EFFECTS OF CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES AND EFFECTS OF ETHANOL EXPOSURE DURING EARLY NEURODEVELOPMENT ON BEHAVIORS IN MICE

Lu Dai
University of Kentucky, lu.dai@uky.edu
Digital Object Identifier: https://doi.org/10.13023/ETD.2017.482

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
Dai, Lu, "EFFECTS OF CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES AND EFFECTS OF ETHANOL EXPOSURE DURING EARLY NEURODEVELOPMENT ON BEHAVIORS IN MICE" (2017). Theses and Dissertations--Toxicology and Cancer Biology. 18.
https://uknowledge.uky.edu/toxicology_etds/18

This Doctoral Dissertation is brought to you for free and open access by the Toxicology and Cancer Biology at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Toxicology and Cancer Biology by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Lu Dai, Student
Dr. Gang Chen, Major Professor
Dr. Isabel Mellon, Director of Graduate Studies
EFFECTS OF CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES

AND

EFFECTS OF ETHANOL EXPOSURE DURING EARLY NEURODEVELOPMENT ON BEHAVIORS IN MICE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Lu Dai

Lexington, Kentucky

Co-Director: Dr. Gang Chen, Associate Professor of Pharmacology and Nutritional Sciences

Dr. Jia Luo, Professor of Pharmacology and Nutritional Sciences

Lexington, Kentucky

Copyright © Lu Dai, 2017
ABSTRACT OF DISSERTATION

EFFECTS OF CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES
AND
EFFECTS OF ETHANOL EXPOSURE DURING EARLY NEURODEVELOPMENT ON BEHAVIORS IN MICE

The dissertation consists of three major projects with the focus on the immunotoxicity of chromium and the behavior disorders caused by early ETOH exposure respectively.

Hexavalent chromium [Cr(VI)] is widely used in various industrial processes and has been recognized as a carcinogen. As the first line of host defense system, the immune system can be a primary target of Cr(VI). T cell population represents a major arm of the immune system that plays a critical role in host anti-tumor immunity. Dysfunction of T cells compromises host anti-tumor immunity resulting in oncogenesis. Using mouse splenic T cells as an in vitro model system, the present study assessed the effects of Cr(VI) on T cell functions, as the first step of our investigation of the mechanism underlying Cr(VI)-inhibited immunosurveillance and carcinogenesis. Our results showed that Cr(VI) decreased the viability of CD4+ and CD8+ T cells, inhibited T cell activation, functions, including cytokine release, and degranulation.

Fetal ethanol (ETOH) exposure can damage the developing central nervous system and lead to cognitive and behavioral deficits, known as fetal alcohol spectrum disorders (FASD). The use of animal models, especially mouse models is essential for investigating the neurogenetic mechanism of fetal ETOH effects and screening pharmacotherapies against it, due to the extensive knowledge of mouse genetics. However, the availability of mouse model is limited. Via adopting various dosage, timing and administration routes of ETOH exposure, we developed two mouse models to assess behavioral or cognitive changes caused by fetal ETOH exposure in pre-weaning and adolescent period. Our results show that high dosage of ETOH exposure (4 g/kg) during PD 4-10 resulted in hyperactivity, disinhibition, and deficits in learning and memory in mouse offspring, which lays the groundwork for the future
FASD research.

Keywords
Cr(VI), T cell immunity, carcinogenesis; FASD, behavioral deficits, mice model

Lu Dai

November 27th, 2017
Date
EFFECTS OF CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES

AND

EFFECTS OF ETHANOL EXPOSURE DURING EARLY NEURODEVELOPMENT ON BEHAVIORS IN MICE

By

Lu Dai

2017

__________________________
Dr. Gang Chen
Co-Director of Dissertation

__________________________
Dr. Jia Luo
Co-Director of Dissertation

__________________________
Dr. Isabel Mellon
Director of Graduate Studies

__________________________
November 27th, 2017
Date
ACKNOWLEDGEMENT

I would like to begin by expressing the deepest appreciation to my advisor, Dr. Gang Chen for all of the support during my Ph.D. study and research. I would like to thank him for guiding me on the research projects and to publish peer review scientific papers. Without his guidance and persistent help, this dissertation would not have been possible. Additionally, I am grateful for the support and encouragement of Dr. Jia Luo, Dr. Xianglin Shi, and Dr. Susan Baron. Their advice was tremendously beneficial for each aspect of my life and my growth as a scientist. I would also like to thank my Director of Graduate Studies, Dr. Isabel Mellon for the help with my graduate study as well as the fellowship application. I must also thank all the faculty members in the Department of Toxicology and Cancer Biology because they have each given me guidance at one point or another. My sincere thanks also go to all my lab members, both past and present, Dr. Wenhua Xu, Dr. Hui Li, Dr. Caigu He, Dr. Jiajun Cui, Dr. Yuanlin Qi, Dr. Mingfang Zhang, and Howard Brim for the intelligent suggestions and training me on new skills. Many thanks to Dr. Andrew Hawkey who has been giving me a large support on animal behavioral testing and analysis. I would also like to thank Dr. Greg Bauman and Dr. Jennifer Strange for their help on flow cytometry analysis. Finally, I would like to express my eternal gratitude to my parents, Qinrong Dai and Jianping Geng, who have been a constant resource of inspiration. Without their unfailing faith, support, and love, I would have never had the courage to overcome the adversities I have faced and accomplished so much today.
TABLE OF CONTENTS

ACKNOWLEDGEMENT ........................................................................................................... III

TABLE OF CONTENTS .......................................................................................................... IV

LIST OF TABLES ................................................................................................................ VI

LIST OF FIGURES .............................................................................................................. VII

CHAPTER 1: EFFECTS OF HEXAVALENT CHROMIUM ON MOUSE SPLENIC T LYMPHOCYTES

Synopsis ................................................................................................................................. 1

Introduction ........................................................................................................................... 3

Materials and Methods ....................................................................................................... 5

Animals ................................................................................................................................. 5

Chemicals ............................................................................................................................. 5

Splenocytes cultures ........................................................................................................... 6

Cell viability assay ............................................................................................................ 6

T cell activation assay ....................................................................................................... 7

T cell proliferation assay ................................................................................................. 7

Determination of cytokine levels ...................................................................................... 8

Detection of the cytolytic function of CD8+ T cells .......................................................... 8

Statistical analysis ............................................................................................................ 8

Results ................................................................................................................................ 9

Cr(VI) decreases T cell viability ....................................................................................... 9

Cr(VI) inhibits T cell activation ....................................................................................... 11

Cr(VI) inhibits T cell proliferation .................................................................................. 14

Cr(VI) inhibits the secretion of IL-2, IL-4, and IL-10 by T cells ....................................... 16

Cr(VI) inhibits the cytolytic function of CD8+ T cells ..................................................... 19

Discussion ......................................................................................................................... 20

CHAPTER 2: NEONATAL ETHANOL EXPOSURE CAUSES BEHAVIORAL DEFICITS IN YOUNG MICE

Synopsis ............................................................................................................................... 24
LIST OF TABLES

TABLE 2.1. OFFSPRING WEIGHT PROFILE DURING PD 4-10 AND AT THE TIME OF TESTING ........................................... 33
TABLE 3.1. TREATMENT STRATEGY .......................................................................................................................... 54
TABLE 3.2. BODY WEIGHTS OF OFFSPRING ON PD 4-10 AND AT THE TIME OF BEHAVIORAL TESTING. .......................................................... 60
LIST OF FIGURES

FIGURE 1.1. CR(VI) DECREASES T CELL VIABILITY. ........................................ 10
FIGURE 1.2. CR(VI) INHIBITS T CELL ACTIVATION. ......................................... 13
FIGURE 1.3. CR(VI) INHIBITS T CELL PROLIFERATION................................. 16
FIGURE 1.4. CR(VI) INHIBITS THE SECRETION OF IL-2, IL-4, AND IL-10 BY T CELLS. ................................................................................................................. 18
FIGURE 1.5. CR(VI) INHIBITS THE CYTOLYTIC FUNCTION OF CD8+ T CELLS. ................................................................. 20
FIGURE 2.1. BEC OF ETOH TREATED PUPS. ....................................................... 32
FIGURE 2.2. TOTAL DISTANCE TRAVELED IN OF. ............................................. 35
FIGURE 2.3. TOTAL DISTANCE TRAVELED IN THE CENTER. ............................ 37
FIGURE 2.4. OPEN FIELD TEST CENTER PREFERENCE. ................................ 37
FIGURE 2.5. ELEVATED PLUS MAZE TEST.......................................................... 39
FIGURE 2.6. MORRIS WATER MAZE ACQUISITION PHASE............................ 41
FIGURE 2.7. MORRIS WATER MAZE PROBE TRIAL………………………………… 42
FIGURE 2.8. MORRIS WATER MAZE REVERSAL LEARNING............................ 43
FIGURE 2.9. MORRIS WATER MAZE VISUAL PLATFORM............................... 44
FIGURE 3.1. BECS OF POSTNATAL ETOH TREATED OFFSPRING .................. 58
FIGURE 3.2. BODY WEIGHT GAIN OF PREGNANT DAMS................................. 59
FIGURE 3.3. DISTANCE TRAVELED ACROSS TIME IN THE OPEN FIELD. .... 62
FIGURE 3.4. DISTANCE TRAVELED IN THE CENTER OF OPEN FIELD........... 63
FIGURE 3.5. THE NUMBER OF ENTRIES INTO THE CENTER OF OPEN FIELD, ................................................................................................................................. 64
FIGURE 3.6. ELEVATED PLUS MAZE TEST.......................................................... 66
FIGURE 3.7. MORRIS WATER MAZE TEST ACQUISITION.............................. 68
FIGURE 3.8. MORRIS WATER MAZE PROBE TRIAL........................................ 69
FIGURE 3.9. MORRIS WATER MAZE REVERSAL LEARNING............................ 70
FIGURE 3.10. MORRIS WATER MAZE A VISUAL PLATFORM TRIAL............. 71
Chapter 1: Effects of hexavalent chromium on mouse splenic T lymphocytes

Synopsis

Background: Hexavalent chromium [Cr(VI)] is widely used in various industrial processes and has been recognized as a carcinogen. As the first line of host defense system, the immune system can be a primary target of Cr(VI). T cell population represents a major arm of the immune system that plays a critical role in host anti-tumor immunity. Dysfunction of T cells, such as exhaustion under the persistent presence of tumor antigen, compromise host anti-tumor immunity resulting in oncogenesis. Previous studies have shown Cr(VI) exposure alters the phenotype of human peripheral blood lymphocytes. However, the mechanism of the alteration and whether such an alteration in immunity affects immunosurveillance and promotes carcinogenicity are not clear.

Methods: Mouse splenic T cells were used in the present study as an *in vitro* model system. With the treatment of 2 or 5 µM Cr(VI), the viability, activation, proliferation, and function of splenic T cells were assessed *in vitro*.

