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ORIGINAL ARTICLE

Inflammatory effect of 2-aminoanthracene (2AA) on adipose tissue gene expression in pregnant Sprague Dawley rats

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ABSTRACT

Adipocyte dysfunction may be a critical link between obesity and insulin resistance as a result of abnormal fat storage and mobilization. Adipocytes uniquely secrete adipokines and cytokines, such as leptin and TNFα, wich promote insulin sensitivity. Previously we reported insulin-signaling related altered gene expression in animals exposed to 2-Aminoanthracene (2AA). 2AA is an amino-substituted polycyclic aromatic hydrocarbon used in manufacturing dyes, chemicals, inks, resins, and polyurethanes. The objective of this study was to examine the inflammation related effects of 2AA exposure from gestation to postnatal period on dams that ingested 2AA. To examine 2AA effects, pregnant dams were assigned into dose regimens of 2AA. Dams were fed 2AA contaminated diet during the period of gestation and postpartum. The expression of key gene transcripts reported to be important in mediating inflammatory processes was examined via quantitative RT-PCR. Histologic examination of adipose tissue (AT) was also carried out to understand the anatomy of AT due to 2AA exposure during gestation and two weeks postpartum. Examination of the adipose tissue for microscopic changes revealed no alterations between control and low-dose animals. However, AT of the high-dose animals was infiltrated by increased numbers of CD68+mononuclear cells (macrophages) and small numbers of eosinophils and mast cells, consistent with inflammation. In addition, analysis of the mRNA expression of cytokines and adipokines demonstrated the importance of inflammation in AT dysfunction. For instance, TNFα, LEPTIN and IL-6 transcripts were relatively more expressed in the low dose animals than in the high dose and control rats. At the protein level, however, high amounts of cytokines were noted. The effects of 2AA on pregnant dams appear to be more pronounced in the high dose group than in the low dose group, possibly indicating increased susceptibility of rat offspring within this group to elicit a diabetic-type response.

KEY WORDS: 2-aminoanthracene; adipose tissue (AT); CD68; TNFα; inflammatory response; susceptibility

ABBREVIATIONS:
2AA: 2-Aminoanthracene; **PAH:** Polycyclic Aromatic Hydrocarbon; **AT:** Adipose Tissue; **CD68:** Cluster of differentiation 68; **CD14:** Cluster of differentiation 14; **sCD14:** soluble CD14; **mCD14:** membrane anchored CD14; **TNFα:** Tumor necrosis factor alpha; **IL-6:** Interleukin – IL-6; **C (0 mg/kg):** Control; **LD (50 mg/kg):** Low dose; **HD (100 mg/kg):** High dose

Introduction

Exposure to environmental contaminants through various routes is known to increase risk-developing disease such as type-2-diabetes and cancer (Diamanti-Kandarakis *et al.*, 2009; Sargis *et al.*, 2012). Environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) directly affect gene expression. This could lead to an increase or reduction in the level of corresponding proteins through either destabilization of the mRNA or inhibition of translation (Walker, 2012). A recent paper from our research laboratory indicated that 2-aminoanthracene (2AA), a PAH modulates the expression of some insulin signaling related genes in a time-dependent manner (Mattis *et al.*, 2014).

2-Aminoanthracene (2AA), a PAH, is a known carcinogen. This aromatic amine occurs naturally and in synthetic form. 2AA is also a teratogen that may cause mutations which may lead to birth defects or malformations (Boudreau *et al.*, 2006). Occupational and non-occupational exposure to 2AA has been noted in the

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past. 2AA can be found in drugs, agricultural chemicals, plastics, inks, and dyes charbroiled and cooked meats (Baker *et al.*, 2001; Boudreau *et al.*, 2006). The compound 2AA is metabolized via an indirect biotransformation pathway, which involves initial oxidation by hepatic (liver) P450 enzymes to create more reactive hydroxylamine derivative (Jemnitz *et al.*, 2004). The hydroxylamine is metabolized by enzyme N-acetyltransferase (NAT) and sulfotransferase to yield a highly reactive O-substituted N-hydroxylamine intermediate. This is then followed by spontaneous heterolysis of the N-O bond to generate arylnitrenium ions. This electrophilic reactive metabolite can form DNA adducts that in turn modulate broad gene expression (Gato *et al.*, 2012).

