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Effects of Nicotine on Chicken Embryonic Development

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Abstract: Studies indicate that 12.7% women are smoking during pregnancy, and a significant proportion of the United States population has been exposed to maternal smoking in utero. Mounting data suggests that nicotine can have a negative impact on neural system development. The goal of this study was to evaluate effects of nicotine exposure on chicken neural system. The early chick embryo is an established model of the first month of embryonic development in mammals. Nicotine (nicotine hydrogen bitartrate) or vehicle (sodium bitartrate monohydrate) solutions were injected in eggs prior to incubation. Three cohorts of 24 eggs distributed between treatment groups were generated. After injections, eggs were sealed and placed in the incubator. Embryos were harvested on day 5 after injections, evaluated, embedded in paraplast, sectioned, and stained with hematoxylin and eosin for histological analysis. Our data indicates that the nicotine treatment at 300 ng/ml does not affect viability, weight, or length of the embryos. Nonetheless, nicotine notably affects the axial rotation of the embryos (defined as a change in the dorsoventral orientation of the head during development). In this study, altered axial rotation was observed in nicotine treated groups 4 times more often than in controls ($p<0.05$). Microscopic analysis demonstrated that atypical axial rotation was associated with incomplete closing of the embryonic neural tube in the cervical region, but not in other areas of the tube. Further research is needed to evaluate the exact mechanisms of the developmental insult onto neural system development that was observed in the present study.

Keywords: Nicotine, Chicken, Neural Development

Introduction

Neural tube defects (NTD) occur in 3000 births per year in the US (Centers for Disease and Prevention, 2010). NTD affecting the brain (anencephaly and craniorachischisis) are typically fatal. NTD affecting the spinal cord (spina bifida) commonly result in neurological dysfunctions below the region of the defect. All congenital defects are associated with increased child mortality during first year of life and a high cost paid by various medical care financiers and society due to various degrees of disabilities (Malcoe et al., 1999; Yi et al., 2011). Improved neonatal medical care makes congenital defects one of the principal causes of negative outcomes of the pregnancies. Consequently, the focus of medical field research is shifting toward more intensive investigation of the causes of developmental abnormalities. Several risk factors have been proposed for NTDs including both first hand and passive smoking during the periconceptual period (Li et al., 2012; Wang et al., 2014).

The principal component of tobacco is nicotine. The chemical underlines addictiveness of tobacco and as both toxic and teratogenic effects on the cells (Lichtensteiger et al., 1988; Joschko et al., 1991; Roy and Sabherwal, 1994; Berger et al., 1998; Roy et al., 1998; Roy et al., 2002). Even relatively low doses of nicotine show strong cytotoxicity, including sharp increase of pyknotic/apoptotic cell counts in the developing brain (Roy et al., 1998). Such neuroteratogenic effects have been revealed as structural disturbances in the cortex and

hippocampus in young animals (Roy and Sabherwal, 1994; Roy and Sabherwal, 1998; Roy et al., 2002; Onal et al., 2004). Some of the nicotine induced developmental impacts, like Purkinje neuron loss in the cerebellum, are persistent and not resolved in later development (Abou-Donia et al., 2006).

Chicks are often used in teratogenic studies because chicks have a short period of development, easily available for injections, and have well-defined developmental course (Hamburger and Hamilton, 1951). Obviously, the human nervous system has a greatly expanded structure, a longer and more complex development than nervous system of birds. Nevertheless, on earliest stages of development, that correspond to the first half of human pregnancy, birds undergo generally parallel development with mammals. At the same time, even though the general outlines of the development course are similar, humans and animals have distinct sensitivity to nicotine (Matta et al., 2007; Navaro et al., 1989), and higher doses are used in animals to model the nicotine exposure experienced by humans. Nicotine plasma levels of ~25-30 ng/ml of plasma nicotine in humans are equivalent to moderate to heavy smoking (~2 packs/day) (Benowitz, 1996; de Leon et al., 2002). In chicks, desirable nicotine levels can be 50 times higher (El-Beltagy-Ael at al., 2015). The dose we use in the current study is 300 ng/ml.