Results: Cr(VI) decreased the viability of CD4+ and CD8+ T cells, inhibited T cell functions, including activation, cytokine release, and degranulation.

Conclusion: The data obtained from the current *in vitro* study set up a foundation for our further investigation of the mechanism underlying Cr(VI)-inhibited immunosurveillance and carcinogenesis.
Keywords

Cr(VI), T cell, immune response, toxicity, cancer
Introduction

Chromium (Cr) exists widely in nature and is used in various industrial processes (Barceloux, 1999). Humans can be exposed to chromium through inhalation, ingestion, or dermal exposure (Shrivastava et al., 2002). In spite of 6 valence states of chromium, only Cr(III) and Cr(VI) are of biological significance with respect to their toxicity and carcinogenicity (Steinhagen et al., 2004). Cr(VI) is considered as a human carcinogen and chronic exposure to Cr(VI) is associated with an increased incidence of various cancers (Costa, 1997, Salnikow and Zhitkovich, 2008).

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines, which together act as the first line of host defense system against infection or cancer (Parkin and Cohen, 2001, Candeias and Gaipl, 2016, Subramanian et al., 2015). The immune system monitors the host body, recognizing and eliminating newly arising cancer cells to stop/control tumor formation in a process termed ‘immunosurveillance’. Cancer cells possess tumor antigens. The encounter between cancer cells and immune system initiates a process known as ‘immunoediting’ that can bring about three outcomes: elimination, equilibrium or escape of cancer cells from immunosurveillance. Hence, the host immunity suppresses tumor development, whereas tumor formation implicates a successful escape of tumor cells from host immunosurveillance. Previous studies have shown that Cr(VI) may impair the immune system (Dangleben et al., 2013, Akbar et al., 2011, Salsano et al., 2004) and occupational exposure to Cr(VI) decreased the number of lymphocytes in peripheral blood of chromate workers (Tanigawa et al.,
1995, Qian et al., 2013). However, the effects of Cr(VI) on host anti-tumor immunity is not clear.

The host anti-tumor immunity can be carried out in a form of cell-mediated immunity. As an important component of cell-mediated immunity, T cells play a critical role in immunosurveillance (Vorhees and Williams, 2006). CD4+ and CD8+ are two major T cell subpopulations. Quiescent naïve T cells mainly reside in secondary lymphoid organs such as the spleen (Mebius and Kraal, 2005). Upon attacked by either exogenous or endogenous insult, T cells are activated and undergo rapidly clonal expansion (proliferation). Activated CD4+ T cells perform their effector functions through secreting cytokines, whereas activated CD8+ T cells mediate lysis of target cells via releasing perforin and granules (Broere(1) et al., 2011). Due to its high content of T cells, the culture of mouse splenocytes has been widely used as an in vitro model system to test immunotoxicity to T cells by a variety of insults (Song et al., 2014, Pestka et al., 1994).

Our goal is to understand the effects of Cr(VI) on host anti-tumor immunity and its contribution to Cr(VI) carcinogenicity. As the first step, in the current study, we aimed to determine whether Cr(VI) at environmentally relevant concentrations affects the viability, activation, proliferation and effector function of T cells. Using mouse splenic T cell culture as an in vitro model, our results showed that Cr(VI) decreased the viability of CD4+ and CD8+ T cells, inhibited the activation, proliferation, cytokine secretion and cytotoxic function of the splenic T cells. These
results set up a foundation for our further investigation of the mechanism underlying Cr(VI)-induced immunosurveillance inhibition and carcinogenesis.

**Materials and Methods**

*Animals*

Male and female C57BL/6 mice (8-week-old) were purchased from Harlan Laboratory (Indianapolis, IN) and maintained under specific pathogen-free conditions at the Division of Laboratory Animal Resources, University of Kentucky Medical Center (Lexington, KY). All protocols were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

*Chemicals*

Potassium dichromate (K₂Cr₂O₇) was obtained from Sigma-Aldrich (St. Louis, MO) and used for Cr(VI) treatment. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Anti-CD3/CD28, APC-conjugated anti-CD4, PE-conjugated anti-CD4, PE-Cy5.5-conjugated anti-CD4, APC-conjugated anti-CD8, FITC-conjugated anti-CD8, PE-conjugated anti-69, APC-conjugated anti-CD8, FITC-conjugated anti-CD25, FTIC-conjugated CD107a were from BioLegend (San Diego, CA). Mouse IL-2, IL-4, IL-10 ELISA MAX™ Deluxe Set and carboxyfluorescein diacetate succinimidyl ester (CFSE) cell division tracker kit were obtained from BioLegend (San Diego, CA).
**Splenocytes cultures**

C57BL/6 mice were sacrificed and their spleens were aseptically removed. A suspension of splenocytes was obtained by mesh desegregation with a cell strainer (Corning Incorporated, Durham, NC). Erythrocytes were lysed in RBC lysis buffer (10X) (BioLegend, San Diego, CA), and the cell pellets were washed with RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/mL of penicillin and streptomycin, and 0.1%β- mercaptoethanol. The cell number was assessed by exclusion of trypan blue (Sigma-Aldrich, St. Louis, MO). Cells were plated at a density of $6 \times 10^6$ cells/ml and maintained in a humidified atmosphere of 5% CO$_2$ at 37$^\circ$C.

**Cell viability assay**

T cell viability was determined via propidium iodide (PI) staining assay. PI is impermeable to intact plasma membranes of living cells, allowing the accurate determination of cell viability in a variety of studies (Yeh et al., 1981, Bank, 1987). In this experiment, the isolated splenocytes were plated on 24-well plates (Costar, Washington) containing 1 ml of RPMI-1640 complete medium. Then cells were treated with 0, 2 or 5 µM Cr(VI). Three independent experiments were conducted and each treatment contained triplicates. After 24 hours, cells were washed and re-suspended in 0.5 ml FACS buffer (PBS, 2% FBS and 0.1% sodium azide). Single cell suspensions were labeled with APC-conjugated anti-CD4 and FITC-conjugated anti-CD8 antibodies on ice for 30 mins in the dark. Shortly before flow cytometric
analysis, 20 µl of the PI solution was added to 0.5 ml of cell suspension. Then, cells were analyzed with BD FACS Calibur flow cytometry (San Jose, California).

**T cell activation assay**

Anti-CD3 and anti-CD28 antibodies were used to induce T cell activation (Frauwirth and Thompson, 2002) which can be detected by the increase of CD69 and CD25 levels on T cell surface (Morzadec et al., 2012). Briefly, splenocytes were treated with anti-CD3 and CD28 antibodies (1 µg/ml) for 24 hours. Cells were stained with APC-conjugated anti-CD4, anti-CD8, PE-conjugated anti-CD69 and FITC-conjugated anti-CD25 antibodies. After 30 mins staining, cells were analyzed using BD FACSCalibur flow cytometry. PI staining was used to exclude dead cells.

**T cell proliferation assay**

The proliferation of splenic T cells was measured as previously described (Quah and Parish, 2010). Briefly, the intracellular fluorescent dye, CFSE, covalently labels intracellular molecules. With each cell division, these fluorescent molecules are equally distributed in daughter cells, shown as the half-reduced CFSE intensity in daughter cells, which allows T cells proliferation to be examined by flow cytometry (Quah and Parish, 2010). Therefore, the splenocytes were labeled with CFSE and cultured in a 24-well plate in medium with or without anti-CD3/CD28 antibodies (1 µg/ml). Cells were then treated with 0, 2 or 5 µM Cr (VI) for 96 hours and stained with PE-Cy5.5 conjugated anti-CD4 and APC-conjugated anti-CD8 antibodies, and cell proliferation was analyzed by flow cytometry. PI staining was used to exclude
dead cells.

**Determination of cytokine levels**

The cytokine levels were determined using ELISA kits as described previously (Katial et al., 1998). Splenocytes were cultured in 24-well plates and treated with either anti-CD3/CD28 antibodies alone or anti-CD3/CD28 antibodies plus Cr (VI) (2 or 5 µM) for 24 hours. Cell-free supernatants were collected for measurement of cytokine levels by ELISA.

**Detection of the cytolytic function of CD8⁺ T cells**

As previously described, CD8⁺ T cell-mediated cytotoxicity was evaluated via measuring the levels of CD107a which is expressed on T cell surface following activation-induced degranulation (Betts et al., 2003). Briefly, 1 ×10⁶ cells were cultured in complete RPMI and treated with 2 or 5 µM Cr(VI). After 18 hours, anti-CD3/CD28, FTIC conjugated CD-107a or hamster IgG isotype control antibodies were added. One hour later, 1 µl monensin solution (1000×) was added to each well. Cells were then cultured in a humidified atmosphere of 5% CO₂ at 37°C for additional 5 hours and then were stained with APC-conjugated CD8 antibodies. The expression of CD107a on T cell surface was detected by flow cytometry. Three independent experiments were conducted with triplicates for each treatment group.

**Statistical analysis**

All experiments were repeated at least three times with triplicates each. Data were expressed as mean ± standard deviation. GraphPad Prism was used to analyze all
data. Comparison of more than two groups was made with a one-way analysis of variance (ANOVA) followed by Tukey’s test. A p value < 0.05 was considered significant.

**Results**

*Cr(VI) decreases T cell viability*

We first determined the effects of Cr(VI) on the viability of CD4⁺ and CD8⁺ T cells. The splenocytes were treated with 2 or 5 µM Cr(VI) *in vitro* and their viability was determined by PI staining followed by flow cytometry. Lymphocytes were first identified by a low forward scatter (FSC) and low side scatter (SSC) gate. Live CD4⁺ and CD8⁺ T cells were then defined based on their cell markers and PI staining. The viability of CD4⁺ and CD8⁺ T cells was calculated based on the percentage of live CD4⁺ or CD8⁺ T cells among total splenocytes as relevance to controls (Figure 1.1). Cr(VI) decreased the viability of CD4⁺ cells, which reached the significant level at 5 µM, but not at 2 µM (Figure 1.1A and B), whereas the viability of CD8⁺ T cells was significantly decreased by Cr(VI) at both 2 and 5 µM (Figure 1.1C and D).
Figure 1.1. Cr(VI) decreases T cell viability.

(A) and (C) show the percentages of live CD4+ and CD8+ among total splenocytes after the splenocytes were treated with 2 or 5 µM Cr(VI) for 24 hours. (B) and (D) show the viability of CD4+ and CD8+, which was calculated by dividing the percentage of live CD4+ and CD8+ in total splenocytes of each treatment group by 10.
that of the control group. Three independent experiments were conducted and each treatment group contained three replicates. Data were shown as mean ± SEM. *: P < 0.05 vs controls.