It has been established in *in vitro* experiments that increased adipose cell size correlates with serum insulin concentrations, insulin resistance, and increased risk of developing type-2-diabetes (Lönn *et al.*, 2010). Enlarged adipose tissue and adipocytes produce a variety of hormones involved in glucose metabolism, inflammation, macrophage activation, and fibrinolysis. Dysfunctional adipocytes provide a critical link between obesity and insulin resistance leading to abnormal fat storage and mobilization (Ferland-McCollough *et al.*, 2010; Jung & Choi, 2014).

Figure 1 provides a schematic of the link between adipokines and cytokines to insulin sensitivity. Adipocytes uniquely secrete adipokines such as *LEPTIN* and *ADIPONECTIN* that promote insulin sensitivity [12]. Adipose tissues contain bone marrow-derived macrophages, and the content of macrophages tracks with degree of obesity. Adipose tissue macrophages (ATM) are a major source of cytokines such as tumor necrosis factor alpha (TNF_a) and interleukin 6 (*IL-6*). These function in a paracrine and potentially an endocrine fashion to cause decreased insulin sensitivity. Activation of macrophages leads to release of variety of cytokines which recruit additional cytokines in a feed forward process that only further increases ATM (Hajer *et al.*, 2008; Makki *et al.*, 2013; Prieto-Hontoria *et al.*, 2011). ATM surface associated *CD14* (cluster of differentiation 14 represented in two forms – soluble or or membrane anchored) on the other hand regulates adipose tissue inflammatory activity and insulin resistance through interaction with toll-like receptors in adipocytes (Hajer *et al.*, 2008; Fernández-Real *et al.*, 2011). Finally the macrophage content can be examined by looking at the CD68 marker for macrophages.

The goal of this study is to examine the effect of 2AA exposure from gestation to postnatal period on adipose tissue of dams that ingested 2AA. The modulation of specific mRNA transcripts and the cellular response were evaluated for altered gene expression and adipose tissue macrophage infiltration. The ultimate goal is to understand the extent to which environmental PAH exposure in mothers affects the offspring with respect to disease susceptibility.

Methods

Experimental Design

Nine timed pregnant dams (Day 1) were purchased from Taconic Hudson, NY and assigned into dose regimens of 0 mg/kg – (control – C), 50 mg/kg – (low dose – LD) and 100 mg/kg diet – (high dose – HD) 2AA. Dams were fed 2AA contaminated diet during the period of gestation and postpartum. Dams were sacrificed post-weaning. AT from the abdomen along with other tissues were sampled

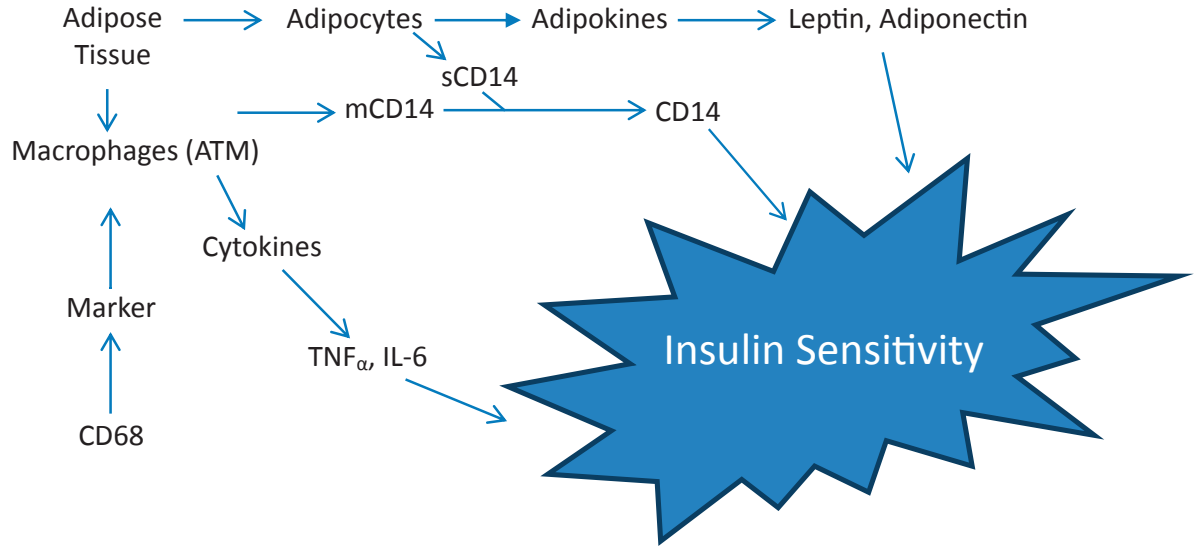

Figure 1. Diagrammatic representation of unique inflammatory proteins that provide a link to insulin sensitivity. The figure shows the importance of the adipose tissue in producing genes that essential to understanding insulin sensitivity.

at necropsy and excised tissues were frozen immediately in liquid nitrogen and stored in −80 °C freezer until analysis. Animals were housed at the Georgia Southern University Animal Facility (1176A Biological Sciences Fieldhouse). This facility is accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were treated according to the principles outlined in the ILAR's (Institute for Laboratory Animal Research) Guide for Care and Use of Laboratory Animals. Our protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC protocol# I13010). We were careful to minimize the number of animals employed in the research as well as minimizing animal discomfort.

