

Methanol Foundation

Grant title: **Molecular Basis of Methanol Developmental Toxicity,  
and  
Implications for Carcinogenic Potential**

# **Final Report**

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## **EXECUTIVE SUMMARY**

Methanol (**MeOH**) is teratogenic in rodents, which unlike humans use catalase to convert both methanol to formaldehyde, and its formic acid (**FA**) metabolite to carbon dioxide and water. It is not known if methanol is developmentally toxic in humans, and it is unclear if rodents can predict human risk in light of their different routes of MeOH metabolism. A more accurate model for predicting human risk might be found in monkeys, or at least in a species that is more like humans than mice in their metabolism of MeOH.

A better understanding of the molecular mechanism of MeOH teratogenesis would also help in understanding the potential human risk. One possible mechanism, involving enhanced oxidative stress and the formation of toxic reactive oxygen species (**ROS**), also would be relevant to the potential for MeOH to cause cancer, which has been suggested by one controversial study in rats.

We have found that the metabolism of MeOH in monkeys, which closely reflect humans, is more similar in rabbits than in mice. In complementary in vitro studies, we found that the activity of catalase in rabbits is only 36% of that in mice, and similar to the activity in humans, consistent with a primary role for catalase in mice in metabolizing MeOH and FA, while rabbits and humans rely upon alcohol dehydrogenase (**ADH**). These results suggest that the rabbit might be a more accurate model than the mouse for predicting the human risk for MeOH developmental toxicity.

In developmental studies, rabbits were shown for the first time to be resistant to MeOH teratogenesis, in contrast to C57BL/6 mice, which were susceptible, as had previously been shown by other laboratories. However, we also found that a different (C3H) mouse strain was resistant, providing the first evidence that not all mice are susceptible. The dose (2 g/kg i.p.) of MeOH administered to both species was high, and substantially above the lethal human dose. Given that rabbits, which more closely reflect human MeOH metabolism, and at least one strain of mice are resistant to MeOH teratogenesis, it is questionable whether the human risk for MeOH developmental toxicity can be accurately assessed in sensitive rodent models.

In mouse strains that are sensitive to the developmental toxicity of methanol, we examined the role of catalase and ROS in the embryopathic mechanism. In addition to the metabolism of MeOH by catalase in rodents, this antioxidative enzyme also detoxifies ROS (**fig. 1**). We used the following respective approaches in vivo and/or in embryo culture: (1) genetically modified mice deficient in catalase (acatalasemic, **aCat**), and transgenic mice expressing human catalase activity (high catalase activity, **hCat**); and, (2) pretreatment with the free radical spin trapping agent phenylbutylnitron (**PBN**). Although the in vivo studies were inconclusive, in embryo culture, MeOH embryopathies were enhanced in aCat mice and reduced in hCat mice, and were blocked by pretreatment with PBN. These results suggest that ROS play an important role in the mechanism of MeOH developmental toxicity in sensitive strains of mice, and that

embryonic catalase plays an important protective role via its antioxidative activity, as distinct from its peroxidative, MeOH-metabolizing role in maternal liver or the embryo.

In addition to its potential role in teratogenesis, ROS-initiated oxidatively damaged DNA, and particularly the 8-oxoguanine (**8-oxoG**) lesion, is mutagenic and carcinogenic. In a novel evaluation of adult mice, rabbits and monkeys, we found that an acute high dose (2 g/kg i.p.) of MeOH did not enhance 8-oxoG formation in any tissue including bone marrow in any species, nor did the same dose of MeOH daily in mice for 15 days. These results suggest that it is unlikely that human environmental exposure to MeOH would cause cancer via a mechanism involving oxidatively damaged DNA.

