New Zealand white rabbit progeny exposed *in utero* to methanol are resistant to skeletal anomalies reported for rodents, but exhibit a novel vertebral defect

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ABSTRACT

Rabbits may serve as a useful model for predicting the human risk for methanol (MeOH) teratogenicity, which currently is unknown. New Zealand white (NZW) rabbits are resistant to the MeOH-initiated gross morphological anomalies characteristically observed in several strains of mice and rats, but skeletal development has not been assessed. Pregnant rabbits were administered 2 doses of 2 g/kg MeOH on gestational day (GD) 7 or 8, and assessed for skeletal abnormalities on GD 29. Variations between treated and control fetuses were observed only in the number of post-lumbar vertebrae, where MeOH-exposed fetuses had fewer ossified vertebrae, which has not been reported for rodents. Furthermore, rabbits did not exhibit the MeOH-initiated skeletal defects characteristically reported for rodent fetuses. These results expand the morphological breadth of the relative species-dependent resistance of rabbits to MeOH teratogenicity compared to rodents, yet reveal a novel skeletal defect or delay in ossification not reported for rodents.

1. Introduction

Interest in the use of methanol (MeOH) as an alternate fuel source raises the potential for increased acute and chronic environmental exposure for humans. Of particular concern is the risk of augmented MeOH exposure for pregnant women. Limited information is available regarding the effects of MeOH on the developing human fetus, and is restricted to case studies of late gestational exposure in which the level and duration of accidental dosage are unknown [1,2]. The potential developmental risk for humans is accordingly unknown and reliant on teratological studies, primarily in rodents.

MeOH is a known teratogen in specific strains of mice and rats, including the inbred C57BL/6J and the outbred CD-1 mouse strains [3]. Gross morphological assessments of these mice reveal strain-specific characteristic craniofacial defects including ophthalmic abnormalities in C57BL/6J mice [4–7], and neural tube defects and cleft palates in CD-1 mice following MeOH exposure [8,9]. Unlike the strain-specific differences in MeOH-initiated external craniofacial defects, MeOH causes similar skeletal anomalies in both strains. In C57BL/6J mice, exposure to upwards of 4.9 g/kg MeOH on gestational day (GD) 7 leads to a variety of skeletal defects, including anomalies to the cervical vertebrae, supernumerary ribs and an abnormal number of pre-sacral vertebrae [6]. Additionally, there is a high incidence of anomalies to the facial bones of these mice, consistent with the external facial abnormalities observed following *in utero* exposure to MeOH [6]. Similarly, exposure of CD-1 mice to 10,000 ppm MeOH during gastrulation leads to a significant increase in the incidence of skeletal anomalies, comparable to those observed in C57BL/6J mice [8].

While rodents are the most common species used to predict the human risk for MeOH teratogenicity, there are significant species differences in MeOH metabolism, which could affect interpretation of the aforementioned rodent teratological observations as they relate to the developing human [3]. MeOH is metabolized into formaldehyde by the peroxidative action of catalase in rodents, but by alcohol dehydrogenase (ADH1) in humans [10,11]. Following the rapid oxidation of formaldehyde into formic acid (FA) by formaldehyde dehydrogenase (ADH3) in all species [12], differences arise again in the breakdown of FA into carbon dioxide and water. Owing

to a limited amount of the co-substrate tetrahydrofolate (THF), FA accumulates in humans, resulting in an increased incidence of acute MeOH toxicity [13,14]. In rodents, however, the manifestations of MeOH exposure do not include acute toxicity, likely a result of increased basal THF stores, higher 10-formyl-THF dehydrogenase activity, and/or the use of catalase for FA oxidation [13,15]. Importantly, catalase also functions as an antioxidant in all species [16,17], which could further confound the interpretation of developmental data in mice, as the formation of reactive oxygen species (ROS) have been implicated in the mechanism of action of MeOH teratogenicity [12,18]. In particular, hydrogen peroxide (H_2O_2) is detoxified by catalase, and its production within the embryo may be enhanced by MeOH [3,17,19]. As such, the extent to which catalase acts as an antioxidative or peroxidative enzyme, particularly within the embryo, is unknown.

Accordingly, we have sought to discover a non-rodent, mammalian animal model that may accurately predict the potential human risk for MeOH teratogenicity, if any. Using gross morphological assessments in the New Zealand white (NZW) rabbit, we discovered a species resistant to the external structural anomalies caused by MeOH exposure that are characteristic for several strains of mice and rats [5]. In light of the use of the NZW rabbit to accurately predict human risk to the teratogenic drugs thalidomide [20] and ethylene glycol [21], as well as the potential use of ADH1 for MeOH metabolism in rabbits [22,23], it is reasonable to consider that the rabbit may be more appropriate for modeling human risk to MeOH exposure. As such, we have assessed the skeletal development of NZW rabbit fetuses following in utero MeOH exposure. It is important to note that the MeOH dose used in this study was chosen in part to match similar studies in rodents, and to ensure that potential effects in rabbits would not be missed. The dose greatly exceeds likely human exposure levels, and may not be directly relevant to human risk assessment. Through an extensive examination of the vertebral column, ribs, sternum and appendicular skeleton of control and treated fetuses, we observed a substantial reduction in the number of post-lumbar vertebrae in MeOH-treated fetuses, which has not been reported for rodents. This anomaly is consistent with the trend towards short tails previously reported for NZW rabbit fetuses [5]. In contrast, rabbits were not susceptible to the spectrum of multiple skeletal anomalies reported for rodents, consistent with the resistance of rabbits to the entire spectrum of external anomalies reported for rodents.

