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The Effects of *In Utero* and Lactational Exposure to Chloroform on Postnatal Growth and Glucose Tolerance in Male Wistar Rats

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Water chlorination results in the formation of trihalomethanes (THMs) including chloroform. In human studies, fetal growth restriction has been associated with exposure to THMs during pregnancy and impaired fetal growth has been associated with an increased risk of type 2 diabetes. Therefore, the objective of this study was to determine the effect of *in utero* and lactational exposure to chloroform on birthweight and postnatal indicators of type 2 diabetes. Female Wistar rats were given chloroform (0 µg/L, 75 µg/L) in their drinking water for 2 wk prior to mating until parturition (*in utero* exposure only) or until weaning (*in utero* + lactational exposure). At postnatal d 1 (PND1) pups of dams exposed to chloroform had significantly higher serum glucose levels and lower insulin levels, but this effect was not due to β-cell depletion in the neonatal pancreas. Glucose homeostasis in response to a glucose challenge was not changed by chloroform treatment. Chloroform exposure did not affect birthweight; however, offspring of dams exposed to chloroform had significantly impaired postnatal growth. Although fetal and neonatal exposure to chloroform did not elicit physiological changes associated with the onset of type 2 diabetes, there were physiological changes resulting in impaired postnatal growth.

Key Words: Chloroform; fetal exposure; glucose homeostasis; postnatal growth.

Introduction

Chlorination has been the major disinfection process for public water supplies for many years and has proven highly effective in destroying and inactivating human pathogenic microorganisms. When chlorine reacts with the natural organic compounds present in the water supply, chemical disinfection byproducts are formed *(1)*. Disinfection byproducts of water chlorination include trihalomethanes, haloacetic acids, chlorophenols, chloral hydrate, and haloacetonitriles *(1)*. The trihalomethanes (THMs) are the most prevalent compounds formed by chlorination and include chloroform, bromodichloromethane, chlorodibromomethane, and bromoform. Of the THMs, chloroform is detected most frequently and at the highest concentrations in Canadian drinking water and accounts for approx 83% of the total THM levels *(2)*. Presently the current acceptable level of total THMs in drinking water in Canada is 100 µg/L based on an annual running average of quarterly samples to account for seasonal variation (Canadian Water Quality Guidelines). However, there have been reports of chloroform concentrations that exceed the current maximum acceptable concentration (MAC) for total THMs *(1)* and total THM concentrations that exceed the MAC have been widely reported across Canada *(2)*. Recently, Graves et al. *(3)* have reported that the weight of evidence from epidemiological studies suggests a positive association between exposure to water disinfection byproducts during pregnancy and fetal growth restriction.

Barker et al. *(4)* were the first to suggest the fetal origins of adult disease hypothesis, in which it is proposed that an adverse intrauterine environment could cause disease in adult life. They proposed that low birthweight babies were at increased risk for developing obesity, hypertension, and type 2 diabetes. There is now substantial epidemiological evidence to support the hypothesis that fetal growth retardation is associated with an increased incidence of adult onset diseases including type 2 diabetes, hypertension, coronary heart disease, dyslipidemia, stroke, and obesity *(5–10)*. Given the association between birthweight and the risk of adverse health outcomes in adulthood in human populations *(5–10)*, and the potential association between exposures *in utero* to the byproducts of water chlorination and intrauterine growth restriction, we propose that exposure to THM *in utero* may have life-long health consequences that have not previously been identified and may represent a significant public health concern. Therefore, the objectives of this study are to deter-

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Table 1
Effect of *In Utero* and *In Utero* + Lactational Exposure to Chloroform on Postnatal Weight and Weight Gain*

Treatment	PND21 (weaning)	26 wk of age	Weight gain (weaning–26 wk)
Control	57.7 ± 0.74[a]	647.2 ± 11.22[c]	589.4 ± 11.45[e]
75 µg/L chloroform (*in utero* only)	49.6 ± 2.64[b]	630.3 ± 19.07[c]	580.7 ± 18.32[e]
75 µg/L chloroform (*in utero* + lactation)	54.3 ± 1.52[ab]	554.4 ± 3.68[d]	500.1 ± 14.07[f]

*Weights (g) are presented as mean ± SEM (n = 12 per group). Values with different superscripts are significantly different ($p < 0.05$) after analysis by one-way analysis of variance followed by pairwise comparisons (Bonferroni's t-test) where significance was indicated.

mine: (1) if maternal exposure to chloroform, at concentrations which are relevant for human populations, during pregnancy and lactation can affect birthweight and postnatal growth of the offspring; and (2) if maternal exposure to chloroform during pregnancy and lactation results in impaired glucose homeostasis, a hallmark of the development of type 2 diabetes in humans.