## Methanol Metabolism

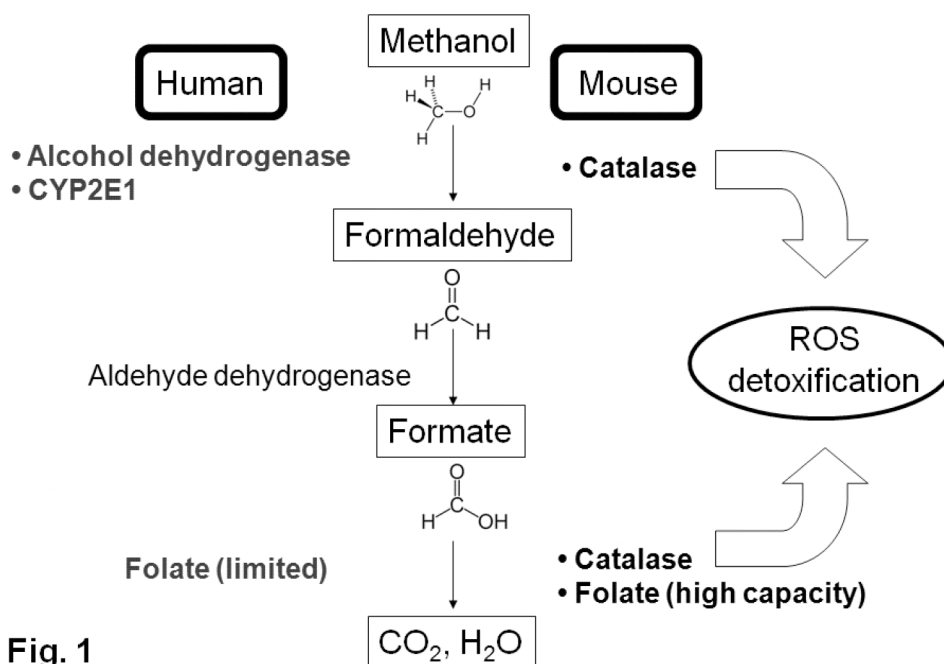


Fig. 1

Potential future studies would include:

- More complete elucidation of the molecular mechanisms of teratogenesis.
- More extensive studies in non-rodent models similar to humans.
- Effects of methanol on the developing fetal brain.
- Basis for oxidative stress in embryos but not adults.
- More rigorous evaluation of carcinogenic potential.

## **SUMMARY LIST OF PUBLICATIONS AND SUBMITTED MANUSCRIPTS**

1. Sweeting, J. N., Siu, M., McCallum, G. P., Miller, L. and Wells, P. G. Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates. **Toxicology and Applied Pharmacology** 247(1): 28-35, 2010.
2. McCallum, G. P., Siu, M., Ondovcik, S. L., Sweeting, J.N. and Wells, P. G. Methanol exposure does not lead to accumulation of oxidative DNA damage in bone marrow or spleen of mice, rabbits or primates. **Molecular Carcinogenesis**: in press, 2010.
3. Sweeting, J. N., Siu, M., Wiley, M. J. and Wells, P. G. Species- and strain-dependent teratogenicity of methanol in rabbits and mice. **Reproductive Toxicology**: in press, 2010.
4. McCallum, G. P., Siu, M., Sweeting, J.N. and Wells, P. G. Methanol exposure does not produce oxidatively damaged DNA in lung, liver or kidney of adult mice, rabbits or primates. **Toxicology and Applied Pharmacology**: in press, 2010.
5. Miller, L. and Wells, P. G. Methanol embryopathies in embryo culture in transgenic mice expressing human catalase, and mutant catalase-deficient mice. Manuscript submitted 2<sup>nd</sup> October 2010 to Toxicology and Applied Pharmacology.
6. Siu, M., Wiley, M. J. and Wells, P. G. Methanol teratogenicity in mutant catalase-deficient mice, and transgenic mice expressing human catalase. Manuscript submitted 30<sup>th</sup> September 2010 to Toxicological Sciences.
7. Miller, L. and Wells, P. G. Protection against methanol embryopathies in mouse embryo culture by the free radical spin trapping agent phenylbutylnitrone. Manuscript submitted on 22<sup>nd</sup> September 2010 to Toxicology and Applied Pharmacology, and declined by the editors for lack of mechanistic data. We will complete studies of oxidative macromolecular damage and resubmit the manuscript with these new data, likely in November or December 2010.

