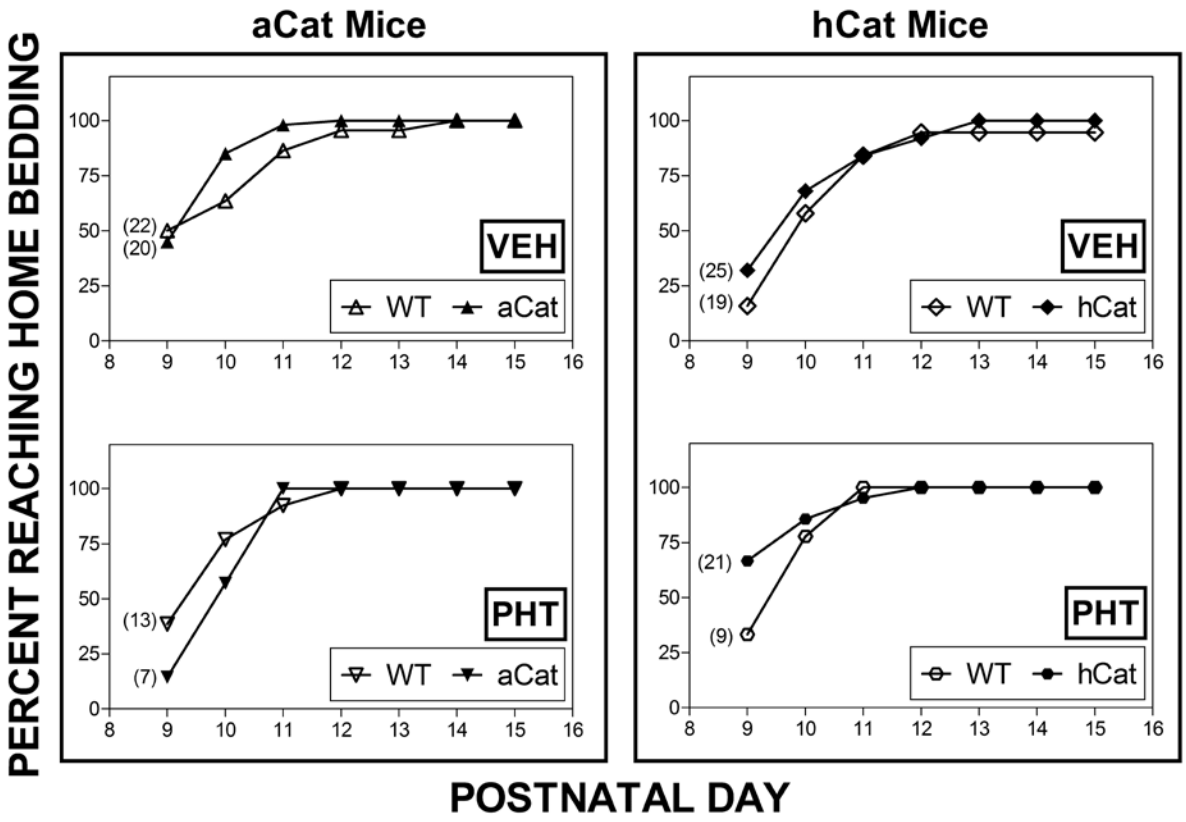

aCat Mice
hCat Mice
PERCENT REACHING HOME BEDDING
POSTNATAL DAY
VEH
PHT
WT
aCat
hCat
(22)
(20)
(25)
(19)
(13)
(7)
(21)
(9)
0
25
50
75
100
8
9
10
11
12
13
14
15
16

Figure 4. Effect of endogenous catalase expression on baseline and phenytoin-initiated changes in offspring olfactory orientation.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and the percent of animals in each group successfully reaching home bedding was recorded. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure 5.

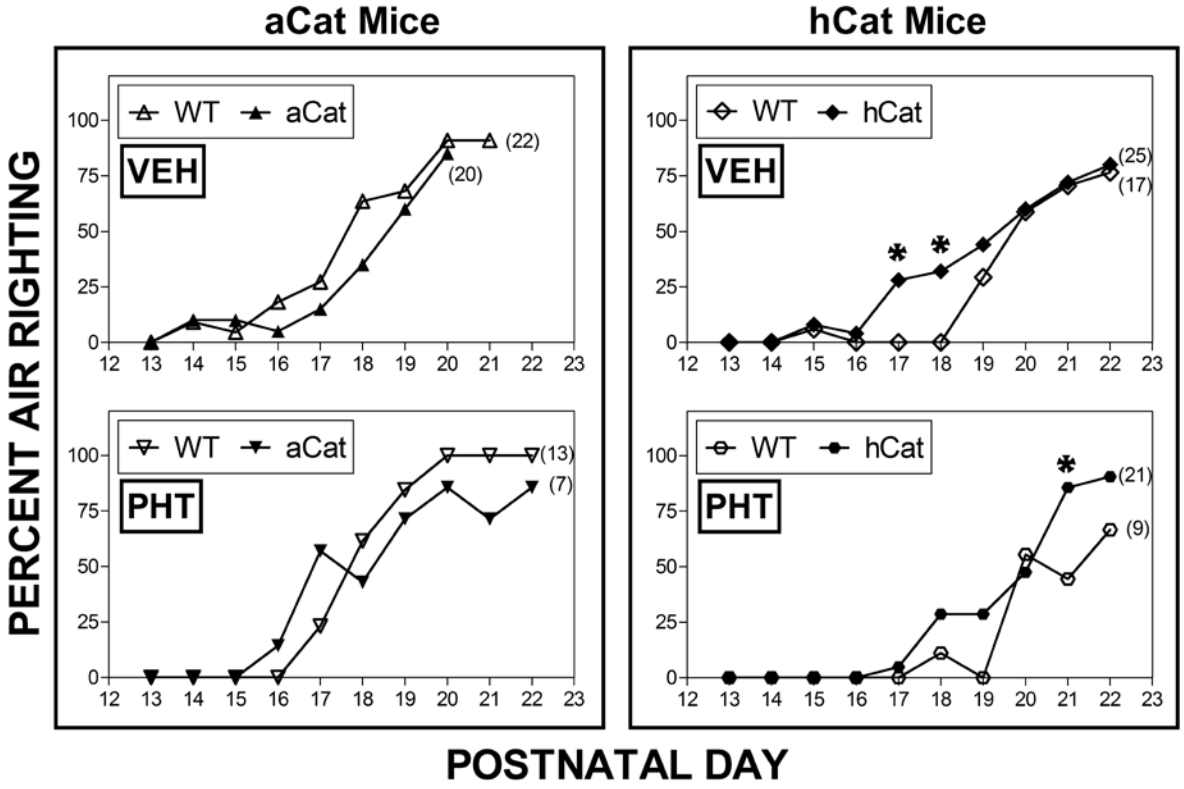

aCat Mice
hCat Mice
WT
aCat
hCat
VEH
PHT
(22)
(20)
(25)
(17)
(13)
(7)
(21)
(9)
*
PERCENT AIR RIGHTING
POSTNATAL DAY

Figure 5. Effect of endogenous catalase expression on baseline and phenytoin-initiated changes in offspring air righting.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and the percent of animals in each group successfully righting themselves by the 3/3 trial criterion was recorded. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parentheses.

2. hCat mice

Phenytoin exposure did not affect the performance of either WT or hCat pups compared with vehicle exposure (**Supplementary Fig. S-4, right panels**); however, 90% of the hCat pups were able to obtain a score of 2 on the final trial, compared to 76% in the WT animals. Similarly, hCat pups showed similar increases in performance on PND 17 to 18 compared with WT when exposed to vehicle ($p<0.05$) and phenytoin respectively, although an increase following phenytoin exposure was significant only on PND 21 (**Fig. 5, right panels**).

Post weaning functional tests - Rotarod

Young mice following in utero exposure

1. aCat mice

In utero exposure to phenytoin did not affect rotarod performance over postnatal weeks 6 to 12 in WT female and male animals compared to vehicle exposure (**Supplementary Fig. S-5**). On the other hand, phenytoin-exposed aCat female mice showed a 34% decrease in performance at 10 weeks of age when compared with female vehicle exposure ($p<0.05$). No such difference was observed in the phenytoin-exposed male aCat mice. aCat female mice also showed a 57% and 50% decrease in performance at 6 and 8 weeks with vehicle and phenytoin exposures respectively compared to WT females ($p<0.05$) (**Fig. 6, left panels**). Male aCat mice showed a similar pattern, with 52% decrease in latency to fall with phenytoin exposure compared to WT males on PND 6 ($p<0.05$) (**Fig. 6, right panels**). Altered performances returned to normal by postnatal week 12.

2. hCat mice

WT females exposed *in utero* to phenytoin showed a 45% decrease in latency to fall at 10 weeks of age compared to vehicle females (**Supplementary Fig. S-6**). When exposed to vehicle, hCat females showed a 2.5-fold increased performance on postnatal week 6 compared with WT females (**Fig. 7, left panels**). Similarly, hCat females when exposed to phenytoin performed 34% better on postnatal week 10 compared with WT females. The above changes were not significantly different by repeated measures ANOVA. Males exposed *in utero* to vehicle did not

show any difference between WT and hCat animals (**Fig. 7, right panels**), while following *in utero* phenytoin exposure, WT males performed 2-fold better than hCat males at 8 weeks of age ($p<0.05$). Altered performances returned to normal by postnatal week 12.

Aged mice, untreated

1. aCat mice

aCat aged females showed significant 67% decrease in motor coordination compared with WT control females ($p<0.05$) (**Fig. 8, left panels**). No deficit was observed in the aged males.

2. hCat mice

hCat aged mice did not show any difference in rotarod performance in either females or males (**Fig. 8, right panels**).

Brain catalase activity

1. aCat mice

Catalase activity in the brain of neonatal aCat pups on PND 4 was 56% lower compared to WT ($p<0.05$) (**Fig. 9, top left panel; Fig. 10, top panel**). From PND 4 to 13, brain catalase activity increased 6- and 8-fold in both WT and aCat pups ($p<0.05$). From PND 13 to 30, brain catalase activity slightly decreased in both WT and aCat pups by 40% and 54% respectively ($p<0.05$), at which point aCat pups still had 57% lower activity compared with WT ($p<0.05$).

aCat male and female young adult mice exhibited 56% and 79% decreased catalase activity in the brain respectively, compared with WT controls ($p<0.05$) (**Fig. 9, bottom left panel**). In both WT and aCat mice, males had 3- and 6-fold higher activities respectively compared with females ($p<0.05$). A similar pattern was observed in aged mice, with aCat male and female mice having 53% and 65% lower catalase activity than in WT controls ($p<0.05$), and male mice still having 5- and 6.7-fold higher catalase activity than females in both WT and aCat animals ($p<0.05$) (**Fig. 9, bottom left panel; Fig. 10, bottom panel**). Going from young to aged mice, catalase activity decreased in aged males for both WT and aCat mice by 65% and 61%

respectively ($p<0.05$), and in aged females for both WT and aCat mice by 80% and 65% respectively ($p<0.05$).

2. hCat mice

Catalase activity in the brain of neonatal hCat pups on PND 4 was 27% higher compared to WT controls ($p<0.05$) (**Fig. 9, top right panel; Fig. 10, top panel**). From PND 4 to 13, brain catalase activity increased 5- and 6-fold in both WT and hCat pups ($p<0.05$). From PND 13 to 30, brain catalase activity slightly decreased in WT pups by 55% and increased by 20% in hCat pups ($p<0.05$), at which point hCat pups still had 4.6-fold higher activity than WT controls ($p<0.05$).

Compared to WT controls, hCat male and female young adult mice exhibited 2- and 4-fold increased brain catalase activity respectively ($p<0.05$) (**Fig. 9, bottom right panel**). In both WT and hCat mice, males had 12- and 6-fold higher activities respectively compared to females ($p<0.05$). A similar pattern was observed in aged mice, with hCat male and female mice having 2- and 8-fold higher brain catalase activity than WT ($p<0.05$), and male mice still having 5- and 1.5-fold higher brain catalase activity than females in both WT and hCat animals ($p<0.05$) (**Fig. 9, bottom left panel; Fig. 10, bottom panel**). Going from young to aged mice, catalase activity decreased in aged males for both WT and hCat mice by 67% and 62% respectively ($p<0.05$). In contrast, the WT female brain catalase activity decreased by 18% with age, although it increased by 37% in the hCat females ($p<0.05$).

Figure 6.

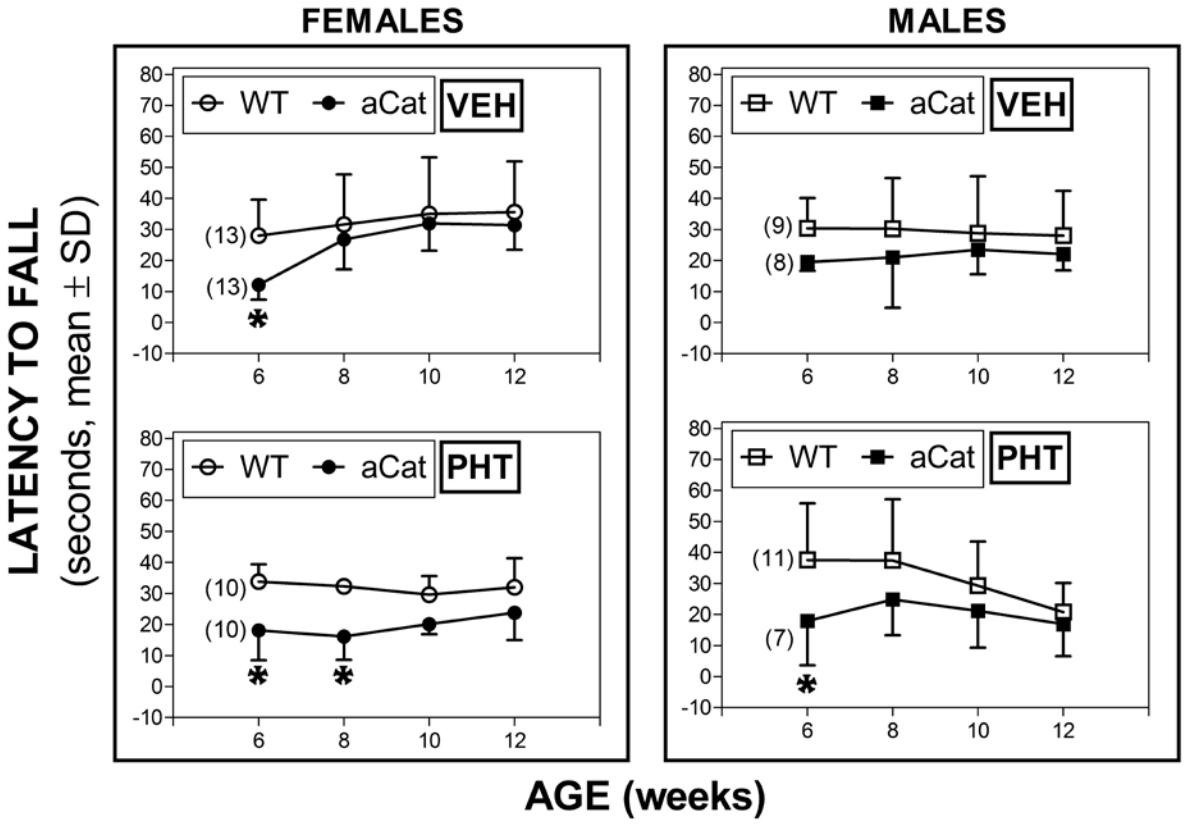

FEMALES
MALES
WT
aCat
VEH
PHT
(13)
(13)
(9)
(8)
(10)
(10)
(11)
(7)
LATENCY TO FALL
(seconds, mean ± SD)
AGE (weeks)

Figure 6. Effect of endogenous catalase deficiency (aCat) on baseline and phenytoin-initiated changes in motor coordination in male and female offspring.

aCat animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and motor coordination impairment was assessed by the rotarod test at 20 rpm beginning at 6 weeks of age. The latency or time at which the mice fell from the rod was recorded. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parentheses.

Figure 7.

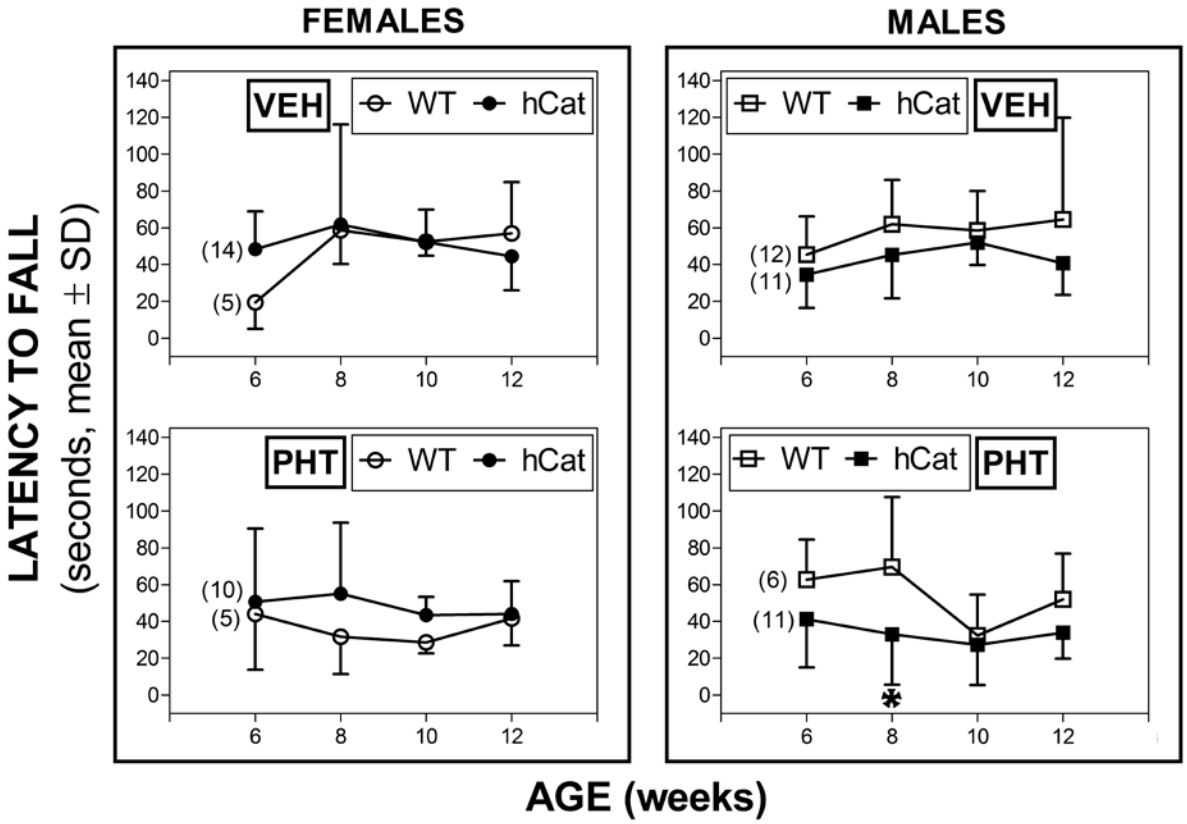

FEMALES
MALES
VEH
PHT
WT
hCat
LATENCY TO FALL
(seconds, mean ± SD)
AGE (weeks)
(14)
(5)
(10)
(5)
(12)
(11)
(6)
(11)
*

Figure 7. Effect of enhanced expression of endogenous catalase (hCat) on baseline and phenytoin-initiated changes in motor coordination in male and female offspring.

hCat were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle as described in fig. 1, and motor coordination impairment was assessed as described in fig. 6. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parentheses.

Figure 8.