Results

Birth Phenotype

Chloroform administration to the dam had no significant effect on litter size (control 15.2 ± 3.15; 75 µg/L chloroform 16.2 ± 1.93), sex ratio (data not shown), or birthweight (control 6.0 ± 0.08 g; 75 µg/L chloroform 5.8 ± 0.04 g, p = 0.15).

Postnatal Growth

During lactation (PND1 to PND21) pups exposed to chloroform *in utero* exhibited impaired neonatal growth such that at weaning these animals were significantly lighter than offspring with no chloroform exposure (control) (Table 1). Once the pups were weaned the animals in the *in utero* exposure group did not show any further impairment in growth such that by 2 wk post-weaning their body weights were not significantly different from the control animals (Fig. 1). Conversely, following weaning, the offspring that were exposed to 75 µg/L chloroform *in utero* and during lactation had significantly impaired weight gain for the remainder of the study resulting in lower body weight at 26 wk of age compared to both control offspring and the offspring of dams who received chloroform in the drinking water during pregnancy alone (Table 1).

Analysis of the total growth response (area under the curve) indicates that the growth of the pups exposed to chloroform *in utero* and during lactation was significantly ($p <$ 0.01) lower than both the control group and the *in utero* exposure group (AUC control: 10685 ± 140; 75 µg/L chloroform *in utero* + lactation 9329 ± 221.1; 75 µg/L chloroform *in utero* only 10416 ± 298.9).

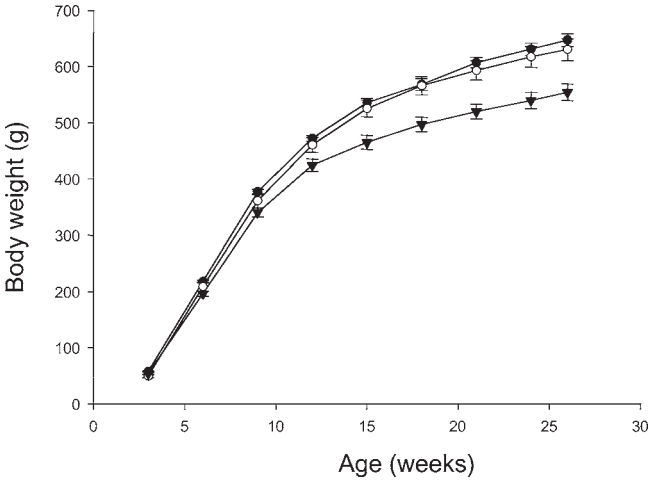

Fig. 1. Postnatal growth of the offspring of dams given distilled water (control: closed circles, n = 12), 75 µg/L chloroform during pregnancy (*in utero* exposure: open circles, n = 12), and 75 µg/L chloroform during pregnancy and lactation (*in utero* + lactation: closed triangles, n = 12). Data are presented as mean ± SEM.

Glucose Homeostasis

Fasting Glucose Concentrations

Serum glucose concentrations were significantly elevated (Table 2) and serum insulin levels significantly reduced at PND1 in pups born to dams given 75 µg/L chloroform in the drinking water (control: 0.56 ± 0.101 ng/mL, n = 45; 75 µg/L chloroform: 0.27 ± 0.032 ng/mL, n = 50, $p < 0.001$). At 4 and 26 wk of age there was no significant effect of treatment on fasting glucose concentrations (Table 2). Furthermore, there was no effect of treatment on fasting serum insulin concentrations at 26 wk of age (control: 0.40 ± 0.079 ng/mL, n = 12; 75 µg/L chloroform *in utero* + lactation: 0.32 ± 0.080 ng/mL, n = 12; 75 µg/L chloroform *in utero* only: 0.38 ± 0.113 ng/mL, n = 9: p = 0.75).

Table 2
Effect of *In Utero* and *In Utero* + Lactational Exposure to Chloroform on Serum Glucose Concentrations*

Treatment	PND1	4 wk	26 wk
Control	13.4 ± 0.85[a] (*n* = 45)	50.5 ± 3.82 (*n* = 12)	124.4 ± 6.31 (*n* = 12)
75 µg/L chloroform (*in utero* only)	16.7 ± 0.88[b] (*n* = 50)	41.6 ± 3.14 (*n* = 12)	105.0 ± 6.67 (*n* = 12)
75 µg/L chloroform (*in utero* + lactation)		39.2 ± 6.54 (*n* = 12)	131.0 ± 8.13 (*n* = 12)

*Glucose concentrations (mg/dL) are presented as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) after analysis by Student's *t*-test.