## **SCIENTIFIC RATIONALE**

### **MOLECULAR BASIS OF METHANOL DEVELOPMENTAL TOXICITY, and IMPLICATIONS FOR CARCINOGENIC POTENTIAL**

#### **The Problem**

Methanol appears to be metabolized quite differently in rodents such as mice than it is in humans. Nevertheless, most studies to estimate the human safety of methanol in the developing embryo and fetus (hereafter collectively referred to as the embryo) have been carried out in rodents, raising the question of the accuracy, if not the relevance, of regulations based upon such studies.

#### **Fundamental Question**

From the regulatory viewpoint, the fundamental question is whether the mechanism by which methanol harms the embryo is similar in rodents and humans. Since the mechanism of methanol toxicity in the embryo remains to be established, it is not clear whether or to what extent the reported differences between rodents and humans in the metabolism of methanol determine its relative toxicity in the embryo of these two species. It is similarly unclear whether the molecular effects of methanol in rodent embryos reflect those in the developing human.

#### **Background**

The mechanism by which methanol causes toxicity in the developing embryo and fetus is not clearly understood, but one contributing factor may be enhanced “oxidative stress”, or increased formation of highly reactive and potentially toxic forms of oxygen termed “reactive oxygen species” (**ROS**). These ROS include highly toxic “free radical” intermediates such as hydroxyl radicals that have been implicated in a number of human diseases and drug toxicities including cancer, neurodegenerative diseases and birth defects.

Using a number of models involving genetically altered animals and drugs that enhance oxidative stress, results from our laboratory and others have shown that ROS can adversely embryonic development in at least two ways (Wells et al., 1997, 2005, 2009a,b). The first is by altering the level of signals, termed “signal transduction”, within embryonic cells that are involved in the activation of genes leading to the production or suppression of proteins ultimately necessary for normal development. The second effect of ROS is to damage cellular macromolecules such as genes and proteins, resulting their inability to perform their normal developmental role.

## Research objectives

We originally proposed to determine:

- (1) If ROS play a substantial role in the embryonic toxicity of methanol; and,
- (2) If the mechanism of this embryotoxicity is the same in the mouse as it is in a non-rodent species that, unlike the mouse, metabolizes methanol like humans.

Subsequent to the submission of our proposal, a similar question of relevance arose in regard to a controversial study suggesting that methanol might be carcinogenic in rodents. Since ROS-mediated oxidative DNA damage is a known molecular mechanism of carcinogenesis, we added a third objective to determine:

- (3) If methanol causes oxidative DNA damage in potential cancer target tissues of adult male mice and adults from non-rodent species that metabolize methanol more like humans.

## Approach

The approach was based upon methods and models, some unique to our laboratory, that we have used over the last 30 years to determine the role of ROS in adverse embryonic effects that either arise spontaneously or are caused by drugs (Wells et al., 2009a). The mouse was used as the basic animal model, and compared to a second non-rodent species that, unlike the mouse, metabolizes methanol like humans, primarily via alcohol dehydrogenase (**ADH**) and cytochrome P450 2E1 (**CYP2E1**), rather than via catalase as in rodents. The toxicological relevance of catalase in rodents is potentially complex, since in addition to its role in methanol metabolism, catalase also plays an antioxidative role in the detoxification of ROS. Rabbits were the first non-rodent species investigated as potentially reflective of humans, based in part upon their remarkable species difference from mice in susceptibility to birth defects caused by the ROS-initiating sedative drug thalidomide (Parman et al., 1999).