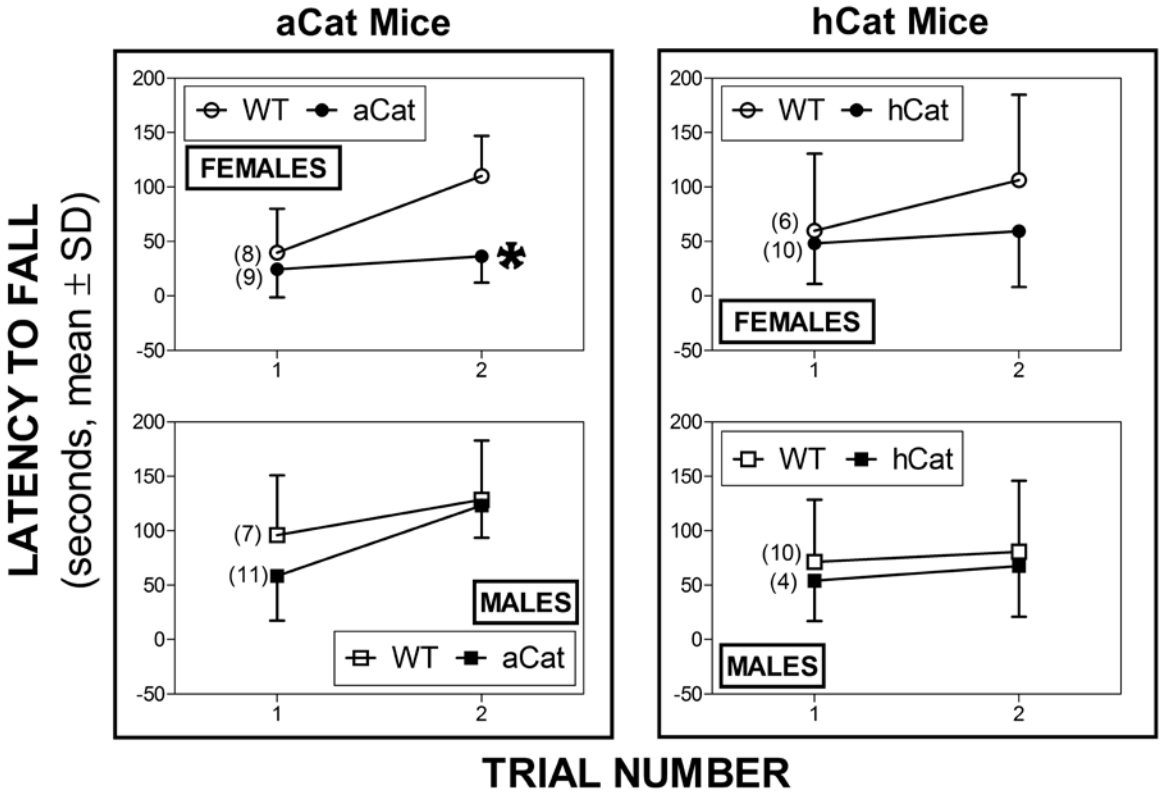

aCat Mice
hCat Mice
WT
aCat
hCat
FEMALES
MALES
(8)
(9)
(6)
(10)
(7)
(11)
(4)
*
LATENCY TO FALL
(seconds, mean ± SD)
TRIAL NUMBER

Figure 8. Effect of altered catalase expression on baseline motor coordination in aged male and female mice.

Motor coordination was assessed in untreated aged aCat and hCat mice as described in fig. 6, beginning at 18 months of age. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The number of pups in each group is given in parentheses.

Figure 9.

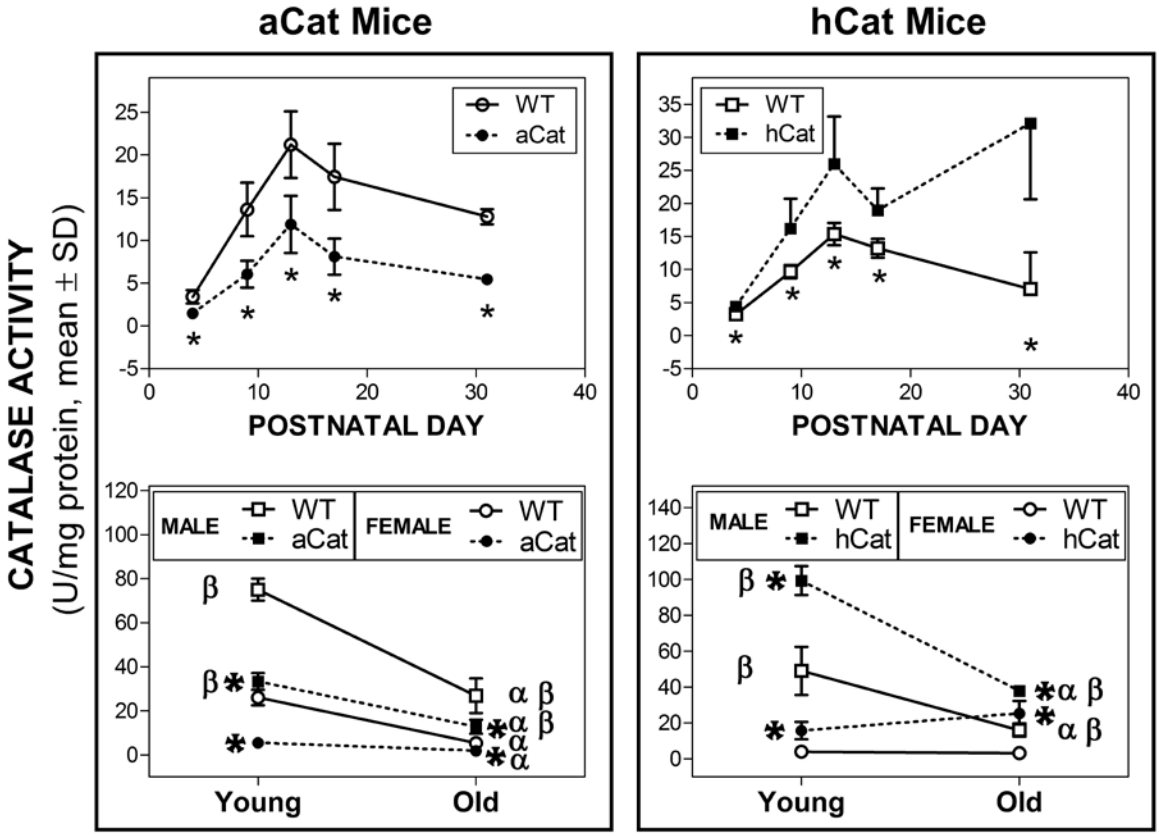

aCat Mice
hCat Mice
CATALASE ACTIVITY
(U/mg protein, mean ± SD)
WT
aCat
hCat
POSTNATAL DAY
MALE
FEMALE
Young
Old

Figure 9. Catalase activity in brains of young pups and aged mice with genetically deficient or enhanced catalase activity.

In aCat and hCat mice, brain samples from young pups, young adults (2-3 month old) and aged mice (18-20 months old) were analyzed for endogenous catalase activity using the FOX assay. Each group consists of 4-7 samples from 2-3 litters. The asterisk indicates a difference from WT mice for both aCat and hCat mice of the same age ($p<0.05$). The alpha symbol indicates a difference from young mice of the same genotype and sex ($p<0.05$). The beta symbol indicates a difference from female mice of the same genotype and age ($p<0.05$).

Figure 10.

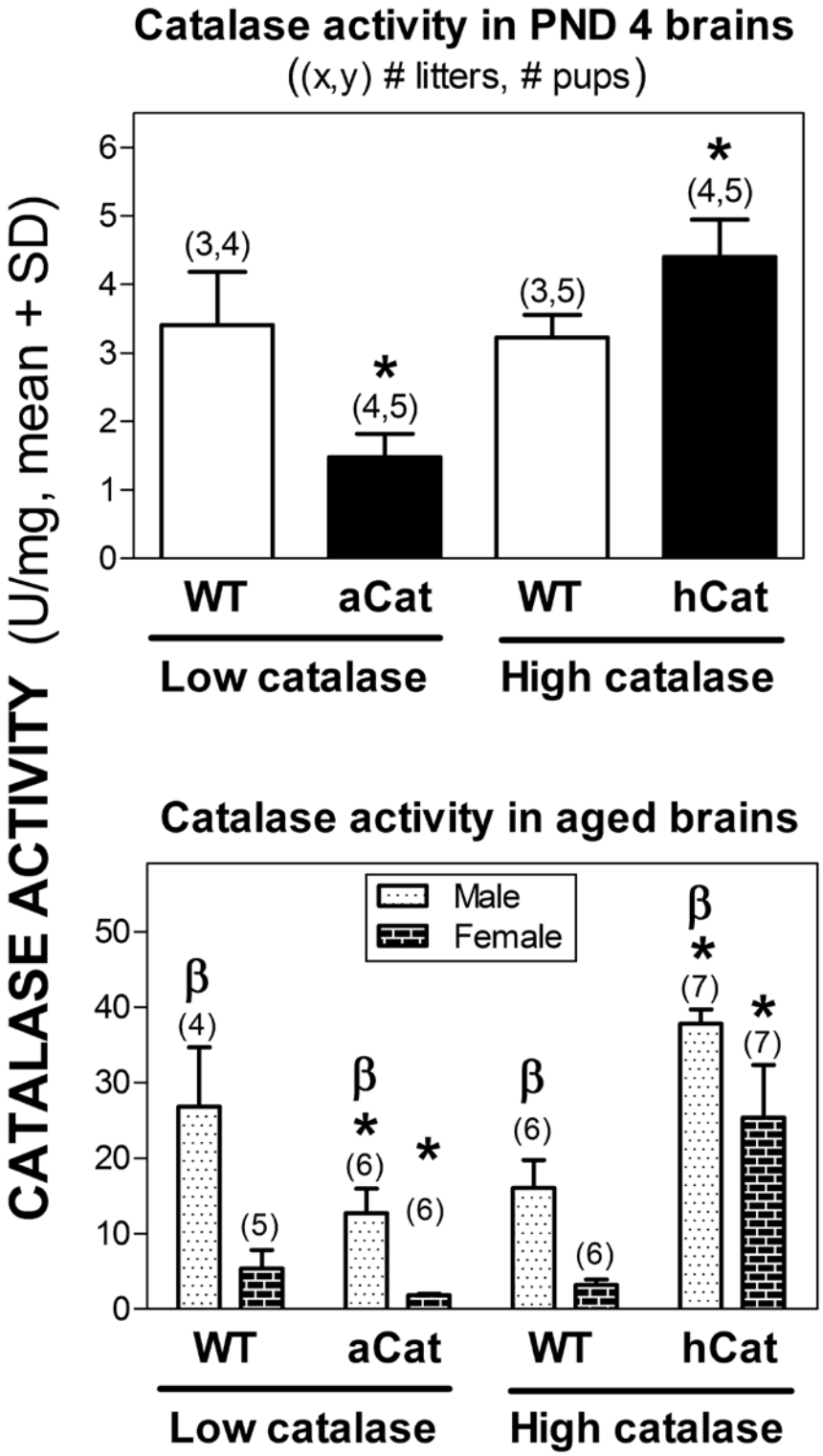

Catalase activity in PND 4 brains
((x,y) # litters, # pups)
CATALASE ACTIVITY (U/mg, mean + SD)
0
1
2
3
4
5
6
(3,4)
*
(4,5)
(3,5)
*
(4,5)
WT
aCat
WT
hCat
Low catalase
High catalase
Catalase activity in aged brains
Male
Female
0
10
20
30
40
50
β
(4)
(5)
β
*
(6)
*
(6)
β
(6)
(6)
β
*
(7)
*
(7)
WT
aCat
WT
hCat
Low catalase
High catalase

Figure 10. Catalase activity in brains of genetically modified pups on postnatal day 4 and aged mice.

Data for catalase activity from fig. 9 for PND 4 (top panel) and aged mice (lower panel) are represented. The asterisk indicates a difference from WT ($p<0.05$). The beta symbol indicates a difference from the respective female group ($p<0.05$).

2.3.5 DISCUSSION

This study provides the first evidence that the low level of catalase in the developing brain modulates susceptibility to postnatal neurodevelopmental deficits following *in utero* exposure to phenytoin-enhanced oxidative stress, thus indicating the importance of endogenous catalase in protecting the fetus from ROS-initiated functional deficits during development. Furthermore, most catalase-dependent behavioral abnormalities (surface righting, negative geotaxis and rotarod performance) were observed in vehicle-treated animals, suggesting that basal levels of *in utero* oxidative stress were sufficient to cause postnatal functional abnormalities when catalase is deficient. In contrast, catalase-deficiencies alone were not sufficient to alterations in negative geotaxis and rotarod performance, which were adversely affected by *in utero* phenytoin exposure. Although all catalase-dependent alterations in behavior, exhibited by offspring exposed *in utero* to vehicle or phenytoin, returned to normal within the study period, it would be worthwhile to determine whether or not these early neurodevelopmental deficits are followed by other neurobehavioral deficits later in life. The relatively transient nature of the phenytoin-initiated neurobehavioral deficits, as well as the apparent lack of an effect of phenytoin on the other behavioral measures, may have been due in part to the use of a single administration of phenytoin, and the lower maternal dose of phenytoin employed to avoid excessive fetal and neonatal death. The confirmation herein of a neuroprotective role for endogenous fetal catalase refutes its previously questionable efficacy based upon the observation that embryonic and fetal catalase levels are only about 3%-13% of maternal activity (Sections 2.1 and 2.2) (Abramov & Wells 2011a; b; El-Hage & Singh 1990; Winn & Wells 1999). Since two animal models used in this study are on different background strains, we compare each genetically modified mouse only to its own control and avoid a direct comparison of the two different genetic modifications.

Catalase activity in the brains of newborn pups was substantially lower in females than males, and the gender-dependent, inverse correlation between this activity and the development of neurobehavioral function suggests that lower brain catalase activity in female offspring contributes to their enhanced susceptibility to the adverse neurodevelopmental effects of oxidative stress. Similar but lesser gender dependence in brain catalase activities was observed in aged mice, due primarily to a substantial decline with age in male mice. These results provide a novel mechanism for the altered gender-dependent susceptibility of female neonates

and aged mice to the baseline and phenytoin-enhanced motor coordination deficits that were observed in aCat and hCat mice compared to their wild-type controls. This mechanism may similarly contribute to gender-dependence in other ROS-mediated neurobehavioral deficits.

Our observations with respect to **endogenous** neonatal brain catalase in aCat and hCat mice are consistent with enhanced protection observed following the administration of an **exogenous** form of this enzyme at the earlier developmental stage of organogenesis in embryo culture and *in vivo* (Winn & Wells 1995b; 1999). The results for fetal exposure extend the gestational range of importance covered in recent studies showing a protective role for endogenous catalase in the same genetically altered models during organogenesis in embryo culture (Section 2.1) (Abramov & Wells 2011b) and *in vivo* (Section 2.2) (Abramov & Wells 2011a), which revealed structural rather than functional consequences of *in utero* developmental and phenytoin-enhanced oxidative stress.

Our studies with aged mice similarly indicate for the first time that endogenous brain levels of catalase also play an important neuroprotective role in the normal process of aging. It has been previously suggested that ROS play an important role in aging (Jeng et al 2011; Phillips et al 2000) but the activity of catalase has not been investigated with relation to aging and accompanying neuronal deficits. Here we show for the first time that aged aCat mice with lower catalase activity exhibit deficits in motor coordination, and particularly that females are uniquely susceptible, possibly due to their substantially lower levels of brain catalase.

In utero exposure to phenytoin on GD 17 resulted in dose-dependent increases in the incidence of neonatal death within the first 4 days of life in all mouse strains, but aCat pups were most susceptible. Conversely, hCat pups appeared to be relatively protected at the lower dose of phenytoin although this protection was not significant. It appeared this protection, as evident by an almost 50% reduction in mortality, may have been statistically significant with a higher enhancement in fetal catalase activity. Similarly, surviving aCat pups continued to have a higher incidence of pre-weaning death, while hCat pups were relatively protected at the lower dose of phenytoin. Lower catalase activity in aCat embryos has been shown to result in increased levels of ROS leading to formation of oxidatively damaged DNA following phenytoin exposure (Section 2.2) (Abramov & Wells 2011a). Similar to the outcomes observed in embryos, it is likely that phenytoin-initiated ROS at the fetal stage contributed to neonatal death. It also has been suggested that ROS play a role by suppressing the activity of numerous CYP450 enzymes

and affecting fatty acid metabolism, which may result in inflammatory tissue damage, disruption of cell membrane integrity and death (Prandota 2004), although CYP-catalyzed metabolism also can directly enhance ROS formation (Wells et al 2009). There was a phenytoin-induced increase in gestational length in aCat mice by one day. Although statistical analysis showed that there was a significant difference in the gestational length, this is still within the normal range for this mouse strain. Weight of the pups did not differ between the two groups, and we observed no other visible differences in the pups, including eating habits, weight gain, fur growth, eye opening. Conversely, we cannot exclude the possibility that the increase in gestational length had an effect on the behavioral measures.

This study tested neonates on the time-specific development of reflexive and spontaneous behaviors that are typical in rodents, and reported to be sensitive to a variety of insults such as *in utero* exposure to phenytoin (McCartney et al 1999; Pizzi & Jersey 1992; Vorhees 1983). Deviation in the developmental course of these behaviors is suggestive of alterations within an organism (Rodier 1980), although the precise relationship between developmental insult and behavioral abnormalities is not fully understood.

The protective role of endogenous catalase was shown by decreased performance of the aCat pups compared to their WT controls. In most of the tests administered, the aCat pups, which had lower brain catalase activity, performed worse than their WT controls, independent of the treatment. With vehicle-treated animals, the most substantial decreases in performance of the aCat pups were in surface righting and early testing on the rotarod. *In utero* exposure to phenytoin decreased performance of the WT pups in surface righting and olfactory orientation, whereas a greater effect and significance were observed in the aCat mice that showed significantly decreased performance in surface righting, negative geotaxis and rotarod performance.

The opposite results were observed in the hCat pups, which consistently performed better than their WT controls. Increased brain catalase activity in the hCat pups was protective against endogenous oxidative stress by increased performance in surface righting, negative geotaxis, and air righting in females. When exposed to phenytoin, hCat pups continued to perform better in olfactory orientation and air righting in females. Similar changes in rotarod performance were not statistically significant, meriting further examination. Compared to vehicle, phenytoin exposure in the hCat mice enhanced olfactory performance, suggesting a developmentally

positive effect of phenytoin. Prenatal phenytoin exposure has been previously reported to enhance performance in some postnatal behavioral tests (Elmazar & Sullivan 1981; McCartney et al 1999), which the authors speculated may have resulted from an increase in either growth-affecting prenatal epidermal growth factor (**EGF**) or thyroid function, or both. *In utero* phenytoin exposure can cause variations in either system, including suppression of pituitary thyroid stimulating hormone secretion (Theodoropoulos et al 1980). This could result in prenatal upregulation of thyroid receptors leading to enhanced responsiveness to the offspring's endogenous thyroid hormone levels, potentially resulting in accelerated early development. Alternatively, EGF concentrations, known to have a role in the development and maintenance of sympathetic and sensory neurons (Strand et al 1989), are increased by *in utero* phenytoin exposure (Modeer & Andersson 1990).

Motor function in aCat and hCat pups treated with either vehicle or phenytoin appeared to have caught up with WT controls, and at the end of the test period all pups showed fully developed reflexes with most tests. There is evidence to support the hypothesis that phenytoin exposure causes Purkinje cell degeneration and weight decrease of the cerebellum (Blank et al 1982; Hatta et al 1999; Ohmori et al 1992). However, it has been reported that recovery of phenytoin-initiated neurological symptoms is fast when the cerebellar lesions occur in childhood, possibly due to other parts of the brain taking over the cerebral function (Ghez 1991). Similar mechanisms may have been involved in motor function and reflex recovery in our experiments.

This protective role of endogenous catalase against endogenous and phenytoin-enhanced oxidative stress was seen in both motor coordination tests as well as in cognition and reflex tests, suggesting the importance of catalase in numerous brain regions and pathways involved in brain development and function. The locomotor tests such as surface and air righting as well as rotarod rely upon the development of functional motor and sensory systems (Elmazar & Sullivan 1981). The required coordination of these systems suggests that both central and peripheral pathways must be necessary for a mature response. Exteroceptive stimuli originate outside or at a distance from the body, while, proprioception is a distinct sensory modality that provides feedback solely on the location of the various parts of the body in relation to each other. The coordination of types of locomotion that require utilization of multiple exteroceptive and proprioceptive cues involves the cerebellum, and it has been suggested that delay in the maturation of the rodent cerebral cortex contributes to delayed development of complex locomotor skills (Altman &

Sudarshan 1975). Other studies suggest that phenytoin exposure results in reduction of total brain weight in neonates (Hatta et al 1999). In the neonatal period in rodents, regions such as the olfactory bulbs, hippocampus and cerebellum are known to be late proliferating regions in the brain and their development extends into the neonatal period, thus making these regions more vulnerable to disorders (Jacobson 1991; Rodier 1980). Effects of phenytoin on late proliferating areas such as the hippocampus may have resulted in weight reduction of the cerebellum observed in one study (Hatta et al 1999), and in our study adversely affected motor coordination tests. The protective effect of increased catalase in the female hCat mice, together with the enhanced motor coordination deficits associated with decreased catalase in female aCat mice, which exhibit substantially lower levels of brain catalase than their male littermates, would suggest that ROS are involved in the mechanism of phenytoin-initiated neurodevelopmental toxicity. It would be interesting to similarly evaluate other ROS-initiating neurodevelopmental teratogens.

In the present study, normal developmental oxidative stress and *in utero* exposure to phenytoin on GD 17 resulted in alterations in the functional performance of genetically modified offspring with altered brain catalase levels in various developmental tests and locomotor response. High-dose (65 mg/kg) phenytoin treatment resulted in increased neonatal and pre-weaning death of offspring in all mouse strains and genotypes, therefore functional tests were investigated only in the lower (55 mg/kg) dose group. In aCat mice, the majority of pups died within the first 4 days of life. Studies done in rats treated *in utero* with phenytoin reported that pups which died prematurely had no milk present in their stomachs, and dams showed evidence of milk retention, suggesting either an inability of the pups to suckle or the dam to release milk (McCartney et al 1999). However, the developmental changes cannot be explained solely by the nutritional status of the offspring, as demonstrated by other studies showing that phenytoin-exposed pups reared by control dams still displayed significant functional deficits (Elmazar & Sullivan 1981; Vorhees et al 1990).

