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Nitrate induces a type 1 diabetic profile in alligator hatchlings

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ABSTRACT

Type 1 diabetes (T1D) is a chronic autoimmune disease that affects 1 in 300 children by age 18. T1D is caused by inflammation-induced loss of insulin-producing pancreatic beta cells, leading to high blood glucose and a host of downstream complications. Although multiple genes are associated with T1D risk, only 5% of genetically susceptible individuals actually develop clinical disease. Moreover, a growing number of T1D cases occur in geographic clusters and among children with low risk genotypes. These observations suggest that environmental factors contribute to T1D etiology. One potential factor, supported primarily by epidemiological studies, is the presence of nitrate and nitrite in drinking water. To test this hypothesis, female hatchling alligators were exposed to environmentally relevant concentrations of nitrate in their tank water (reference, 10 mg/L, or 100 mg/L NO_3-N) from hatch through 5 weeks or 5 months of age. At each time point, endpoints related to T1D were investigated: plasma levels of glucose, triglycerides, testosterone, estradiol, and thyroxine; pancreas, fat body, and thyroid weights; weight gain or loss; presence of immune cells in the pancreas; and pancreatic beta cell number, assessed by antibody staining of nkx6.1 protein. Internal dosing of nitrate was confirmed by measuring plasma and urine nitrate levels and whole blood methemoglobin. Cluster analysis indicated that high nitrate exposure (most animals exposed to 100 mg/L NO3-N and one alligator exposed to 10 mg/L NO3-N) induced a profile of endpoints consistent with early T1D that could be detected after 5 weeks and was more strongly present after 5 months. Our study supports epidemiological data correlating elevated nitrate with T1D onset in humans, and highlights nitrate as a possible environmental contributor to the etiology of T1D, possibly through its role as a nitric oxide precursor.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which T lymphocytes destroy insulin producing beta cells in the pancreas. The insulin deficit prevents cellular glucose uptake, leading to high blood sugar levels. If not treated, ongoing hyperglycemia leads to a host of downstream complications. T1D was rapidly fatal until the introduction of insulin treatment in 1923. On average, T1D affects boys and girls equally, although regional gender biases in both directions are observed, suggesting that some factors affecting the disease in males and females may be different (Gale, 2002).

Today, one in 300 children will develop T1D by age 18, and this rate is rising (Maahs et al., 2010). Historical mortality records indicate that T1D incidence in the U.S. rose from 1.3/100,000/year in 1890 to 3.1/100,000/year in 1920 (Joslin, 1923). Similar rates were documented in Denmark with 2/100,000/year under age 15 years in 1905–1909 and 4/100,000/year in 1915–1919 (Gale, 2002). However, since the mid 1950's, the number of T1D cases have been rising by 2–5% per year in Asia, Europe, and North America, particularly among children under 5 years old (Gale, 2002; Maahs et al., 2010). In 2015, the highest number of new T1D cases among children aged 0 – 14 years occurred in Finland and Sweden (62.3/100,000/year and 43.2/100,000/year, respectively). The number of new cases in the U.S. was 23.7/100,000/year in 2015 following a 25% increase in prevalence between 2001 and 2009 (Dabelea et al., 2014; International Diabetes Federation, 2015).

That said, there is considerable geographic variation in T1D incidence with 2015 rates as low as 0.1/100,000/year in Papua New Guinea and Venezuela (International Diabetes Federation, 2015). There are also large parts of the world, notably sub-Saharan Africa, for which few data exist and where diabetes registries do not operate.

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Consequently, impacts of the disease on many populations remain unknown.

The cause(s) of increasing T1D incidence have not been clearly identified, but it is widely agreed that changing environmental factors play a significant role in disease initiation and progression (as reviewed in Bahadoran et al. (2016)). In 1923, the influential clinician and diabetes researcher Elliott Joslin wrote that all his patients with juvenile diabetes appeared to have inherited the disease. Consequently, in the past two decades, considerable effort has been directed to the molecular characterization of T1D and several susceptibility genes have been identified. Mutations in certain susceptibility genes, such as glucokinase, are a strong risk factor for inherited cases of Maturity-Onset Diabetes of the Young (MODY). However, since Joslin's time, the landscape of T1D has changed. Today, only 5% of genetically susceptible individuals actually develop clinical disease and there are a growing number of T1D cases among children with low risk genotypes (Maahs et al., 2010). These observations support a substantial role for environmental factors during fetal development and early childhood in T1D etiology.

Several environmental factors have been investigated in relation to T1D: early introduction of cow's milk or gluten and/or premature cessation of breast-feeding, vitamin D deficiency during gestation and the first year of life, viral infections, rapid growth in childhood, increased caloric intake, obesity, intestinal permeability, and dysfunction of the gut immune system (Akerblom et al., 2002; Gale, 2002; Howard and Lee, 2012; Maahs et al., 2010). Occupational exposure to polychlorinated biphenyls (PCB's) and childhood exposure to air-borne ozone and sulfate are also associated with T1D (Hathout et al., 2006; Langer et al., 2002). Alternatively, T1D could be caused, not so much by the addition of industrial era artifacts but by the loss of historically normal exposures that promote development of the immune system. The “hygiene hypothesis” suggests that 20th century reduction in childhood exposures to microbes and parasites may underlie the increased susceptibility at both ends of the immune disease spectrum: allergic disease and auto-immune disorders, including T1D (Okada et al., 2010).