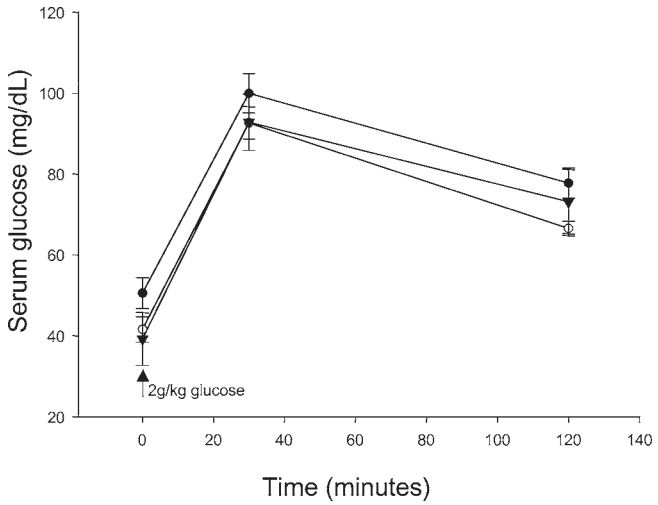

Fig. 2. Serum glucose concentrations (mg/dL) following administration of an oral glucose load (2 g/kg body weight) at 4 wk of age for the offspring of dams given distilled water (control: closed circles, *n* = 12), 75 µg/L chloroform during pregnancy (*in utero* exposure: open circles, *n* = 12), and 75 µg/L chloroform during pregnancy and lactation (*in utero* + lactation: closed triangles, *n* = 12). Data are presented as mean ± SEM. There was no effect of treatment on glucose concentrations at either 30 or 120 minutes following the administration of a glucose challenge or in the total glucose response (area under the curve).

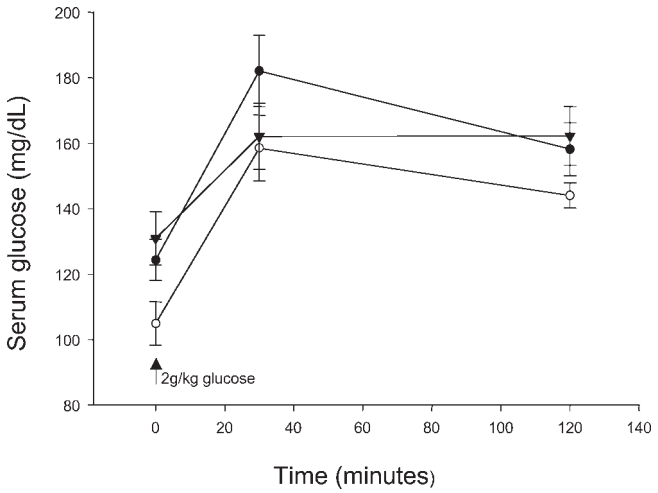

Fig. 3. Serum glucose concentrations (mg/dL) following administration of an oral glucose load (2 g/kg body weight) at 26 wk of age for the offspring of dams given distilled water (control: closed circles, *n* = 12) 75 µg/L chloroform during pregnancy (*in utero* exposure: open circles, *n* = 12), and 75 µg/L chloroform during pregnancy and lactation (*in utero* + lactation: closed triangles, *n* = 12). Data are presented as mean ± SEM. There was no effect of treatment on glucose concentrations at either 30 or 120 minutes following the administration of a glucose challenge or in the total glucose response (area under the curve).

Oral Glucose Tolerance Test

At 4 and 26 wk of age an oral glucose challenge was administered to all offspring. Analysis by one-way ANOVA showed no significant effect of fetal exposure to chloroform with or without lactational exposure on the peak serum glucose values (30 min) following the glucose challenge or the 120-min glucose concentration at either 4 (Fig. 2) or 26 (Fig. 3) wk of age. There was no difference between the treatment groups in the total glucose response (area under the curve) at either age. Furthermore, there was no difference between the treatment groups in the peak insulin response (30 min) or 120-min insulin concentration following the glucose challenge at 26 wk of age (data not shown).

Pancreas β-Cell Area

To assess whether *in utero* exposure to chloroform altered the development of the fetal pancreas, the pancreatic β-cells were immunolocalized with an insulin antibody, and the percentage of islet cell area was calculated. There was no significant effect of chloroform exposure *in utero* on pancreas weight, the pancreas:body weight ratio or the percentage of β-cell area in the neonatal pancreas (Table 3).