The results of the postnatal reflex testing are comparable to previous studies examining the effect of *in utero* phenytoin in the offspring of rats (Elmazar & Sullivan 1981; McCartney et al 1999; Vorhees 1983). However, the focus of this study was to elucidate the importance of endogenous catalase in protecting the embryo from developmental and phenytoin-enhanced oxidative stress, therefore we administered only one dose of phenytoin, on GD 17. Published studies investigating the neurodevelopmental deficits caused by phenytoin employ a multiple

dosing regimen for phenytoin administration, therefore a direct comparison between the results of this study and others is not possible. This study was focused on investigating the effect on each individual pup, as distinct from the litter as a whole, since there is variation in both catalase activity and performance on the test among pups of the same litter. The importance of this intra-litter variability was evident in embryo culture studies, where we have found that developmental anomalies in individual littermates varies significantly with their catalase activity (Section 2.1) (Abramov & Wells 2011b).

Surface righting tests showed that aCat mice performed worse than WT controls when exposed to both normal developmental and phenytoin-enhanced oxidative stress, suggesting that low endogenous catalase increased susceptibility of these pups. This is consistent with the protection observed with enhanced levels of catalase in the hCat pups. Previous studies showed a delay in surface righting in rats following *in utero* phenytoin exposure (Vorhees 1983), but no such differences were seen in this study, similar to another report in rats (Pizzi & Jersey 1992), possibly due to the relatively low dose of phenytoin used to avoid neonatal death.

aCat offspring exposed *in* utero to vehicle performed normally in the negative geotaxis test, in contrast to aCat offspring exposed to phenytoin, which exhibited a substantial decrease in this task compared to WT controls, suggesting a role for oxidative stress in this effect of phenytoin. Conversely, vehicle-exposed hCat mice performed better than their WT controls, suggesting that catalase was protective even against normal developmental oxidative stress. The absence of any enhancement by phenytoin in hCat mice compared to their WT controls may have been similarly due to the protective effect of enhanced catalase. It is interesting to note that exposure to phenytoin in the WT controls for the hCat mice actually resulted in enhanced performance in these mice, similarly to previously published studies (McCartney et al 1999).

Olfactory orientation was previously shown to be variably affected by *in utero* phenytoin exposure, with performance unaffected in some offspring (Vorhees 1983) and accelerated in others (McCartney et al 1999). We observed similar results where hCat pups exposed to phenytoin showed enhanced performance, while vehicle-exposed offspring were unaffected. This behavior was not affected by genetic modulation of endogenous catalase, suggesting mechanisms of olfactory development unrelated to oxidative stress.

Previous reports of air righting studies in rats indicate that offspring exposed *in utero* to phenytoin have balance abnormalities reflected by delayed developmental acquisition of the air

righting reflex (Elmazar & Sullivan 1981; McCartney et al 1999; Minck et al 1991; Vorhees 1987). No such effects were seen in this study, possibly due to the low dose of phenytoin or the administration of only a single dose. On the contrary, air righting ability was improved by exposure to both vehicle and phenytoin in hCat mice compared to WT, and by vehicle in aCat offspring, suggesting that the development of this function is not altered by oxidative stress, at least with the dosing regimen employed herein. Although there was no statistical significance, aCat mice appeared slower in developing the dynamic righting reflex and were not able to reach the levels achieved by their controls.

The substantial 5- to 8-fold increases in catalase activity in neonatal brains of pups from PND 4 to 13 may be at least partially in response to increased oxidative stress from respiration, and the resulting increased requirement for antioxidative enzymes. Similar increases in postnatal catalase activity have been observed as the fetus moves from a relatively hypoxic *in utero* environment to a more hyperoxic environment with an approximate 4-fold elevation in oxygen concentration (El-Hage & Singh 1990; Khan & Black 2003). Also remarkable was the 3-fold to 12-fold higher brain catalase activities in male versus female offspring, which could explain the gender-dependent differences observed herein, as well as contribute to gender-dependent differences in susceptibility to other ROS-mediated pathologies. This gender disparity, along with the associated risks, were carried through to old age, albeit somewhat reduced due largely to a substantial decline in brain catalase activities in aged male brains, and provide a likely mechanism for the gender-specific susceptibility of untreated aged female aCat mice to motor coordination deficits. The basis for gender-dependent effects is unknown. Potential mechanisms could include gender differences in other ROS-related pathways involved in signal transduction, and/or in other pathways like those involving EGF activity. EGF activity in fetal brain has been found to be different between genders (Rosenblum et al 1998), which could alter brain development during oxidative stress, and/or windows of susceptibility. A similar unexplained gender-dependence in neurodevelopmental deficits has been observed following in utero exposure to the ROS-initiating drug methamphetamine in some strains (McCallum et al 2011; Wong et al 2008), but not others (Jeng et al 2005).

In summary, this study is the first to demonstrate that endogenous catalase contributes to the protection of fetus against endogenous and phenytoin-enhanced ROS-mediated

neurodevelopmental deficits, as well as to the gender-dependent nature of risk. The same gender-dependent neuroprotective role was evident in untreated aged mice.

2.3.6 ACKNOWLEDGEMENTS

We would like to thank Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX) for generously providing a breeding pair of the transgenic hCat mice.

SECTION 3: SUMMARY, CONCLUSIONS AND FUTURE STUDIES

3.1 SUMMARY AND CONCLUSIONS

About 20,000 children born annually in Canada exhibit a structural and/or functional birth defect (teratogenesis), including intellectual deficits (Harper 1998), but the mechanisms and determinants of risk are for the most part unknown. ROS including H_2O_2 have been implicated in the mechanism of birth defects (Nicol et al 2000; Wells et al 2009), and the developing embryo and fetus are uniquely susceptible due to their low levels of most antioxidative enzymes. In particular, little is known about the antioxidative enzyme catalase, which detoxifies H_2O_2, with regard to its embryonic expression, inter-individual variability in catalytic activity or protective importance.

Catalase is one of the ROS-detoxifying enzymes found in all adult organs (Ogata 1991) and the embryo (Wells et al 2009). If not detoxified by catalase, H_2O_2 can initiate signal transduction pathways, or react with iron to form highly reactive hydroxyl radicals, which can oxidatively damage cellular macromolecules (lipids, proteins, DNA, etc.), altering their function (Halliwell & Gutteridge 2007). Enhanced ROS-mediated signal transduction and/or oxidative damage to cellular macromolecules like DNA have been implicated in a spectrum of adverse structural and functional developmental consequences (Wells et al 2009). Numerous studies have demonstrated a protective role for exogenous catalase against ROS-mediated damage, including ischemic-reperfusion injury (Liu et al 1989; Oda et al 1992; Padmanabhan et al 1985), cold injury (Das et al 1991), favism (Gaetani et al 1996) and diabetic teratology (Cederberg & Eriksson 1997; Erickson & Borg 1991). Oxidative stress also has been implicated in neurodegeneration in the aging brain (Jeng et al 2011; Lee et al 2000; Simpson et al 2010), where catalase activity is similarly low (Chen et al 2004; Yang & Lin 2002). However, it is not clear whether the low levels of endogenous catalase in the embryo and adult brain are sufficient to contribute to protection against the respective neurodevelopmental and aging effects of baseline and drug-enhanced ROS formation in brain.

In the embryo, the activity of catalase is only about 5% of that in the adult liver (Abramov & Wells 2011b; El-Hage & Singh 1990; Winn & Wells 1999), which may contribute to an increased embryonic risk from developmental and xenobiotic-enhanced ROS-mediated damage. A potential protective role for catalase against teratogenesis has been demonstrated in embryo culture, where the addition of exogenous catalase enhanced embryonic antioxidative activity and protected against phenytoin-initiated DNA oxidation and embryopathy (Winn &

Wells 1995b). A subsequent *in vivo* study showed that maternal administration of PEG-catalase could increase embryonic catalase activity, decrease phenytoin-initiated protein oxidation and protect the embryo against phenytoin teratogenicity (Winn & Wells 1999).

In addition to the mechanistic insights gained by protein therapy with exogenous catalase, manipulation of catalase activity may eventually prove useful for treating acatalasemia, a homozygous hereditary condition resulting in a catalase deficiency. Acatalasemia in humans appears in several variant forms, the most widely known of which is the Japanese variant, where the deficiency is most apparent in red blood cells (**Table 4**) (Ogata 1991). Little is known about the pathogenic consequences of catalase deficiencies other than Takahara's disease, an oral gangrene attributed to H_2O_2 produced by microorganisms in the mouth, especially in cases of poor oral hygiene (Ogata 1991). Similar susceptibilities in adult animals have been reported for acatalasemic mice, which exhibit increased susceptibility to carbon tetrachloride-initiated liver damage (Wang et al 1996), increased formation of mammary tumors (Ishii et al 1996) and increased sensitivity to hepatic tumorigenesis after exposure to ionizing radiation (Yamada et al 1997).

Given the pivotal role of catalase in ROS detoxification, we anticipated that a deficiency in this enzyme could be an important risk factor for adverse development mediated by either endogenous or drug-enhanced oxidative stress, as was found for another antioxidative enzyme, G6PD (Nicol et al 2000). On the other hand, the low embryonic levels of catalase could render this enzyme relatively unimportant at least until later in the fetal period or during postnatal development when expression is increased substantially. Although exogenous catalase therapy is cytoprotective in ROS-mediated teratogenesis, the role of the endogenous enzyme in protecting the embryo remained unanswered.

Herein, we employed genetically modified aCat (low catalase) and hCat (high catalase) mice, along with protein therapy using PEG-catalase in aCat mice, to assess the pathogenic role of physiological and drug-enhanced ROS formation within the embryo, and the developmental importance of endogenous catalase in protecting the embryo from developmental and drug-enhanced oxidative stress.

It has been shown *in vivo*, in embryo culture and *in vitro* that phenytoin and other xenobiotics can be bioactivated by embryonic enzymes such as PHS and LPO to free radical intermediates that enhance ROS formation (Kubow & Wells 1989; Miranda et al 1994; Parman

& Wells 2002; Yu & Wells 1995). In contrast to low embryonic activities of alternative bioactivating enzymes like the cytochromes P450 during organogenesis (Hines 2008; Juchau et al 1992; Wells & Winn 2010), levels and activities of PHS and LPO are relatively high (Datta & Kulkarni 1994; Mitchell et al 1985; Parman & Wells 2002).

Our studies are the first to directly investigate the embryoprotective role of endogenous embryonic catalase against developmental and phenytoin-enhanced oxidative stress. We hypothesized that aCat mice would be more sensitive to endogenous embryonic and phenytoin-enhanced ROS production, oxidative DNA damage, embryopathies and neurodevelopmental deficits in comparison to their wild-type littermates. Conversely, hCat embryos would be less sensitive than wild-type littermates to ROS-initiated defects listed above. The results are summarized as follows:

1. Endogenous catalase is embryoprotective in embryos exposed in culture to developmental and phenytoin-enhanced oxidative stress. Vehicle-exposed aCat and hCat embryos respectively exhibited reduced and enhanced catalase activity compared to wild-type controls, with conversely enhanced and reduced spontaneous embryopathies. Phenytoin was embryopathic in all strains without altering catalase activity, but less so in the wild-type embryos for the aCat strain, which exhibited about half the catalase activity of CD-1 embryos. Exposure to phenytoin in aCat and hCat embryos resulted in enhanced and reduced embryopathies, respectively, compared to their WT controls. Among aCat embryos exposed to phenytoin, embryopathies increased with decreasing catalase activity, and were completely blocked by addition of exogenous catalase, which increased embryonic catalase activity to wild-type levels (**Study 1**).

2. Embryonic catalase protects against endogenous and phenytoin-enhanced DNA oxidation and embryopathies *in vivo*. Compared to WT catalase-normal controls, both untreated and phenytoin-exposed aCat mice exhibited an increase in embryonic DNA oxidation and embryopathies, both of which were completely blocked by protein therapy with exogenous catalase. Conversely, compared to WT controls, untreated and to a lesser extent phenytoin-exposed hCat mice were protected, with untreated hCat embryos exhibiting a decrease in embryonic DNA oxidation and embryopathies. Higher concentrations of phenytoin in fetal brain compared to fetal liver and maternal tissues may explain the high frequency of neurodevelopmental deficits compared to other fetal anomalies caused by this drug (**Study 2**).

3. Protective role of endogenous catalase in baseline and phenytoin-enhanced neurodevelopmental and behavioral deficits. Postnatal death of offspring was enhanced in aCat mice and reduced in hCat mice following phenytoin exposure. Compared to wild-type controls, catalase deficiency in the offspring reduced postnatal surface righting, negative geotaxis and rotarod performances independent of drug treatment, and catalase deficiency enhanced phenytoin-initiated negative geotaxis and rotarod deficits in aCat females. Untreated aged female but not male aCat mice exhibited loss in motor coordination. Conversely, hCat offspring showed treatment-independent increased surface righting, negative geotaxis, air righting and, in females, improved baseline and phenytoin-impaired rotarod performance. Gender dependencies were consistent with higher brain catalase activities in male than female offspring, providing a novel mechanism for gender dependency (**Study 3**).

Results from this thesis provide the most direct evidence to date that the relatively low level of endogenous embryonic and fetal catalase provides important developmental protection against physiological and drug-enhanced oxidative stress. Similarly, endogenous catalase contributes to fetal protection from ROS-mediated neurodevelopmental deficits, as well as to the gender-dependent nature of risk. These findings also emphasize that interindividual differences in the expression of embryonic and fetal catalase likely constitute an important determinant of risk for ROS-mediated developmental pathologies.

3.2 FUTURE STUDIES

Our embryo culture studies showed that low levels of endogenous catalase were protective for embryonic development against both, endogenous and phenytoin-enhanced, oxidative stress. Not all developmental outcomes appeared to be modulated by catalase, at least at this gestational stage. Such outcomes may not be affected by ROS, or unaffected outcomes may reflect cell-specific or tissue-specific variations in embryonic catalase and/or other ROS-relevant protective pathways not detected by our measurements in whole-embryo homogenates. It would be interesting to further investigate this issue in greater detail.

The mechanisms underlying the relative resistance of the C3H strain to phenytoin embryotoxicity in embryo culture are unknown, but could include differences in embryonic drug bioactivation, other pathways for ROS detoxification and/or repair of oxidative macromolecular damage. Despite this apparent strain resistance, the enhanced dysmorphogenesis in aCat embryos exposed to phenytoin compared to phenytoin-exposed wild-type embryos provides the first direct evidence that the relatively low level of constitutive embryonic catalase provides important protection against ROS-initiating teratogens. A more detailed study of this mouse strain focused upon phenytoin bioactivation, ROS detoxification and repair of oxidative macromolecular damage, with direct comparison to the phenytoin-sensitive C57BL/6J strain, would provide insights into the mechanism of strain resistance.

Exogenous PEG-catalase was employed with aCat mice both in embryo culture and *in vivo* to determine the protective role of catalase by increasing catalase activity in the developing embryo and fetus. This protein therapy showed that enhancing embryonic and fetal catalase activity was protective against embryo and fetal toxicity. Additional studies using siRNA in the hCat mouse strain expressing human catalase to lower endogenous levels of catalase would be useful to confirm that the protection observed in the hCat embryos was due to their enhanced catalase activity.

In the *in vivo* study, phenytoin caused a remarkable and substantial dose-dependent increase in the incidence of exencephaly, which was not observed in the vehicle controls of any strain in this study. The cases of exencephaly were distributed across all litters, and could not be attributed to a litter effect involving a single predisposed dam. The mechanism for this phenytoin-initiated birth defect is not known, but it was completely blocked by pretreatment with PEG-catalase, which restored embryonic catalase activity in the aCat embryos to normal levels,

suggesting that oxidative stress plays an important role. This birth defect should be examined at greater detail with respect to the ROS-initiated mechanism involved and the protection that catalase offered.

To ensure that pharmacokinetic differences did not confound our interpretation of the effects of genetic modulation of endogenous catalase activity on the embryopathic effects of phenytoin, we measured the concentrations of phenytoin in maternal plasma and in maternal and fetal liver and brain tissues. Our results showed that phenytoin freely crossed the placenta and achieved similar concentrations in fetal and adult liver, while higher concentrations were seen in fetal brain compared to fetal liver, and in most cases to adult brain. Since fetal and adult liver concentrations were similar, this suggests a role for fetal brain transporters, as distinct from placental transporters. Phenytoin is reported to be a substrate for the placental P-glycoprotein efflux transporter, which may actively pump phenytoin from the placenta back to the maternal circulation and thus protect the embryo. The ontogeny of this and other potential phenytoin transporters in fetal brain remains to be determined, and the contribution of fetal brain transporters to the determinants of risk for neurodevelopmental deficits warrants a detailed investigation.

The formation of ROS within the embryo can alter signal transduction in addition to causing oxidative damage to cellular macromolecules (Janssen-Heininger et al 2008), either of which can adversely affect development. In this thesis I have investigated the effects of varying endogenous catalase activities only with respect to oxidative damage to DNA and the resultant developmental toxicity. It would be interesting to expand the study to look at the potential embryopathic contribution of alterations in ROS-mediated signal transduction in catalase-modified embryos.

A catalase knockout mouse with no catalase expression has been recently engineered and reported to develop normally (Ho et al 2004). However, no detailed developmental studies appeared to have been conducted in this mouse and a more detailed evaluation of this knockout mouse and its susceptibility to ROS-initiating teratogens might prove enlightening.

SECTION 4: REFERENCES

Abad F, Maroto R, Lopez GM, Garcia P, Garcia AG. 1995. Pharmacological protection against the cytotoxicity induced by 6-hydroxy-dopamine and H_2O_2 in chromaffin cells. *European Journal of Pharmacology* 293:55-64

Abel KM, Wicks S, Susser ES, Dalman C, Pedersen MG, et al. 2010. Birth weight, schizophrenia, and adult mental disorder: is risk confined to the smallest babies? *Archives of General Psychiatry* 67:923-30

Abramov JP, Wells PG. 2011a. Embryonic catalase protects aginst endogenous and phenytoin-enhanced DNA oxidation and embryopathies in acatalasemic and human catalase-expressing mice. *FASEB Journal* 25:21288-2200

Abramov JP, Wells PG. 2011b. Embryoprotective role of endogenous catalase in acatalasemic and human catalase-expressing mouse embryos exposed in culture to developmental and phenytoin-enhanced oxidative stress. *Toxicological Sciences* 120:428-38

Adams J, Vorhees CV, Middaugh LD. 1990. Developmental neurotoxicity of anticonvulsants: human and animal evidence on phenytoin. *Neurotoxicology and Teratology* 12:203-14

Aebi H, Suter H, Feinstein RN. 1968. Activity and stability of catalase in blood and tissues of normal and acatalasemic mice. *Biochemical Genetics* 2:245-51

Aebi H, Wyss SR, Scherz B, Gross J. 1976. Properties of erythrocyte catalase from homozygotes and heterozygotes for Swiss-type acatalasemia. *Biochemical Genetics* 14:791-807

Allen RG, Tresini M. 2000. Oxidative stress and gene regulation. *Free Radical Biology and Medicine* 28:463-99

Altman J, Sudarshan K. 1975. Postnatal development of locomotion in the laboratory rat. *Animal Behaviour* 23:896-920

Amstad P, Peskin A, Shah G, Mirault M-E, Moret R, et al. 1991. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to to oxidative stress. *Biochemistry* 30:9305-13

Arlen RR, Wells PG. 1996. Inhibition of thalidomide teratogenicity by acetylsalicylic acid: evidence for prostaglandin H synthase-catalyzed bioactivation of thalidomide to a teratogenic reactive intermediate. *Journal of Pharmacology and Experimental Therapeutics* 277:1649-58

Atlas SA, Zweier JL, Nebert DW. 1980. Genetic differences in phenytoin pharmacokinetics: *in vivo* clearance and *in vitro* metabolism among inbred strains of mice *Developmental Pharmacology and Therapy* 1:281-304

Azarbayjani F, Danielsson BR. 1998. Pharmacologically induced embryonic dysrhythmia and episodes of hypoxia followed by reoxygenation: a common teratogenic mechanism for antiepileptic drugs? *Teratology* 57:117-26

Ballabh P, Braun A, Nedergaard M. 2004. The blood-brain barrier: an overview Structure, regulation, and clinical implications. *Neurobiology of Disease* 16:1-13

Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, et al. 1999. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38:6699-705

Barrow MV, Taylor WJ. 1969. A rapid method for detecting malformations in rat fetuses. *Journal of Morphology* 127:291-306

Becker BF. 1993. Towards the physiological function of uric acid. *Free Radical Biology and Medicine* 14:615-31

Beyerlein A, Ness AR, Streuling I, Hadders-Algra M, von KR. 2010. Early rapid growth: no association with later cognitive functions in children born not small for gestational age. *American Journal of Clinical Nutrition* 92:585-93

Bjornstedt M, Xue J, Huang W, Akesson B, Holmgren A. 1994. The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *Journal of Biological Chemistry* 269:29382-4