It was shown previously, that injection of high doses of cotinine (the principle metabolite of nicotine) *in ovo* induces malformations at the cranial part of the thoracic neural tube (Dalgic et al., 2009). The goal of this study was to evaluate the effect of nicotine injections onto development of chicken embryos.

Materials and Methods

Three independently produced groups of 24 fertile specific pathogen-free eggs of the domestic chicken were obtained from Charles River Laboratories (Catskill, New York, USA). Within each group the eggs were labelled and then randomly distributed between treatments to avoid the bias. Nicotine (nicotine hydrogen bitartrate calculated as free base) and vehicle chemicals (sodium bitartrate monohydrate) were obtained from Sigma-Aldrich (St. Louise, Missouri, USA). The nicotine doses of 300 ng/ml were calculated according to the weight of the egg, and matching vehicle controls were produced. The chemicals were dissolved in Ringer's solution and sterilized before the injections using .22µm Millex® syringe filter units (Sigma-Aldrich, St. Louice, Missouri, USA). Then the outer egg shell was wiped with alcohol, the injection was delivered from the blunt pole of the egg, and the hole was sealed with scotch tape (Dalgic et al., 2009). After injections, eggs were placed in the incubator.

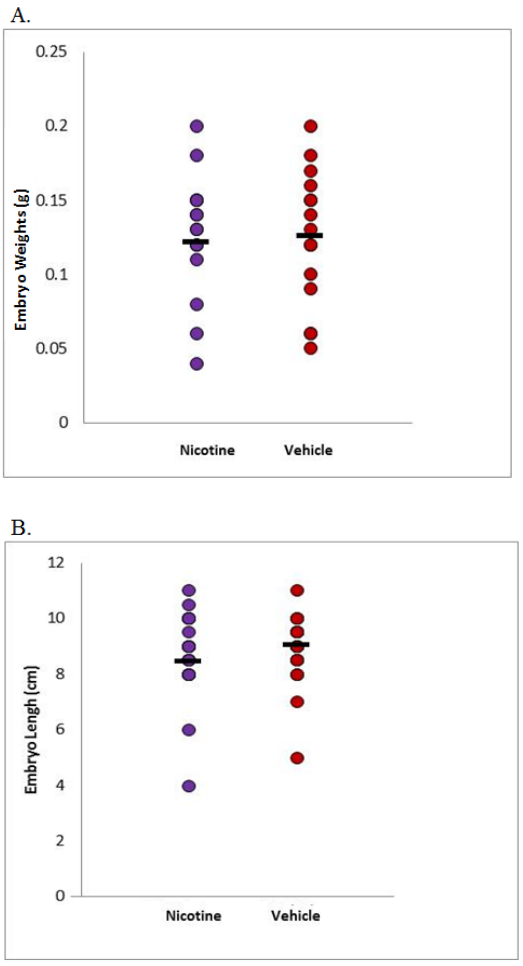

Figure 1. Range plots illustrating the weight (A) and length (B) of the harvested embryos. Values for weight are in grams; values for length are in centimeters.

Embryos were harvested on day 5 after injections. First, the shell was chipped out to observe the animals in the egg. The viability of the embryo was assessed based on the heart activity using a stereomicroscope. The appropriate development

stage was verified (Hamburger and Hamilton, 1951) and 55 embryos were admitted to the study (29 nicotine treated and 26 vehicle controls). Following the initial evaluation within the egg, the embryos were moved to a petri dish, and the extraembryonic membranes were removed.