Our first series of studies found that the metabolism of methanol in monkeys was reflected more accurately by rabbits than by mice, so rabbits were used along with mice in our developmental studies. In studies of adult animals to assess the ability of methanol to cause DNA oxidation in cancer target tissues, mice were compared to rabbits and monkeys. For in vivo developmental studies, methanol teratogenicity was compared in mice and rabbits, the latter of which had not previously been studied. Additional in vitro studies in mice involved embryo culture, which permits more precise manipulation of factors potentially involved in the mechanism of adverse effects, and reveals embryonic mechanisms independent of the mother. The high reactivity of ROS usually means that the critical pathways involved in both their formation and detoxification reside within the embryo, since ROS are too unstable to travel from the mother across the placenta and into embryonic target tissues. The role of embryonic

catalase and ROS in methanol embryopathies in mice were investigated respectively using: (1) genetically modified mice either deficient in catalase (acatalasemic) or expressing human catalase (high catalase activity); and, (2) pretreatment with the free radical spin trapping agent phenylbutylnitron (**PBN**).

## **SUMMARY OF CONCLUSIONS**

A detailed presentation of the research results and conclusions is presented in the next section.

The following is a brief summary of our conclusions:

### 1. Species differences in the metabolism and pharmacokinetics of methanol

We have found that the in vivo metabolism of MeOH in monkeys, which closely reflect humans, is more similar in rabbits than in mice, with a great accumulation of FA in rabbits and monkeys. In complementary in vitro studies, we found that the activity of catalase in rabbits is only 36% of that in mice, and similar to the activity in humans, consistent with a primary role for catalase in mice in metabolizing MeOH and FA, while rabbits and humans rely upon alcohol dehydrogenase (**ADH**). These results suggest that the rabbit might be a more accurate model than the mouse for predicting the human risk for MeOH developmental toxicity.

### 2. Species- and strain-dependent susceptibility to the developmental toxicity of methanol

In developmental studies, rabbits were shown for the first time to be resistant to MeOH teratogenesis, in contrast to C57BL/6 mice, which were susceptible, as had previously been shown by other laboratories. However, we also found that a different (C3H) mouse strain was resistant, providing the first evidence that not all mice are susceptible. The dose (2 g/kg i.p.) of MeOH administered to both species was high, and substantially above the lethal human dose. Given that rabbits, which more closely reflect human MeOH metabolism, and at least one strain of mice are resistant to MeOH teratogenesis, it is questionable whether the human risk for MeOH developmental toxicity can be accurately assessed in sensitive rodent models.

### 3. ROS in the mechanism of methanol developmental toxicity

In mouse strains that are sensitive to the developmental toxicity of methanol, we examined the role of catalase and ROS in the embryopathic mechanism. In addition to the metabolism of methanol by catalase in rodents, this antioxidative enzyme also detoxifies ROS in all species (**fig. 1**). We used the following respective approaches in vivo and/or in embryo culture: (1) genetically modified mice deficient in catalase (acatalasemic, **aCat**), and transgenic mice expressing human catalase activity (high

catalase activity, **hCat**); and, (2) pretreatment with the free radical spin trapping agent phenylbutylnitrone (**PBN**). The in vivo studies were inconclusive, since the C3H strain for the aCat mice was resistant to MeOH teratogenicity, possibly due to such maternal factors as maternal metabolism and/or placental transporters, and the hCat strain was not protected compared to its catalase-normal wild-type controls, again possibly due to maternal factors. However, in embryo culture, MeOH embryopathies were enhanced in aCat mice and reduced in hCat mice, and were blocked by pretreatment with PBN. These results suggest that ROS play an important role in the mechanism of MeOH developmental toxicity in sensitive strains of mice, and that embryonic catalase plays an important protective role via its antioxidative activity, as distinct from its peroxidative, MeOH-metabolizing role in maternal liver or the embryo.