Diet preparation

The 2AA (CAS# 613-13-8) with 98 % was purchased from Sigma Aldrich (St Louis, MO) and used without further purification. The appropriate amount of 2AA was initially mixed with sucrose and shipped to Harlan Laboratories Inc. for incorporation into the Global Rodent Diet 2020. 2AA was incorporated into the 2020 diet at Harlan Laboratories Inc., Madison WI. Sucrose blended 2AA was premixed with some of the powdered-diet and then mixed with the rest of the diet for even distribution. Approximately 10% of water was added to the diet and then pelleted. No heat was added during the pelleting process. The diet is finally dried at 50 °C for 8 hours in order to reduce moisture and possible mold contamination. The diet was then packaged and shipped. Control diet was pelleted similar to 2AA adulterated diet to ensure uniformity in diet preparation.

Total RNA isolation

The RNeasy Plus Universal Mini by Qiagen Inc. was used for total RNA isolation. The procedures were followed as listed in the protocol. Approximately 20–30 mg of adipose tissue sample was added to a QIAzol lysis reagent, homogenized and allowed to bind to RNA spin column. Total RNA concentration and quality were examined using Nanodrop 2000c spectrophotometer, RNA electrophoretic gels and ExperionTM RNA StdSens analysis kit according to the manufacturer's specifications (Bio-Rad Laboratories Inc., Hercules, CA USA).

H&E and other immunohistochemistry

AT were fixed in 10% neutral buffered formalin for at least 48 hours, trimmed, routinely processed for histology, sectioned at 4-µm thickness, and stained with hematoxylin and eosin.

Immunohistoc hemical staining (IHC) for the macrophage marker CD68 was performed on unstained 4-mm thick sections of the AT. The primary antibody was a mouse monoclonal antibody (clone ED1. Serotec, Oxford, UK) at a dilution of 1:5 000 with an incubation period of 60 min. Multiple tissues from a domesticated brown rat (*Rattus norvegicus*) were used as controls.

Photomicrographs of each sample were acquired using a camera (Olympus DP70) mounted on a light microscope (Olympus BX41) with a commercial software program (cellSens, Olympus Corporation, Tokyo, Japan). Up to 13 photomicrographs were captured for each sample of AT at 200× and 400× magnification at a resolution of 4080×3072 pixels.

Up to 12 photomicrographs of each sample were randomly selected for quantitative analyses using a commercial software program (Photoshop CS6, Adobe Systems Inc., San Jose, CA). In sections of the AT, the number of positive-staining cells for CD68 was recorded for each selected photomicrograph at 200× magnification, and the mean was calculated per sample. Similarly, the surface areas of 10 randomly-selected adipocytes from each group were measured at 400× magnification, and the mean was calculated per sample.

Adipokine and cytokine mRNA quantification by RT-PCR

The expression of key gene transcripts reported to be important in mediating inflammatory processes was examined via quantitative RT-PCR. Genes whose expression levels were quantified included: ADIPONECTIN, TNF-α, IL-6, CD14, CD68, LEPTIN, and GAPDH as a housekeeping gene. FASTA mRNA sequences of these mRNA transcripts were obtained for *Rattus norvegicus* using the National Center for the Biotechnology Information (NCBI) database. Forward and reverse primers for the genes were then generated using NCBI Primer-Blast. Primer sequences were shown in Table 1. Primers were bought from Integrated DNA Technologies Inc (IDT), Coralville IA USA.

An iScript cDNA synthesis kit was employed to synthesize cDNAs from total RNA extract samples of dam adipose tissue. These were then combined with primers and SsoFast EvaGreen supermix for the qPCR reaction. The product was quantified via a Bio-Rad CFX96TM instrument (Bio-Rad Laboratories Inc.) using the manufacturer's guidelines. The normalized relative gene expression values were determined via delta Ct parameter.