Table 3
Effect of *In Utero* Exposure to Chloroform on Pancreas Weight and β-Cell Area*

Treatment	Control	75 μg/L chloroform
Pancreas weight (mg)	14.1 ± 0.04	14.3 ± 0.04
Pancreas weight:body weight	2.7 ± 0.22	2.3 ± 0.19
% β cell area	3.0 ± 0.30	2.7 ± 0.30

*Values are presented as mean ± SEM (n = 10 per group).

Discussion

There is compelling evidence from human epidemiological studies and animal experiments to support the hypothesis that exposure of the fetus and neonate to certain hormonal, nutritional, metabolic, and environmental conditions may permanently alter the physiology of the resulting offspring and increase the risk of adverse postnatal health outcomes. The goal of this study was to determine if maternal exposure to chloroform during pregnancy and lactation, at concentrations that are commonly found in North American drinking water supplies, is associated with physiological alterations in the offspring that are associated with an increased health risk in later life. In this study *in utero* exposure to chloroform did not alter birthweight in Wistar rats; however, postnatal growth was significantly altered by the chloroform exposure. Offspring of dams given chloroform during pregnancy alone (i.e., fetal exposure only) weighed significantly less at weaning and had reduced growth during the lactational period compared to controls. Interestingly, in the 2 wk following weaning these animals had a similar rate of growth such that from 5 to 26 wk of age their body weights were not significantly different from the controls. Conversely, when the chloroform exposure occurred during the fetal and lactational period, postnatal weight gain was not altered during the lactational period but was significantly reduced following weaning, an effect that persisted until 26 wk of age. These results suggest that, in the rat, exposure to chloroform during lactation may have a more significant impact on future growth than fetal exposures. This persistent effect of *in utero* and lactational exposure to 75 μg/L chloroform to inhibit postnatal growth is unlike the effect of chloroform exposure in adult animals. Chu et al. *(11)* reported that adult rats receiving 2500 ppm chloroform had a suppressed growth rate, an effect that was reversible after cessation of treatment.

Longnecker and Daniels *(12)* propose that since incidence rates of type 2 diabetes have increased over short periods of time and vary widely over geographic areas that environmental factors may have a role in the etiology of diabetes. Given that the incidence of type 2 diabetes is higher in adults who had a low birthweight *(4)* and that *in utero* exposures to water disinfection byproducts have been associated with intrauterine growth restriction *(3,13,14)*, we therefore hypothesized that measuring metabolic changes associated with the onset of type 2 diabetes would be an appropriate measure of the postnatal health consequences of fetal and lactational exposure to chloroform. We have shown that chloroform exposure *in utero* did alter glycemic control at PND1, but this effect did not persist past weaning. We hypothesized that the observed decrease in insulin concentrations and resultant increase in serum glucose concentration at PND1 may have been a result in a reduction in the number of β-cells in response to chloroform exposure. Chloroform administered in drinking water has been shown to decrease cell proliferation in mouse liver *(15)*, and chloroform has been shown to induce DNA strand breaks in human lung epithelial cells *(16)*. Taken together these data suggest that exposure to THMs *in utero* may have altered cell cycle regulation in fetal pancreatic β-cells leading to altered pancreatic development and a reduction in the number of insulin secreting β-cells. However, when β-cell area was calculated, there was no significant effect of chloroform to alter β-cell area suggesting that chloroform exposure *in utero* did not alter fetal pancreatic development. We speculate that the observed reduction in serum insulin concentrations at PND1 may reflect a defect in insulin secretion from the pancreas at this time, although this remains to be examined. The effect of fetal chloroform exposure to alter glycemic control in the neonate did not persist into adulthood as there were no significant differences between glycemic control in response to an OGTT at either 4 or 26 wk of age.

Results from this study suggest that although the animals exposed to chloroform during fetal and neonatal development do not exhibit persistent metabolic changes associated with the onset of type 2 diabetes, there have been alterations in the physiology of the offspring resulting in impaired postnatal growth. This study is the first to identify a long-term effect of chloroform exposure during gestation and lactation at levels that are relevant for North Americans. Furthermore, we suggest that based on the results of our study that the postnatal health consequences of fetal exposure to THMs warrants further investigation and may represent a previously unidentified public health concern.