#### 4. ROS-initiated oxidative DNA damage and implications for carcinogenic potential

In addition to its potential role in teratogenesis, ROS-initiated oxidatively damaged DNA, and particularly the 8-oxoguanine (**8-oxoG**) lesion, is mutagenic and carcinogenic. In a novel evaluation of adult mice, rabbits and monkeys, we found that an acute high dose (2 g/kg i.p.) of MeOH did not enhance 8-oxoG formation in any tissue including bone marrow in any species, nor did the same dose of MeOH daily in mice for 15 days. These results suggest that it is unlikely that human environmental exposure to MeOH would cause cancer via a mechanism involving oxidatively damaged DNA.

## **DETAILS OF COMPLETED RESEARCH**

(Abstracts of papers and manuscripts)

1. **Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates.** Sweeting, J. N., Siu, M., McCallum, G. P., Miller, L. and Wells, P. G. Toxicology and Applied Pharmacology 247(1): 28-35, 2010.

Methanol (**MeOH**) is metabolized primarily by alcohol dehydrogenase in humans, but by catalase in rodents, with species variations in the pharmacokinetics of its formic acid (**FA**) metabolite. The teratogenic potential of MeOH in humans is unknown, and its teratogenicity in rodents may not accurately reflect human developmental risk due to differential species metabolism, as for some other teratogens. To determine if human MeOH metabolism might be better reflected in rabbits than rodents, the plasma pharmacokinetics of MeOH and FA were compared in male CD-1 mice, New Zealand white rabbits and cynomolgus monkeys over time (24, 48 and 6 hr, respectively) following a single intraperitoneal injection of 0.5 or 2 g/kg MeOH or its saline vehicle. Following the high dose, MeOH exhibited saturated elimination kinetics in all 3 species, with similar peak concentrations and a 2.5-fold higher clearance in mice than rabbits. FA accumulation within 6 hr in primates was 5-fold and 43-fold higher than in rabbits and mice respectively, with accumulation being 10-fold higher in rabbits than mice. Over 48 hr, FA accumulation was nearly 5-fold higher in rabbits than mice. Low-dose MeOH in mice and rabbits resulted in similarly saturated MeOH elimination in both species, but with approximately 2-fold higher clearance rates in mice. FA accumulation was 3.8-fold higher in rabbits than mice. Rabbits more closely than mice reflected primates for *in vivo* MeOH metabolism, and particularly FA accumulation, suggesting developmental studies in rabbits may be useful for assessing potential human teratological risk.

2. **Methanol exposure does not lead to accumulation of oxidative DNA damage in bone marrow or spleen of mice, rabbits or primates.** McCallum, G. P., Siu, M., Ondovcik, S. L., Sweeting, J.N. and Wells, P. G. Molecular Carcinogenesis: in press, 2010.

Genotoxicity tests indicate methanol (**MeOH**) is not mutagenic, but a rodent study has suggested carcinogenic potential, which could result from free radical-initiated oxidative DNA damage. To investigate this possibility we treated male CD-1 mice, New Zealand white rabbits, and cynomolgus monkeys with MeOH (2.0 g/kg ip) and assessed tissue oxidative DNA damage 6 h post dose, measured as 8-oxo-2'-deoxyguanosine (**8-oxodG**). We found no MeOH-dependent increases in 8-oxodG in bone marrow or spleen of any species. Chronic treatment of CD-1 mice with MeOH (2.0 g/kg ip) daily for 15 days also did not increase 8-oxodG levels in these organs. Further studies in the DNA repair deficient oxoguanine glycosylase 1 (**Ogg1**) knockout (**KO**) mice supported these findings. Fibroblasts from *Ogg1* KO mice accumulated 8-oxodG following acute exposure to the renal carcinogen potassium bromate (**KBrO<sub>3</sub>**; 2.0 mM) but did not

accumulate 8-oxodG following exposure to 125 mM MeOH 6 h post treatment. *Ogg1* KO mice accumulated 8-oxodG in bone marrow and spleen with age but not following exposure to MeOH. In addition, free radical-mediated hydroxynonenal-histidine protein adducts were not enhanced by MeOH in primate bone marrow or spleen, or in rabbit bone marrow or mouse spleen, although modest increases were observed in rabbit spleen and mouse bone marrow. Taken together these observations suggest that MeOH exposure does not promote the accumulation of oxidative DNA damage in bone marrow and spleen, and it is unlikely that human environmental exposure to MeOH would lead to lymphomas via this mechanism.