Blake DA, Martz F. 1980. Covalent binding of phenytoin metabolites in fetal tissue. In *Phenytoin-induced Teratology and Gingival Pathology*, ed. TM Hassel, NC Johnston, KH Dudley, pp. 75-80. New York: Raven Press

Blank NK, Nishimura RN, Seil FJ. 1982. Phenytoin neurotoxicity in developing mouse cerebellum in tissue culture. *Journal of Neurological Sciences* 55:91-7

Bologa M, Koren G, Fassos F, McGuigan M, Reider M. 1994. Drugs and chemicals most commonly used by pregnant women. In *Maternal-fetal toxicology: a clinician's guide*, ed. G Koren, pp. 89-114. New-York: M. Dekker

Bromley RL, Baker GA, Meador KJ. 2009. Cognitive abilities and behaviour of children exposed to antiepileptic drugs in utero. *Current Opinion in Neurology* 22:162-6

Buehler BA. 1984. Epoxide hydrolase activity and fetal hydantoin syndrome. *Proceedings of Greenwood Genetics Center* 3:109-10

Buehler BA, Delimont D, van Waes M, Finnell RH. 1990. Prenatal prediction of risk of the fetal hydantoin syndrome. *New England Journal of Medicine* 322:1567-72

Buttke TM, Sandstorm PA. 1995. Redox regulation of programmed cell death in lymphocytes. *Free Radical Research* 22:389-97

Ceckova-Novotna M, Pavek P, Staud F. 2006. P-glycoprotein in the placenta: expression, localization, regulation and function. *Reproductive Toxicology* 22:400-10

Cederberg J, Eriksson UJ. 1997. Decreased catalase activity in malformation-prone embryos of diabetic rats. *Teratology* 56:350-6

Cederberg J, Galli J, Luthman H, Eriksson UJ. 2000. Increased mRNA levels of Mn-SOD and catalase in embryos of diabetic rats from a malformation-resistant strain. *Diabetes* 49:101-7

Chance B, Sies H, Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* 59:527-605

Chen X, Liang H, Van Remmen H, Vijg J, Richardson A. 2004. Catalase transgenic mice: characterization and sensitivity to oxidative stress. *Archives of Biochemistry & Biophysics* 422:197-210

Chen X, Mele J, Giese H, Van Remmen H, Dolle M, et al. 2003. A strategy for the ubiquitous overexpression of human catalase and CuZn superoxide dismutase genes in transgenic mice. *Mechanisms of Ageing and Development* 124:219-27

Chung CS, Myrianthopoulos NC. 1975. *Factors affecting risks of congenital malformations* The National Foundation March of Dimes

Claiborne A, Yeh JI, Mallett C, Luba J, Crane EJ, et al. 1999. Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38:15407-16

Collins MD, Fradkin R, Scott WJJ. 1990. Induction of pastaxial forelimb ectrodactyly with anticonvulsant agents in A/J mice. *Teratology* 41:61-70

Crawford DR, Mirault ME, Moret R, Zbinden I, Cerutti PA. 1988. Molecular defect in human acatalasia fibroblasts. *Biochemical & Biophysical Research Communications* 153:59-66

Danielsson BR, Azarbayjani F, Skold AC, Webster WS. 1997. Initiation of phenytoin teratogenesis: pharmacologically induced embryonic bradycardia and arrhythmia

resulting in hypoxia and possible free radical damage at reoxygenation. *Teratology* 56:271-81

Das DK, Russell JC, Jones RM. 1991. Reduction of cold injury by superoxide dismutase and catalase. *Free Radical Research Communications* 12/13:653-62

Datta K, Kulkarni AP. 1994. Oxidative metabolism of aflatoxin B_1 by lipoxygenase purified from human term placenta and intrauterine conceptal tissues. *Teratology* 50:311-7

Del Bello B, Paolicchi A, Comporti M, Pompella A, Maellaro E. 1999. Hydrogen peroxide produced during gamma-glutamyl transpeptidase activity is involved in prevention of apoptosis and maintainance of proliferation in U937 cells. *FASEB Journal* 13:69-79

Denu JM, Tanner KG. 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37:5633-42

DeSesso JM, Scialli AR, Goeringer GC. 1994. D-Mannitol, a specific hydroxyl free radical scavenger, reduces the developmental toxicity of hydroxyurea in rabbits. *Teratology* 49:248-59

Dickerman RC, Feinstein RN, Grahn D. 1968. Position of the acatalasemic gene in linkage group V of the mouse. *Journal of Heredity* 59:177-8

Dizdaroglu M. 2005. Base-excision repair of oxidative DNA damage by DNA glycosylates. *Mutation Research* 591:45-59

Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. 2002. Free radical-induced damage to DNA: mechanisms and measurement *Free Radical Biology and Medicine* 32:1102-15

Dominici S, Valentini M, Maellaro E, Del Bello B, Paolicchi A, et al. 1999. Redox modulation of cell surface protein thiols in U937 lymphoma cells: the role of gammaglutamyl transpeptidase-dependent H_2O_2 production and S-thiolation. *Free Radical Biology and Medicine* 27:623-35

El-Hage S, Singh SM. 1989. Regulation of catalase-specific mRNA and its processing during development in mice. *Developmental Genetics* 10:339-44

El-Hage S, Singh SM. 1990. Temporal expression of genes encoding free radical-metabolizing enzymes is associated with higher mRNA levels during in utero development in mice. *Devevlopmental Genetics* 11:149-59

Elmazar MM, Sullivan FM. 1981. Effect of prenatal phenytoin administration on postnatal development of the rat: a behavioral teratology study. *Teratology* 24:115-24

Erickson UJ, Borg LAH. 1991. Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. *Diabetologia* 34:325-31

Espeel M, Briere N, De Craemer D, Jauniaux E, Roels F. 1993. Catalase-negative peroxisomes in human embryonic liver. *Cell & Tissue Research* 272:89-92

Espeel M, Depreter M, Nardacci R, D'Herde K, Kerckaert I, et al. 1997. Biogenesis of peroxisomes in fetal liver. *Microscopy Research & Technique* 39:453-66

Fantel AG. 1982. Culture of whole rodent embryos in teratogen screening. *Teratogenesis, Carcinogenesis, and Mutagenesis* 2:231-4

Fantel AG. 1996. Reactive oxygen species in developmental toxicity: review and hypothesis *Teratology* 53:196-217

Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R, Harel S. 2009. Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *Journal of Child Neurology* 24:846-51

Feinstein RN, Seaholm JE, Howard JB, Russell WL. 1964. ACATALASEMIC MICE. *Proceedings of the National Academy of Sciences of the United States of America* 52:661-2

Finnell RH. 1980. Phenytoin-induced teratogenesis: a mouse model. *Science* 211:483-4

Finnell RH, Abbott LC, Taylor SM. 1989. The fetal hydantoin syndrome: answers from a mouse model. *Reproductive toxicology* 3:127-33

Finnell RH, Chernoff GF. 1982. Mouse fetal hydantoin syndrome: effects of maternal seizures. *Epilepsia* 23:423-9

Finnell RH, Kerr BM, van Waes M, Steward RL, Levy RH. 1994. Protection from phenytoin-induced congenital malformations by coadministration of the antiepileptic drug stiripentol in a mouse model. *Epilepsia* 35:141-8

Fort DJ, Bantle JA. 1990. Use of frog embryo teratogenesis assay-xenopus and an exogenous metabolic activation system to evaluate the developmental toxicity of dyphenylhydantoin. *Fundamental and Applied Toxicology* 14:720-33

Frei B, England L, Ames BN. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America* 86:6377-81

Fridovich I. 1986. Superoxide dismutases. *Advances in Enzymology & Related Areas of Molecular Biology* 58:61-97

Fromm MF. 2002. The influence of *MDR1* polymorphisms on P-glycoprotein expression and function in humans. *Advanced Drug Delivery Reviews* 54:1295-310

Gaetani GF, Rolfo M, Arena S, Mangerini R, Meloni GF, Ferraris AM. 1996. Active involvement of catalase during hemolytic crises of favism. *Blood* 88:1084-8

Ghez C. 1991. The Cerebellum. In *Principles of neural science*, ed. ER Kandel, JH Schwartz, TM Jessel, pp. 626-46. Norwalk, CT: Appleton & Lange

Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, et al. 2005. Electron transfer between cytochrome *c* and $p66^{Shc}$ generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell* 122:221-33

Giorgio M, Trinei M, Migliaccio E, Pelicci PG. 2007. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nature Reviews: Molecular Cell Biology* 8:722-8

Goncalves LL, Ramkissoon A, Wells PG. 2009. Prostaglandin H synthase-1-catalyzed bioactivation of neurotransmitters, their precursors and metabolites: oxidative DNA damage and electron spin resonance spectroscopy studies. *Chemical Research in Toxicology* 22:842-52

Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, Busse R. 2000. A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circulation Research* 87:26-32

Goth L. 2000. Lipid and carbohydrate metabolism in acatalasemia. *Clinical Chemistry* 46:564-6

Goth L. 2001. A new type of inherited catalase deficiencies: its characterization and comparison to the Japanese and Swiss type of acatalasemia. *Blood Cells, Molecules & Diseases* 27:512-7

Goth L, Eaton JW. 2000. Hereditary catalase deficiencies and increased risk of diabetes. *Lancet* 356:1820-1

Graham DG. 1978. Oxidative pathways for catecholamines in the genesis ofneuromelanin and cytotoxic quinones. *Molecular Pharmacology* 14:633-43

Gupta BL. 1973. Microdetermination techniques for H_2O_2 in irradiated solutions. *Journal of Microchemistry* 18:363-74

Hailer-Morrison MK, Kotler JM, Martin BD, Sugden KD. 2003. Oxidized guanine lesions as modulators of gene transcription. Altered p50 binding affinity and repair shielding by 7,8-dihydro-8-oxo-20-deoxyguanosine lesions in the NFkappaB promoter element. *Biochemistry* 42:9761-70

Halliwell B, Gutteridge LMC. 1991. Reactive oxygen species in living systems: sources, biochemistry, and role in human disease. *The American Journal of Medicine* 91:14-22

Halliwell B, Gutteridge LMC. 2007. *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press

Hansen DK. 1991. The embryotoxicity of phenytoin: an update on possible mechanisms. *Proceedings of the Society for Experimental Biology & Medicine* 197:361-8

Hansen DK, Branham WS, Sheehan DM, Holson RR. 1992. Embryotoxicity of phenytoin in adrenalectomized CD-1 mice. *Proceedings of the Society for Experimental Biology and Medicine* 199:501-8

Hansen DK, Hodes ME. 1983. Metabolism of phenytoin in teratogenesis-susceptible and -resistant strains of mice. *Drug Metabolism and Disposition* 11:21-4

Hansen JM. 2006. Oxidative stress as a mechanism of teratogenesis. *Birth Defects Research Part C: Embryo Today Reviews* 78:293-307

Hanson JW, Myrianthopoulos NC, Harvey MAS, Smith DW. 1976. Risks to the offspring of women treated with hydantoin anticonvulsants with emphasis on fetal hydantoin syndrome. *Journal of Pediatrics* 89:662-8

Hanson JW, Smith DW. 1975. The fetal hydantoin syndrome. *Journal of Pediatrics* 87:285-90

Harbison RD. 1978. Chemical-biological reactions common to teratogenesis and mutagenesis *Environmental Health Perspectives* 24:87-100

Harbison RD, Becker BA. 1969. Relation of dosage and time of administration of diphenylhydantoin to its teratogenic effect in mice. *Teratology* 2:305-11

Harbison RD, Becker BA. 1970. Effect of phenobarbital and SKF525A pretreatment on diphenylhydantoin teratogenicity in mice. *Journal of Pharmacology and Experimental Therapeutics* 175:283-8

Harbison RD, Becker BA. 1972. Diphenylhydantoin teratogenicity in rats. *Toxicology and Applied Pharmacology* 22:193-200

Harbison RD, MacDonald JS, Sweetman BJ, Taber D. 1977. Proposed mechanisms of diphenylhydantoin-induced teratogenicity. *Pharmacologist* 19:179

Harden CL, Sethi NK. 2008. Epileptic disorders in pregnancy: an overview. *Current opinion in obstetrics & gynecology* 20:557-62

Harper PS. 1998. *Practical Genetic Counselling. 5th Edition*. Boston: Butterworth Heinemann

Hatta T, Ohmori H, Murakami T, Takano M, Yamashita K, Yasuda M. 1999. Neurotoxic effects of phenytoin on postnatal mouse brain development following neonatal administration. *Neurotoxicology and Teratology* 21:21-8

Himpel S, Bartels J, Zimdars K, Huether G, Adler L, et al. 2006. Association between body weight of newborn rats and density of serotonin transporters in the frontal cortex at adulthood. *Journal of Neural Transmission* 113:295-302

Hines RN. 2008. The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacology & Therapeutics* 118:250-67

Ho YS, Xiong Y, Ma W, Spector A, Ho DS. 2004. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *The Journal of Biological Chemistry* 279:32804-12

Holmgren A. 1988. Thioredoxin and glutaredoxin: small multi-functional redox proteins with active-site disulfide bonds. *Biochemical Society Transactions* 16:95-6

Horwitz DL, Leff JA. 1995. Catalase and hydrogen peroxide cytotoxicity in cultured cardiac myocytes. *Journal of Molecular and Cellular Cardiology* 27:909-15

Indredavik MS, Skranes JS, Vik T, Heyerdahl S, Romundstad P, et al. 2005. Low-birth-weight adolescents: psychiatric symptoms and cerebral MRI abnormalities. *Pediatric Neurology* 33:259-66

Ishii K, Zhen LX, Wang DH, Funamori Y, Ogawa K, Taketa K. 1996. Prevention of mammary tumorigenesis in acatalasemic mice by vitamin E supplementation. *Japanese Journal of Cancer Research* 87:680-4

Jacobson M. 1991. Histogenesis and morphogenesis of cortical structures. In *Developmental Neurobiology*, ed. M Jacobson, pp. 401-51. New York: Plenum Press

Janssen-Heininger YMW, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, et al. 2008. Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radical Biology and Medicine* 45:1-17

Jeng W, Ramkissoon A, Parman T, Wells PG. 2006. Prostaglandin H synthase-catalyzed bioactivation of amphetamines to free radical intermediates that cause CNS regional DNA oxidation and nerve terminal degeneration. *FASEB Journal* 20:638-50

Jeng W, Ramkissoon A, Wells PG. 2011. Reduced DNA oxidation in aged prostaglandin H synthase-1 (PHS-1) knockout mice *Free Radical Biology and Medicine* 50:550-6

Jeng W, Wong AW, Ting-A-Kee R, Wells PG. 2005. Methamphetamine-enhanced embryonic oxidative DNA damage and neurodevelopmental deficits. *Free Radical Biology and Medicine* 39:317-26

Jiang ZY, Hunt JV, Wolff SP. 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Analytical Biochemistry* 202:384-9

Juchau MR, Fantel AG, Harris C, Beyer BK. 1986. The potential role of redox cycling as a mechanism for chemical teratogenesis. *Enviromental Health Perspectives* 70:131-6

Juchau MR, Lee QP, Fantel AG. 1992. Xenobiotic biotransformation/bioactivation in organogenesis-stage conceptal tissues: implications for embryotoxicity and teratogenesis. *Drug Metabolism Reviews* 24:195-238

Kappus H. 1986. Overview of enzyme systems involved in bio-reduction of drugs and in redox cycling. *Biochemical Pharmacology* 35:1-6

Katsumata M, Gupta C, Baker MK, Sussdorf CE, Goldman AS. 1982. Diphenylhydantoin: an alternative ligand of a glucucorticoid receptor affecting prostaglandin generation in A/J mice. *Science* 218:1313-5

Kennedy JC, Memet S, Wells PG. 2004. Antisense evidence for nuclear factor-kappaB-dependent embryopathies initiated by phenytoin-enhanced oxidative stress. *Molecular Pharmacology* 66:404-12

Khan JY, Black SM. 2003. Developmental changes in murine brain antioxidant enzymes. *Pediatric Research* 54:77-82

Khobta A, Anderhub S, Kitsera N, Epe B. 2010. Gene silencing induced by oxidative DNA base damage: association with local decrease of histone H4 acetylation in the promoter region. *Nucleic Acids Research* 38:4285-95

Kim PM, Wells PG. 1996. Phenytoin-initiated hydroxyl radical formation: characterization by enhanced salicylate hydroxylation. *Molecular Pharmacology* 49:172-81

Kim YS, Kim SU. 1991. Oligodentroglial cell death induced by oxygen radicals and its protection by catalase. *Journal of Neuroscience Research* 29:100-6

Kitsera N, Stathis D, Luhnsdorf B, Muller H, Carell T, et al. 2011. 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription blocking damage by OGG1. *Nucleic Acids Research* 39:5926-34

Knobloch J, Rüther U. 2008. Shedding light on an old mystery: thalidomide suppresses survival pathways to induce limb defecs. *Cell Cycle* 7:1121-7

Kubow S, Wells PG. 1989. In vitro bioactivation of phenytoin to a reactive free radical intermediate by prostaglandin synthetase, horseradish peroxidase, and thyroid peroxidase. *Molecular Pharmacology* 35:504-11

Lee CJJ, Goncalves LL, Wells PG. 2011. Embryopathic effects of thalidomide and its hydrolysis products in rabit embryo culture: evidence for a prostaglandin H synthase (PHS)-dependent, reactive oxygen species (ROS)-mediated mechanism. *FASEB Journal* 25:2468-83

Lee CK, Weindruch R, Prolla TA. 2000. Gene-expression profile of the ageing brain in mice. *Nature Genetics* 25:294-7

Lee SR, Kwon KS, Kim SR, Rheei SG. 1998. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *The Journal of Biological Chemistry* 273:15366-72

Lenzen S. 2008. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* 51:216-26

Liu L, Wells PG. 1994a. *In vitro* and *in vivo* cytochromes P450- and peroxidase-catalysed formation of phenytoin-DNA adducts in murine maternal hepatic and embryonic tissues. *Proceedings of the 10th International Symposium on Microsomes and Drug Oxidations*, p. 555. Toronto, Canada

Liu L, Wells PG. 1994b. In vivo phenytoin-initiated oxidative damage to proteins and lipids in murine maternal hepatic and embryonic tissue organelles: potential molecular targets of chemical teratogenesis. *Toxicology and Applied Pharmacology* 125:247-55

Liu L, Wells PG. 1995a. DNA oxidation as a potential molecular mechanism mediating drug-induced birth defects: phenytoin and structurally related teratogens initiate the formation of 8-hydroxy-2'-deoxyguanosine in vitro and in vivo in murine maternal hepatic and embryonic tissues. *Free Radical Biology and Medicine* 19:639-48

Liu L, Wells PG. 1995b. Potential molecular targets mediating chemical teratogenesis: *In vitro* peroxidase-catalyzed phenytoin bioactivation and oxidative damage to proteins and lipids in murine maternal hepatic microsomes and embryonic 9000 g supernatant. *Toxicology and Applied Pharmacology* 134:71-80

Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY. 1989. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *American Journal of Physiology* 256:H589-93

Lum JT, Vassanji NA, Wells PG. 1985. Analysis of the toxicologically relevant metabolites of phenytoin in biological samples by high-performance liquid chromatography. *Journal of Chromatography* 338:242-8

Lum JT, Wells PG. 1986. Pharmacological studies on the potentiation of phenytoin teratogenicity by acetaminophen. *Teratology* 33:53-72

Mahmood B, Daood MJ, Hart C, Hansen TWR, Watchko JF. 2001. Ontogeny of P-glycoprotein in mouse intestine, liver and kidney. *Journal of Investigative Medicine* 49:250-7

Mann H, McCoy MT, Subramaniam J, Van Remmen H, Cadet JL. 1997. Over expression of superoxide dismutase and catalase in immortalized neural calls: toxic effects of hydrogen peroxide. *Brain Research* 770:163-8

Marnett LJ. 2000. Oxyradicals and DNA damage. *Carcinogenesis* 21:361-70

Martz F, Failinger C, Blake DA. 1977. Phenytoin teratogenicity, correlation betwee embryopathic effect and covalent binding of putative arene oxide metabolite in gestational mouse. *Journal of Pharmacology and Experimental Therapeutics* 203:231-9

Massey KM. 1966. Teratogenic effects of diphenylhydantoin sodium. *Journal of Oral Therapeutics and Pharmacology* 2:380-5

McCallum GP, Wong AW, Wells PG. 2011. Cockayne Syndrome B protects against methamphetamine-enhanced oxidative DNA damage in murine fetal brain and postnatal neurodevelopmental deficits. *Antioxidants and Redox Signaling* 14:747-56

McCartney MA, Scinto PL, Wang SS, Altan S. 1999. Developmental effects of phenytoin may differ depending on sex of offspring. *Neurotoxicology & Teratology* 21:119-28

McClain RM, Langhoff L. 1980. Teratogenicity of dyphenyhydantoin in the New Zealand white rabbit. *Teratology* 21:371-9

Meador KJ, Penovich P, Baker GA, Pennell PB, Bromfield E, et al. 2009. Antiepileptic drug use in women of childbearing age. *Epilepsy and Behavior* 15:339-43