**Cr(VI) inhibits T cell activation**

To determine the effect of Cr(VI) on T cell activation, splenocytes were treated with 2 or 5 µM Cr(VI) plus anti-CD3/anti-CD28 antibodies *in vitro* and the expression of CD69 and CD25 on the surface of CD4⁺ and CD8⁺ T cells were measured by flow cytometry. CD4 and CD8 populations were identified via CD4 and CD8 markers within live lymphocytes gate, respectively. The percentage of activated T cells (CD69⁺ or CD25⁺) among total T cells was derived from their percentages within live lymphocytes, shown as in Figure 1.2. Compared to isotype control and non-stimulation group, anti-CD3/anti-CD28 antibodies treatment significantly activated the T cells, as evidenced by the increase of CD69⁺ or CD25⁺ T cells. Co-treatment of 2 or 5 µM Cr(VI) significantly decreased CD69⁺ T cells among total T cells (Figure 1.2A and B), while CD25⁺ T cells were significantly decreased by 5 µM but not 2 µM Cr(VI) treatment (Figure 1.2C and D).
A

CD69

non-stimulation

isotype control

aCD3/aCD28

aCD3/aCD28 + 2μM Cr(VI)

aCD3/aCD28 + 5μM Cr(VI)

T cells

B

CD69

% of CD69+ T cells in total T cells

non-stimulation

isotype control

CD3/CD28

2μM Cr(VI) + CD3/CD28

5μM Cr(VI) + CD3/CD28

*  

*  

+  

+
Figure 1.2. Cr(VI) inhibits T cell activation.

(A) and (C) show the percentage of CD69+ and CD25+ T cells within live lymphocyte gate after they were treated with 2 or 5 µM Cr(VI) plus anti-CD3 and anti-CD28 antibodies for 24 hours. (B) and (D) show the percentages of CD69+ or CD25+ T cells among total live T cells, which were calculated by dividing the percentage of CD69+ or CD25+ T cells within live lymphocyte by total T in that gate. Three independent experiments were conducted with triplicates for each treatment.
group. Data were shown as mean ± SEM. *: P < 0.05 as compared with aCD3/aCD28 treatment group.

**Cr(VI) inhibits T cell proliferation**

Following activation, T cells undergo rapid proliferation. We then determined T cell proliferation by CFSE assay. Mouse splenocytes were pre-stained with CFSE and then treated with 2 or 5 µM Cr(VI) plus anti-CD3/anti-CD28 before flow cytometry analysis. The proliferation of CD4 or CD8 T cells was determined by the percentage of divided CD4⁺ and CD8⁺ T cells among total live CD4⁺ and CD8⁺ T cells, respectively, as shown in Figure 1.3. Cr(VI) significantly decreased the proliferation of CD4⁺ T cells at both 2 and 5 µM (Figure 1.3A and B), while the proliferation of CD8⁺ T cells was only significantly inhibited by 5 µM Cr(VI), but not 2 µM (Figure 1.3C and D).
Figure 1.3. Cr(VI) inhibits T cell proliferation.

After the splenocytes were treated with 2 or 5 µM Cr(VI) plus anti-CD3 and anti-CD28 antibodies for 96 hours, the percentages of divided CD4+ among all live CD4+ cells (A) and the percentages of divided CD8+ among all live CD8+ cells (C) were marked with M1. The percentages of proliferative CD4+ and CD8+ cells were shown in (B) and (D). Four independent experiments were conducted with triplicates for each treatment per experiment. Data were shown as mean ± SEM. *: P < 0.05 vs aCD3/aCD28 treatment groups.

**Cr(VI) inhibits the secretion of IL-2, IL-4, and IL-10 by T cells**

Once activated, T cells, mostly CD4+ T cells, secrete cytokines to regulate the immune response. Certain cytokines, such as IL-2, IL-4, and IL-10, are critical in anti-tumor immunity (Dennis et al., 2013, Fearon et al., 1990, Lee and Margolin, 2011). Therefore, the effects of Cr(VI) on their secretion were evaluated. As shown in Figure 1.4, the levels of IL-2, IL-4, and IL-10 in the cell supernatant were
increased after stimulation with anti-CD3/anti-CD28 antibodies. Co-treatment of 2 or 5 µM Cr(VI) inhibited their secretion.
Figure 1.4. Cr(VI) inhibits the secretion of IL-2, IL-4, and IL-10 by T cells.

The splenocytes were treated with 2 or 5 µM Cr(VI) plus anti-CD3 and anti-CD28 antibodies for 24 hours, the levels of IL-2 (A), IL-4 (B) and IL-10 (C) in cell supernatant were measured by ELISA. Data were shown as mean ± SEM. *: P < 0.05 vs aCD3/aCD28 treatment groups.
**Cr(VI) inhibits the cytolytic function of CD8$^+$ T cells**

Upon activation, CD8$^+$ T cells exert their cytolytic function via degranulation, which can be measured by their surface levels of CD107a via flow cytometry (Betts et al., 2003). The same gating strategy was used as described above. Our data showed that both 2 and 5 µM Cr(VI) significantly reduced CD107a levels on CD8$^+$ T cells compared to the those treated with only anti-CD3/CD28 antibodies, indicating an inhibitive effect of Cr(VI) on the cytolytic function of CD8$^+$ T cells (Figure 1.5).
Figure 1.5. Cr(VI) inhibits the cytolytic function of CD8⁺ T cells.

The splenocytes were treated with 2 or 5 µM Cr(VI) plus anti-CD3 and anti-CD28 antibodies for 24 hours, the levels of CD107a on CD8⁺ T cells were determined by flow cytometry (A). Three independent experiments were conducted with triplicates for each treatment group (B). Data were shown as mean ± SEM. *: P < 0.05 VS aCD3/aCD28 treatment groups.

Discussion

In the present study, using the culture of mouse splenocytes as an in vitro model system, we examined the effects of Cr(VI) on the viability as well as activation, cytokine secretion and degranulation of splenic T cells. The dosages of Cr(VI), which have been shown to affect human cells (Wise et al., 2016) (Vasant et al., 2001), were chosen due to their relevance to occupational and environmental exposures (Nemec et al., 2010). Our data show that Cr(VI) at 5 µM significantly decreased the viability, inhibited activation, proliferation, cytokine secretion and
degranulation of T cells, which were marginally inhibited by 2 µM Cr(VI). The results may reflect a threshold level of Cr toxicity considering that the maximum contaminant level of Cr in drinking water was set by USEPA at 100 ppb (~1.92 µM). T cell is a core component of host anti-tumor immunity. An efficient immune response to destroy tumor cells requires the clonal expansion of both CD4+ and CD8+ T cells after their activation. Increased numbers of CD4+ and CD8+ T cells are found at the tumor site (Hadrup et al., 2013). Particularly, high numbers of CD8+ T cells are linked to a better prognosis of patients with various types of cancer (Sato et al., 2005, Fukunaga et al., 2004). Our results showed that Cr(VI) decreased the expression of CD69 and CD25 on T cells in response to anti-CD3/CD28 antibodies compared to non-treatment group, suggesting a compromised T cell activation by Cr(VI) treatment (Figure 1.2). The data in Figure 1.3 further indicate that the proliferation of both CD4+ and CD8+ T cells upon activation was inhibited by Cr(VI) exposure, which is consistent with a previous report that cobalt-chromium-molybdenum (CoCrMo) disc samples inhibited the proliferation of human peripheral blood T lymphocytes in vitro (Faleiro et al., 1996).

Cytokines regulate the growth, differentiation, and activation of immune cells and are involved in host anti-tumor immunity (Dranoff, 2004). Within T cell population, they are predominantly secreted by CD4+ T cells (Green et al., 2007). Recently, numerous studies have demonstrated that certain cytokines have broad anti-tumor activity due to their capacity to directly stimulate immune effector cells at the tumor site and enhance tumor cell recognition by cytotoxic effector T cells (Lee and
Margolin, 2011). For example, IL-2 is known to stimulate the proliferation and differentiation of CD4+ T cells and can enhance NK cell and CD8+ T cells function, which engenders systemic anti-tumor immunity (Rosenberg, 2014). Other cytokines such as IL-4 and IL-10 may also exert antitumor effects by enhancing immunosurveillance and suppressing cancer-associated inflammation (Dennis et al., 2013). Our data show that the secretion of IL-2, IL-4, and IL-10 was decreased by 48%, 57% and 35% upon 2 µM, and 65%, 81% and 51% upon 5 µM Cr(VI) exposure, respectively (Figure 1.4). The decreased cell viability by Cr(VI) treatment may potentially reduce the secretion of these cytokines, however, the viability of CD4+ T cells was decreased by 13% and 17% by 2 or 5 µM Cr(VI), respectively (Figure 1.1). These results suggest that Cr(VI) inhibit the secretion of the cytokines in addition to inhibiting T cell viability.

Most tumor cells bear antigens that can be recognized by CD8+ T cells (Gajewski et al., 2013). Upon activation, effector CD8+ T cells target the tumor cells and release cytolytic granules containing perforin and granzyme to induce the apoptosis of these cells, which represents an important anti-tumor effector function of CD8+ T cells and is marked by an up-regulation of cell membrane level of glycoprotein CD107a (Betts et al., 2003). Our data showed that Cr(VI) significantly decreased the expression of CD107a on CD8+ T cells (Figure 1.5), suggesting an impaired anti-tumor effector function of CD8+ T cells by Cr(VI).

In summary, our data suggest an immunotoxicity property of Cr(VI) as it decreased the viability as well as inhibited the activation, proliferation, cytokine secretion and
cytotoxic function of T cells. The suppression of T cell effector function by Cr(VI) may induce immune escape and facilitate cancer development. The present \textit{in vitro} study set up a foundation for our further study to understand the mechanism underlying Cr(VI)-induced immunosurveillance inhibition and carcinogenicity.

\textbf{Acknowledgement}

This article has been published in the journal \textit{Toxicology In Vitro} in September, 2017.

Chapter 2: Neonatal ethanol exposure causes behavioral deficits in young mice

Synopsis

Background: Fetal ethanol (ETOH) exposure can damage the developing central nervous system and lead to cognitive and behavioral deficits, known as fetal alcohol spectrum disorders (FASD). ETOH exposure to mouse pups during early postnatal days was used as a model of ETOH exposure that overlaps the human third-trimester “brain growth spurt” - a model that has been widely used to study FASD in rats.

Methods: C57BL/6 male and female mice were exposed to ETOH (4 g/kg/day) on postnatal days (PD) 4-10 by oral intubation. Behavioral testing of the offspring included open field, elevated plus maze and Morris water maze with offspring tested on PD 20 – 45.

Results: ETOH exposure during PD 4-10 resulted in hyperactivity, disinhibition, and deficits in learning and memory in offspring with no apparent sex differences.

Conclusion: Based on these data, this neonatal mouse model may be useful for future mechanistic and genetic studies of FASD and for the screening of novel therapeautic agents.