Table 1. Nucleotide sequences designed as forward and reverse primers of each specific gene.

Adiponectin	Forward	5′ CCGCTTACATGTATCACTC 3′
	Reverse	5′ ATACTGGTCGTAGGTGAAGA 3′
CD68	Forward	5′ AAGTCCTAGTCCAAGCTCTA 3′
	Reverse	5′ AGGACACATTGTATTCCACT 3′
CD14	Forward	5′ CTCAGAATCTACCGACCA 3′
	Reverse	5′ ATAGATTGAGCGAGTTTAGC 3′
IL-6	Forward	5′ GGAGTTTGTGAAGAACAACT 3′
	Reverse	5′ CTAGGGTTTCAGTATTGCTC 3′
Leptin	Forward	5′ CTGTCGTGACTGACTCTATG 3
	Reverse	5′ GCTAAGTGATTTCTCATTCC 3′
TNF-α	Forward	5′ GAACACCCTGGTACTAACTC 3′
	Reverse	5′ TAGATAAGGTACAGCCCATC 3′

Serum adipokine quantification via ELISA

Thermo Scientific's rat TNFα ELISA kit was used to determine the TNFα amount in blood serum of dams that consumed 2AA via the diet. The protocol was followed according to procedures as listed in the manufacturer's assay instruction booklet. Approximately 50 µl of the 1:1 diluted blood serum was added anti-rat TNFα precoated wells. Loosely adherent antibodies were removed with series of buffer washes followed by incubation with biotinylated antibody. After another wash, enzyme substrate was added to wells and the content read at 450 nm and 550 nm on a microplate reader (Spectra Max 190, Molecular Devices Corporation, Sunnyvale, CA, USA). TNFα amount was determined using standard curve of a plot of absorbance at 450 nm minus absorbance at 550 nm against known TNFα concentrations ranging from 0 to 2 500 pg/mL. Significant difference in the concentration of TNFα in serum was determined using one-way ANOVA.

Data analysis

Body weight and feeding data were analyzed using SAS 9.3 statistical software package for Windows (SAS Institute Inc., Cary, NC, USA). Random intercept mixed model was applied to the dataset. The model is: WEIGHT=β_0+β_1W1 + β_2 eat + β_3 date+ β_4 group + β_5 group*date; W1: initial dam's weight; The weight at April, 04, 2014; Eat: how much dam's eat at each date; Date: date record dam's feeding; Group: 1=control, 2=low dose, 3=high dose; and Group*date: group and date interaction term.

Statistical significant differences in the quantity of TNFα proteins in serum of 2AA treated and untreated animals were calculated via analysis of variance (ANOVA). Also, significant differences in the amount of CD68+ cells and adipocyte size data were handled similar to the TNFα proteins results. Data was presented as mean±SE. Significant differences were indicated as either $^{*}p<0.05$ or $^{**}p<0.01$.

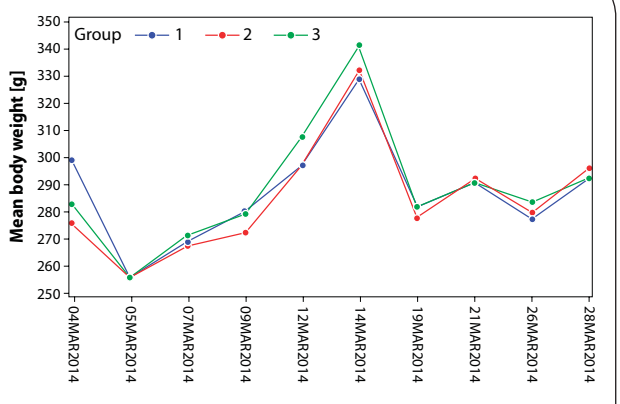

Figure 2. Body weight gain of Sprague Dawley rats during gestation and postnatal period. Animals were exposed to 0 mg/kg 2AA diet (group 1), 50 mg/kg 2AA diet (group 2) and 100 mg/kg 2AA diet (group 3). No significant differences were noted for body weight gain of dams via comparison of the treated group with control rats.

Results

Effect of 2AA on body weight gain

As part of the feeding study, the weights of the animals were monitored over a period of time. The statistical model showed that the initial weight was different between the three groups though the dam's weight changed over time. Also, there was no group effect in the model examined. Similarly, no group or date interaction was noted in the model. That means the weight at each date can be considered as not significantly different. The means of weight is parallel over the time (Figure 2).

