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Examining the Hepatic Effects of In Utero Exposure to 2-Amionanthracene

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Abstract

The extreme prevalence of diabetes in the United States has not always existed; the number of cases has dramatically risen in recent history. Previous research has indicated 2-aminoanthracene (2AA) causing alteration of gene expression and systemic injury in adult rats. This project determines the effects of 2AA on the *in utero* pups after being ingested by the dams. The goal was to identify any systemic injuries or effects on the *in utero* pups after ingestion of 2AA by their dams. This information is useful to determine the possible effects of pregnant humans ingesting 2AA, which is a compound that could easily be ingested in large quantities because of its prevalence in products and prepared food. 2AA could have potential effects on the pregnant mother and her unborn child. 2AA may cause alteration in gene expression or systemic injury that could last into adulthood and encourage or even cause insulin resistance or Type-II diabetes (T2D). Weight gain differed between the test groups and the control group. The low dose of 2AA promoted weight gain and the high dose inhibited. No significant difference in CD68+ (cluster differentiation 68) cell count between the treated and untreated groups occurred. Alteration in gene expression because of 2AA exposure was found. mRNA quantification revealed that adenosine monophosphate-activated protein kinase (AMPK), fatty acid synthase(FASN) and peroxisome proliferator-activated receptor alpha (PPARA) were not expressed, while cluster of differentiation 36 (CD36), hepatocyte nuclear factor 4 (HNF4), phosphoenolpyruvate carboxykinase 1(PCK1) and suppressor of cytokine signaling 1(SOCS1) were up-regulated. Findings from this study suggest intrauterine exposure to polycyclic aromatic hydrocarbons (PAHs), particular 2-aminoanthracene, may lead to susceptibility to T2D.

Keywords: 2-aminoanthracene, gene expression, in utero, PAH, cd68+

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Introduction

Anthropogenic activity has increased the incidence and types of toxins in the environment. Mining, industrial waste, incomplete combustion, and agricultural runoff are a few of the means by which humans directly create toxins that enter into the environment. Some of these substances are found only in certain areas whereas others like heavy metals and polycyclic aromatic hydrocarbons (PAHs) are more pervasive, promoting their exposure to humans. Toxins can cause a multitude of health issues in humans including the gastrointestinal disorders such as, vomiting, diarrhea, depression; and paralysis caused by heavy metals;[1] and skin, lung, and bladder cancer along with cardiovascular diseases, respiratory disorders; and fetal growth retardation associated with exposure to PAHs[-6]

The effect of toxins on early development are not isolated in time. Rather, these stressors while *in utero* can cause downstream health issues later in life through methylation and histone modification of genes. Studies have shown that undernutrition of pregnant mothers can genetically influence the bone mineral density of their offspring[7], and pregnant, obese women have offspring

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that have a higher risk of developing metabolic disorders later in life[8]. Other studies indicate a later onset of insulin resistance in offspring of women who had folate and Vitamin B12 imbalances during their pregnancy[9]. These, along with many other studies, indicate a relationship between stressors during a pregnancy and disease development in the offspring later in life. Some of these stressors can even be environmental toxins exposed to the pregnant mother.

Aromatic amines are one class of toxin that can occur naturally in the environment and are also synthesized for household and industrial products. These chemicals, belong to a class of compounds referred to as PAHs,are commonly used in the manufacturing of dyes, inks, plastics, and agricultural products. They are also used in polyurethanes and epoxies, and are present in road tars and fuels[10]. These compoundsare even found in cooked meat. Because of its widespread presence in everyday products, and even in products that humans eat on a regular basis or use to store food, it is probable that ingestion of this compound is occurring in large quantities and at a fairly high frequency. Ingestion of aromatic amines is a cause for alarm since these are very potent and direct acting carcinogens that cause mutations in many types of cells[11-12].