In addition to possible dietary causes listed above, consumption of elevated levels of nitrite and nitrate in food and drinking water has been linked epidemiologically and experimentally with development of T1D, although the total number of studies remains small and results are often conflicting (Longnecker and Daniels, 2001). For example, studies in Colorado, USA and Yorkshire, England found a positive correlation between nitrate levels in drinking water and T1D incidence, particularly when nitrate levels exceeded 15 mg/L NO_3 (equivalent to 3.4 mg/L NO_3-N) (Kostraba et al., 1992; Parslow et al., 1997). Similarly, risk of T1D increased among Finnish children who consumed more nitrite (but not nitrate) in their diets and/or whose mother's consumed more dietary nitrite during pregnancy (Virtanen et al., 1994). Conversely, either no relationship or a significant inverse relationship between drinking water nitrate and T1D was observed in Sardinia, Italy, a recognized hotspot of T1D prevalence (Casu et al., 2000; Muntoni et al., 2006, respectively). In the Netherlands, van Maanen et al. (2000) reported no relationship between drinking water nitrate and T1D, although they concluded that their study lacked the sample size to detect T1D effects at nitrate levels above 25 mg/L.

To date, just one experimental study tests the effect of nitrate exposure on induction of T1D. In that study, male and female mice were fed a diet rich in cured mutton containing nitrate as a preservative before mating and during pregnancy. Their offspring were also fed the mutton from day 19 until 1–5 weeks later. This regimen produced diabetes in 16% of male progeny and 4.2% of female progeny, with greater effect among mice from the low dose nitrate group than the high dose group (Helgason et al., 1982). This experiment was inspired by the observation that, in Iceland, there was a seasonal spike in T1D among October-born boys whose parents consumed traditional nitrate-cured mutton around the time of conception, during December holiday celebrations (Helgason and Jonasson, 1981). The mutton also contained N-nitrosamines, which are metabolically related to nitrate and nitrite. In a case-control study of Swedish children, childhood consumption of nitrosamines in food was significantly positively correlated with T1D (Dahlquist et al., 1990). It is relevant that the drug streptozotocin, a specific beta cell toxin used to induce type 1 diabetes in animal models is an N-nitroso compound (Longnecker and Daniels, 2001).

To summarize, T1D incidence is rising, with substantial geographic variation. Although T1D has historically been a genetic disease, modern cases exhibit a strong environmental etiology that may include nitrate exposure. To test this hypothesis experimentally, we exposed female alligator hatchlings to three levels of nitrate (reference, 10, 100 mg/L NO_3-N) in their tank water for the period of five weeks or 5 months post-hatch. We used alligators because they are one of the main biomedical research animals investigated in our laboratory. They share many physiological features with humans while also offering a long-lived aquatic model for studying endocrine disruption. We also study wild alligator populations and this experimental project informs future work in natural ecosystems that are impacted by nitrate contamination. Doses used in this study represent the range of nitrate concentrations that can occur in ground and surface waters (Rouse et al., 1999). The 10 mg/L NO_3-N treatment matches drinking water standards in the United States and Europe, which are 10 mg/L NO_3-N and 11.4 mg/L NO_3-N, respectively.

The mechanisms of action for nitrate are only partially known at this time. We have previously identified nitrate as an endocrine disruptor that alters steroid hormone levels either directly or possibly via the actions of nitric oxide (Guillette and Edwards, 2005). Once ingested, nitrate (NO_3^-) can be reversibly converted to nitrite (NO_2^-) in the digestive tract and then nitric oxide in the gut or blood vessels (Guillette and Edwards, 2005; Panesar and Chan, 2000; Umbreit, 2007). Nitric oxide can bind the heme region of steroidogenic P450 enzymes and alter enzymatic action (Del Punta et al., 1996). By this same mechanism, nitric oxide binds heme on hemoglobin to form methemoglobin, a form of hemoglobin that cannot carry oxygen. For this reason, methemoglobinemia is also called blue baby syndrome in reference to the life threatening hypoxia that can affect bottle-fed infants who consume high concentrations of nitrate when contaminated water is used to make infant formula. Deoxy-hemoglobin can also reduce nitrite to form methemoglobin and NO. This latter mechanism is used in part to toggle NO concentrations in the endothelium of blood vessels and subsequent vasodilation (Umbreit, 2007).

In the present study, animals were screened for effects of nitrate on steroidogenesis and other physiological and anatomical endpoints relevant to T1D. The results for steroidogenesis are already published (Hamlin et al., 2016). In people, important diabetes markers also include plasma insulin, glucose stimulated insulin, and glucose tolerance. These markers were not assessed in the present alligator study for a couple of reasons. Alligator feeding needs are very different from people. They sometimes eat only weekly or even less frequently as they get older. In this study, we did not track food consumption patterns. In tandem, insulin-glucose dynamics have not yet been characterized for alligators in the context of their feeding patterns, making glucose tolerance difficult to accurately assess. Understanding these dynamics is a research need for future diabetes studies with alligators.

That said, the mid-20th century rise in T1D incidence coincides with a marked increase in global nitrate contamination from fertilizers, sewage, and fossil fuel combustion (Kaiser, 2001). Today, anthropogenically fixed nitrogen equals all naturally fixed nitrogen sources combined (Kaiser, 2001). It is therefore of great interest to determine experimentally if nitrogen contamination of freshwater systems is diabetogenic and this study contributes to that goal.

2. Methods

The experimental design, animal husbandry, and methods for