Keywords

Fetal ethanol, behavior, development
Introduction

Ethanol (ETOH) consumption during pregnancy can result in damage to the developing central nervous system and lead to a variety of cognitive and behavioral deficits including hyperactivity, disinhibition and deficits in learning, attention and executive functions collectively known as the fetal alcohol spectrum disorders (FASD) (Jacobson, 1998, Riley and McGee, 2005). Many mechanisms have been proposed to help explain the consequences of EtOH's effects on the developing brain, but there are still many unanswered questions.

Numerous animal models have been utilized in the study of FASD. Mouse models have become increasingly useful due to the extensive knowledge of mouse genetics for studies on the etiology of a variety of neurological disorders. Currently, the number of studies using mice to study fetal ETOH effects has been limited and the results are less reliable than in rat studies. In studies using a prenatal ETOH exposure model, voluntary drinking throughout gestation has produced behavioral impairments and deficits in tasks that tap into hippocampal function or sensorimotor integration, although these data are not consistent (Allan et al., 2003, Becker et al., 1993, Brady et al., 2012, Abbott et al., 2016). Prenatal ETOH exposure has also been assessed by oral intubation to the pregnant dam with some studies reporting hyperactivity in ETOH-exposed adolescent mice (Sanchez Vega et al., 2013, Fish et al., 2016), while others report minimal effects on activity (Downing et al., 2009). Some studies have focused on ETOH exposure during the early postnatal period. Olney and colleagues have shown that a single postnatal ETOH injection can induce neuronal apoptosis
(Ikonomidou et al., 2000) and may produce spatial deficits (Wozniak et al., 2004), although there are some inconsistencies in this literature. Multiple injections of ETOH to neonatal mice has been shown to alter performance on hippocampal and cerebellar tasks (Bearer et al., 2015, Wagner et al., 2014) but this literature is far more limited than that available with rat models.

The aim of the current study was to examine the effects of ETOH exposure during the ‘3rd trimester-equivalent’ in terms of human brain growth (Dobbing and Sands, 1979) by oral intubation on FASD-related behaviors in C57BL/6 mice. ETOH was delivered to the mouse pups on PD 4-10 by intubation. The offspring were then tested on PD 20-21 for activity levels using an open field, on PD 25-26 for exploration on an elevated plus maze and on PD 35-45 for spatial learning and memory in a Morris water maze.

**Materials and Methods**

**Animals**

Adult C57BL/6 mice were obtained from Harlan Labs (Indianapolis, IN). Offspring were generated at the University of Kentucky Medical Center’s breeding colony. Male and female pups, representing at least 8 litters from each treatment group, were used in this study. Mice were maintained on a 14:10 h light-dark cycle (lights on at 07:00 h, off at 21:00 h). All procedures were approved by the NIH and the Animal Care and Use Committee of the University of Kentucky.
Breeding

Adult female and male C57BL/6 mice were caged in a ratio of 2:1 and seminal plugs were examined the next morning as evidence that copulation occurred. If a seminal plug was detected, it was designated gestational day (GD) 0 and the pregnant female was singly housed.

Ethanol administration

ETOH was administered at 09:00 h to mouse pups on postnatal day (PD) 4-10 (as a “3rd-trimester exposure” model) (Kelly et al., 1988). The day of birth was designated PD 0. On PD 4, litters were weighed and pseudo-randomly culled to 6 pups maintaining 3 males and 3 females when possible. These litters were then assigned to one of three treatment groups: ETOH, intubated control (Maltose) or non-intubated control (NTC). Pups in the ETOH group received 4 g/kg/day of ETOH on PD 4-10, delivered via oral intubation (0.02 ml/g body weight) in an artificial milk solution developed to nutritionally mimic rodent milk (Kelly and Lawrence, 2008). This dose of ETOH was chosen because it has been shown to produce significant neurotoxicity during the third-trimester equivalent and may lead to neurobehavioral deficits (Dursun et al., 2013). Pups in the intubated control group received isocaloric maltose (in the same milk solution) on PD 4-10. Pups were weighed daily and returned to their dam immediately after intubations. Mortality from intubation was low (~ 5%).
**BEC measurement**

For BEC of the pups, separate groups of pups were treated and trunk blood of pups was collected on PD 7 following decapitation 30, 60, 90, 120, 180, 240, 360 and 480 min after ETOH intubation. BECs were determined using an assay kit from Sigma-Aldrich, St. Louis, MO (product number: MAK076). The data were collected from multiple samples (n=4) per time point.

**Behavioral testing**

Offspring were tested for activity in an open field (OF) for 2 days (between 08:00 – 12:00 h) on PD 20-22 (Mei et al., 2016), and for activity, anxiety and exploration in an elevated plus maze (EPM) at similar times in the morning on PD 25–27 (Mei et al., 2016). Offspring were then weaned on PD 28, housed with 2–3 same-sex littermates and allowed to acclimate for one week prior to testing in the Morris water maze (MWM). Spatial learning and memory in a MWM testing was conducted between 12:00– 16:00 h on PD 35-45 (Chen et al., 2013). These tasks and ages were selected to assess the presence of behavioral impairments in both preweaning and adolescence, as have been reported in clinical populations with FASD (Mattson et al., 2011). All behavioral testing was conducted under low ambient light conditions with white noise to reduce extraneous auditory stimuli. Surfaces and holding cages were cleaned before and after testing with Nature’s Miracle© enzymatic cleaning solution to remove animal odors. When males and females were tested on the same day, the males were tested prior to females. Animal movements were recorded using the AnyMaze tracking system (Stoelting Co.).
Open Field (OF): OF testing is commonly used to measure levels of activity, habituation to novel environments and patterns of exploration which may indicate impulse control or anxiety (Bailey and Crawley, 2009). Each mouse was removed from its home cage and brought into the test room in a clean holding cage for a 10-min habituation period. The OF was a round chamber (diameter 39.4 cm) with opaque white walls and floor. Subjects were tested 30 min daily for two consecutive days. The dependent measures included total distance traveled and distance traveled in the center. The center was defined as a circular zone in the center of the OF with a diameter half of the width of the OF. Measurements related to activity in the center are often used as a measure of anxiety and/or inhibitory control since mice typically display thigmotaxis and avoid the center of open areas. Additional analyses were run on center exploration when controlling for total distance traveled to produce a preference score.

Elevated Plus Maze (EPM): EPM is primarily used as a test for anxiolytic agents although it can also be used to measure exploration (Bailey and Crawley, 2009). The EPM apparatus consisted of plus-shaped (+) Plexiglas maze with clear walls bordering two of the four arms (30 x 6 cm). The mouse was placed halfway down one open arm of the maze, facing away from the center, and was then allowed to explore the maze for 5 min. Subjects were tested in a single session between PD 25-27. The dependent measures included overall distance traveled and distance traveled in the open arms. Additional analyses were conducted looking at open arm exploration controlling for total distance traveled.
Morris Water Maze (MWM): MWM is used to measure spatial learning and retention (Vorhees and Williams, 2006). The MWM consisted of a round plastic tub (diameter 107.6 cm) filled with water (22-23 °C) made opaque using white non-toxic water-based paint. A platform (15.2 x 15.2 cm) was submerged 0.75cm under the surface of the water in a fixed location. Four visible extra-maze cues were placed at various points around the maze. Each mouse was placed in one of four starting positions on the far side of the pool (120, 150, 210 and 240° from the platform) and allowed to swim until either they found the submerged platform or they reached a ceiling of 60 sec. If they did not find the platform, they were gently guided to the platform. Subjects remained on the platform for 5 sec before being removed for a 5 min intertrial interval (ITI). During maze acquisition, four trials were completed per day for four days. On the fifth day, the platform was removed for a single probe trial. The mouse was placed in the opposite quadrant and the swim pattern was recorded for 60 sec. Four hours after the probe trial, subjects were tested for reversal learning which consisted of four trials with the platform replaced one quadrant away from its original location. Following reversal learning (ITI 5 min), a single visible platform trial was conducted where the platform’s location was indicated by a visible rod above the surface of the water. The visible platform component was included to ensure that there were no visual or motor deficits potentially contributing to performance on this task following developmental ETOH exposure. MWM testing was conducted over 5 days between PD 35-45.
The dependent measures during acquisition included latency to reach the platform and distance traveled for each trial. For the probe trial, annulus crossings (the number of crossings in the maze location where the platform was placed during acquisition training) and the distance traveled in this region were recorded. For the reversal phase, the dependent measures included latency to reach and distance traveled to the new platform location. Similar measures were also recorded in the visible platform trial.

**Statistical analysis**

Data were analyzed using SPSS software version 21 (IBM). To avoid potential litter effects, behavioral data from same-sex siblings were averaged together to produce a single data point per sex, per treatment, per litter for each measure (Abbey and Howard, 1973, Wainwright, 1998). Thus, each data point represented the mean of 2-3 same-sex siblings. For each analysis, univariate or mixed-factors analysis of variance (ANOVA) was performed with treatment and sex as grouping factors and repeated measures when warranted. Significant interactions were broken down by simple main effect and/or post hoc Tukey test. The Greenhouse-Geisser correction was used to correct for violations of homogeneity of variance when necessary. In some cases, this correction resulted in degrees of freedom that were not whole numbers. If there was no main effect or interaction with sex or across multiple time points (e.g. days, time bins, etc), the data were collapsed across this variable for ease of presentation.
Results

BEC and body weight

BEC levels of the separate group of pups were determined on PD 7 (see Figure 2.1). BEC curves show a peak at 325.9 ± 18.8 mg/dl for the pups with ETOH exposure.

Figure 2.1. BEC of ETOH treated pups.

BECs were measured at 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours after the pups were exposed to ethanol (4 g/kg, intragastric intubation) on PD 7. Data are expressed as the mean ± S.E.M (n=4).

Offspring body weights on PD 4-10 are presented in Table 2.1. A mixed-factors ANOVA demonstrated a significant interaction between treatment and age on weight, \( F (3.06, 32.17) =10.84, p < 0.05 \). Post hoc tests indicated no significant difference in body weights across groups on PD 4 – PD 9. ETOH exposure significantly decreased body weight when compared with NTC only on PD 10.
Table 2.1. Offspring weight profile during PD 4-10 and at the time of testing.

<table>
<thead>
<tr>
<th>Postnatal Days (PD)</th>
<th>NTC</th>
<th>Maltose Control</th>
<th>ETOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.57 ± 0.22</td>
<td>2.47 ± 0.23</td>
<td>2.48 ± 0.19</td>
</tr>
<tr>
<td>5</td>
<td>3.04 ± 0.25</td>
<td>2.93 ± 0.28</td>
<td>2.86 ± 0.20</td>
</tr>
<tr>
<td>6</td>
<td>3.49 ± 0.23</td>
<td>3.35 ± 0.30</td>
<td>3.20 ± 0.25</td>
</tr>
<tr>
<td>7</td>
<td>3.95 ± 0.21</td>
<td>3.79 ± 0.29</td>
<td>3.65 ± 0.22</td>
</tr>
<tr>
<td>8</td>
<td>4.39 ± 0.22</td>
<td>4.20 ± 0.35</td>
<td>4.02 ± 0.28</td>
</tr>
<tr>
<td>9</td>
<td>4.85 ± 0.22</td>
<td>4.64 ± 0.42</td>
<td>4.46 ± 0.32</td>
</tr>
<tr>
<td>10</td>
<td>5.30 ± 0.22</td>
<td>5.08 ± 0.45</td>
<td>4.76 ± 0.32*</td>
</tr>
</tbody>
</table>

Table 2.1. The body weights of the pups in NTC, maltose and ETOH groups were measured. *: p < 0.05 from the same sex counterparts in NTC. #: p < 0.05 versus maltose-treated controls. Data are expressed as the mean (g) ± S.E.M, (n=8).