Miller L, Wells PG. 2011. Altered methanol embryopathies in embryo culture with mutant catalase-deficient mice and transgenic mice expressing human catalase. *Toxicology and Applied Pharmacology* 252:55-61

Minck DR, Acuff-Smith KD, Vorhees CV. 1991. Comparison of the behavioral teratogenic potential of phenytoin, mephenytoin, ethotoin, and hydantoin in rats. *Teratology* 43:279-93

Ming M, Shea CR, Guo X, Li X, Soltani K, et al. 2010. Regulation of global genome nucleotide excision repair by SIRT1 through xeroderma pigmentosum C. *Proceedings of the National Academy of Sciences of the United States of America* 107:22623-8

Miranda AF, Wiley MJ, Wells PG. 1994. Evidence for embryonic peroxidase-catalyzed bioactivation and glutathione-dependent cytoprotection in phenytoin teratogenicity: modulation by eicosatetraynoic acid and buthionine sulfoximine in murine embryo culture. *Toxicology and Applied Pharmacology* 124:230-41

Mitchell MD, Brennecke SP, Saed SP, Strickland DM. 1985. Arachidonic acid metabolsm in the fetus and neonate. In *Biolgical Protection with Prostaglandins*, ed. MM Cohen, pp. 27-44. Boca Raton, FL: CRC Press

Modeer T, Andersson G. 1990. Regulation of epidermal growth factor receptor metabolism in gingival fibroblasts by phenyoin in vitro. *Journal of Oral Pathology and Medicine* 19:188-91

Mohazzab KM, Kaminski PM, Wolin MS. 1994. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Americal Journal of Physiology* 266:H2568-H72

Mohazzab KM, Wolin MS. 1994. Properties of a superoxide anion-generating microsomal NADH oxidoreductase, a potential pulmonary artery PO_2 sensor. *Americal Journal of Physiology* 267:L823-L31

Monson RR, Rosenberg L, Hartz SC, Shapiro S, Heinonen OP, Slone D. 1973. Diphenylhydantoin and selected congnital malformations. *New England Journal of Medicine* 289:1049-52

Moriscot C, Candel S, Sauret V, Kerr-Conte J, Richard MJ, et al. 2007. MnTMPyP, a metalloporphyrin-based superoxide dismutase/catalase mimetic, protects INS-1 cells and human pancreatic islets from an in vitro oxidative challenge. *Diabetes & Metabolism* 33:44-53

Muppala V, Lin CS, Lee YH. 2000. The role of HNF-1α in controlling hepatic catalase activity. *Molecular Pharmacology* 57:93-100

Nair MK, Chacko DS, Paul MK, Nair L, George B, Kumar GS. 2009. Low birthweight babies--outcome at 13 years. *Indian Pediatrics* 46

Nakashima T, Sato E, Niwano Y, Kohno M, Muraoka W, Oda T. 2007. Inhibitory or scavenging action of ketoconazole and ciclopiroxolamine against reactive oxygen species released by primed inflammatory cells. *British Journal of Dermatology* 156:720-7

New DAT. 1978. Whole embryo culture and the study of mammalian embryos during organogenesis. *Biological Reviews* 53:81-122

Nicol CJ, Zielenski J, Tsui LC, Wells PG. 2000. An embryoprotective role for glucose-6-phosphate dehydrogenase in developmental oxidative stress and chemical teratogenesis. *FASEB Journal* 14:111-27

Noble PG, Antel JP, Yong VW. 1994. Astrocytes and catalase prevent the toxicity of catecholamines to oligodendrocytes. *Brain Research* 633:83-90

Nulman I, Scolnik D, Chitayat D, Farkas LD, Koren G. 1997. Findings in children exposed in utero to phenytoin and carbamazepine monotherapy: Independent efects of epilepsy and medications. *American Journal of Medical Genetics* 68:18-24

Oda T, Nakai I, Mituo M, Yamagishi H, Oka T, Yoshikawa T. 1992. Role of oxygen radicals and synergistic effect of superoxide dismutase and catalase on ischemia-reperfusion injury of the rat pancreas. *Transplantation Proceedings* 24:797-8

Ogata M. 1991. Acatalasemia. *Human Genetics* 86:331-40

Ohmori H, Kobayashi T, Yasuda M. 1992. Neurotoxicity of phenytoin administered to newborn mice on developing cerebellum *Neurotoxicology and Teratology* 14:159-65

Ou P, Wolff SP. 1993. Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide in vitro. *Biochemical Pharmacology* 465:1139-44

Ou P, Wolff SP. 1996. A discontinuous method for catalase determination at "near physiological" concentrations of H_2O_2 and its application to the study of H_2O_2 fluxes within cells. *Journal of Pharmacology and Experimental Therapeutics* 277:945-53

Ouchi A, Nagaoka S-i, Mukai K. 2010. Tunneling effect in regeneration reaction of vitamin E by ubiquinol. *Journal of Physical Chemistry B, Condensed Matter, Materials, Surfaces, Interfaces & Biophysical* 114:6601-7

Ozolins TR, Siksay DLA, Wells PG. 1996. Modulation of embryonic glutathione peroxidase activity and phentyoin teratogenicity by dietary deprivation of selenium in CD-1 mice. *Journal of Pharmacology and Experimental Therapeutics* 277:945-53

Packer L, Witt EH, Tritschler HJ. 1995. Alpha-lipoic acid as a biological antioxidant. *Free Radical Biology and Medicine* 19:227-50

Padmanabhan RV, Gudapaty R, Liener IE, Schwartz BA, Hoidal JR. 1985. Protection against pulmonary oxygen toxicity in rats by the intratracheal administration of liposome-encapsulated superoxide dismutase or catalase. *The American Review of Respiratory Disease* 132:264-7

Palace VP, Khaper N, Qin Q, Singal PK. 1999. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. *Free Radical Biology and Medicine* 26:746-61

Parman T, Chen G, Wells PG. 1998. Free radical intermediates of phenytoin and related teratogens. Prostaglandin H synthase-catalyzed bioactivation, electron paramagnetic resonance spectrometry, and photochemical product analysis. *Journal of Biological Chemistry.* 273:25079-88

Parman T, Wells PG. 2002. Embryonic prostaglandin H synthase-2 (PHS-2) expression and benzo[a]pyrene teratogenicity in PHS-2 knockout mice. *FASEB Journal* 16:1001-9

Pastore A, Piemonte F, Locatelli M, Russo AL, Gaeta LM, et al. 2003. Determination of blood total, reduced, and oxidized glutathione in pediatric subjects. *Clinical Chemistry* 47:1467-9

Percy ME. 1984. Catalase: an old enzyme with a new role? *Canadian Journal of Biochemistry & Cell Biology* 62:1006-14

Perstin JP, Wells PG. 2010. Embryoprotective role of constitutive antioxidative catalase in wild-type and acatalasemic mice in vivo and in embryo culture. *Toxicology Letters* 196S:P204-003

Phillips JP, Parkes TL, Hilliker AJ. 2000. Targeted neuronal gene expression and longevity in *Drosophila*. *Experimental Gerontology* 35:1157-64

Pizzi WJ, Jersey RM. 1992. Effects of prenatal diphenylhydantoin treatment on reproductive outcome, development, and behavior in rats. *Neurotoxicology and Teratology* 14:111-7

Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. 2003. The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology* 66:1499-503

Potschka H, Loscher W. 2001. In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* 42:1231-40

Prandota J. 2004. Possible pathomechanisms of sudden infant death syndrome: key role of chronic hypoxia, infection/inflammation states, cytokine irregularities, and metabolic trauma in genetically predisposed infants. *American Journal of Therapeutics* 11:517-46

Putnam CD, Arvai AS, Bourne Y, Tainer JA. 2000. Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. *Journal of Molecular Biology* 296:295-309

Radogna F, Sestili P, Martinelli C, Paolillo M, Paternoster L, et al. 2009. Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. *Toxicology & Applied Pharmacology* 238:170-7

Ramkissoon A, Wells PG. 2011. Human prostaglandin H synthase (hPHS)-1- and hPHS-2-dependent bioactivation, oxidative macromolecular damage and cytotoxicity of dopamine, its precursor and metabolites. *Free Radical Biology and Medicine* 50:295-304

Raucy JL, Carpenter SJ. 1993. The expression of xenobiotic-metabolizing cytochromes P450 in fetal tissues. *Journal of Pharmacological and Toxicological Methods* 29:121-8

Ravanat JL, Douki T, Duez P, Gremaud E, Herbert K, et al. 2002. Cellular background level of 8-oxo-7,8-dihydro-2′-deoxyguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up. *Carcinogenesis* 23:1911-8

Reed DJ. 1986. Regulation of reductive processes by glutahione. *Biochemical Pharmacology* 35:7-13

Reimer DL, Bailley J, Singh SM. 1994. Complete cDNA and 5' genomic sequences and multilevel regulation of the mouse catalase gene. *Genomics* 21:325-36

Reuner G, Hassenpflug A, Pietz J, Philippi H. 2009. Long-term development of low-risk low birth weight preterm born infants: neurodevelopmental aspects from childhood to late adolescence. *Early Human Development* 85:409-13

Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, Woo HA. 2005. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Current Opinion in Cell Biology* 17:183-9

Riley RJ, Roberts P, Kitteringham NR, Park BK. 1990. Formation of cytotoxic metabolites from phenytoin, imipramine, desipramine, amitriptyline, and mianserin by mouse and human hepatic microsomes. *Biochemical Pharmacology* 39:1951-8

Roberts CJ, Lowe CR. 1975. Where have all the conceptions gone? *Lancet* 1:498-9

Roberts G, Bellinger D, McCormick MC. 2007. A cumulative risk factor model for early identification of academic difficulties in premature and low birth weight infants. *Maternal & Child Health Journal* 11:161-72

Rodier PM. 1980. Chronology of neuron development: Animal studies and their clinical implications. *Developmental Medicine and Child Neurology* 22:525-45

Rogawski MA, Porter RJ. 1990. Antiepileptic drugs: Pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. *Pharmacological Review* 42:233-86

Rolfe BE. 1982. Detection of fetal wastage. *Fertility & Sterility* 37:655-60

Rosenblum DA, Volpe MV, Dammann CEL, Lo YS, Thompson JF, Nielson HC. 1998. Expression and activity of epidermal growth factor receptor on late fetal rat lung are cell- and sex-specific. *Experimental Cell Research* 239:69-81

Rowley DA, Halliwell B. 1983. Formation of hydroxyl radicals from hydrogen peroxide and iron salts by superoxide- and ascorbate-dependen mechanisms: relevance to the pathology of rheumatoid disease *Clinical Science* 64:649-53

Salomon DS, Pratt RM. 1979. Involvement of glucucorticoids in the development of the secondary palate. *Differentiation* 13:141-54

Sauer H, Gunther J, Hescheler J, Wartenberg M. 2000. Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. *American Journal of Pathology* 156:151-8

Sauer H, Wartenberg M, Hescheler J. 2001. Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cellular Physiology and Biochemistry* 11:173-86

Savaskan NE, Ufer C, Kühn H, Borchert A. 2007. Molecular biology of glutathione peroxidase 4: from genomic structure to developmental expression and neural function. *Biological Chemistry* 388:1007-17

Scolnik D, Nulman I, Rovet J, Gladstone D, Czuchta D, et al. 1994. Neurodevelopment of children exposed in utero to phenytoin and carbamazepine monotherapy. *JAMA* 271:767-70

Shaffer JB, Preston KE. 1990. Molecular analysis of an acatalasemic mouse mutant. *Biochemical and Biophysical Research Communications* 173:1043-50

Shaffer JB, Preston KE, Shepard BA. 1990. Nucleotide and deduced amino acid sequences of mouse catalase: molecular analysis of a low activity mutant. *Nucleic Acids Research* 18:4941

Shaffer JB, Sutton RB, Bewley GC. 1987. Isolation of a cDNA clone for murine catalase and analysis of an acatalasemic mutant. *Journal of Biological Chemistry* 262:12908-11

Shanks MJ, Wiley MJ, Kubow S, Wells PG. 1989. Phenytoin teratogenicity: role of enzymatic bioactivation in a murine embryo culture model. *Teratology* 40:311-20

Simon HU, Haj-Yehia A, Levi-Schaffer F. 2000. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5:415-8

Simpson JE, Ince PG, Haynes LJ, Theaker R, Gelsthorpe C, et al. 2010. Population variation in oxidative stress and astrocyte DNA damage in relation to Alzheimer-type pathology in the ageing brain. *Neuropathology & Applied Neurobiology* 36:25-40

Singh M, Shah GL. 1989. Teratogenic effects of phenytoin on chick embryos. *Teratology* 40:453-8

Sivan E, Lee YC, Wu YK, Reece EA. 1997. Free radical scavenging enzymes in fetal dysmorphogenesis among offspring of diabetic rats. *Teratology* 56:343-9

Solaini G, Landi L, Pasquali P, Rossi CA. 1987. Protective effect of endogenous Coenzyme Q on both lipid peroxidation and respiratory chain inactivation induced by an adriamycin-iron complex. *Biochemical & Biophysical Research Communications* 147:572-80

Strand FL, Rose KJ, King JA, Sergarra AC, Zuccarelli LA. 1989. ACTH modulation of nerve development and regeneration. *Progress in Neurobiology* 33:45-85

Strickler SM, Dansky LV, Miller MA, Seni MH, Andermann E, Spielberg SP. 1985. Genetic predisposition to phenytoin-induced birth defects. *Lancet* 2:746-9

Takahara S. 1952. Progressive oral gangrene probably due to lack of catalase in the blood (acatalasaemia); report of nine cases. *Lancet* 2:1101-4

Takahara S. 1971. Actalasemia and hypocatalasemia in the Orient. *Seminars in Hematology* 8:397-416

Theodoropoulos T, Fang S, Azizi F, Ingbar S, H, Vagenakis A, G, Braverman L, E. 1980. Effect of diphenylhydantoin on hypothalmic-pituitary-thyroid function in the rat. *Americal Journal of Physiology* 239:E468-E73

Tishler DM, Weinberg KT, Hinton DR, Barbaro N, Annett GM, Raffel C. 1995. MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* 36:1-6

Tsai CE, Daood MJ, Lane RH, Hansen TWR, Gruetzmacher EM, Watchko JF. 2002. P-glycoprotein expression in mouse brain increases with maturation. *Biology of the Neonate* 81:58-64

Turleau C, De Grouchy J, Tournade M, Gagnadoux M, Junien C. 1984. Del 11p/aniridia complex. Report of three patients and review of 37 observations from the literature. *Clinical Genetics* 26:356-62

Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Molecular & Cellular Biochemistry* 266:37-56

Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease *International Journal of Biochemistry and Cell Biology* 39:44-84

Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions* 160:1-40

van den Dobbelsteen DJ, Nobel CS, Schlegel J, Cotgreave IA, Orrenius S, Slater AF. 1996. Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *Journal of Biological Chemistry* 271:15420-7

Van Dyke D, Hodge SE, Heide F, Hill LR. 1988. Family studies in fetal phenytoin exposure. *Journal of Pediatrics* 113:301-6

Vanoverloop D, Schnell RR, Harvey EA, Holmes LB. 1992. The effects of prenatal exposure to phenytoin and other anticonvulsants on intellectual function at 4 to 8 years of age. *Neurotoxicology and Teratology* 14:329-35

Varga SJ, Matkovics B, Pataki L, Molnar A, Novak Z. 1985. Comparison of antioxidant red blood cell enzymes in premature and full-term neonates. *Clinica Chimica Acta* 147:191-5

von Sonntag C. 1987. *The chemical basis of radiation biology*. New York: Taylor and Francis

Vorhees CV. 1983. Fetal anticonvulsant syndrome in rats: dose- and period-response relationships of prenatal diphenylhydantoin, trimethadione and phenobarbital exposure on the structural and functional development of the offspring. *Journal of Pharmacology and Experimental Therapeutics* 227:274-87

Vorhees CV. 1987. Fetal hydantoin syndrome in rats: dose-effect relationships of prenatal phenytoin on postnatal development and behavior. *Teratology* 35:287-303

Vorhees CV. 1994. Developmental neurotoxicity induced by therapeutic and illicit drugs. *Environmental Health Perspectives* 102:145-53

Vorhees CV, Rindler JM, Minck DR. 1990. Effects of exposure period and nutrition of the developmental neurotoxicity of anticonvulsants in rats: short and long-term effects. *Neurotoxicology* 11:273-84

Vuillaume M, Calvayrac R, Best-Belpomme M, Tarroux P, Hubert M, et al. 1986. Deficiency in catalase activity of xeroderma pigmentosum cell and simian virus 40-transformed human cell extracts. *Cancer Research* 46:538-44

Vuillaume M, Decroix Y, Calvayrac R, Vallot R, Best-Belpomme M. 1983. Xeroderma pigmentosum: H_2O_2 formation and catalytic activity during the evolution of the skin cells: application to an early diagnosis and to a tentative tretament. *Comptes Rendus des Seances de l Academie des Sciences* 296:845-50

Wang DH, Ishii K, Zhen LX, Taketa K. 1996. Enhanced liver injury in acatalasemic mice following exposure to carbon tetrachloride. *Archives of Toxicology* 70:189-94

Wang Y, Zhang SXL, Gozal D. 2010. Reactive oxygen species and the brain in sleep apnea. *Respiratory Physiology & Neurobiology* 174:307-16

Watson RE, DeSesso JM, Hurtt ME, Cappon GD. 2006. Postnatal growth and morphological development of the brain: a species comparison. *Birth Defects Research Part B* 77:471-84

Wells PG, Bhuller Y, Chen CS, Jeng W, Kasapinovic S, et al. 2005. Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species. *Toxicology & Applied Pharmacology* 207:354-66

Wells PG, Gesicki JB. 1984. Modulation of phenytoin teratogenicity by exogenous and endogenous inhibitors of oxidative drug metabolism. *Proceedings of the 9th International Congress of Pharmacology*, p. 1772

Wells PG, Kim PM, Nicol CJ, Parman T, Winn LM. 1997. Reactive Intermediates. In *Handbook of Experimental Pharmacology: Drug Toxicity in Embryonic Development*, ed. KR J, GP Daston, pp. 453-518: Springer-Verlag, Heidelberg

Wells PG, Kupfer A, Lawson JA, Harbison RD. 1982. Relation of *in vivo* drug metabolism to stereoselective fetal hydantoin toxicity in mouse: evaluation of mephenytoin and its metabolite nirvanol. *Journal of Pharmacology and Experimental Therapeutics* 221:228-34

Wells PG, Lee CJJ, McCallum GP, Perstin J, Harper P. 2010a. Receptor- and reactive intermediate-mediated mechanisms of teratogenesis. In *Handbook of Experimental Pharmacology*, ed. JP Uetrecht, pp. 131-62: Springer, Heidelberg

Wells PG, Mackenzie PI, Roy Chowdhury J, Guillemette C, Gregory PA, et al. 2004. Glucuronidation and the UDP-glucuronosltransferases in drug theray and disease. *Drug Metabolism and Disposition* 32:281-90

Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJJ, et al. 2009. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits and cancer. *Toxicological Sciences* 108:4-18

Wells PG, McCallum GP, Lam KCH, Henderson JT, Ondovcik SL. 2010b. Oxidative DNA damage and repair in teratogenesis and neurodevelopmental deficits. *Birth Defects Research Part C: Embryo Today: Reviews* 90:103-9

Wells PG, Nagai MK, Spano Greco G. 1989. Inhibition of trimethadione and dimethadione teratogenicity by the cyclooxygenase inhibitor acetylsalicylic acid: a unifying hypothesis for the teratologic effects of hydantoin anticonvulsants and structurally related compounds. *Toxicology and Applied Pharmacology* 97:406-14

Wells PG, Vo HP. 1989. Effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate on phenytoin-induced embryopathy in mice. *Toxicology and Applied Pharmacology* 97:398-405

Wells PG, Winn LM. 1996. Biochemical toxicology of chemical teratogenesis. *Critical Reviews in Biochemistry and Molecular Biology* 31:1-40

Wells PG, Winn LM. 2010. Developmental Toxicology, Section 1: Concepts. In *Chapter 40. The role of biotransformation in developmental toxicity. In: Comprehensive Toxicology, 2nd edition, CA McQueen (ed.)*, ed. GP Daston, TB Knudsen, p. (in press). Oxford: Elsevier Ltd.