Histopathology via H&E and other immunohistochemistry

Histologic examination of the AT from all three groups revealed sheets of mature adipocytes, containing small numbers of scattered, elongate to round cells, with small to moderate amounts of eosinophilic granular cytoplasm, and a single, centrally-located nucleus with finely-stippled chromatin (macrophages) (Figures 3A, B, C). In addition, histologic examination of the AT from the high dose group (Figure 3F revealed small numbers of mast cells, eosinophils, lymphocytes, and plasma cells, which frequently clustered into loose aggregates adjacent to blood vessels. No other significant differences are evident between the groups on histologic examination.

Immunohistochemical staining for CD68 revealed frequent, moderate to strong, and cytoplasmic staining of the macrophages within the AT of all three groups. Quantitative analysis revealed fewer numbers of CD68-positive cells among the low dose group, compared to that of the control and high dose groups (Figure 4). With regards to the size of adipocytes (Figure 5), there was a large variation within each animal (data not shown), but when compared across groups, the mean size of adipocytes was similar.

Adipokine and cytokine mRNA quantification by RT-PCR

For the present study, the expression of mRNAs that might suggest susceptibility to metabolic syndrome was examined. Six genes including Adiponectin, CD14, CD68, IL-6, Leptin and TNFα were quantified in the AT of dams. These dams were fed 2AA contaminated diets during gestation and postnatal periods. Adiponectin gene was not expressed in any of the treatment groups (Figure 6). CD14 and CD68 mRNA was more highly expressed in AT of the high dose (100 mg/kg) group relative to the control. Genes IL-6 and TNFα were differentially up-regulated in both the high and low dose (50 mg/kg) rats relative to control animals. Relative expression of leptin was unchanged between control and high dose groups and significantly higher in the low dose animals. Similarly, markedly increased levels of IL-6 and TNFα were observed in the low dose group.

Quantification of TNFα levels in serum via ELISA Assay

To validate adipokine and cytokine mRNA values quantified by qRT-PCR, ELISA was employed to calculate the level of TNFα in the serum. The concentration of TNFα in both treatment and control groups was minute, that