ETOH exposure also reduced body weight at the time of open-field and EPM testing (Table 2.1). There were significant interactions between treatment and age, $F(4, 54) = 3.69, p < 0.05$, and sex and age, $F(2, 54) = 4.86, p < 0.05$. Therefore, post hoc tests were conducted for males and females separately. For males, ETOH treatment resulted in lower body weights for both OF and MWM, but not for EPM, relative to maltose-treated controls ($p < 0.05$). Ethanol-treatment did not result in significant differences in body weight compared to NTC. Additionally, maltose treated offspring weighed more immediately prior to testing in MWM but did not differ from NTC before OF and EPM testing. Among females, ETOH treatment resulted in a reduction
in body weight at the time of OF testing in both NTC and maltose controls with no body weight differences before EPM and MWM.

**Behavioral testing**

**Open field:** Neonatal ETOH exposure increased activity in the open field (see Figure 2.2). These data are shown collapsed across sex due to the lack of sex interactions with treatment and the main effects of sex. The repeated measures ANOVA of distance traveled revealed significant main effects of treatment, \( F(2, 43) = 11.99, p < 0.05 \) and 5 min block, \( F(2.67, 114.7) = 79.16, p < 0.05 \), with significant interactions of day by block, \( F(3.89, 167.2) = 22.66, p < 0.05 \). Post hoc tests showed that on day 1 offspring with ETOH exposure traveled further in OF across all time blocks relative to maltose-treated controls \( (p < 0.05) \), and from bin 2 to 5 compared to NTC (see Figure 2.2A). On day 2, ETOH treated offspring were more active (i.e. traveled more distance) across all time blocks than maltose-treated controls, while they were traveled a greater distance from bin 2 to 6 relative to non-treated controls \( (p < 0.05) \) (See Figure 2.2B).
Figure 2.2. Total distance traveled in OF.

OF activity was assessed as distance traveled. Data is shown collapsed across sex, displayed as two days with six 5-min time bins for each day. Asterisks (*) indicate a significant difference from both NTC and maltose-treated controls. (#) indicates a significant difference from ET0H.
significant difference from maltose-treated animals. (n=8 - 9) refers to the number of litters represented per treatment group, with same-sex siblings averaged to represent one data point.

Additional analyses assessed the effects of ETOH on center exploration and preference, respectively measured as the total distance traveled in the center zone and adjusted for the total amount of activity \([(\text{distance traveled in the center zone/total distance traveled}) \times 100]\). As expected, control mice avoided the center zone, with only 19-20% of exploration typically occurring in this zone. ETOH treatment was associated with increased distances traveled in the center zone, although this did not reflect a greater preference for open spaces than controls but more reflected the increased activity the ETOH exposed animals were displaying (see Figure 2.3). The ANOVA on distance traveled in the center revealed a main effect of treatment, \(F(2, 43) = 7.99, p < 0.05\), sex, \(F(1, 43) = 5.33, p < 0.05\), day, \(F(1, 43) = 11.02, p < 0.05\), and a day by 5 min block interaction, \(F(5, 215) = 16.96, p < 0.05\). Male mice explored greater distance in the center than the females, but there was no sex by treatment interaction. Post hoc tests showed that offspring with ETOH exposure explored significantly more distance in the center than NTC or maltose-treated controls (\(p < 0.05\)) on day 1, but not on day 2 (\(p > 0.05\)). When adjusting these scores for the total level of activity to create a preference score, a different pattern was apparent. There were no significant differences across groups in center preference on either day 1 or day 2 (see Figure 2.4).
Figure 2.3. Total distance traveled in the center.

OF center exploration was assessed as distance traveled in the center zone. Data are shown collapsed across sex and six 5-min time blocks. Asterisks (*) indicate a significant difference from both controls. (n=8 - 9) refers to the number of litters represented per treatment group.

Figure 2.4. Open field test center preference.

OF center preference was assessed as [(distance traveled in the center zone/total distance traveled) x 100]. Data are shown collapsed across sex and six 5-min time blocks. Asterisks (*) indicate a significant difference from both controls. (n=8 - 9) refers to the number of litters represented per treatment group.
Elevated plus maze: Neonatal ETOH exposure also produced hyperactivity in the elevated plus maze relative to both the NTC and the maltose-treated controls (see Figure 2.5A). The univariate ANOVA of distance traveled revealed a significant main effect of treatment, \( F(2, 43) = 5.92, p < 0.05 \), with post hoc tests confirming these findings.

The ETOH exposed offspring also showed increased exploration of open arms in the EPM (see Figure 2.5B) and preference for the open arms of the maze (see Figure 2.5C), respectively. Univariate ANOVA for distance traveled in the open arms showed a main effect of treatment, \( F(2, 43) = 19.19, p < 0.05 \), with post hoc tests showing that ETOH treatment increased the distance traveled in the open arms relative to both controls \( (p < 0.05) \). The univariate ANOVA of preference scores also yielded a significant main effect of treatment, \( F(2, 43) = 15.40, p < 0.05 \). As expected, control mice avoided the open arms, with 27% (NTC) of exploration typically occurring in these areas of the maze. ETOH treatment increased open arm preference relative to both controls \( (p < 0.05) \). This effect consisted of a ~15% increase in open arm preference with ~42% of all exploration occurring in the open arms.
Figure 2.5. Elevated plus maze test.

EPM activity and exploration were assessed as the total distance traveled (A), the distance traveled in open arms (B) and open arm preference (C), measured as (the distance traveled in the open arms/the total distance traveled) x 100. Data are shown collapsed across sex. Asterisks (*) indicate a significant difference from both controls (n=8).

Morris water maze: Both the distance (see Figure 2.6A) and the latency (see Figure 2.6B) traveled to reach the platform were impaired following neonatal ETOH exposure. The mixed-factors ANOVA of distance traveled to the platform revealed a significant main effect of day, $F(3, 123) = 34.82, p < 0.05$, and treatment, $F(2, 41) =$
18.73, $p < 0.05$. Post hoc tests confirmed that offspring with ETOH treatment traveled a greater distance to find the platform ($p < 0.05$) relative to both controls. The mixed-factors ANOVA of escape latency revealed a significant main effect of treatment, $F(2, 41) = 11.30, p < 0.05$, and day, $F(3, 123) = 17.47, p < 0.05$. Post hoc tests confirmed that ETOH exposed group took longer to find the platform than controls ($p < 0.05$). A subset of mice failed to learn to swim to the platform during acquisition, often floating rather than searching for the platform. Failure to acquire the task was defined as the failure to reach the platform on all 4 trials on acquisition day 4. Neonatal treatment did not significantly influence the number of mice that failed to meet the criterion. Adjusted sample sizes for MWM analyses with the removal of subjects unable to acquire the task still represented data from 8-9 litters of mice.
Figure 2.6. Morris water maze acquisition phase.

Spatial learning in the MWM was measured as a reduction in the distance (A) and latency (B) traveled prior to escaping onto the platform over four days of training. Asterisks (*) indicate a significant group difference from both controls across time points. (n=8-9) refers to the number of litters represented per treatment group.

For the probe trial, there was also a significant main effect of treatment, \( F (2, 41) = 3.66, p < 0.05 \). Post-hoc analyses revealed a reduction in the number of annulus crossings in the ETOH exposed offspring compared to maltose controls \( (p < 0.05) \), but not NTC \( (p > 0.05) \) (see Figure 2.7).
Figure 2.7. Morris water maze probe trial.

The number of annulus crossings was measured in the probe trial. (#) indicates a significant difference from maltose-treated animals. (n=8-9) refers to the number of litters represented per treatment group.

Performance on the reversal component of the MWM is shown in Figure 2.8. There was a main effect of treatment, $F(2, 41) = 6.47, p < 0.05$, and day, $F(3, 123) = 14.79, p < 0.05$ with the ETOH exposed offspring taking longer to reach the platform than the controls (see Figure 2.8A). There were no significant interactions or main effects on the distance traveled to reach the platform (see Figure 2.8B).
Figure 2.8. Morris water maze reversal learning.

Reversal learning of MWM was tested by shifting the platform to a novel location and the latency (A) and distance (B) of escape onto the platform was measured. Asterisks (*) indicate a significant difference from controls (n=8).

All treatment groups performed similarly on the visual platform component of the MWM. Figure 2.9. showed the swimming speed of all groups in the visual platform trial.
Discussion

Behavioural defects are typically observed in children with FASD and serve as an important tool for diagnosis and treatment of FASD. Different timing and dosages of alcohol exposure will produce different behavioral phenotypes in children. For example, the brain growth spurt is a critical period when the central nervous system undergoes rapid growth and synaptogenesis (Girard et al., 2001). Alcohol exposure during this period has been reported to impair spatial learning in both rats and mice (Lewis et al., 2007, Furumiya and Hashimoto, 2011). Of interest, there is a disparity in brain development between human and rodents. In human, brain growth spurt happens during the third-trimester, while in rodents, it happens during the early postnatal days (Semple et al., 2013). Therefore, this study was designed to investigate the effects of early postnatal ETOH exposure on activity in an open-field, the elevated plus maze and spatial learning and retention in a Morris Water Maze in male and
female C57BL/6 mice. Our results showed that neonatal ETOH exposure resulted in hyperactivity, disinhibition, and deficits in learning and memory in the offspring with no sex differences observed. These results are similar to those in clinical studies and so provide strong face validity for this mouse model of FASD. The effects observed in our study are also very similar to those that have been reported in a variety of rat models for fetal ETOH effects, including those based on gestational exposure alone (Carneiro et al., 2005, Hofmann et al., 2005), postnatal exposure alone (Green et al., 2007, Lewis et al., 2012), and combined pre and postnatal exposure (Cronise et al., 2001, Brocardo et al., 2012), and so provide strong support for this mouse model.

Hyperactivity appeared to be a consistent finding in two different paradigms (OF and EPM) and at two different ages in the current study. This suggests that this effect is persistent at least in the young mouse. Hyperactivity is one of the more frequently observed behavioral characteristics in both rodent models and in clinical populations with FASD and represents a key focus of drug development efforts for FASD (Koren, 2015).