Previous research has indicated that exposure to an

Table 1. A brief description of the mRNA transcripts that had expressions quantified by quantitative RT-PCR in this experiment. The brief description is a summary of each gene from genecards.org[20]

Gene	Description
SOCS1	Codes for a STAT-induced STAT inhibitor (SSI) protein. SSI's regulate cytokine signaling by acting as a negative feedback loop. This is induced by cytokines, and as a result, reduces the effects of cytokines.
CD36	Codes for a glycoprotein that acts as a receptor for thrombospondin, making it involved in various adhesive processes. The protein also binds collagen, anionic phospholipids, and oxidized LDL. This protein binds to long chain fatty acids and may act in their transport.
AMPK	Codes for a protein that is a regulatory subunit of the AMP-activated protein kinase (AMPK). AMPK is activated in response to cellular metabolic stress. It inactivates several enzymes involved in the synthesis of fatty acids and cholesterol. Activation of this gene results in inhibition of protein, carbohydrate, and lipid synthesis. This gene codes for a positive regulator of the AMPK activity.
PCK1	Codes for a cytosolic enzyme that regulates gluconeogenesis. It catalyzes oxaloacetate to phosphoenolpyruvate, which is the rate limiting step when producing glucose from lactate. Expression can be regulated by insulin and glucagon.
FASN	Codes for an enzyme that catalyzes the creation of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH.
HNF4A	Codes for a protein with a transcription factor that binds DNA at sites required for the expression of several hepatic genes. These genes may be essential for the development of the liver, kidney and intestine.
PPARA	Located primarily in the liver, fat, kidney, heart, skeletal muscle, and large intestine to regulate fatty acid synthesis and oxidation, gluconeogenesis, ketogenesis, and lipoprotein creation. Codes for a protein that is a regulator of lipid metabolism and is activated by ligands.
SREBP1C	Codes for a transcription factor that binds to the sterol regulatory element-1 (SRE1). It regulates transcription of the LDL receptor gene and the cholesterol synthesis pathway. It is required for lipid homeostasis and regulates fatty acid production.

aromatic amine, 2-aminoanthracene (2AA), has altered the mRNA expression in the pancreas andliver of rats. The gene expression that was altered included mRNA involved in energy metabolism by the pancreas, such as genes for insulin, chymotrypsin B1, and chymotrypsin C. This suggeststhat 2AA causesderegulation of genes via a feedback mechanism that may eventually lead to insulin resistance[11](Gato and Means, 2011).Existing research has greatly indicated altered gene expression in hepatic tissue as well, including genes involved in energy metabolism and insulin signaling pathway[13](Mattis et al., 2014).

This project examines the effects of *in utero* 2AA exposure on fetal rats. The goal was to determine if the same systemic injuries caused by 2AA on adult rats would also occur in the *in utero* pups after ingestion by their dams. Any effects of *in utero* exposure to 2AA in the rats is indicative of a possible risk to humans and could cause lasting injury, even into adulthood. These injuries could promote or cause metabolic diseases, such as T2D. Evaluation of specific gene expression of suppressor of cytokine signaling 1 (SOCS1), sterol regulatory element-binding protein 1 (SREBP1C), cluster of differentiation 36 (CD36), adenosine monophosphate-activated protein kinase (AMPK), phosphoenolpyruvate carboxykinase 1 (PCK1), fatty acid synthase(FASN), hepatocyte nuclear factor 4 (HNF4A), and peroxisome proliferator-activated receptor alpha(PPARA) (Table 1) in addition to histopathological and inflammatory responses was conducted. The outcome will enable us determine whether intrauterine exposure to 2AA leads to susceptibility to diabetes later in life.

Materials and Methods

Experimental Design

Pregnant dams, all at the same point in the gestational period, were ordered. They were assigned to three dose regimens of 2AA: 0mg/kg (control), 50 mg/kg (low dose-LD) and 100mg/kg (high dose-HD). The dams were fed the 2AA contaminated diets throughout the gestational period and postpartum. The pups were sacrificed 14 d post-birth and 2 d post-wean (IACUC protocol #Il3010).

Some of the offspring were kept for a future dietary study. At three months post-wean, the animals were assigned to regular rat chow (Mazuri Rodent 6F diet, 6.5 % calorie by fat) and moderate high fat chow (Adjusted Fat Diet TD.88137 ~42% calorie by fat, from Harlan Laboratories, Inc) for 6wk. The moderate high fat diet has been previously employed to study atherosclerosis, and metabolic syndrome related conditions[14-15].