### 3. **Species- and strain-dependent teratogenicity of methanol in rabbits and mice.**

Sweeting, J. N., Siu, M., Wiley, M. J. and Wells, P. G. Reproductive Toxicology: in press, 2010.

Estimates of human risk for developmental toxicity of methanol (**MeOH**) are based on studies in rodents, which unlike humans use catalase to metabolize MeOH. Rabbits, like humans, may largely use alcohol dehydrogenase (**ADH**), and more accurately than rodents reflect primate MeOH and formic acid (**FA**) pharmacokinetic profiles. Here we show that New Zealand white rabbits and one strain of mouse (C3H) are resistant to MeOH teratogenicity, whereas C57BL/6J mice are susceptible. Neither rabbits nor mice were susceptible to the acute MeOH toxicity observed in humans. The strain-dependent teratological susceptibility in mice could not be explained by differences in MeOH or FA disposition, nor could the resistance of rabbits, which exhibited more prolonged FA accumulation, suggesting that different mechanisms underlie MeOH teratogenesis and the FA-mediated acute toxicity in humans. It is not clear if the human risk for MeOH developmental toxicity can be accurately estimated using sensitive rodent strains.

### 4. **Methanol exposure does not produce oxidatively damaged DNA in lung, liver or kidney of adult mice, rabbits or primates.**

McCallum, G. P., Siu, M., Sweeting, J.N. and Wells, P. G. Toxicology and Applied Pharmacology: in press, 2010.

*In vitro* and *in vivo* genotoxicity tests indicate methanol (**MeOH**) is not mutagenic, but carcinogenic potential has been claimed in one controversial long-term rodent cancer bioassay that has not been replicated. To determine whether MeOH could indirectly damage DNA via reactive oxygen species (**ROS**)-mediated mechanisms, we treated male CD-1 mice, New Zealand white rabbits and cynomolgus monkeys with MeOH (2.0 g/kg ip) and 6 hr later assessed oxidative damage to DNA, measured as 8-oxo-2'-deoxyguanosine (**8-oxodG**) by HPLC with electrochemical detection. We found no MeOH-dependent increases in 8-oxodG in lung, liver or kidney of any species. Chronic treatment of CD-1 mice with MeOH (2.0 g/kg ip) daily for 15 days also did not increase 8-oxodG levels in these organs. These results were corroborated in DNA repair-deficient oxoguanine glycosylase 1 (*Ogg1*) knockout (**KO**) mice, which accumulated 8-

oxodG in lung, kidney and liver with age, but exhibited no increase following MeOH, despite a 2-fold increase in renal 8-oxodG in *Ogg1* KO mice following treatment with a ROS-initiating positive control, the renal carcinogen potassium bromate (**KBrO<sub>3</sub>**; 100 mg/kg ip). These observations suggest that MeOH exposure does not promote the accumulation of oxidatively damaged DNA in lung, kidney or liver, and that environmental exposure to MeOH is unlikely to initiate carcinogenesis in these organs by DNA oxidation.

**5. Methanol embryopathies in embryo culture in transgenic mice expressing human catalase, and mutant catalase-deficient mice.** Miller, L. and Wells, P. G. Manuscript submitted 2<sup>nd</sup> October 2010 to Toxicology and Applied Pharmacology.