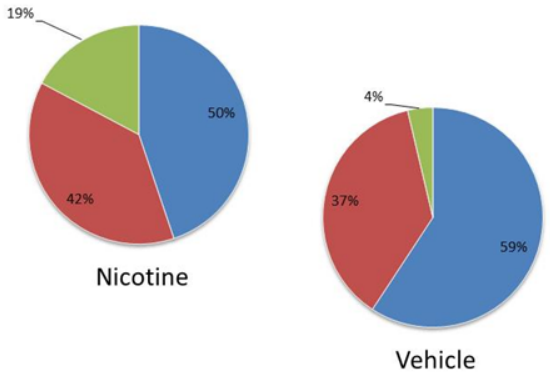

Figure 2. Circle charts demonstrate the frequencies of the embryos that have the head and body turn completed (blue), the embryos with the angle between head and body plains less than 90^{o} (red), and embryo with visually obvious problems in the cervical region such as an angle of the head turn of more than 90^{o} (green) for nicotine and vehicle treatment groups.

Following the measures of weight and length (measured from crown to rump), the embryos were fixed in 4% paraformaldehyde, and embedded in paraplast. The anterior portions of the embryos were sliced, stained with hematoxylin and eosin for light microscopic analysis. The measures of the tissue separation between spinal cord and the developing epidermis of the skin were performed on the coronal sections through the cervical region of the 32 embryos (16 nicotine and 16 vehicle treated animals) using Leica EC3 camera. The treatment groups were compared using Z-test (axial rotation) and an independent-samples t-test (the rest of the comparisons).

Results

Our data indicated that the nicotine treatment does not affect viability (animal loss in treatment groups), weight, and length of the embryos (Fig. 1). Nonetheless, nicotine notably affects the axial rotation ("head turning") of the embryos. It is known that during early development the embryo initially lies with its head facing down toward the yolk. Later in development the head starts to rotate and the rotation spreads down the body. Eventually, the entire embryo lies on its left side on top of the yolk (Roebroek et al., 1998; Manca et al., 2012). In our study, atypical axial rotation (defined by excessive rotation and uneven appearance of dorsal part of the cervical regions) was observed in nicotine treated groups 4 times more often than in controls ($p<0.05$, Fig. 2).

The microscopic evaluation indicates that altered axial rotation in nicotine treated animals was associated with defects of the spinal cord development in this treatment group. On the coronal sections taken in the cervical regions of the affected animals, we observed incomplete development of separation between the roof of the spinal cord and the surface of the embryo (Fig. 3). However, the difference in the thickness of the tissue above the dorsal surface of the spinal cord across all animals between treatment groups was not significantly different (Fig. 4).

Discussion

The study demonstrated a non-systemic, specific insult on the process of neurulation following a dose of nicotine at which animal growth is not affected. Although the mechanisms of observed insults are unclear, the data suggests that nicotine exposure at early development may lead to alteration in the CNS development in the cervical region.

There are important differences in nicotine metabolism in chick and human embryos. Human embryo exposed to drug that crossing the placenta, and pregnant smokers demonstrate high rates of nicotine metabolism (Lambers and Clark, 1996; Dempsey et al., 2002). In sharp contrast, during the early development of birds, at the time when liver and kidneys are not functional, the concentration of injected drug in embryonic tissues is likely to remain steady for a long period of time, as nicotine will not be excreted or effectively metabolized (Bolin and Burggren, 2013; Wong and Cavey, 1992). It is also possible that nicotinic metabolic pathway in chicks will have important distinctions from the one described in humans (Hukkanen et al., 2005), but it will happen later in development and is

not likely to be relevant for this study.