The pups were sacrificed using CO_2 chamber and the livers, in addition to other tissues, were collected and weighed. For this particular project, the only organ that was examined in detail was the liver. The livers were immediately harvested, labeled, and placed in liquid nitrogen. Small samples of the liver were fixed in 10% neutral buffered formalin for histopathological analysis.

Diet Preparation

The 2AA (CAS# 613-13-8) with 98% purity was purchased from Sigma Aldrich (St Louis, MO).The appropriate amount of 2AA was initially mixed with sucrose and shipped to Harlan Laboratories Inc. to be addedto the Global Rodent Diet 2020. Sucrose-blended 2AA was first premixed with some of the powdered diet and then incorporated into the rest of the diet for homogeneity. Approximately 10% of water was added, then the diet was pelleted without heat. The diet was dried at 50 °C for 8 hr to reduce the risk of possible mold contamination. Control diet was pelleted similar to 2AA adulterated diet to ensure uniformity in diet preparation.

At 12 wkpost-wean, the progeny were assessed for their susceptibility to T2D via dietary changes. The rat offspring wererandomized into normal rat chow and moderately high fat diet (Adjusted Fat Diet TD.88137 ~42 % by fat, from Harlan Laboratories, Inc) for 6 wk.

Extraction of Total RNA

Total RNA was extracted from the livers of the rats using a RNeasy Plus Universal Mini by Qiagen Inc. Approximately 20-30 mg of liver was homogenized in RLT buffer to denature and inactivate RNases. The RNA was then allowed to bind to a silica-gel membrane and finally was eluted with RNase-free water. Total RNA was quantified using the UV-visNanodrop 2000c spectrophotometer (Thermo Scientific). RNA electrophoretic gels were run to determine the RNA quality.

Quantification of Selected mRNA Transcripts Essential to Understanding the Diabetic Condition

The mRNA expression of six transcripts were quantified by real-time quantitative polymerase chain reaction (RT-qPCR). The expression of SOCS1,SREBP1C, CD36,

AMPK, PCK1, FASN, HNF4A, and PPARA were measured with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control gene. Measurement of mRNA levels was found using a CFX96™ Real-Time System (Bio-Rad Laboratories, Inc). FASTAmRNA sequence of the selected genes were obtained for *Rattus norvegicus* from the National Center for the Biotechnology Information (NCBI). Using NCBI (National Center for Biotechnology Information) Primer-Blast, forward and reverse primers for each mRNA transcript were created(Table 2).

Histopathology and Immunohistochemical Staining Analysis

Damage done to the liver cells were examined using histopathology. The samples were fixed overnight in buffered formalin, then embedded in paraffin and placed on glass. Hemotoxylin and eosin stain was used to make the morphology of the tissue more visible for examination. Immunohistochemical staining (IHC) for CD68 was performed on the liver. The primary antibody used for CD68 IHC was a mouse monoclonal antibody (clone ED1. Serotec, Oxford, UK). It was used at a dilution of 1:5000 with an incubation period of 1 hr. Multiple tissues from a domesticated brown rat (*Rattus norvegicus*) were used as controls.

A camera (Olympus DP70) mounted on a light microscope (Olympus BX41) with a commercial software program (cellSens, Olympus Corporation, Tokyo, Japan) was used to take photomicrographs. As many as 13 photomicrographs were captured for each sample of liver at 200x magnification at a resolution of 4080 x 3072 pixels.

As many as 12 photomicrographs of each sample liver were randomly selected for quantitative analyses using a commercial software (Photoshop CS6, Adobe Systems Inc., San Jose, CA). The number of positive stained CD68 cells was recorded for each photomicrograph and the mean was calculated for each sample.