2. Methods

2.1. Chemicals

Chloroform and HPLC grade MeOH were purchased from EMD Sereno Canada, Inc. (Mississauga, ON). Saline (0.9%, sterile) was obtained from Baxter Corporation (Mississauga, ON). Isoflurane was purchased from Abbott Laboratories Ltd. (Saint-Laurent, QC). Compressed oxygen (99%) and CO_2 were purchased from BOC Gases (Mississauga, ON). Ethanol (EtOH) (100%) was purchased from Commercial Alcohols (Brampton, ON). Glacial acetic acid was purchased from Caledon Laboratories, Ltd. (Georgetown, ON). Potassium hydroxide (KOH) and Alizarin red stain were obtained from Sigma–Aldrich (St. Louis, MO). Glycerol was purchased from Bioshop Canada Inc. (Burlington, ON).

2.2. Animal housing and mating

All animal protocols were approved by the University of Toronto Animal Care Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

Female NZW rabbits were purchased from Charles River Laboratories (CRL) (Saint-Constant, QC), and were housed in cages from Allentown, Inc. (Allentown, NJ). Animal rooms were maintained at 20 °C and 60% humidity, and placed on an automated 12 h light/dark cycle. Rabbits were fed standard high-fiber rabbit chow (Lab Diet: 5236Hi-Fiber, PMI Nutrition International LLC, Brentwood, MO), and provided with water ad libitum. Diets were supplemented weekly with vegetables.

Rabbits were time-mated between 07:00 and 09:00 h at CRL, and a successful mating was designated as gestational day (GD) 0. Rabbits were transported to the university's animal facility on GD 4 or 5 and acclimatized for 2–3 days prior to MeOH exposure.

2.3. Dosing and fetal preservation

Rabbits were administered 2 g/kg MeOH (20% [w/v] in sterile 0.9% saline) or its saline vehicle control by intraperitoneal (ip) injection at 09:00 and 17:00 h, as previously described [5]. A total daily dose of 4 g/kg was administered to each rabbit. Injections were carried out on GD 7 or 8, following anaesthetization with 3% isoflurane in 2 L of oxygen. Subsequent to each injection, rabbits were exposed to 100% oxygen for approximately 2 min to facilitate recovery from the anaesthetic. Pregnant does were not visibly affected by MeOH treatment, and there was no change in food intake.

We have previously demonstrated that a single dose of 2 g/kg MeOH in rabbits results in peak MeOH concentrations that are achieved within 1 h, with saturable elimination that is maintained until approximately 24 h post injection, and complete elimination by 42 h [24]. By dosing with 2 g/kg twice, at an 8 h interval, we ensured a sustained and saturating MeOH concentration beyond 24 h. In mice, skeletal defects have been observed following a comparable dosing regimen, in which MeOH was administered by intraperitoneal injection at a 4 h interval, for a total daily dose of 3.4 or 4.9 g/kg [6]. The 8 h interval was chosen for our rabbit study to account for the 2-fold slower clearance rate of MeOH in rabbits compared to mice [24]. Additionally, the dosing regimen was chosen so as to encompass the period of organogenesis, which commences on GD 8 in both mice and rabbits [25,26]. We have previously shown that this dosing regimen causes teratogenic effects in multiple strains of mice [4,5]. Accordingly, we administered MeOH to pregnant rabbits on GD 7 or 8, to allow for a more relevant species comparison.

On GD 29, does were euthanized by CO_2 asphyxiation. Period out of place fetuses were delivered by Caesarean section, exsanguinated through the jugular vein, and preserved in Carnoy's fixative (6 parts EtOH, 3 part chloroform, 1 part glacial acetic acid). Fetuses were obtained from studies described previously [5]. All does were selected for euthanasia and fetal extraction at random, so as to avoid any potential variability in ossification patterns that can arise from the timing of caesarian sections [27].

2.4. Skeletal staining and assessment

Following preservation, each fetus was skinned and eviscerated, and subsequently dehydrated in increasing concentrations of EtOH (70, 80 and 95%) over the span of 3 days. Fetal soft tissue was macerated in 1% KOH over 3 days, with fresh solution added daily. Each fetus was then placed in an individual sealable glass container and immersed in Alizarin red stain (6 mg/L in 1% KOH; approximately 200 mL of stain solution was used per specimen). The Alizarin stain was replaced daily for 3 days. On completion of the skeletal staining, specimens were cleared with 100% glycerol until visualization of the skeletons was attained, at which point the fetuses were placed in a solution of 1:1 glycerol to 100% EtOH for a maximum of 1 week. For long-term storage, fetuses were placed in 100% EtOH.