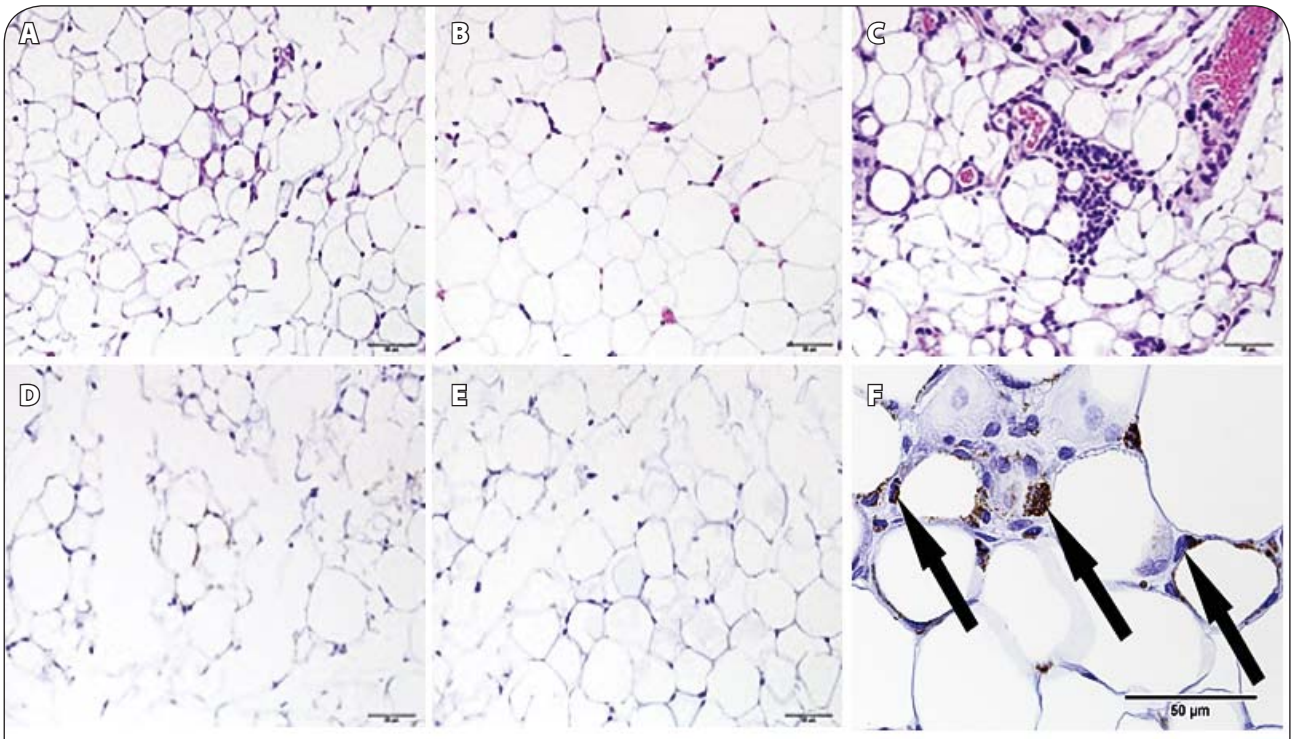

Figure 3. A, B & C. Histopathological changes in AT of dams fed 2AA from gestation through postnatal period (H&E stain). **D, E & F** indicate selected CD68 immunohistochemical staining in AT of dams that ingested 2AA from gestation through postweaning. The arrows in Figure 3F represent locations of CD68 positive cells. A, D were control (0 mg/kg); followed by B, E being low dose (50 mg/kg) and C, F high dose (100 mg/kg-2AA)

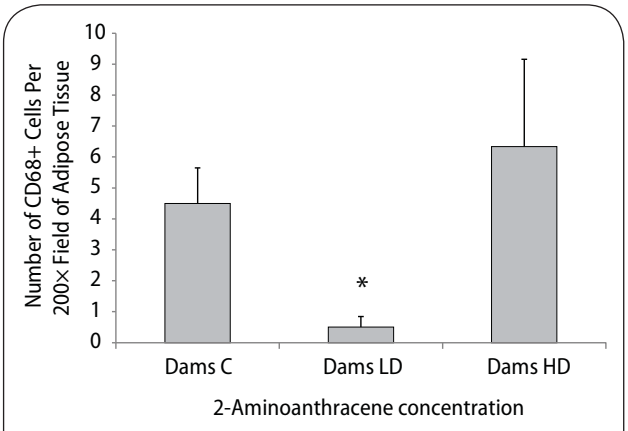

Figure 4. Levels of CD68+ cells in Adipose Tissue. Dams C – Control (0 mg/kg diet), Dams LD – Low Dose (50 mg/kg diet), Dams HD – High Dose (100 mg/kg diet). Significant decreases in CD68 was observed in the low dose group, *$p<0.05$.

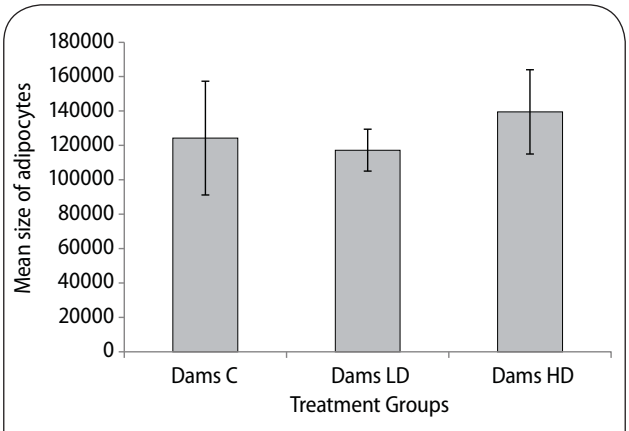

Figure 5. Mean size of adipocytes. Photoshop was employed to measure the surface areas of 10 adipocytes for each treatment group at 400× magnification, measured in numbers of pixel. Dams C – Control (0 mg/kg diet), Dams LD – Low Dose (50 mg/kg diet), Dams HD – High Dose (100 mg/kg diet). No significant difference between treatment groups was noted.

is, in picogram amounts. TNFα concentration was dose dependently elevated in the blood; dams that ingested 2AA had significantly increased TNFα levels in serum.

Discussion

A recent diabetes report indicated an ever increasing diabetes incidence in the American population particularly in children (CDC, 2014). Albeit, with much effort invested in creating public awareness, encouragement to eat right and to exercise, diabetes incidence continues to rise. Researchers believe exposure to environmental chemicals during early years of life or *in utero* may play a significant contributing role in the process (Heindel, 2006; Patriarca *et al.*, 2000; Newbold, 2010). A recent study reported an association between endocrine-disrupting environmental toxicants and obesity and diabetes (Newbold, 2010). The