In addition to activity, both the OF and EPM tasks used in the current study included a component typically used to assess anxiety; the open arms of the EPM and the center region of the OF. Mice generally avoid these open areas and control mice displayed this species-typical behavior. ETOH exposure increased exploration in these open areas, although interpretation was complicated by the fact that these offspring were hyperactive. To better understand this pattern, activity in the open areas was assessed
relative to total activity for each subject and this lead to different patterns on the two tasks. For the OF, the ETOH exposed mice were equally hyperactive in the periphery and in the center and so the increased exploration in the center was probably best explained as hyperactivity. In contrast, EPM exploration showed open arm preference approaching 50%, suggesting no real preference or avoidance for the open arms. These EPM findings are consistent with previous studies using young rats with early ETOH exposure (Carneiro et al., 2005).

The disparity in ETOH effects on open area exploration in the OF and EPM is interesting. Although these open areas are both aversive, previous studies suggest that the EPM may be more sensitive to anxiolytic or disinhibitory effects than the OF (Schmitt and Hiemke, 1998, Acevedo et al., 2014), possibly due to the unique combination of open spaces and raised elevation in the EPM (Schmitt and Hiemke, 1998). The results from the current study provide support for the hypothesis that hyperactivity and reduced open space avoidance may represent functionally distinct forms of inhibitory deficits in mice (Carola et al., 2002). From an ecological perspective, the failure of a rodent to avoid open spaces would lead to a higher “risk” of predation, suggesting that the observed ETOH effects could have real implications for natural consequences. These inhibitory failures may be an important feature of the present mouse model, as those with FASD have difficulty avoiding risky behaviors or aversive consequences, leading to poorer outcomes through childhood and adolescence (Green et al., 2007) into adulthood (Moore and Riley, 2015). Although the present data do not implicate specific inhibitory control mechanisms which impact
decision making (Bari and Robbins, 2013), future testing with this model may use more sophisticated tests of executive function to parse out these factors.

ETOH exposure also impaired acquisition of the MWM spatial task. Offspring with ETOH exposure had longer swim paths and greater latencies to reach the platform during acquisition of the task. These differences were not observed in the visible platform trial, so the differences in performance displayed by the ETOH exposed offspring relative to the controls could not be due to swimming deficits. Treatment effects were not evident in the first trial, but once evident, were consistent across acquisition. This did not translate to altered behavior during the probe trial relative to non-treated controls. Given this pattern, the observed performance impairments may be interpreted as impairments in spatial learning. These findings are consistent with previous 3rd-trimester ETOH exposure rodent models reporting deficits in MWM acquisition (Goodlett and Peterson, 1995, Banuelos et al., 2012, Wagner et al., 2014).

Deficits in memory and spatial abilities are also commonly reported among clinical populations with fetal ETOH exposure (Doyle and Mattson, 2015) and also represent key targets for intervention.

There were no observable sex differences in outcome as a function of neonatal ETOH exposure. Previous mouse studies with relatively brief, 1 or 3 days, postnatal ETOH exposure paradigms have similarly shown little or no sex differences with one exception after a 2-day binge that was limited to the probe trial (Wagner et al., 2014). In addition to the overlap in behavioral characteristics displayed by these offspring to rat and clinical studies, the advantage of this mouse intubation model over injection is
that it avoids potential tissue damage and ETOH leakage due to the porous skin (Moser et al., 2005). Moreover, intubation allows for a more consistent and better controlled BEC level in the offspring. It should be noted that a relatively high BEC was used in the current study as previous studies have emphasized the importance of peak BECs in ETOH’s behavioral teratogenicity (Kelly et al., 1987) and that higher BECs, as the BECs observed in this study, are typically required for persistent behavioral effects (Allan et al., 2003). Nevertheless, these high BEC levels are comparable to those reported in human alcoholics (Urso et al., 1981, Jones and Harding, 2013) and those used in rodent models (Goodlett et al., 1990).

In conclusion, the current early postnatal ETOH intubation paradigm resulted in hyperactivity, disinhibition, and deficits in learning and memory in the young mice with no apparent sex difference. These behavioral deficits are most likely the consequences of ethanol-induced damage to the developing brain. Normal developmental signaling in neurons (Luo, 2009) and glial cells (Costa et al., 2004) has been shown to be disrupted by neonatal ethanol exposure. The disruption may lead to mal-development of certain brain areas that are sensitive to ethanol exposure, such as the prefrontal cortex, corpus callosum, hippocampus and cerebellum, which control the functions of activity, learning, and memory (Alfonso-Loeches and Guerri, 2011). Therefore, the current mouse model can be useful for further studying the molecular, cellular and genetic mechanisms underlying ethanol-induced behavioral deficits in FASD.
Acknowledgement

The manuscript is currently under the review of the journal *Alcoholism: Clinical and Experimental Research*. First authorship of this manuscript is shared between Wenhua Xu, Lu Dai, Andrew Hawkey, and Hui Li.
Chapter 3: The effects of early ethanol exposure on behavioral consequence in young mice

Synopsis

Background: Fetal alcohol spectrum disorder (FASD) is an umbrella term for a range of cognitive and behavioral defects in children with prenatal alcohol exposure. The behavioral consequences resulted from the early ETOH exposure are important for diagnosis and drug development of FASD and largely depend on the timing, dose, and avenue of ETOH administration.

Methods: The current study utilized prenatal DID (Drinking in the Dark) and postnatal intubation procedures alone or in combination to produce five ETOH exposure paradigms. Behaviors of C57BL/6 mice were assessed through an open field, elevated plus maze and Morris water maze tests on PD 20 – 45.

Results: Our results showed that postnatal ETOH exposure with or without prenatal treatment resulted in hyperactivity, disinhibition, and deficits in spatial learning and memory in C57BL/6 mouse offspring.

Conclusion: By employing different timing, dosages, and methods of ETOH delivery, our results provided an inclusive aspect on how early ETOH exposure may affect behavioral consequences in mice, which serves as a solid base for the further investigation on the neurogenesis mechanism underlying the FASD.

Keywords

FASD, C57BL/6 mice, DID, behavioral deficits
Introduction

Maternal alcohol ingestion during pregnancy may cause a spectrum of morphological anomalies and behavioral deficits in the offspring, known as fetal alcohol spectrum disorders (FASD) (Wilhelm and Guizzetti, 2015). The children affected by fetal alcohol exposure display a series of cognitive and behavioral abnormalities such as impulsive behaviors and hyperactivity, as well as deficits in learning and memory (Kable et al., 2016).

In order to investigate the neurogenetic mechanisms underlying FASD and seek the therapeutic targets, a number of animal models, including mice and rats, have been developed. Compared to rats, mice better meet the demands for genetic manipulation and testing, given the broad understanding of mouse genetics. Additionally, behavioral consequences of fetal alcohol exposure vary by timing, dosage, and methods of ETOH delivery (Kelly et al., 2009). For example, M.L. Kleiber exposed C57BL/6J dams to 10% ETOH in water via the two-bottle method across the gestational period (Kleiber et al., 2011). ETOH treated pups showed less activity than the controls, while no behavioral abnormalities were detected among ETOH exposed offspring when mice were treated with 3 g/kg ETOH from G7-G18 through intragastric intubation (Downing et al., 2009).

The purpose of the current study was to test the effects of early ETOH exposure on C57BL/6 mice behaviors by manipulation of timing, dose and ETOH administration. ETOH was delivered to the pregnant dams via a method named “Drinking in the Dark” (DID) (Moore et al., 2007, Rhodes et al., 2005), to the pups through
intubation from PD 4-10, or both combined. The activity, exploration and spatial learning of the offspring were then tested using an open field and an elevated plus maze for preweaning animals and Morris water maze when they reached adolescence.

**Materials and Methods**

**Animals**

The adult male and female C57BL/6 mice were purchased from Harlan Labs (Indianapolis, IN) and used as breeders for this study. All animals were housed in the animal facility of the University of Kentucky Medical Center. All procedures were approved by the NIH and the Animal Care and Use Committee of the University of Kentucky. The breeding facility was maintained on a reversed light cycle, so experimental procedures including drinking in the dark and postnatal exposure were carried out in the dark. Dim red lighting was used when experimenters were present to avoid disruptions to the light cycles of the mice. A dark anti-room adjacent was used to allow experimenters to enter the dark procedure room without outside light exposure. Behavior tests were conducted in the light.

**Breeding and maternal treatment**

Approximately 10 weeks old, female mice were individually housed and acclimated to a 12-hour reverse light/dark cycle (lights on at 2100 h, off at 0900 h) for one week prior to the drinking in the dark session. Once the session started, female mice were randomly assigned to the water or ethanol group. Three hours into the dark cycle
(0900), mice in the ethanol group were only given access to ethanol (20% v/v in water) in a sipper-tube for 2 hours (1200 h – 1400 h) per day for the first three days and then for 4 hours (1200 h – 1600 h) per day for a week. The control group mice were given access to water only. Following one week of 4-hour DID adaptation, individual females were placed into the cage of singly housed males for mating for two hours from 1600 h – 1800 h and returned to their home cages. The DID sessions continued throughout the entire gestation. Vaginal plugs were examined early the next morning as evidence that copulation occurred.

To avoid the possible negative effects of continued maternal ethanol intake and withdrawal from maternal ethanol intake on litter care, the period of ethanol intake was shortened to 90 min on the postnatal day 0 (the day of birth), 60 min on PD 1, and 30 min on PD 2. DID sessions no longer employed on PD 3, or any day thereafter (Boehm et al., 2008).

**Postnatal treatment**

On PD 4, offspring with or without prenatal ETOH treatment were weighed and randomly assigned to either a postnatal ETOH intubation or a non-intubated group. To preclude potential litter effects, male and female pups, representing at least 8 litter for each treatment group were used. Pups in the postnatal ETOH group received either 3 g/kg/day or 4 g/kg/day of ETOH delivered via oral intubation in the water on PD 4–10. These doses were chosen because they have been shown to produce significant neurotoxicity during the third-trimester equivalent and may lead to neurobehavioral deficits (Dursun et al., 2013). The non-intubated group received
no treatment. The pups were returned to the dam and allowed to nurse immediately after intubations. Pup body weights were recorded daily. Mortality from intubation was low (approximately 5%). The maternal and postnatal ETOH treatments were shown in Table below.

Table 3.1. Treatment Strategy.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DID</th>
<th>3 g/kg</th>
<th>4 g/kg</th>
<th>DID+3 g/kg</th>
<th>DID+4 g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dams</td>
<td>Water</td>
<td>Water</td>
<td>20% v/v in water</td>
<td>Water</td>
<td>20% v/v in water</td>
<td>20% v/v in water</td>
</tr>
<tr>
<td>Pups (PD 4-10)</td>
<td>Non-treated</td>
<td>Non-treated</td>
<td>i.g.3 g/kg/day</td>
<td>i.g.4 g/kg/day</td>
<td>i.g.3 g/kg/day</td>
<td>i.g.4 g/kg/day</td>
</tr>
<tr>
<td>Exposure window</td>
<td>Non-exposure</td>
<td>1st and 2nd trimester exposure</td>
<td>3rd trimester exposure</td>
<td>3rd trimester exposure</td>
<td>Full trimesters exposure</td>
<td>Full trimesters exposure</td>
</tr>
</tbody>
</table>

Table 3.1. Pregnant dams received either no treatment or DID during the entire gestational period. Their offspring were treated with water, 3 g/kg, or 4 g/kg ETOH from PD 4-10, which produced 5 treatment paradigms in total.