Wide K, Henning E, Tomson T, Winbladh B. 2002. Psychomotor development in preschool children exposed to antiepileptic drugs in utero. *Acta Paediatrica* 91:409-14

Wieacker P, Mueller CR, Maycrova KH, Grzeschik KH, Rogers HH. 1980. Assignment of the gene coding for human catalase to the short arm of chromosome 11. *Annales de Genetique* 23:73-7

Winn LM, Wells PG. 1995a. Free radical-mediated mechanisms of chemical teratogenesis. *European Journal of Neurology* 2:5-29

Winn LM, Wells PG. 1995b. 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. *Molecular Pharmacology* 48:112-20

Winn LM, Wells PG. 1997. Evidence for embryonic prostaglandin H synthase-catalysed bioactivation and reactive oxygen species-mediated oxidation of macromolecules in phenytoin and benzo[a]pyrene teratogenesis. *Free Radical Biology and Medicine* 22:607-21

Winn LM, Wells PG. 1999. Maternal administration of superoxide dismutase and catalase in phenytoin teratogenicity. *Free Radical Biology and Medicine* 26:266-74

Winn LM, Wells PG. 2002. Evidence for Ras-dependent signal transduction in phenytoin teratogenicity. *Toxicology and Applied Pharmacology* 184:144-52

Wong AW, McCallum GP, Wells PG. 2008. Oxoguanine glycosylase 1 protects against methamphetamine-enhanced fetal brain oxidative DNA damage and neurodevelopmental deficits. *Journal of Neuroscience* 28:9047-54

Wong M, Helston LMJ, Wells PG. 1989. Enhancement of murine phenytoin teratogenicity by the γ-glutamylcysteine synthetase inhibitor L-buthionine-(SR)-sulfoximine and by the glutathione depletor diethyl maleate. *Teratology* 40:127-41

Xu D, Rovira II, Finkel T. 2002. Oxidants painting the cysteine chapel: redox regulation of PTPs. *Developmental Cell* 2:251-2

Yamada K, Ito A, Watanabe H, Takahashi T, Basaran NH, Gotoh T. 1997. High sensitivity to hepato-tumorigenesis in hypocatalasemic C3H/C(s)b/Gen mice exposed to low doses of 252Cf fission neutrons and 60Co gamma-rays. *Anticancer Research* 17:2041-7

Yang CY, Lin MT. 2002. Oxidative stress in rats with heatstroke-induced cerebral ischemia. *Stroke* 33:790-4

Yu WK, Wells PG. 1995. Evidence for lipoxygenase-catalyzed bioactivation of phenytoin to a teratogenic reactive intermediate: in vitro studies using linoleic acid-dependent soybean lipoxygenase, and in vivo studies using pregnant CD-1 mice. *Toxicology and Applied Pharmacology* 131:1-12

APPENDICES

I. SUPPLEMENTAL FIGURES FOR STUDY 1:

EMBRYOPROTECTIVE ROLE OF ENDOGENOUS CATALASE IN ACATALASEMIC AND HUMAN CATALASE-EXPRESSING MOUSE EMBRYOS EXPOSED IN CULTURE TO DEVELOPMENTAL AND PHENYTOIN-ENHANCED OXIDATIVE STRESS

Running title: Protection by embryonic catalase

Julia P. Abramov[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Figure S-1.

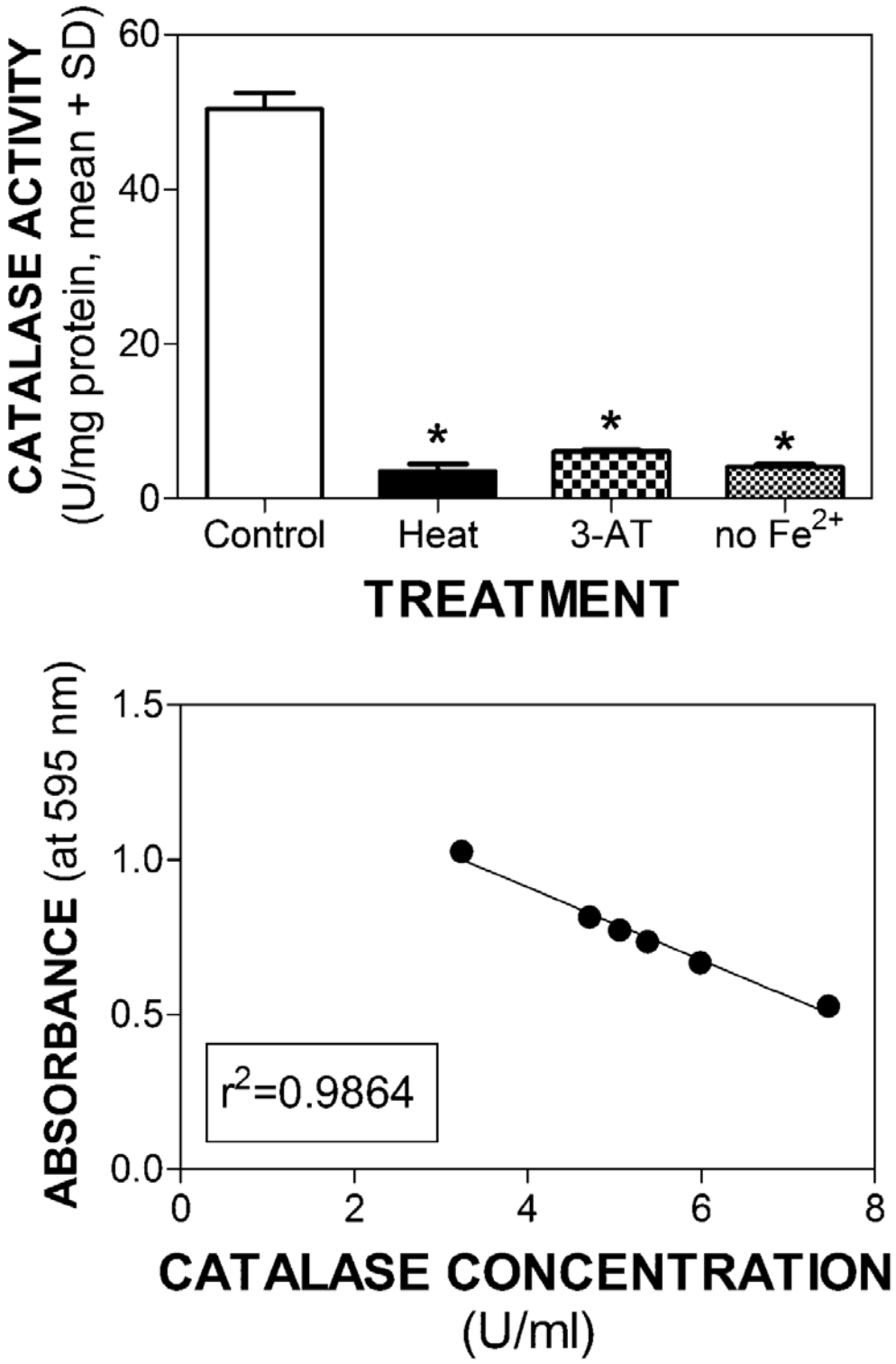

CATALASE ACTIVITY
(U/mg protein, mean + SD)
60
40
20
0
*
*
*
Control
Heat
3-AT
no Fe2+
TREATMENT
ABSORBANCE (at 595 nm)
1.5
1.0
0.5
0.0
r2=0.9864
0
2
4
6
8
CATALASE CONCENTRATION
(U/ml)

Figure S-1. Various controls for the FOX assay measuring catalase activity.

Catalase activity was measured using the FOX assay[1,2] modified for catalase measurement by using an additional catalase standard curve. ***Upper panel****:* Various controls were used to demonstrate that this assay measured the decomposition of H_2O_2 catalyzed only by catalase. Embryonic gestational day (**GD**) 12 samples from CD-1 mice were used, with 5 samples for each group. To confirm the role of catalase, three controls were tested: (1) samples were heat-inactivated at 60°C for 20 min to deactivate the catalase protein; (2) the catalase inhibitor 3-AT (3-amino-1,2,4-triazol, 20 mM) was added to the incubation; or, (3) iron ions (Fe^{2+}) were omitted from the reaction mixture to control for endogenous iron interferences. Asterisks indicate a difference from the control ($p<0.05$). ***Lower panel****:* A high correlation was seen between different catalase concentrations and the absorbance readings at 595 nm ($p<0.05$), indicating that the decomposition of H_2O_2 that was observed by the decrease in the absorption values was catalyzed by catalase alone.

Another antioxidative enzyme, glutathione peroxidase (**GPx**), can catalyze the decomposition of H_2O_2 in the cell, therefore the above controls were employed to confirm that this assay was specific to catalase alone. Also, since GPx requires the addition of the co-factor glutathione (**GSH**) for catalytic activity, and this assay did not include GSH, GPx did not play a role in the observed removal of H_2O_2. Also, GPx-dependent removal of H_2O_2 is saturable at high concentrations[3], and the assay herein required the addition of 200 μM of H_2O_2. At this higher concentration of H_2O_2, removal is dependent primarily upon catalase.

---

[1] Gupta, B. L. (1973). Microdetermination techniques for H_2O_2 in irradiated solutions. *Journal of Microchemistry* **18**, 363-374.

[2] Ou, P., and Wolff, S. P. (1996). A discontinuous method for catalase determination at "near physiological" concentrations of H_2O_2 and its application to the study of H_2O_2 fluxes within cells. *Journal of Pharmacology and Experimental Therapeutics* **277**, 945-953.

[3] Di Ilio, C., Del Boccio, G., Casalone, E., Aceto, A., and Sacchetta, P. (1986). Activities of enzymes associated with the metabolism of glutathione in fetal rat liver and placenta. *Biol Neonate* **49**, 96-101.

II. SUPPLEMENTAL FIGURES FOR STUDY 2:

ANTIOXIDATIVE EMBRYONIC CATALASE PROTECTS AGAINST PHENYTOIN-ENHANCED OXIDATIVE DNA DAMAGE AND TERATOGENESIS IN ACATALASEMIC AND HUMAN CATALASE-EXPRESSING MICE

Running title: Embryoprotective role of catalase

Julia P. Abramov[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Figure S-1.

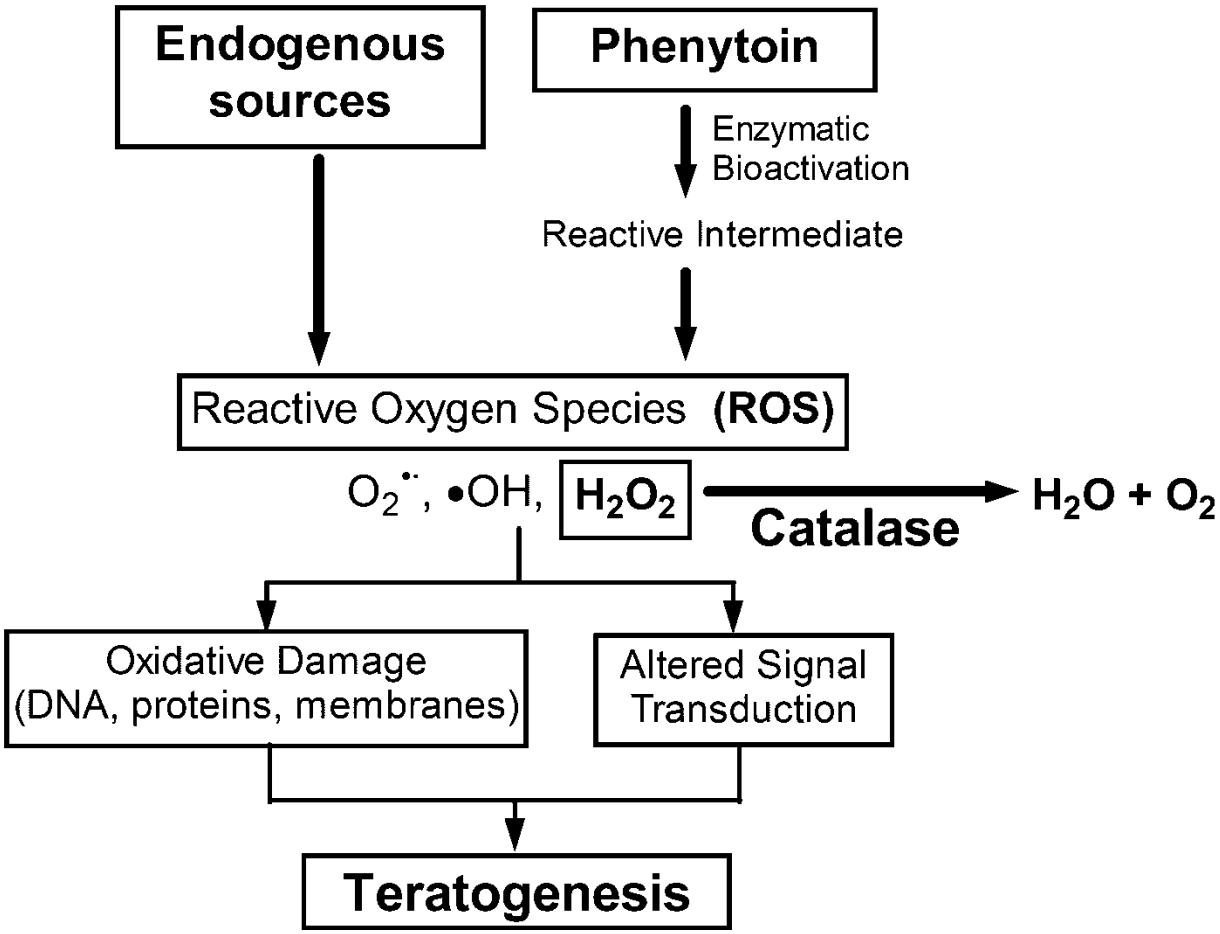

Endogenous sources
Phenytoin
Enzymatic Bioactivation
Reactive Intermediate
Reactive Oxygen Species (ROS)
$O_2^{\bullet-}$, •OH, H_2O_2
Catalase
$H_2O + O_2$
Oxidative Damage (DNA, proteins, membranes)
Altered Signal Transduction
Teratogenesis

Figure S-1. Postulated embryoprotective role of catalase.

Toxic reactive oxygen species (**ROS**) such as hydrogen peroxide (H_2O_2) are formed endogenously and can be substantially enhanced by xenobiotic stimulation of endogenous pathways (e.g. phenytoin, methamphetamine) and/or bioactivation to free radical intermediates. The highly reactive ROS can alter signal transduction and/or cause irreversible oxidative damage to cellular macromolecules (DNA, proteins, lipid membranes), adversely affecting developmental processes. H2O2 can be detoxified by catalase, producing water and oxygen. The risk for teratogenesis is determined by the balance among pathways for embryonic ROS formation and detoxification, and repair of oxidatively damaged macromolecules.

Figure S-2.

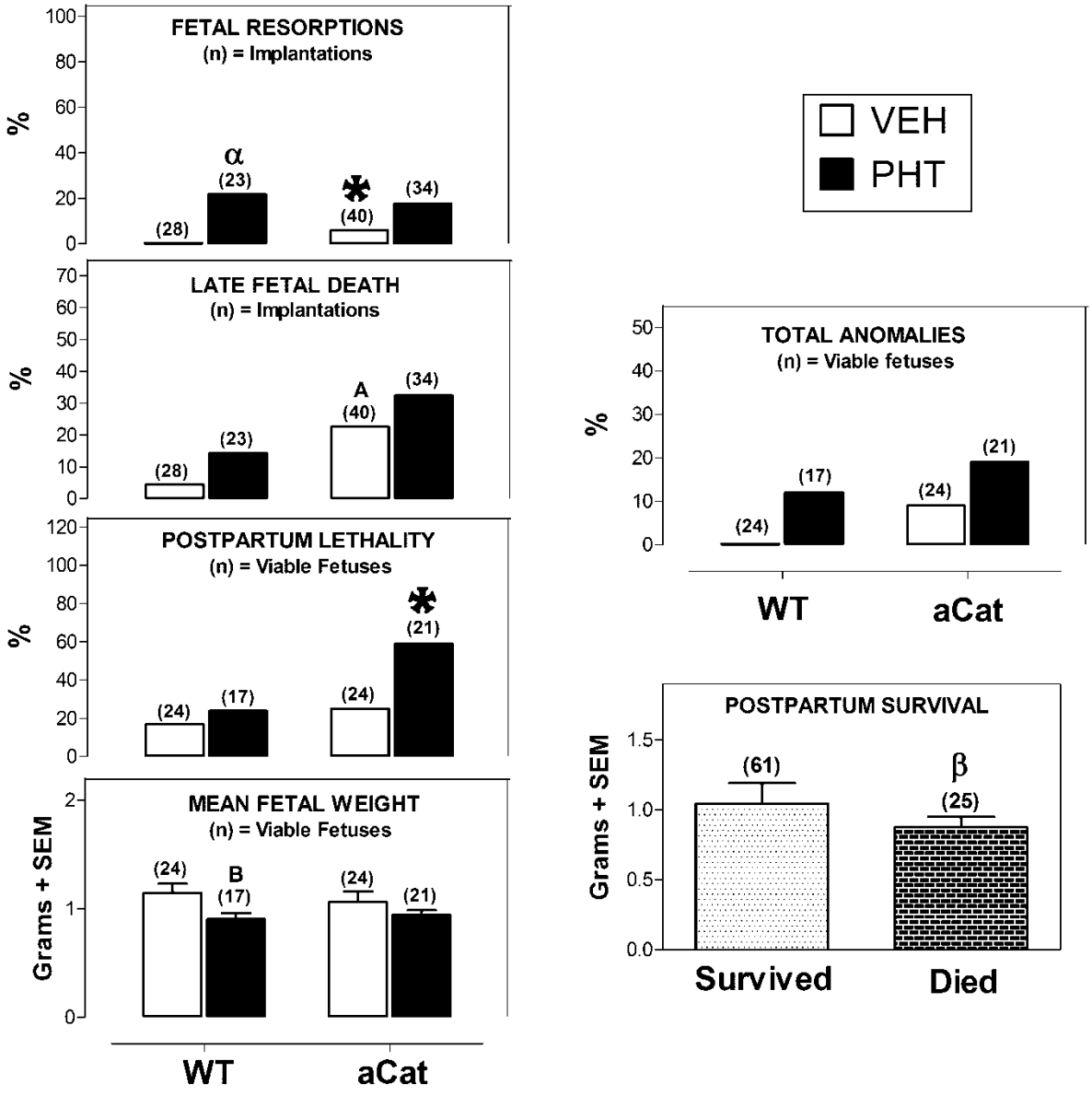

FETAL RESORPTIONS
(n) = Implantations
%
α
(23)
(28)
✱
(40)
(34)
LATE FETAL DEATH
(n) = Implantations
%
A
(40)
(34)
(23)
(28)
POSTPARTUM LETHALITY
(n) = Viable Fetuses
%
✱
(21)
(24)
(17)
(24)
MEAN FETAL WEIGHT
(n) = Viable Fetuses
Grams + SEM
(24)
B
(17)
(24)
(21)
WT
aCat
VEH
PHT
TOTAL ANOMALIES
(n) = Viable fetuses
%
(24)
(17)
(24)
(21)
WT
aCat
POSTPARTUM SURVIVAL
Grams + SEM
(61)
β
(25)
Survived
Died

Figure S-2. Embryopathies in acatalasemic mice treated with phenytoin on gestational days (GDs) 12 and 13.

Pregnant catalase-deficient (acatalasemic, **aCat**) dams were treated intraperitoneally (**i.p.**) on gestational days (**GDs**) 12 and 13 with either 65 mg/kg phenytoin (**PHT**) or its vehicle (**VEH**), and sacrificed on GD 19. Fetal resorptions (*in utero* deaths) were analyzed as described in Fig. 1. Late resorptions were the remnants of fetuses that died *in utero* sufficiently late in gestation that a dissectible mass remained. Incidence was calculated by dividing the number of typeable resorptions for a given genotype by the total number of implantations (fetuses and resorptions) for that embryonic genotype. Postpartum lethality was calculated by dividing the total number of fetuses born live and dying within 2 hr by the total number of viable fetuses. Total anomalies included kinky tail and abnormal lower jaw. The percentage was calculated by dividing the number of fetuses affected with at least one anomaly by the number of total live fetuses. For postpartum survival, mean fetal weight was calculated for all surviving and dying fetuses, independent of the genotype. The asterisk indicates a difference from wild-type (**WT**) mice in the same treatment group ($p<0.05$), with a marginal difference indicated by the letter A ($0.05<p<0.1$). The alpha symbol indicates a difference from VEH-treated mice of the same genotype ($p<0.05$), with a marginal difference indicated by the letter B ($0.05<p<0.1$). The beta symbol indicates a difference from the group that survived ($p<0.05$).

Figure S-3.