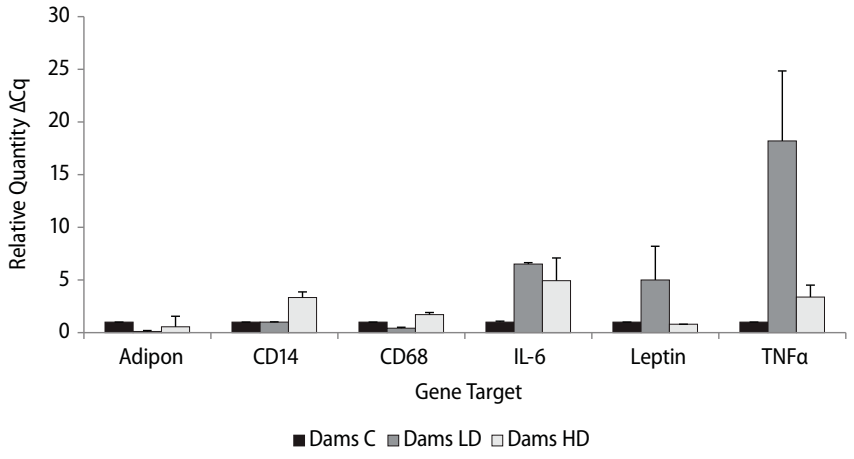

Figure 6. Relative Expression (ΔCq) of Adipose Tissue Genes Involved in Diabetic-Related Conditions. Dams were fed C – Control (0 mg/kg diet), Dams LD – Low Dose (50 mg/kg diet), Dams HD – High Dose (100 mg/kg diet) from Gestation through weaning.

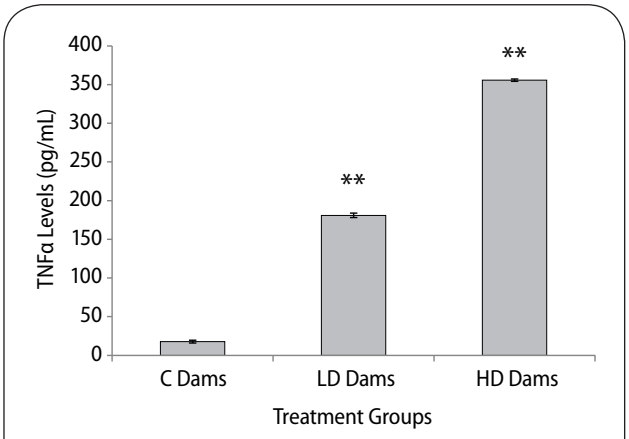

Figure 7. The quantification of TNF_{α} levels in the serum of dams via ELISA. TNFα concentration was significantly (**p<0.01) elevated in dams that were exposed to 2AA from gestation through postnatal period. Treatment groups included: 0 mg/kg – (C Dams); 50 mg/kg – (LD Dams) and 100 mg/kg-2AA diet (HD Dams)

ultimate goal of our research group is to examine the link between exposure to polycyclic aromatic hydrocarbon (2AA) exposure *in utero* and diseases such as diabetes. Dams cannot be directly compared with offspring in their responses to contaminant exposure because of epigenetic differences. Nevertheless, much information can be gleaned from evaluating the dams' reaction during one of their most vulnerable periods.

Weight gain of dams during the course of the study showed no significant difference. Mean weight gain was parallel over time with no group effect observed in the model used to evaluate 2AA effect on weight gain. This observation is consistent with a previous study involving the toxicity of 2AA in fisher-344 rats (Gato & Means, 2011). To examine specific gene expression data, understanding the anatomy of the AT is essential. Similar to the body mass data, microscopic evaluation of AT indicated no architectural changes between the control and low dose group. However, in the AT of animals fed 100 mg/kg of 2AA there were clusters of mononuclear cells infiltrating the AT, as well as small numbers of eosinophils and mast cells. The adipocyte size among the groups were not significantly different though slightly greater in exposed groups. The adipocyte size has been previously linked to diabetes and obesity, as enlarged adipocytes are reported to promote the recruitment of macrophages that consequently increase inflammation (Greenberg & Obin, 2006).