**BEC measurement**

For BEC of pregnant dams, blood samples were obtained from a 1-2 mm tail clip following 4 hours DID period. For BEC of the pups, separate groups of pups were treated and trunk blood of pups was collected on PD 4 or PD 10, following decapitation 30, 60, 90, 120, 180, 240, 360 and 480 min after ETOH intubation. BEC was determined using an assay kit from Sigma-Aldrich, St. Louis, MO (product number: MAK076). The data were collected from multiple samples (n=4) per time.
Behavioral testing

Open Field (OF): The OF was a round chamber (diameter 39.4 cm) with opaque white walls and floor, which is commonly used to test levels of activity, impulse control or anxiety (Bailey and Crawley, 2009). The center was defined as a circular zone in the center of the OF with a diameter half of the width of the OF. Since mice typically display thigmotaxis and avoid the center of open arenas, OF center exploration is often used as a measure of anxiety and/or inhibitory control. On PD 20–22, the activity of offspring in an open field was tested 30 mins daily for two consecutive days in the morning between 08:00 – 12:00 (Mei et al., 2016). The measurements included the total distance traveled, distance traveled in the center and the number of entries into the center.

Elevated Plus Maze (EPM): The EPM is a plus-shaped (+) Plexiglas maze with clear walls bordering two of the four arms (30 x 6 cm). It is typically used to evaluate the impulse control and/or anxiety issues of mice (Bailey and Crawley, 2009). On PD 25–27, the exploration of each subject in the EPM was tested for 5 mins. The distance traveled overall, distance traveled in the open arms and the number of open arm entries was measured.

Morris Water Maze (MWM): MWM is a round plastic tub with a diameter of 107.6 cm, which is used to measure spatial learning and memory (Vorhees and Williams, 2006). Four visible extra-maze cues were placed around the tub at various points. A platform (15.2 x 15.2 cm) was submerged 0.75 cm under the surface of the water in a
fixed location. On PD 35 -45, the performance of offspring in MWM was tested between at 12:00 – 16:00 h (Chen et al., 2013). During maze acquisition, four trials were completed per day for four consecutive days. For each trial, each mouse was placed in one of four starting positions on the far side of the pool (120, 150, 210 and 240° from the platform) and allowed to swim until either it found the submerged platform or reached a ceiling of 60 secs. If it did not find the platform, it was gently guided to the platform. Subjects remained on the platform for 5 sec before being removed for a 5 min intertrial interval (ITI). The latency and distance traveled for each mouse per trail were recorded. On the fifth day, a single probe trial was conducted. The platform was removed and the mouse was placed in the opposite quadrant. The number of annulus crossings was recorded for 60 sec. Four hours after the probe trial, the platform was moved to one quadrant away from its original location for a reversal learning test. Four trials in total were conducted as described in the acquisition phase. The distance and latency traveled before each mouse escaped on the platform were recorded. Following reversal learning (ITI 5 min), a single visible platform trial was conducted with a visible rod placed on the top of the platform to indicate its location. The visible platform component was included to ensure that there were no visual or motor deficits potentially contributing to performance on this task following developmental ETOH exposure.

All behavioral tests above were conducted under low ambient light conditions with white noise to reduce extraneous auditory stimuli. Nature’s Miracle© enzymatic cleaning solution was used to clean the surfaces and holding cages to remove animal
odors before and after testing. When males and females were tested on the same day for the same behavioral tests, the males were tested first. Animal movements were recorded using the AnyMaze tracking system (Stoelting Co.)

**Statistical Analysis**

SPSS software version 21 (IBM) was used to analyze all data. To avoid potential litter effects, behavioral data from same-sex siblings were averaged together to produce a single data point per sex, per treatment, per litter for each measure (Abbey and Howard, 1973, Wainwright, 1998). Thus, each data represents the mean of 2-3 same-sex siblings. Univariate or mixed-factors analysis of variance (ANOVA) was performed with treatment and sex as grouping factors followed by post hoc Tukey test. The Greenhouse-Geisser correction was used to correct for violations of homogeneity of variance when necessary. In some cases, this correction resulted in degrees of freedom that were not whole numbers. If there was no main effect or no interaction between treatment and sex or multiple time points (e.g. days, time bins, etc), the data were averaged across this variable for ease of presentation and higher statistical power.

**Results**

**BEC and body weight**

Over the course of gestation, pregnant dams in the DID group had free access to 20% EtOH solution for 4 hours during the early dark cycle. They consumed a range of 5.3 – 8.2 g/kg with an average of 6.58 g/kg EtOH per day and had an average
BEC of 0.751 mg/ml. BEC levels of pups that received 3 g/kg or 4 g/kg ETOH alone were determined on PD 4 and PD 10, respectively. All BEC curves peaked at 1 hour post-intubation with a value of 235.3 ± 17.63 and a value of 325.7 ± 22.49 for postnatal 3 g/kg or 4 g/kg ETOH treatment, respectively on PD 4 (Figure 3.1A), a value of 210.5 ± 16.5 and a value of 307.2 ± 25.6 correspondingly on PD 10 (Figure 3.1B).

Figure 3.1. BECs of postnatal ETOH treated offspring.

BECs were measured at 30, 60, 90, 120, 180, 240, 360 and 480 after ETOH
intubation (3 g/kg or 4 g/kg) on PD 4 (Figure 3.1A) and PD 10 (Figure 3.1B). BECs peaked at approximately 1 hour post intubation. Data are expressed as mean ± S.E.M (n=5).

Body weights of pregnant dams were recorded every 5 days from GD 0 to GD 20. A mixed factors ANOVA showed a significant main effect of day, F (4, 70) = 1291.92, P < 0.05, and a day by treatment interaction, F (4, 70) = 8.98, P < 0.05. No significant effects of dam treatment on body weight gain (Figure 3.2) or litter size were observed.

![Body weight gain of pregnant dams](image)

Figure 3.2. Body weight gain of pregnant dams.

Body weights of pregnant dams in both control and DID treated groups were recorded every 5 days from GD 0 to GD 20. Data are expressed as mean ± S.E.M (n=14).

Body weights of newborn offspring were recorded daily from PD 4 to PD 10. There
was a significant effect of treatment, $F (5, 42) = 5.53$, $P < 0.05$, age, $F (1.75, 73.31) = 17.971.57$ and an interaction between age and treatment, $F (8.73, 73.31) = 10.65$ $P < 0.05$. Post hoc analysis showed combined maternal DID and postnatal 4 g/kg EtOH weighed less than control on PD 5-10. Mice treated with 4 g/kg ETOH alone weighed less than control on PD 8-10 (Table 3.2). ETOH treatment at 3 g/kg dose alone or in combination with maternal DID did not affect the body weights of the offspring.

Offspring were so weighed on the day of behavioral testing. There were significant effects of treatment, $F (5, 86) = 4.65$, $P < 0.05$ and sex, $F (1, 86) = 34.18$, $P < 0.05$ on body weights. Post hoc tests showed that female offspring with combined maternal DID and postnatal 4 g/kg EtOH weighed less than 3 g/kg treatment alone on the day of MWM testing.

Table 3.2. Body weights of offspring on PD 4-10 and at the time of behavioral testing.

<table>
<thead>
<tr>
<th>Postnatal Day (PD)</th>
<th>Control (g)/- SEM</th>
<th>DID+3 g/kg (g)/- SEM</th>
<th>DID+4 g/kg (g)/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.64±0.06</td>
<td>2.68±0.03</td>
<td>2.64±0.03</td>
</tr>
<tr>
<td>5</td>
<td>3.08±0.08</td>
<td>3.14±0.04</td>
<td>3.06±0.03</td>
</tr>
<tr>
<td>6</td>
<td>3.51±0.10</td>
<td>3.56±0.03</td>
<td>3.50±0.02</td>
</tr>
<tr>
<td>7</td>
<td>3.95±0.11</td>
<td>4.04±0.04</td>
<td>3.91±0.02</td>
</tr>
<tr>
<td>8</td>
<td>4.39±0.08</td>
<td>4.49±0.03</td>
<td>4.36±0.02</td>
</tr>
<tr>
<td>9</td>
<td>4.84±0.09</td>
<td>4.89±0.03</td>
<td>4.83±0.03</td>
</tr>
<tr>
<td>10</td>
<td>5.30±0.10</td>
<td>5.36±0.03</td>
<td>5.22±0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>9.64±0.43</td>
<td>9.55±0.40</td>
<td>10.08±0.46</td>
<td>9.72±0.27</td>
<td>10.33±0.47</td>
<td>9.98±0.46</td>
<td>9.01±0.40</td>
<td>8.61±0.45</td>
<td>9.20±0.34</td>
</tr>
<tr>
<td>24</td>
<td>12.65±0.43</td>
<td>12.15±0.51</td>
<td>13.11±0.51</td>
<td>11.70±0.49</td>
<td>12.65±0.48</td>
<td>11.83±0.91</td>
<td>12.56±0.48</td>
<td>11.46±0.57</td>
<td>11.74±0.58</td>
</tr>
<tr>
<td>35</td>
<td>20.50±0.48</td>
<td>17.48±0.59</td>
<td>21.20±0.45</td>
<td>17.78±0.31</td>
<td>21.20±0.63</td>
<td>18.48±0.29</td>
<td>19.36±0.48</td>
<td>16.28±0.29</td>
<td>19.80±0.65</td>
</tr>
</tbody>
</table>
Table 3.2. The body weights of offspring were recorded every day from PD 4 to PD 10, as well as on the day of each behavioral test. The data are presented as mean ± S.E.M. *: p <0.05 as compared with controls (n=8).

**Behavioral testing**

**Open field:** An analysis of OF exploration revealed that offspring in both groups that received postnatal 4 g/kg ETOH were more active relative to controls. Maternal DID did not affect activity levels alone or alter the effect of postnatal exposure. Figure 3.3 shows activity in the OF as measured by distance traveled over two OF sessions. The mixed factors ANOVA of distance traveled revealed significant main effects of treatment, F (5, 100) = 14.33, p < 0.05, day, F (1, 100) = 27.15, p < 0.05, and time bin, F (3.62, 362.24) = 248.18, p < 0.05, with significant interactions of day by treatment, F (5, 100) = 2.35, p < 0.05, and bin by treatment, F (18.12, 362.24) = 1.83, p < 0.05, as well as an interaction of day, bin and treatment, F (20.68, 413.51) = 1.91, p < 0.05. Post hoc tests showed that offspring with postnatal ETOH exposure of 4 g/kg, with and without maternal DID, traveled more distance across multiple time blocks within each session relative to controls (p < 0.05). Maternal DID, postnatal 3 g/kg, and their combination did not significantly alter distance traveled in the OF.
Figure 3.3. Distance traveled across time in the open field.