Results

Body Weight Gain

At week 1, all the groups had different mean weights. The mean animal weight for high fat diet group showed continuous increase over time. However, the mean animal weight for regular fat diet group did not show a similar trend. The low dose group for both diet types demonstrated higher mean weights for all weeks as compared to other groups, followed by the control group for both diet types and lowest being the high dose group for both diet types. These summaries suggest that the high dose has negative effects on animal weight and the low dose has positive effects on animal weight while

Table 2. Primer sequences of specific genes quantified in liver tissues of 2AA exposed neonates

GENE	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
SOCS1	ACTCCGTGACTACCTGAGTT	AGATACTGGGAACATGTAAAC
SREBP1C	GTTAACGTGGGTCTCCT	GAGAAGAGAAGCTCTCAGG
CD36	GCTAGCTGATTACTTCTGTGTAG	GGGGTCCTGAGTTATATTTT
AMPK	AGGCTGTGAAAGAAGTATGT	GCGGTAATAATTTGAAGAGT
PCK1	CCAAGATTGGTATTGAGC	AGCTGAGAGCTTCGTAGAC
FASN	CAGAGATCCTGAGACTCTTC	CATTTTCTAGGGATAACAGC
HNF4A	AGCTGGGTATACTTGGTCAT	ACTGGTTCCTCTTATCTTTG
PPARA	CTGTTATGGGTGAAACTCTG	GGCATACTCTGTGATCTCTT

the control is in the middle. Regarding the time effect, all agent groups on a high fat diet showed considerable progression in mean weight over time while all agent groups on a regular fat diet showed minimal progression in mean weight (Figure 1).

Histopathology and Immunohistochemical Staining Analysis

For the young pups, there were great individual differences in the amount of glycogen and lipid in the livers of the animals. However, there were not significant differences in these parameters between treated and untreated groups. The similarity in the glycogen and lipid levels can probably be attributed to how long the animals nursed before they were sacrificed (Figure 2).

For CD68, there were rare and small clusters of CD68+ macrophages near portal areas (Figure 3). Randomized measured aggregates of hepatic CD68+ cells seemed to be slightly increased in the 2AA exposure of young rats (Figure 4A). This change was not observed to be significantly altered relative to the control groups. With respect to the older rats, the female progeny on a regular diet and exposed to low or high levels of 2AA *in utero* showed the greatest levels of CD68+ cells (Figure 4B). The control groups appeared to indicate lower amounts of CD68+ positive cells.

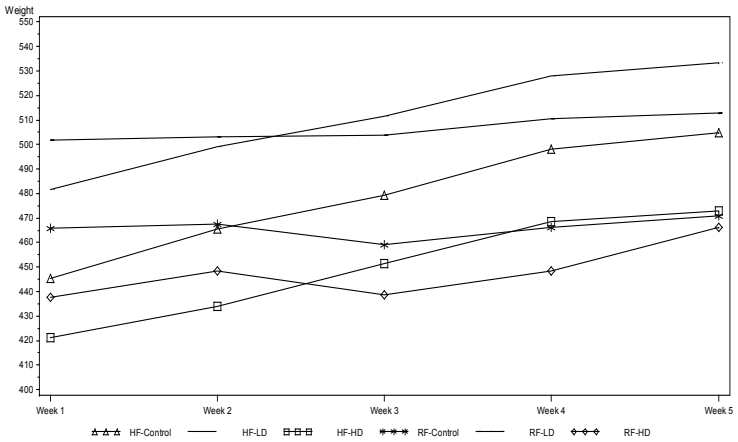


Figure 1. Sprague Dawley body weight showing the mean animal weight for the diet subgroups and 2AA subgroups for five weeks. Treatment groups include HF-Control (moderate high fat control); HF-LD (moderate high fat low dose, 50 mg/kg-2AA); HF-HD (moderate high fat high dose, 100 mg/kg-2AA); RF-Control (regular diet control); RF-LD (regular diet low dose, 50 mg/kg-2AA); and RF-HD (regular diet high dose, 100 mg/kg-2AA).