To further determine the effect of 2AA on dams during gestation and postnatal, the mRNA expression of selected adipokines and cytokines in AT was analyzed. Adipose tissue is the primary site of triglycerides and lipid storage (Arner *et al.*, 2010). Certain types of AT will act as a "sink" for fatty acids storing lipids that would otherwise be detrimental in high concentrations if in the plasma or ectopic organs (Ferland-McCollough *et al.*, 2010). AT does not just provide storage for excess calories but also secretes fatty acid and a variety of polypeptides (Arner *et al.*, 2010). Adipose tissue consists of a variety of cells that control the unique protein signatures functions such as ADIPONECTIN, TNFα, IL-6, CD14, CD68, and LEPTIN. Adipocytes uniquely secrete adipokines such as LEPTIN and ADIPONECTIN that promote insulin (Kwon, 2013). Immune cells such as macrophages are bone marrow derived that are reported to correlate with the degree of obesity (Linehan *et al.*, 2014; Tilg & Moschen, 2006; Trouplin *et al.*, 2013). AT macrophages (ATM) are major source of cytokines such as TNFα and IL-6 (Guilerme *et al.*, 2008; Olefsky & Glass, 2010), which function as paracrine and potentially an endocrine fashion to cause decreased insulin sensitivity. Activation of macrophages leads to release of variety of cytokines that recruit additional cytokines in a feed forward process that further increases ATM number. CD14 on the other hand modulates AT inflammatory activity and insulin resistance through interaction with toll-like receptor cells ((Hajer *et al.*, 2008; Prieto-Hontoria *et al.*, 201). For the present study, there was a rise in the amount of macrophage infiltration in the high dose group compared to the low dose and control dams. Similar patterns of CD68 staining in tissues were noted in the gene expression of CD68. The mRNA expression of *CD68* in AT seems to follow the patterns observed in quantified CD68 positive cells.

In addition to *CD68* mRNA expression, adipokines such as leptin and *ADIPONECTIN* and proinflammatory cytokines *TNFα* and *IL-6* were examined for their activity

in AT. Gene expression results indicate that *TNFα*, *LEPTIN* and *IL-6* were up-regulated in the low dose dams more so than the control and high dose animals. This implies that this group as well as the high dose animals may be susceptible to a diabetic phenotype in the offspring. The expression of *ADIPONECTIN* was relatively higher in the control than the treated dams. On the contrary, expression of the transcript *CD14* was greater in the high dose animals than both control and low dose groups. Clearly, dams that ingested 2AA show inflammatory response in varying degrees. It appears ingestion of 50 mg/kg-2AA diet by dams affects the activity of TNFα, leptin and IL-6 in a proinflammatory fashion. Whereas animals that were fed 100 mg/kg-2AA had CD14, CD68, *TNFα, LEPTIN* and *IL-6* affected.

We further examined the level of TNFα protein in serum of dams. The level of this cytokine in serum was dose dependent. That is dams fed 100 mg/kg-2AA diet had highest TNFα content in serum followed by the 50 mg/kg-2AA and the control. Previously, TNFα activity in serum has been found to reveal the pattern of TNFα amount in fat tissues (Lönn *et al.*, 2010; Winkler *et al.*, 2003). We believe the aggregate TNFα in serum reflects relative quantity in adipose tissues.

The data from Figures 4, 6 and 7 might seem contradictory. This is not the case at all. For instance, CD68+ cells, which indicate the level of macrophages in the cell seem to be less in the low dose group. On the contrary, two transcripts IL-6 and TNFα released by macrophages were over-expressed in the low dose animals. This seeming anomaly might be due to the fact that CD68+ counts were performed on tissue sections randomized while the tissues used in the gene expression were whole. Also, serum protein TNFα amount was elevated correspondingly in the 2AA treated groups opposite of the TNFα mRNA level. It is well known fact that cellular protein abundance do not always positively correlate with mRNA expression (Vélez-Bermúdez & Schmidt, 2014). This might explain the apparent contradiction in the serum TNFα concentration and gene expression.

Conclusions

The effect of ingestion of 2AA a PAH was evaluated in pregnant Sprague Dawley dams. Body weight gain during gestation and postnatal period indicated no significant differences in animals. Examination of the AT for microscopic changes suggests no architectural alterations between control and low dose animals. However, there was an inflammatory response in the AT of the high dose animals with clusters of mononuclear cells and small numbers of eosinophils and mast cells. In addition, analysis of the mRNA expression of cytokines and adipokines demonstrate the importance of inflammation in ATs. For instance, TNFα, LEPTIN and IL-6 transcripts were relatively more expressed in the low dose animals than the high dose and control rats. At the protein level however, high amounts of cytokines were noted. It appears the effects of 2AA on pregnant dams were more pronounced in the high dose group than the low dose group, possibly indicating increased susceptibility of rat offspring within this group to elicit diabetic-type response. Future investigations will include examining the progeny for diabetic-susceptibility symptomatology.

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Competing Interests. *The authors declare no conflict of interest.*

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