Materials and Methods

Animals and Tissue Collection

All animal experiments were approved by the Animal Research Ethics Board at McMaster University. Nulliparous 200–250 g female Wistar rats were maintained under controlled lighting (lights on 0700 to 1900 h) and temperature with *ad libitum* access to food and water. Two weeks prior to mating, the dams were randomly assigned to three treatment groups (n = 4 per group): (1) distilled water (control); (2) distilled water containing 75 μg/L chloroform (75

ppb) for the duration of the pregnancy and until weaning (PND21); or (3) distilled water containing 75 µg/L chloroform (75 ppb) for the duration of the pregnancy until parturition at which point the dams were switched to distilled water alone (control). Chloroform was added to the drinking water on a daily basis to minimize evaporation or degradation. The concentration of chloroform (75 µg/L) was chosen to represent a level below 83% of the MAC for total THM concentrations in Canadian water supplies and a level that that has frequently been reported in chlorinated water supplies across Canada *(2)*. Dams were allowed to deliver and pups were weighed after birth (PND1) and litters culled to three males to ensure homogeneity of litter size. Trunk blood for insulin and glucose measurements was collected from the culled pups, allowed to clot at 4°C, centrifuged and stored at −80°C until analysis. Serum glucose concentrations were measured by a commercially available kit using the glucose oxidase method (Pointe Scientific Inc., Lincoln Park, NJ), and insulin levels were measured by an ultrasensitive rat insulin ELISA designed to use small volumes of serum (Crystal Chem Inc., Downers Grove, IL). The insulin ELISA kit had a detection limit of 5 pg/mL with intra- and interassay variabilities of 5.5% and 4.8%, respectively. After weaning, pups were removed from their respective mothers, housed in pairs, and given distilled water (control) for drinking water. From PND1 up to wk 26, pups were weighed weekly to assess postnatal growth.

Oral Glucose Tolerance Tests

At 4 and 26 wk of age, glucose tolerance in the offspring was assessed by an oral glucose tolerance test (OGTT). Following an overnight fast, glucose (2 g/kg) was administered by oral gavage. Blood samples were taken from the saphenous vein at 0, 30 min, and 120 min following the administration of the glucose load. Blood samples were allowed to clot at 4°C, centrifuged, and stored at −80°C until needed for measurement of serum glucose concentrations as described above. Serum insulin concentrations were measured as described above in samples from the 26 wk OGTT.

Immunohistochemistry

To assess whether chloroform exposure *in utero* can alter the fetal development of the pancreatic β-cells, pancreas tissue was removed from all culled male pups at birth. The pancreas from each animal was weighed and then fixed by immersion in 10% neutral buffered formalin (EM Science, Gibbstown, NJ) at 4°C overnight, washed in PBS, and embedded in paraffin. To assess the effects of chloroform exposure *in utero* on pancreatic β-cell development, immunohistochemical detection of insulin was performed on 5 µm sections of neonatal pancreatic tissue (n = 10 per group). Tissue sections were deparaffinized in xylene, rehydrated, and washed in PBS. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% hydrogen peroxide (in methanol) for 30 min. Following the blocking step, sections were incubated for 30 min at 37°C in citrate buffer (pH 3.0) followed by an incubation with 10% normal goat serum and 1% BSA. Sections were then incubated with the primary antibody, a polyclonal, guinea pig anti-human insulin antibody (1:150 dilution) (DakoCytomation, Carpinteria, CA), which has been shown by the manufacturer to cross react with rat insulin peptides overnight at 4°C. Sections were then washed in PBS, and immunostaining was identified by the avidin–biotin–peroxidase technique, using the Vectastain kit (Vector Laboratories, Burlinghame, CA) with diaminobenzadine as the chromogen. Tissue sections were then counterstained with Carazzi's hematoxylin, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Control sections were incubated with 1% BSA in PBS in place of the primary antibody.

Morphometric Analysis

Morphometric analysis was performed with an Olympus IX2-UCB light microscope and Metamorph Software (Universal Imaging Corp. Downingtown, PA). All measurements were performed at 10× magnification, and the whole pancreas was analyzed (minimum of 14 fields per section). The total area of each pancreatic section was measured along with the areas of positively stained regions, and the ratio of total positive stained area to total pancreatic tissue area was obtained for each section to yield a percent positive stained area which represents the β-cell area.

Statistical Analysis

All statistical analyses were performed using SigmaStat (v.2.03, SPSS, Chicago, IL), and t-test or analyses of variance (ANOVA) followed by appropriate *post hoc* tests when significance was indicated by ANOVA (p = 0.05). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using Mann-Whitney rank sum test or Kruskal–Wallis one-way ANOVA on ranks. Area under the curve for the total glucose response following the glucose tolerance test and postnatal growth was assessed using the trapezoidal rule.

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