The mechanisms underlying the teratogenicity of methanol (**MeOH**) in rodents, unlike its acute toxicity in humans, is unclear, but may involve reactive oxygen species (**ROS**). Embryonic catalase, although less than 10% of maternal activity, may protect the embryo by detoxifying ROS. This hypothesis was investigated in whole embryo culture to remove confounding maternal factors, including metabolism of MeOH by maternal catalase. C57BL/6 (**C57**) mouse embryos expressing human catalase (**hCat**) or their wild-type (**C57 WT**) controls, and C3Ga.Cg-Catb/J acatalasemic (**aCat**) mouse embryos or their wild-type C3HeB/FeJ (**C3H WT**) controls, were explanted on gestational day (**GD**) 9 (plug = GD 1), exposed for 24 hr to 4 mg/mL MeOH or vehicle, and evaluated for functional and morphological changes. hCat and C57 WT vehicle-exposed embryos developed normally. MeOH was embryopathic in C57 WT embryos, evidenced by decreases in anterior neuropore closure, somites developed and turning, whereas. hCat embryos were protected. Vehicle-exposed aCat mouse embryos had lower yolk sac diameters compared to C3H WT controls, suggesting endogenous ROS are embryopathic. MeOH was more embryopathic in aCat embryos than WT controls, with reduced anterior neuropore closure and head length only in catalase-deficient embryos. These data suggest that ROS may be involved in the embryopathic mechanism of methanol, and that embryonic catalase activity may be a determinant of teratological risk.

**6. Methanol teratogenicity in mutant catalase-deficient mice, and transgenic mice expressing human catalase.** Siu, M., Wiley, M. J. and Wells, P. G. Manuscript submitted 30<sup>th</sup> September 2010 to Toxicological Sciences.

Reactive oxygen species (**ROS**) are implicated in rodent methanol (**MeOH**) teratogenesis, but the mechanisms remain unclear, particularly regarding catalase, which in rodents both detoxifies ROS and metabolizes MeOH and its formic acid (**FA**) metabolite. Herein, we treated pregnant mice that express either high catalase activity (transgenic mice expressing human catalase, **hCat**) or low activity (mutant acatalasemic mice, **aCat**), or their respective C57BL/6J or C3H wild-type (**WT**) controls, with either MeOH (2 g/kg ip) or saline. hCat mice and WT controls were similarly susceptible to

MeOH-initiated ophthalmic abnormalities, with respective incidences of 66% and 44%, vs. 0% in saline controls ( $p < 0.01$ ). MeOH caused cleft palates in WT ( $p < 0.05$ ) and hCat ( $p > 0.05$ ) mice, with respective similar incidences of 17% and 11%, vs. 0% in saline controls. MeOH was not teratogenic in Acat mice or their WT controls, precluding an assessment of the developmental impact of catalase deficiency. Catalase activity was increased by 1.5-fold and  $> 2$ -fold in hCat embryos and maternal livers, respectively, and conversely decreased by 35% and  $> 40\%$  in Acat embryos and maternal livers. MeOH and FA plasma concentrations over 12 hr were similar in all strains. For the fetal outcomes investigated, the lack of significant protection from MeOH teratogenicity in hCat mice *in vivo*, in contrast to related studies in embryo culture, suggests that an *in vivo* modulatory effect of endogenous catalase on MeOH teratogenicity, if any, would require an increase in activity beyond the 1.5-fold magnitude achieved in hCat embryos.

**7. Protection against methanol embryopathies in mouse embryo culture by the free radical spin trapping agent phenylbutylnitrone.** Miller, L. and Wells, P. G. Manuscript submitted on 22<sup>nd</sup> September 2010 to Toxicology and Applied Pharmacology, and declined by the editors for lack of mechanistic data. We will complete studies of oxidative macromolecular damage and resubmit the manuscript with these new data, likely in November or December 2010.