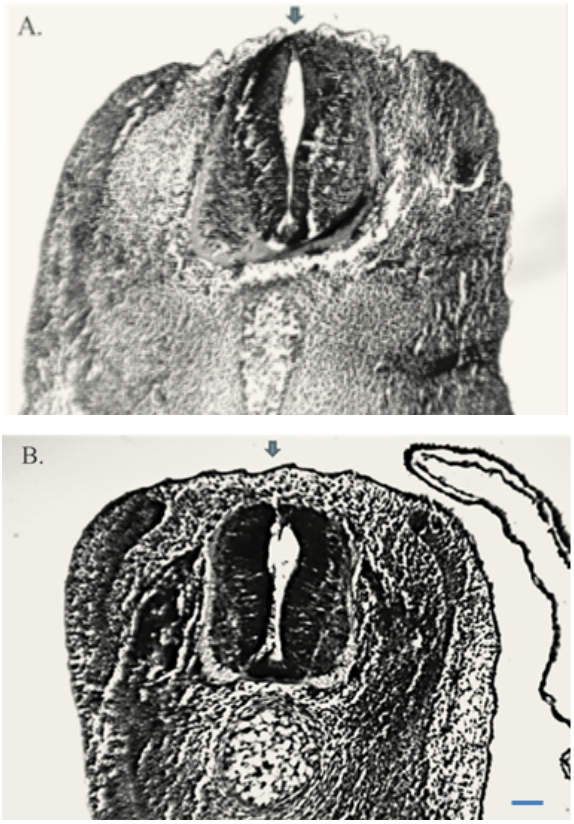

Figure 3. Cross-sections of cervical chicken spinal cords stained with hematoxylin and eosin from nicotine treated (A) and nicotine naïve (B) animals. The spinal cord of nicotine treated embryo failed to separate from the dorsal surface (arrows). At the time of sacrifice, this embryo had more than 90º angle between head and body plains. Bar: 100 µm

In our study the embryos were exposed to the higher nicotine doses than observed in human smokers. The nicotine levels observed in heavy smokers is typically reported to be around 30 ng/ml. At this or even lower doses, the maternal smoking is associated with a lower birth weight (Chiolero et al., 2005). In sharp contrast to humans, our exposure did not resulted in decrease of animal weight, but rather discrete effect on development of specific part of the neural system in a significant number of exposed animals. Mounting data indicate differential sensitivity of different species to nicotine (Navarro et al., 1989). More research is required to fully appreciate the differences in nicotine promoted insults across different species and different regimens of the drug exposure.

The exact mechanisms of observed effects also remain unclear. The recognized targets for nicotine are receptors for acetylcholine that are expressed starting from early stages of neurulation (Zoli et al., 1995; Atluri et al., 2001; Schneider et al., 2002; Tribollet et al., 2004). After migration from the mitotic zone, future neurons express mRNA for various nicotinic receptors subunits in a certain order (Atluri et al., 2001; Schneider et al., 2002). This suggests that there is a precise regulation of cholinergic actions on developmental stages and multiple ways for nicotine to disrupt this process and to provoke structural damage.

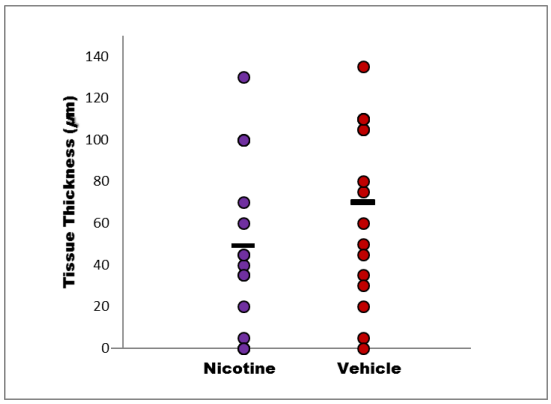

Figure 4. Range plots illustrating the thickness of the tissue measured from the developing epidermis to the roof plate of the spinal cord in the nicotine and vehicle treatment groups.

In its actions on nicotinic receptors, acetylcholine at early developmental stages acts not as a neurotransmitter but in a morphogenetic capacity by controlling and coordinating proper assembly of the brain (Seidler et al., 1994; Smith, 1994; Buznikov et al., 1996; Nguyen et al., 2001; Hohmann, 2003). Therefore, cholinergic ligands like nicotine have the ability to act as neuroteratogens (Joschko et al., 1991; Chen et al., 1999) by disrupting the timing and intensity of acetylcholine-mediated commands, most likely via alterations in gene expression (Greenberg et al., 1986; Slotkin et al., 1997; Trauth et al., 1999). Currently we are working on identification of molecular mechanisms of nicotine induced developmental defects using RNA expression analysis.

Acknowledgements

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