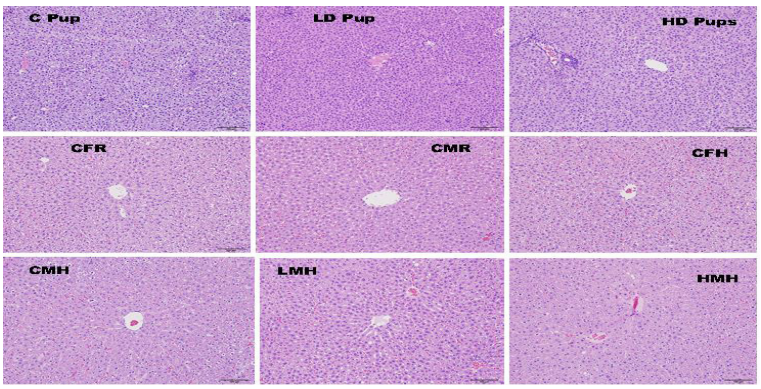


Figure 2. Histologic depiction of hepatic architecture of Sprague Dawley rat offspring that were exposed to 0 mg/kg- (C Pup), 50 mg/kg- (LD Pup) and 100 mg/kg-2AA (HD Pup) in the intrauterine environment. Three months postwean rats were assigned into moderate high and regular diet groups for six weeks. Select images include: CFR – control female regular diet; CMR – control male regular diet; CFH – control high fat diet; CMH – control male high fat diet; LMH – low dose male high fat; and HMH – high dose male high fat diet.

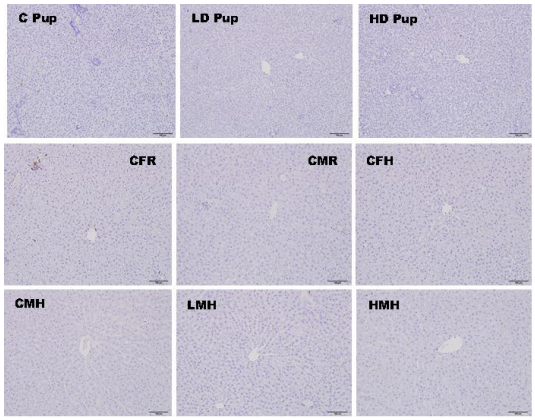


Figure 3. Hepatic CD68 immunohistochemical (IHC) staining. Progeny were from dams ingested 2AA from gestation through postweaning. C Pup – 0 mg/kg-; LD Pup – 50 mg/kg- and HD Pup – 100 mg/kg-2AA. Older pups were fed moderate high and regular diet for six weeks. Select images include: CFR – control female regular diet; CMR – control male regular diet; CFH – control high fat diet; CMH – control male high fat diet; LMH – low dose male high fat; and HMH – high dose male high fat diet.

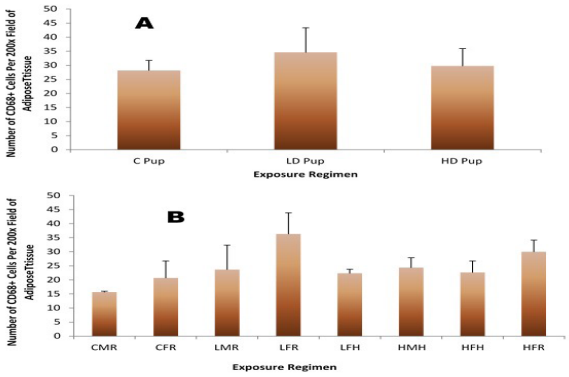


Figure 4. Randomized CD68 positive cells in the liver**A**. Intrauterine treatment to 2AA groups included; C Pup-Control (mg/kg diet), LD Pup-Low Dose (50mg/kg diet), HD Pup-High Dose (100 mg/kg diet). **B**.*In utero* exposure was combined with dietary ingestions of regular diet (5M30 rodent diet) and high fat diet (Adjusted Fat Diet TD.96132) for 6 weeks 3 months postwean; CMH-control male high fat; CMR- control male regular diet; LMH-low dose male high fat; CFR-control female regular diet; LFR-low dose female regular diet; HFR-high dose female regular diet; HFR-high dose female regular diet; andHMH-high dose high fat. Though the treated exposed rats indicated higher CD68 values, statistical inference showed they were not significant.