The teratogenicity of methanol (**MeOH**) in rodents may be mediated in part by reactive oxygen species (**ROS**), which can be scavenged by the free radical spin trap phenylbutylnitrone (**PBN**). The involvement of ROS in MeOH embryopathies was investigated in mouse whole embryo culture to remove confounding maternal factors, including metabolism of MeOH by maternal catalase. CD-1 mouse embryos were explanted on gestational day (**GD**) 8.5 (plug = GD 0), exposed for 24 hr to 4 or 6 mg/mL (124 or 187 mM) MeOH or vehicle with or without pretreatment with PBN (0.125-0.22 mM) and evaluated for functional and morphological changes. Vehicle-exposed embryos developed normally, whereas MeOH was embryopathic, causing concentration-dependent decreases in anterior neuropore closure (**ANC**) ( $p < 0.001$ ), somite development ( $p < 0.01$ ), turning ( $p < 0.001$ ) and head length ( $p < 0.01$ ), while increasing heart rate ( $p < 0.001$ ), yolk sac diameter ( $p < 0.05$ ) and embryonic lethality ( $p < 0.05$ ), the latter in a concentration-dependent fashion. PBN pretreatment protected against MeOH-initiated reductions in ANC ( $p < 0.01$ ), turning ( $p < 0.05$ ), somite development ( $p < 0.01$ ) and head length ( $p < 0.05$ ) at the lower MeOH concentration, and protected somite development ( $p < 0.01$ ) at the higher MeOH concentration. The protection by PBN suggests that embryonic ROS formation may be involved in the mechanism of methanol embryopathies.

## **POTENTIAL FUTURE STUDIES**

1. More complete elucidation of the molecular mechanisms of teratogenesis.

Although we and others have found evidence for the involvement of ROS, the molecular determinants of risk remain to be established. This would include the mechanisms of ROS formation, the role of other antioxidative enzymes, DNA repair and other ROS-relevant pathways including ROS-dependent signal transduction. ROS-dependent mechanisms do not preclude the involvement of non-ROS-related mechanisms, which also should be evaluated. Such evaluations would include an initial interrogation using gene expression arrays.

2. More extensive studies in non-rodent models similar to humans.

More information is needed from non-rodent models more metabolically and developmentally similar to humans. This includes a more complete evaluation of the molecular mechanisms and developmental consequences in rabbits. Also, another model potentially closer to humans, but less ethically, technically and financially challenging than monkeys, should be explored. One possibility would be mini-pigs.

3. Effects of methanol on the developing fetal brain.

In addition to structural teratogenesis resulting from methanol exposure during the embryonic period, the effects of methanol on the developing fetal brain resulting from exposure later in pregnancy should be evaluated, including effects on postnatal functions such as cognition, learning, memory and related behaviors.

4. Basis for oxidative stress in embryos but not adults.

Our studies in mice found evidence for ROS-mediated developmental toxicity in the embryo, but not for ROS-mediated DNA damage in adult tissues of male mice, rabbits or monkeys. The determinants of this difference between embryonic and adult methanol exposures need to be established.

5. More rigorous evaluation of carcinogenic potential.

More rigorous studies are needed to determine whether methanol exposure, particularly in realistic amounts, can cause cancer. These studies should include non-rodent models more metabolically similar to humans.

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Wells PG, Bhuller, Y, Chen CS, Jeng, W, Kasapinovic S, Kennedy JC, Kim PM, Laposa RR, McCallum GP, Nicol CJ, Parman T, Wiley MJ and Wong AW. (2005) Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species. *Toxicol. Appl. Pharmacol.* 207(2S): 348-359. [review paper]

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

Wells PG, Lee CJJ, McCallum GP, Perstin J and Harper PA. (2009b) Chapter 6. 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.

## **APPENDIX**

(Copies of reprint of published paper and proofs of papers in press)

1. Sweeting, J. N., Siu, M., McCallum, G. P., Miller, L. and Wells, P. G. Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates. **Toxicology and Applied Pharmacology** 247(1): 28-35, 2010.
2. McCallum, G. P., Siu, M., Ondovcik, S. L., Sweeting, J.N. and Wells, P. G. Methanol exposure does not lead to accumulation of oxidative DNA damage in bone marrow or spleen of mice, rabbits or primates. **Molecular Carcinogenesis**: in press, 2010.
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