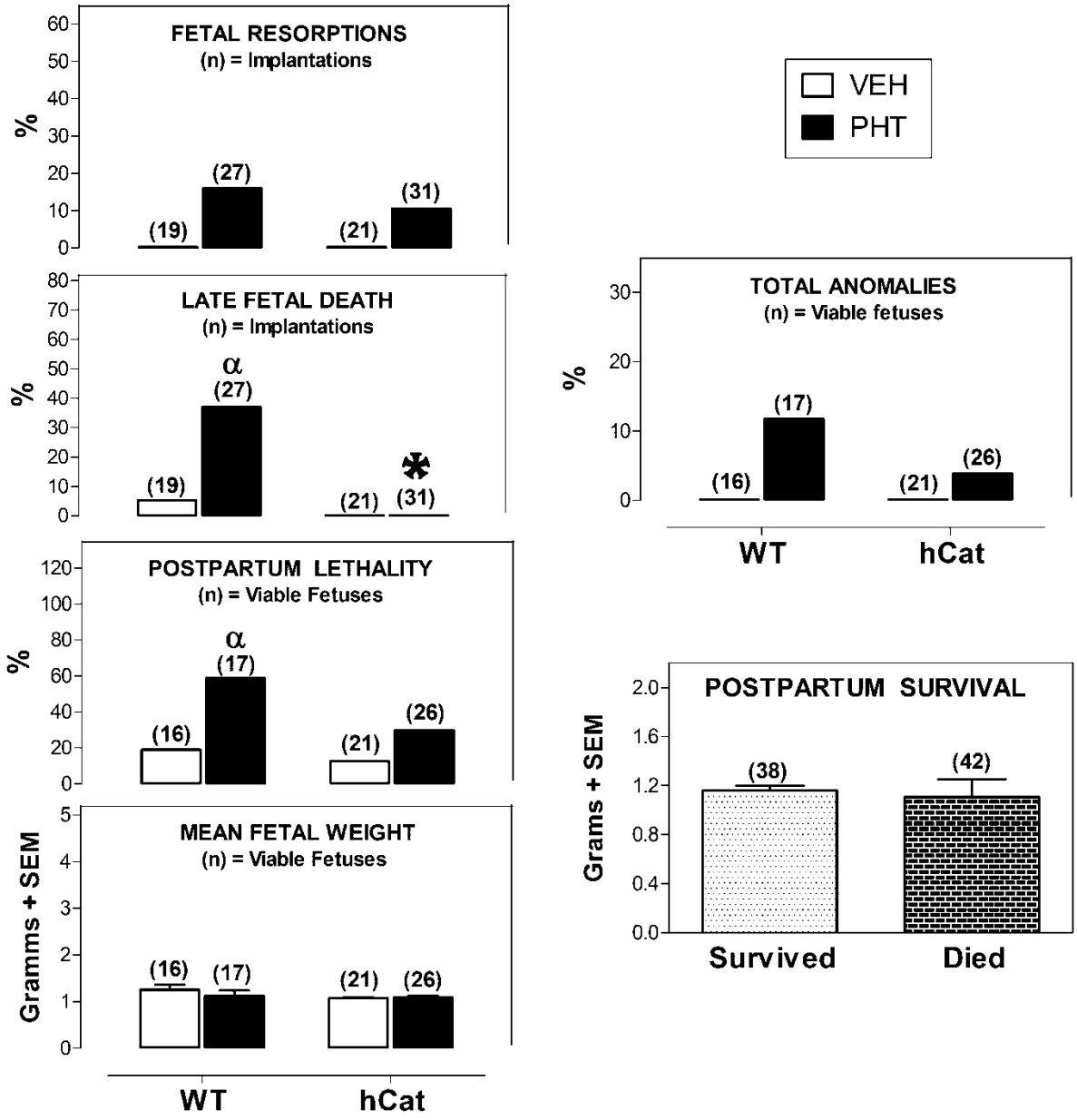

FETAL RESORPTIONS
(n) = Implantations
%
(19)
(27)
(21)
(31)
LATE FETAL DEATH
(n) = Implantations
α
*
POSTPARTUM LETHALITY
(n) = Viable Fetuses
MEAN FETAL WEIGHT
(n) = Viable Fetuses
Gramms + SEM
WT
hCat
VEH
PHT
TOTAL ANOMALIES
(n) = Viable fetuses
(16)
(17)
(21)
(26)
POSTPARTUM SURVIVAL
Grams + SEM
(38)
(42)
Survived
Died

Figure S-3. Embryopathies in mice expressing human catalase treated with phenytoin on GDs 12 and 13.

Pregnant transgenic dams expressing human catalase (**hCat**) were treated i.p. on GDs 12 and 13 with either 65 mg/kg phenytoin or its vehicle, and sacrificed on GD 19. Fetal resorptions (*in utero* deaths) were analyzed as described in Fig. 1. Late resorptions were the remnants of fetuses that died *in utero* sufficiently late in gestation that a dissectible mass remained. Incidences and mean fetal weights were calculated as described in fig. S1. Total anomalies included kinky tail and underdeveloped eye. The asterisk indicates a difference from WT mice in the same treatment group ($p<0.05$). The alpha symbol indicates a difference from VEH-treated mice of the same genotype ($p<0.05$).

Figure S-4.

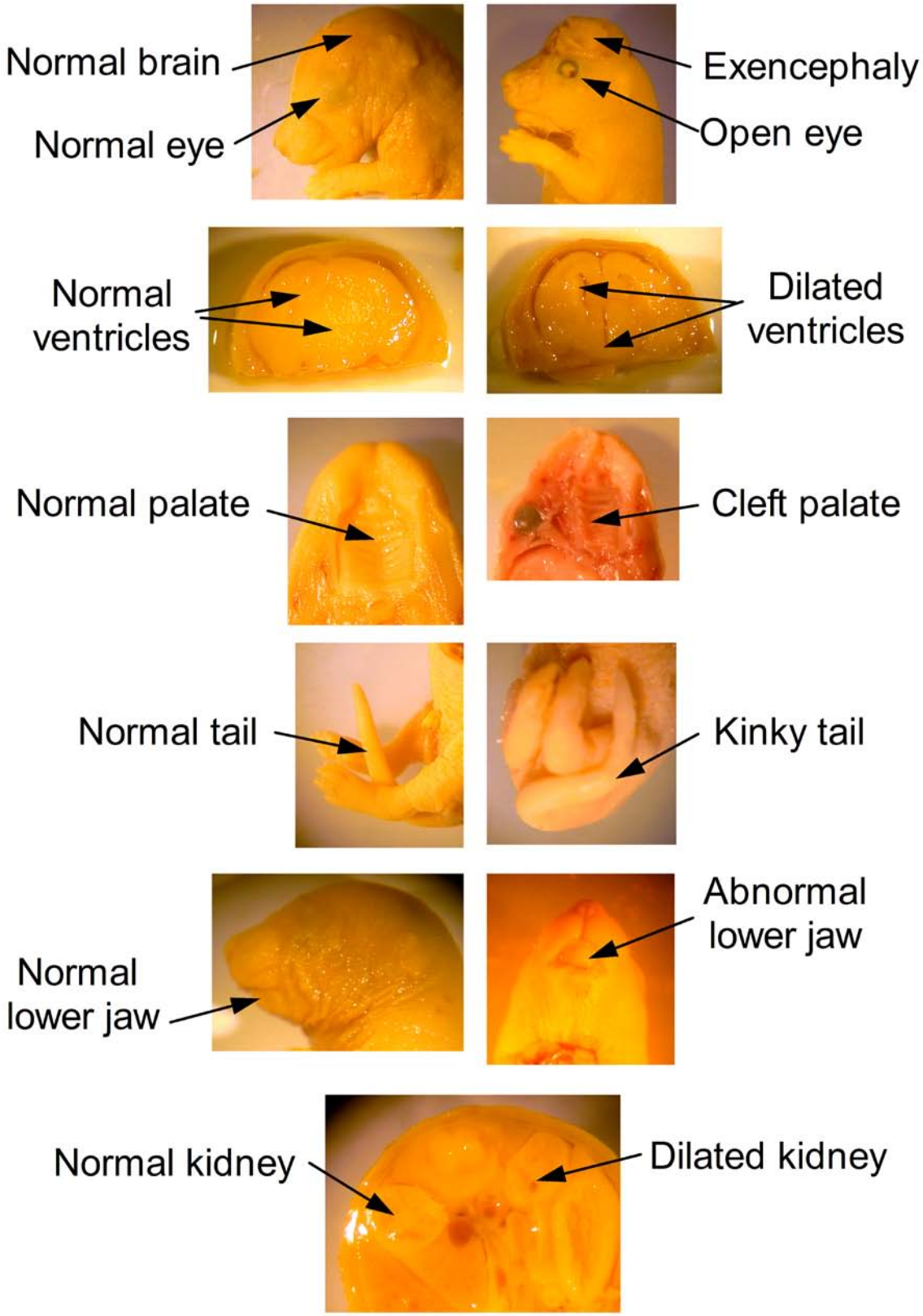

Normal brain
Normal eye
Exencephaly
Open eye
Normal ventricles
Dilated ventricles
Normal palate
Cleft palate
Normal tail
Kinky tail
Normal lower jaw
Abnormal lower jaw
Normal kidney
Dilated kidney

Figure S-4. Phenytoin embryopathies in acatalasemic mice and mice expressing human catalase.

Representative major anomalies for the data described in figs. 2, 3, S-2 and S-3.

III. SUPPLEMENTAL FIGURES FOR STUDY 3:

PROTECTIVE ROLE OF ENDOGENOUS CATALASE IN BASELINE AND PHENYTOIN-ENHANCED NEURODEVELOPMENTAL AND BEHAVIORAL DEFICITS INITIATED *IN UTERO* AND IN AGED MICE

Running title: Neuroprotective role of endogenous catalase

Julia P. Abramov[1], Audrey Tran[2], Aaron Shapiro[1] and Peter G. Wells[1,2]

[1]Faculty of Pharmacy and [2]Department of Pharmacology and Toxicology
University of Toronto
Toronto, Ontario, Canada

Figure S-1.

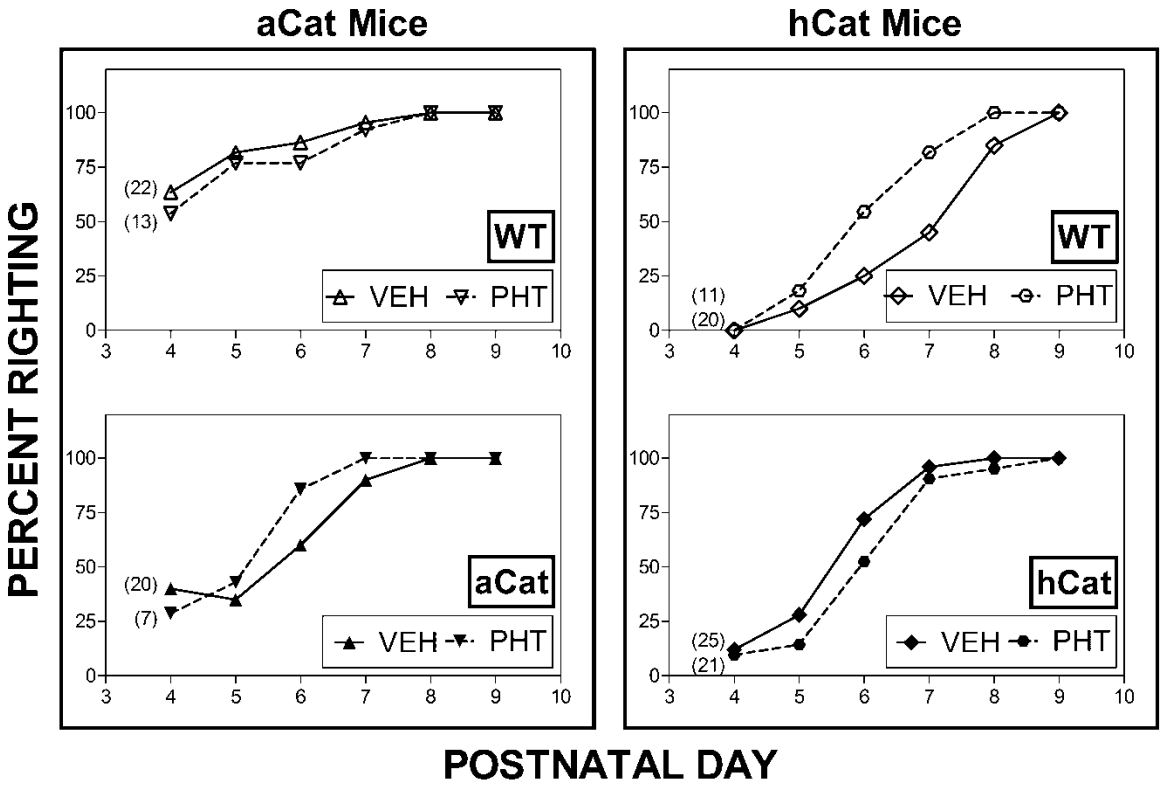

aCat Mice
hCat Mice
PERCENT RIGHTING
POSTNATAL DAY
WT
aCat
hCat
VEH
PHT
(22)
(13)
(11)
(20)
(25)
(21)
(7)
0
25
50
75
100
3
4
5
6
7
8
9
10

Figure S-1. Effect of phenytoin in aCat and hCat offspring on surface righting.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and the percent of animals in each group successfully righting themselves was recorded. The number of pups in each group is given in parentheses.

Figure S-2.

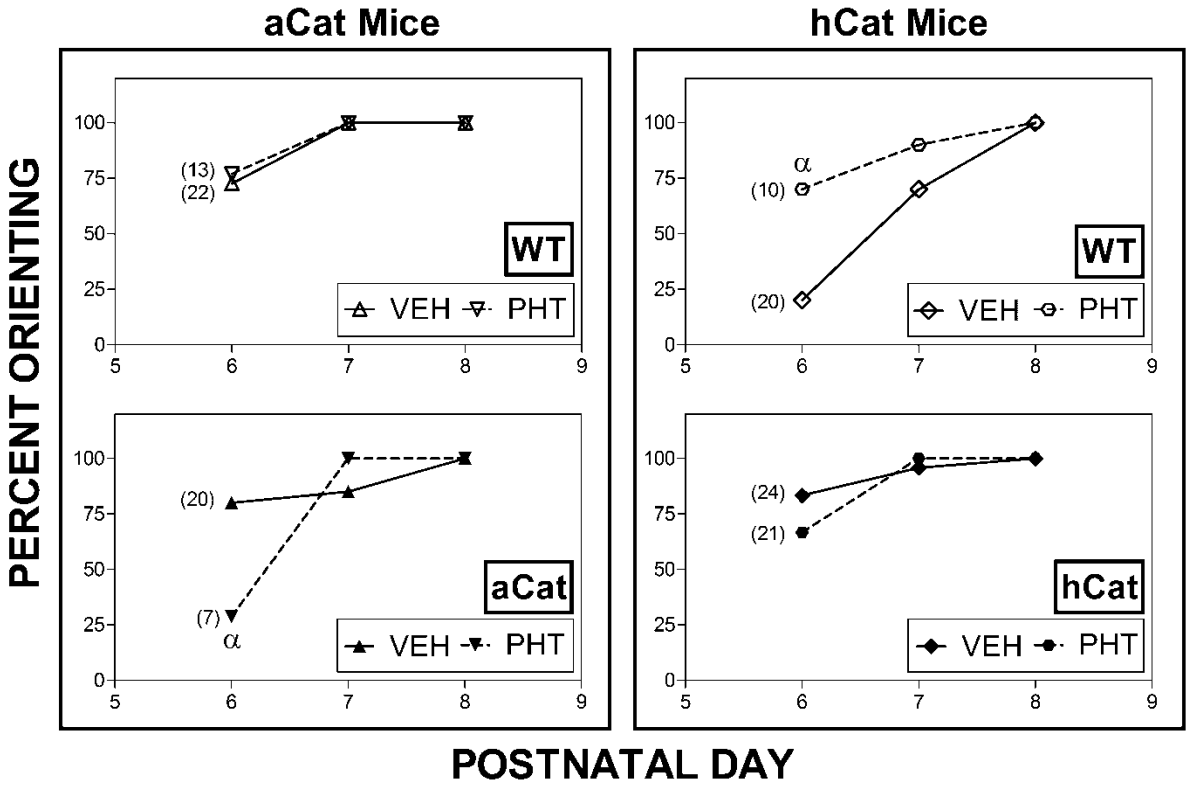

aCat Mice
hCat Mice
PERCENT ORIENTING
POSTNATAL DAY
WT
aCat
hCat
VEH
PHT
(13)
(22)
(20)
(7)
α
(10)
(24)
(21)
0
25
50
75
100
5
6
7
8
9

Figure S-2. Effect of phenytoin in aCat and hCat offspring on negative geotaxis.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and the percent of animals in each group successfully orienting themselves upward on the incline was recorded. The alpha symbol indicates a difference from VEH mice in the same genotype group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure S-3.

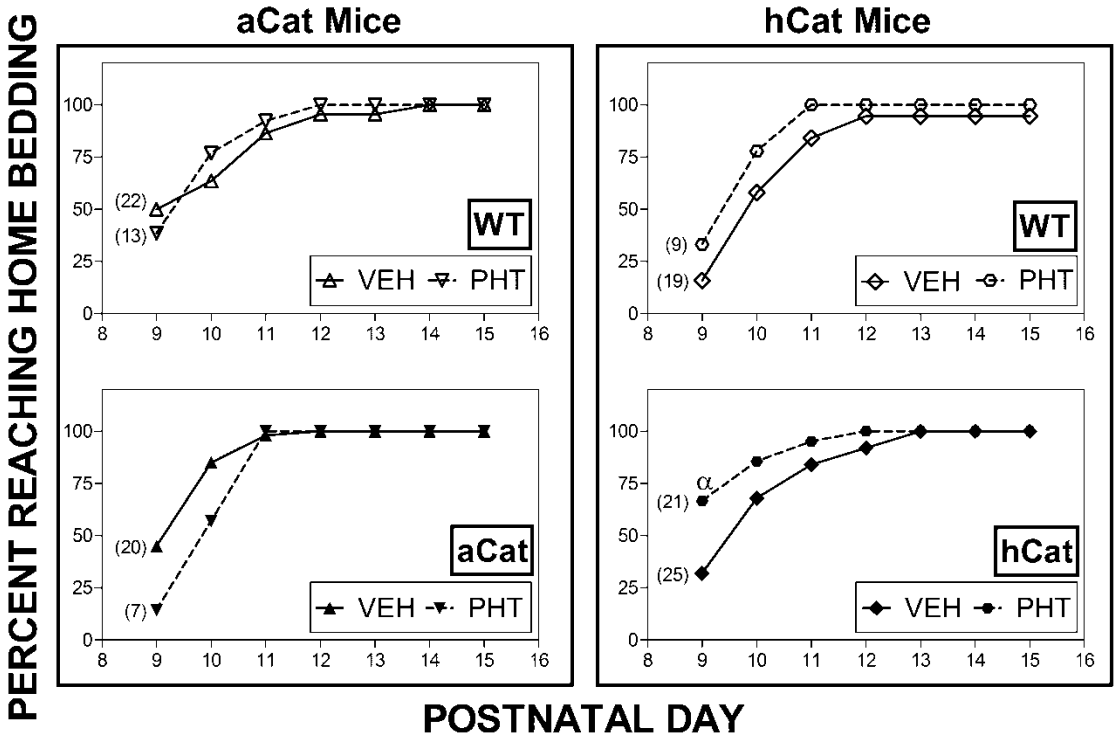

aCat Mice
hCat Mice
PERCENT REACHING HOME BEDDING
POSTNATAL DAY
WT
aCat
hCat
VEH
PHT
(22)
(13)
(9)
(19)
(20)
(7)
(21)
(25)
α

Figure S-3. Effect of phenytoin in aCat and hCat offspring on olfactory orientation.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and the percent of animals in each group successfully reaching home bedding was recorded. The alpha symbol indicates a difference from VEH mice in the same genotype group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure S-4.

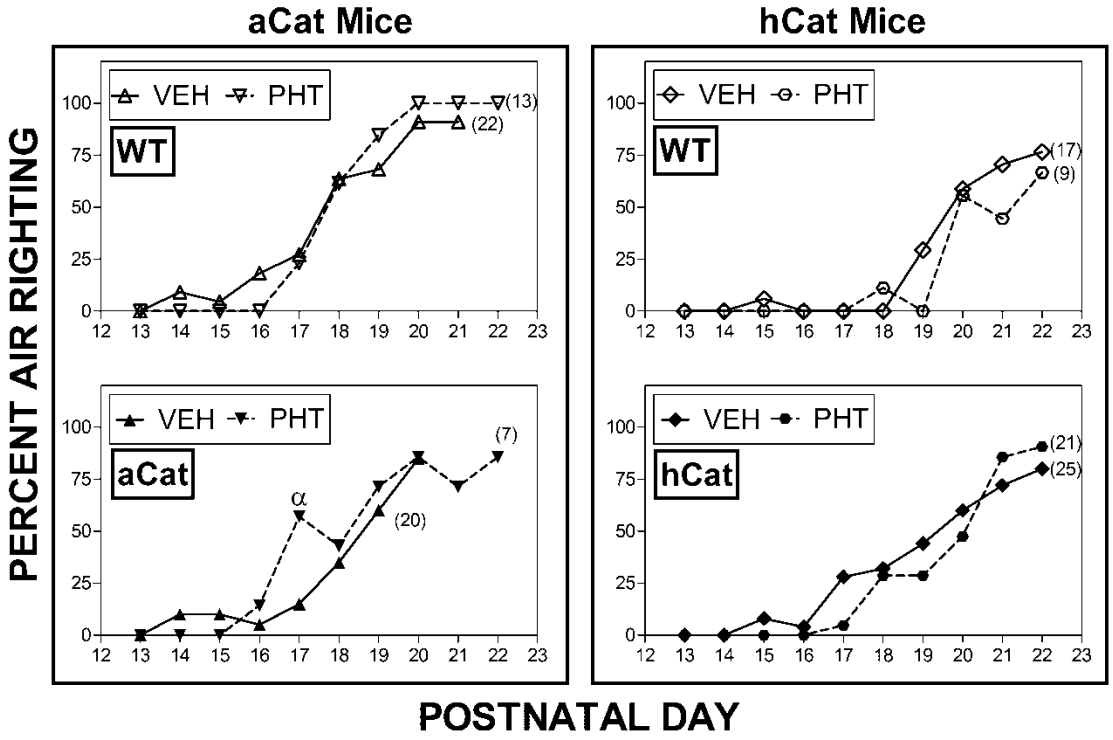

aCat Mice
hCat Mice
VEH
PHT
WT
aCat
hCat
(13)
(22)
(17)
(9)
(7)
(20)
(21)
(25)
α
PERCENT AIR Rrighting
POSTNATAL DAY

Figure S-4. Effect of phenytoin in aCat and hCat offspring on air righting.

Animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and the percent of animals in each group successfully righting themselves by the 3/3 trial criterion was recorded. The alpha symbol indicates a difference from VEH mice in the same genotype group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure S-5.

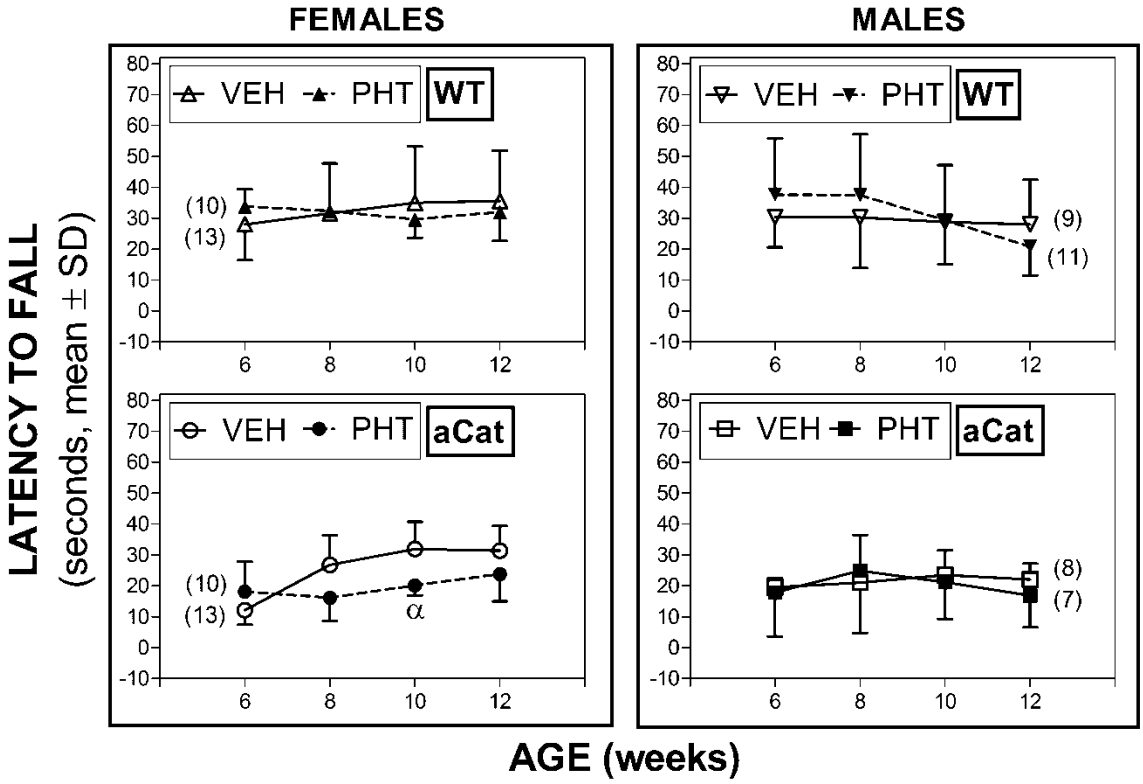

FEMALES
MALES
VEH
PHT
WT
aCat
(10)
(13)
(9)
(11)
(8)
(7)
α
LATENCY TO FALL
(seconds, mean ± SD)
AGE (weeks)

Figure S-5. Effect of phenytoin on motor coordination in male and female offspring of aCat mice.

aCat animals were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and motor coordination impairment was assessed by the rotarod test at 20 rpm beginning at 6 weeks of age. The latency or time at which the mice fell from the rod was recorded. The alpha symbol indicates a difference from VEH mice in the same genotype group ($p<0.05$). The number of pups in each group is given in parenthesis.

Figure S-6.

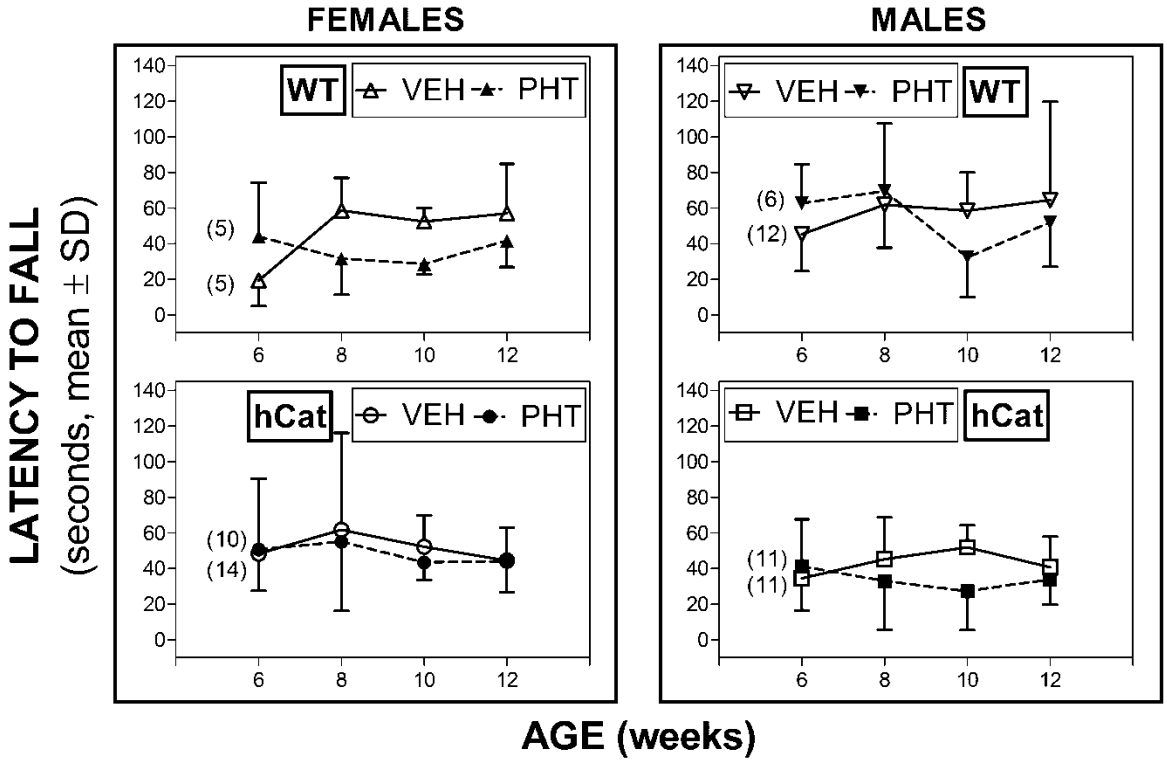

FEMALES
MALES
WT
VEH
PHT
hCat
(5)
(5)
(6)
(12)
(10)
(14)
(11)
(11)
LATENCY TO FALL
(seconds, mean ± SD)
AGE (weeks)

Figure S-6. Effect of phenytoin on motor coordination in male and female offspring of hCat mice.

hCat were exposed *in utero* to phenytoin, 55 mg/kg i.p., or its vehicle on GD 17, and motor coordination impairment was assessed by the rotarod test at 20 rpm beginning at 6 weeks of age. The latency or time at which the mice fell from the rod was recorded. The number of pups in each group is given in parenthesis.

IV. FOX ASSAY PROTOCOL

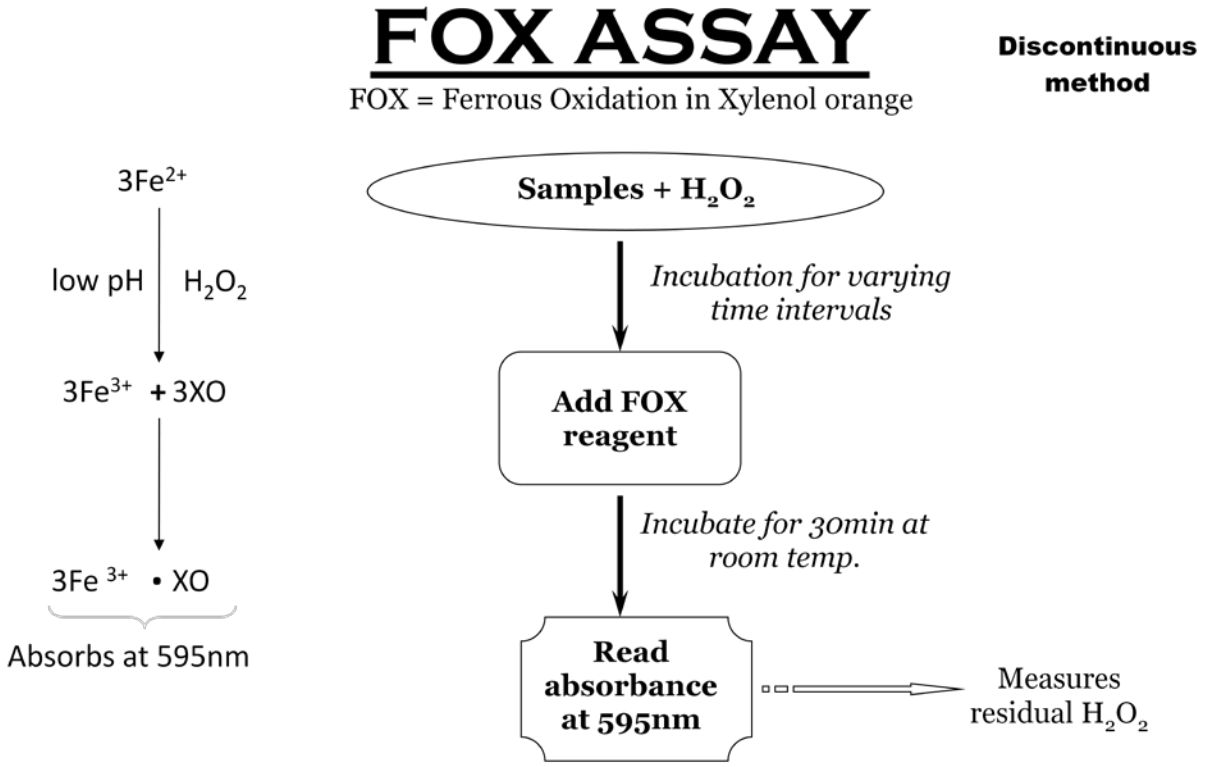


Figure 1. FOX assay technique diagram.

Assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acidic conditions. The ferric ion binds with the indicator dye Xylenol orange (3,3'-bis[N,N-di(Carboxymethyl)-aminomethyl]-o-cresolsulfone-phthalein, sodium salt) (**XO**) to form a stable colored complex which can be measured at 595 nm. Sorbitol is added for substantial chain oxidation of ferrous ions, increasing the color yield. Approximately 18 moles of Fe^{2+} are oxidized to Fe^{3+} for every mole of hydrogen peroxide present, increasing the sensitivity of the assay. This discontinuous method allows catalase to be measured in the presence of high levels of other materials as well as at low, 'near physiological' levels of H_2O_2.

From: Ou and Wolff, 1996.

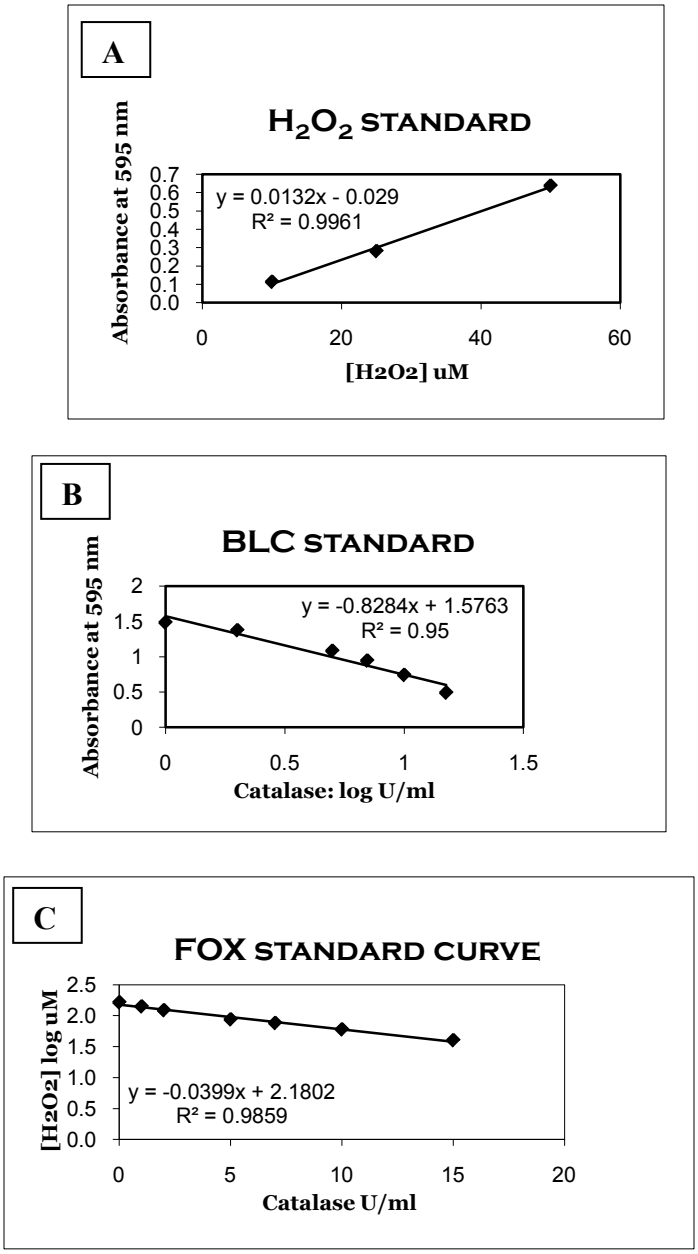


Figure 2. Construction of standard curves for the FOX assay.

A. Standard curve for H_2O_2 is constructed using various H_2O_2 standards and plotted against absorbance at 595 nm. **B.** Standard curve for bovine liver catalase (BLC) is constructed using BLC standards converted to logU/ml and plotted against absorbance at 595 nm. **C.** Standard curve for the FOX reagent is constructed using the previous two standards curves combined. Logarithmic scale was used to generate a straight line and R^2 calculation. During experiments only the linear portion of the original curve was used for calculations.

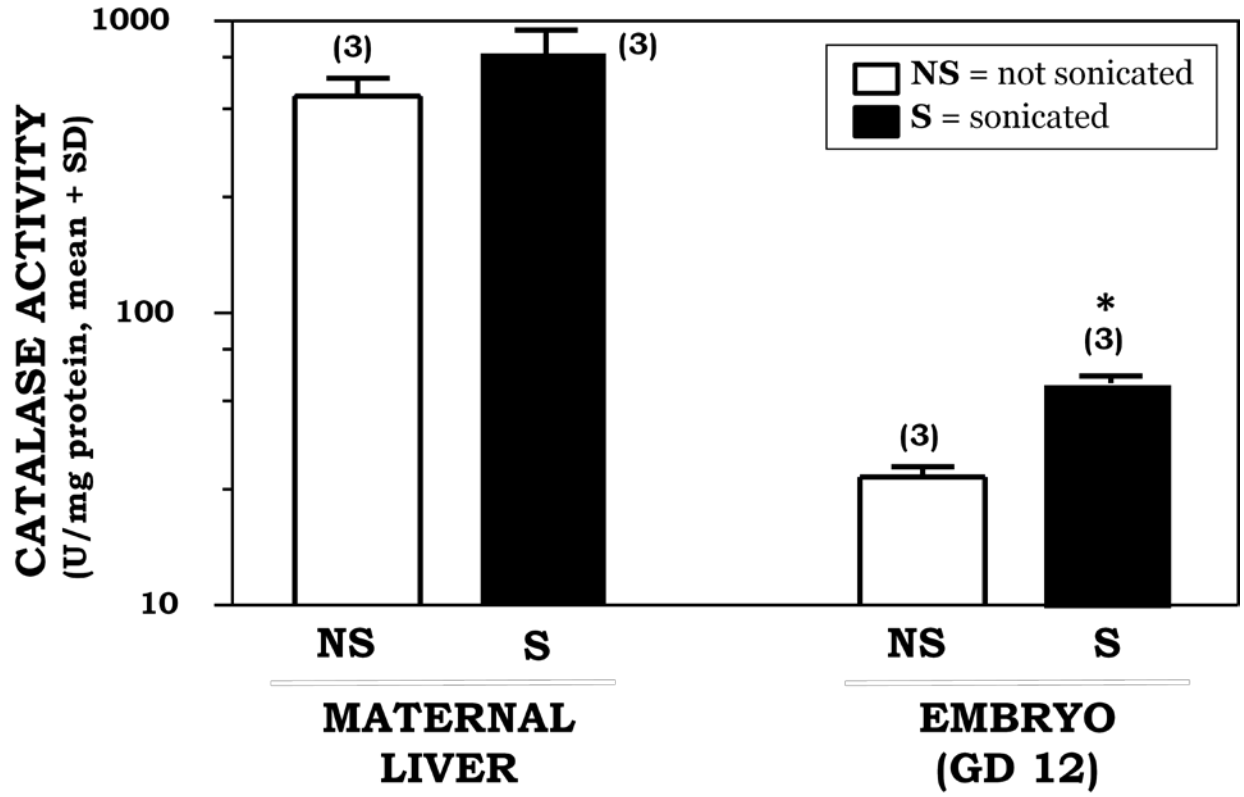


Figure 3 Effect of sonication on measurable endogenous catalase activity.

Embryonic and adult liver samples were collected from CD-1 mice on GD 12 and homogenized in PBS buffer. Homogenates were either sonicated for 15 sec on ice or not sonicated. Catalase activity was determined using the FOX assay, standardized for protein content. Asterisk indicates a difference from non-sonicated sample ($p<0.05$).

Sonication of the homogenates increased the measurable embryonic catalase activity by 47%. A smaller and non-significant increase was observed in maternal liver samples. Sonication did not change the relative embryonic activity compared to the maternal values. The increase in measurable catalase activity detected with sonication of the embryonic homogenates indicates that this procedure is helpful in releasing catalase from peroxisomes without affecting the embryonic to maternal ratio.

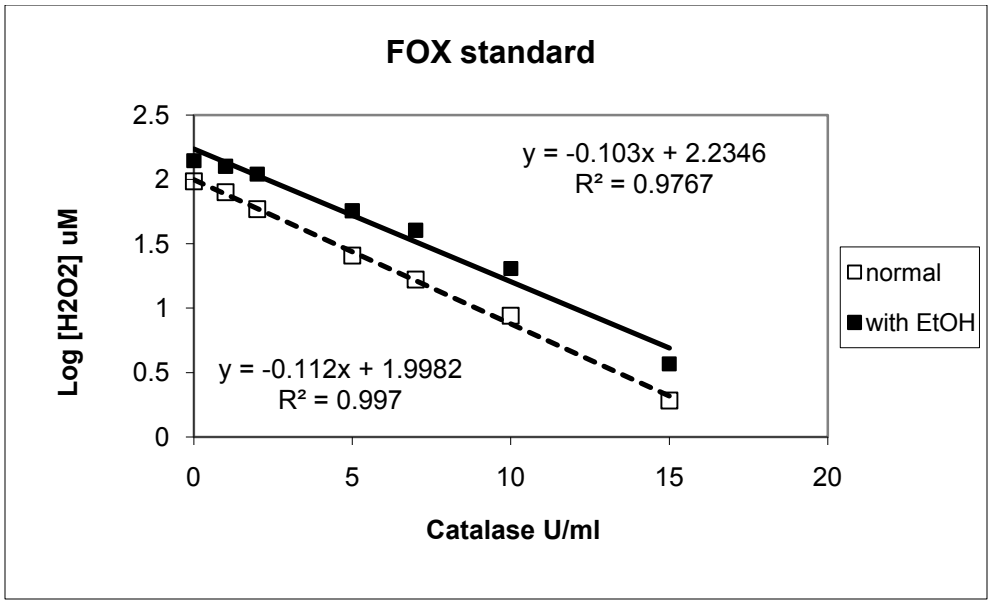


Figure 4. Effect of incubation with ethanol on measurable endogenous catalase activity.

FOX standard curve was constructed as described in fig. 2. Bovine liver catalase standards were incubated with 0.17 M ethanol (EtOH) for 30 min on ice. Ethanol provided additional source of H^+ for catalase enzyme to initiate the peroxidatic function of catalase which allows conversion from the inactive complex I to an active enzyme thus liberating it for the FOX assay.

From: Percy, 1984.