Quantification of Selected mRNA Transcripts Essential to Understanding the Diabetic Condition

To further investigate intrauterine effects on the development of diabetic condition, specific gene expression related to these processes were examined. AMPK, FASN, and PPARA genes were not shown to be expressed at all in the treated groups. On the contrary, two-week neonates whose mothers ingested 2AA during gestation revealed the up-regulation of CD36, HNF4, PCK1, and SOCS1 in their livers (Figure 5).

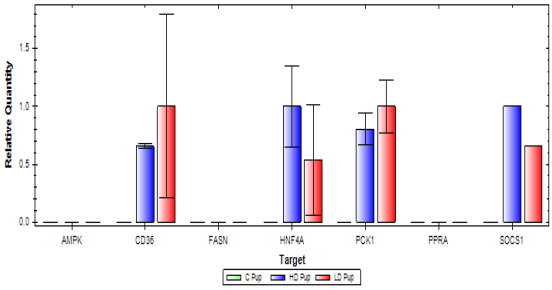


Figure 5. Relative hepatic mRNA quantification of selected transcripts that play crucial roles in understanding the diabetic disease condition. Rats were exposed to 2AA *in utero*. Exposure groups were 0 mg/kg- (C Pup); 50 mg/kg- (LD Pup) and 100 mg/kg-2AA (HD Pup).

Discussion

The present study was undertaken to investigate the contribution of non-traditional risk factors such as exposure to a PAHs, 2AA, during early years of life to T2D. Exposure to 2AA seemed to have a modulating effect on the body weight gain of the rats studied. Overall, animals exposed to 2AA *in utero* and fed moderate high fat diets for three months post-wean indicated higher weight gains than 2AA control animals. This finding seems to support the hypothesis of the study that intrauterine disturbance via chemical exposure will cause effects in adulthood related to T2D. Others studies have found similar outcomes when humans and laboratory rats were exposed to chemicals classified as obesogens[16] (Thayer et al, 2012).

Another parameter examined in the present study was anatomy of the hepatocytes via histopathology. Qualitative analysis of the liver of both neonates and three month old Sprague Dawley rats on moderate high fat diets showed no significant differences in glycogen

or lipids between the treated and untreated rats. There was also no significant difference in staining of CD68+ cells in the livers between the treated and untreated rats. However, randomized counting of the amount of CD68+ cells denote slightly increased aggregates of these cells in low and high dose animals on regular diet. Comparing the animals on control diet with the animals on high fat diets, the control male and females had the lowest levels of CD68+ cells. Cd68+ provides crucial information related to the level of macrophages, which in turn cpan be correlated with cellular inflammatory activity[17].

Finally, we quantified the abundance of mRNA in the livers of neonates to provide clues as to which gene would be the most essential to understanding the diabetic condition during adulthood. For instance, deletion of PCK1 in both specific tissues and whole bodies were found to lead to T2D in rats[18], whereas the overexpression of SOCS1 was previously noted to have a protective role in diabetes[19]. Quantification of hepatic gene expression in the two week old pups revealed up-regulation of CD36, HNF4, PCK1 and SOCS1 transcripts. These animals were from dams that ingested 2AA during gestation and postpartum period. On the contrary AMPK, FASN and PPRA were not expressed at all. Overall, there were significant changes between pups exposed to 2-aminoanthracene *in utero* and the control pups.

In conclusion, findings from this study suggest intrauterine exposure to PAHs, particularly 2-aminoanthracene, may lead to susceptibility to T2D. Though anatomic evaluation of the liver displayed no alterations, several genes such as SOCS1 and PCK1 were up-regulated in a dose-dependent manner. Changes in body weight gains seem to support this assertion. It was determined that the low dose of 2AA acted to increase weight gain, the high dose negatively affected weight gain, and the high fat diet was shown to cause more weight gain. CD68 indicate the level of inflammatory activity in the tissues. It appears the exposed pups had a slightly higher levels of CD68 positive cells. When 2AA exposure is combined with moderate high fat, the CD68 positive cells were greater in these set of animals.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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