Postnatal ETOH exposure of 4 g/kg, with and without maternal DID were hyperactive on both OF sessions. Data is presented, collapsed across sex, as total distance traveled in the OF (mean ± S.E.M). *: p <0.05 as compared with controls (n=8-12 liters per treatment group).
Additional analyses assessed ETOH effects on center exploration, measured as the total distance traveled in the center zone and number entries into the center. Postnatal ETOH treatment of 4 g/kg ETOH was associated with a greater distance traveled in the center zone, although this did not reflect a greater likelihood of entering the center. For center exploration, there were significant main effects of treatment, F (5, 100) = 8.21, p < 0.05, sex, F (1, 100) = 7.22, p < 0.05, and day, F (1, 100) = 30.74, p < 0.05, but no interactions with day. Post hoc tests showed that offspring with postnatal exposure to 4 g/kg ETOH explored a significantly greater distance in the center than controls (p < 0.05) (Figure 3.4). No group differences or interactions were detected on the number of entries into the center (Figure 3.5).

![Figure 3.4. Distance traveled in the center of open field.](image)

Postnatal ETOH treatment of 4 g/kg, regardless of prenatal treatment, traveled a greater distance in the center zone relative to controls on both test days. Data is shown, collapsed across sex and 5-min time block for each day (mean ± S.E.M). *: p <0.05 as compared with controls (n=8-12 liters per treatment group).
Figure 3.5. The number of entries into the center of open field.

No difference was observed among groups in respect to the number of entries into the center. Data is shown, collapsed across sex and 5-min time block for each day (mean ± S.E.M).

**Elevated plus maze:** ETOH effects were observed on both total activity level and the tendency to explore open areas in the EPM, with combined DID and postnatal 4 g/kg ETOH treatments showing the most robust effects across these measures. Figure 3.6 shows performance in the EPM, as measured by distance traveled (Figure 3.6A) and open arm exploration (Figure 3.6B and C). The univariate ANOVA of distance traveled in the EPM revealed a significant main effect of treatment, $F (5, 97) = 5.83$, $p < 0.05$, with post hoc tests confirming that offspring with combined maternal DID and postnatal 4 g/kg ETOH were more active ($p < 0.05$) relative to controls. No other treatment groups reached significance on this measure.

Additional analyses assessed ETOH effects on open arm exploration, measured as the distance traveled in (Figure 3.6B) and the number of entries into (Figure 3.6C) the open arms of the maze. ETOH exposure dose-and time-dependently increased
both explorations of the open arms and the number of entries, although the patterns for these two measures differed somewhat. Univariate ANOVA for distance traveled in the open arms showed a main effect of treatment, $F(5, 97) = 12.28$, $p < 0.05$, with post hoc tests showing that the combination of maternal DID and postnatal ETOH treatment (either 3 or 4 g/kg) increased distance traveled in the open arms relative to controls, ($p < 0.05$). Of interests, offspring with combined DID and postnatal treatment of 4 g/kg also traveled greater distances in the open arms than offspring with postnatal 4 g/kg alone. The univariate ANOVA of open arm entries yielded a significant main effect of treatment, $F(5, 97) = 4.70$, $p < 0.05$. Post hocs showed that postnatal 4 g/kg ETOH treatment increased open arm entries relative to controls ($p < 0.05$), with and without maternal DID.

![Graph showing total distance and treatment effects](image)
Figure 3.6. Elevated plus maze test.

Offspring exposed to the combined maternal DID and postnatal 4 g/kg ETOH were more active relative to controls (Figure 3.6A). Offspring with the combination of maternal DID and postnatal ETOH treatment (either 3 or 4 g/kg) traveled a greater distance in the open arms relative to controls (Figure 3.6B). Postnatal ETOH treatment of 4 g/kg increased open arm entries relative to controls (Figure 3.6C). The data is presented, collapsed across sex (mean ± S.E.M). *: p < 0.05 as compared with controls (n=8-11 liters per treatment group).

Morris water maze: Offspring exposed to postnatal ETOH exposure, independent of
prenatal exposure, showed impaired acquisition in the MWM. Acquisition performance was measured by the latency and distance traveled prior to escape (Figure 3.7) onto the platform. The mixed-factors ANOVA of escape latency revealed a significant main effect of treatment, $F(5, 97) = 8.07$, $p < 0.05$, and day, $F(2.60, 251.70) = 57.62$, $p < 0.05$. Post hoc tests confirmed that those groups exposed to 4 g/kg ETOH postnatally, with or without maternal DID, as well as those receiving 3 g/kg postnatally, required more time to find the platform ($p < 0.05$) relative to controls. A similar trend among those with combined maternal DID and postnatal 3 g/kg failed to reach significance. The mixed-factors ANOVA of distance traveled to the platform revealed a significant main effect of treatment, $F(4, 67) = 4.78$, $p < 0.05$, and day, $F(2.68, 259.80) = 104.88$, $p < 0.05$. Post hoc tests confirmed that those groups with postnatal ETOH exposure with 4 g/kg ETOH, with or without maternal DID, traveled further to reach the platform than controls ($p < 0.05$).
Figure 3.7. Morris water maze test acquisition.

Offspring exposed to 4 g/kg ETOH postnatally, regardless of prenatal treatment, as well as those receiving 3 g/kg, took longer to find the platform (A). Mice with postnatal 4 g/kg ETOH treatment also traveled a longer distance than controls prior to escape onto the platform (B). The data is presented, collapsed across sex and 4 daily acquisition trials (mean ± S.E.M). *: p <0.05 as compared with controls (n=8-12 liters per treatment group).

For the probe trial, there was a significant main effect of treatment, F (5, 109) = 4.11, p < 0.05. Post-hoc analysis revealed that there was a reduction in the number of annulus crossing in offspring with postnatal 4 g/kg ETOH treatment alone and when combined with maternal DID (p < 0.05). A similar trend among offspring with postnatal exposure to 3 g/kg ETOH failed to reach significance (p=.06) (Figure 3.8).
Offspring exposed to 4 g/kg ETOH with or without prenatal treatment showed a reduction in the number of annulus crossing (mean ± S.E.M). *: p <0.05 as compared with controls (n=8-12 liters per treatment group).

Performance on the reversal component of the MWM is shown in Figure 3.9. Postnatal ETOH exposure alone increased escape latencies in the reversal phase relative controls. Escape latency is presented in Figure 3.9A. There was a main effect of treatment, F (5, 97) = 5.62, p < 0.05. Post-hoc analysis revealed that that offspring with postnatal 4g/kg ETOH treatment alone and when combined with maternal DID took longer to reach the platform than controls (p < 0.05). There were no significant treatment effects on the distance traveled to the platform (see Figure 3.9B).
Figure 3.9. Morris water maze reversal learning.

Offspring with postnatal 4 g/kg ETOH treatment alone or combined with maternal DID took longer to reach the platform than controls during the reversal phase of the MWM (A), but no difference was observed among groups in respect to the distance traveled to the platform (B). The data is presented, collapsed across sex (mean ± S.E.M). *: p <0.05 as compared with controls (n=8-12 liters per treatment group).

To assess whether early ETOH exposure altered motor performance or visual ability, swim speed was measured in a visible platform probe (Figure 3.10). There were no significant effects of treatment on swim speed to reach the visible platform. A sex
effect was detected, $F(5, 109) = 5.62, p < 0.05$, whereby females swam faster than males.

Figure 3.10. Morris water maze a visual platform trial.

No difference was shown in the swimming speed among groups in the single visual platform trial. The data is presented, collapsed across sex (mean ± S.E.M).

**Discussion**

The current study aimed to characterize key behavioral phenotypes across early development with different methods, dosage and timing of ETOH delivery. In doing so, maternal DID and postnatal intubation procedures were used alone or in combination to produce five ETOH exposure paradigms. ETOH exposure effects on behavioral performance were detected. Since our previous study showed that there was no significant difference between non-treated control and maltose intubated control in respect to behavioral consequences (seen in project 2), this current study did not include the maltose-intubated group as an isocaloric control.

Maternal DID was found to lead to a BEC of approximately 100 mg/dl. However, the moderate levels of ETOH exposure produced by DID have not been shown to produce robust behavioral impairments across in juvenile or adolescent mice, either
in the current study or in previous work by Boehm et al (Boehm et al., 2008). By contrast, each of the doses of ETOH selected for postnatal intubation resulted in peak BECs exceeding 200 mg/dl. Of these, only the 4 g/kg dose produced high BECs that remained above 200 mg/dl for over two hours, a pattern associated with behavioral impairments in existing models. Postnatal ETOH intubation paradigms have rarely been reported in mice, although they are preferable methods to repeated injections which may lead to increased ETOH leakage from the pups’ porous skin (Moser et al., 2005). The pharmacokinetic and behavioral data presented here, as well as strong rates of pup survival (<5% mortality), support the continued use of postnatal intubations to model 3rd-trimester ETOH exposure in mice. Additionally, the weight gain of dams and pups was unaffected by maternal DID alone. Offspring exposed to 4 g/kg or combination of DID with 4 g/kg ETOH weighed less than controls on early postnatal ages. Even so, the present data do suggest that these sub-threshold ETOH exposures may contribute to greater effects for postnatal treatments, as DID and 4 g/kg showed weight reductions earlier than pups with postnatal 4 g/kg ETOH alone.

In the current study, prenatal treatment, postnatal treatment or their combination may alter performance on a number of behavioral outcomes. OF and EPM were conducted in prewean ing mice to assess the presence of hyperactivity and/or alterations in exploratory behavior, as have been demonstrated in other rodent models (Mei et al., 2016, Fish et al., 2016). Postnatal exposure to 4 g/kg ETOH, regardless of maternal DID, led to hyperactivity in the OF. Similar patterns of
hyperactivity were found in the EPM, although this effect was only detectable in offspring with combined maternal DID and postnatal intubations of 4 g/kg ETOH. These findings are in line with the previous mouse and rat models of FASD showing hyperactivity in young ETOH-exposed animals (Melcer et al., 1994, Kim et al., 2013). To better understand these changes, activity in the center zone of the OF and open arms of the EPM were assessed alongside the number of entries into the center zone or open arms. For the OF, offspring receiving postnatal intubations of 4 g/kg traveled further in the center but did not differ in the number of entries into the center from the controls, suggesting that increased distance traveled observed in these offspring reflect hyperactivity that is unrelated to center avoidance. In contrast, ETOH exposure was shown to influence both explorations of the open arms and the number of entries into open arms, but the effects were different based on dose- and paradigm of exposure. Distance traveled in the open arms was significantly increased in both groups receiving combined DID and postnatal intubations, regardless of postnatal dose (3 or 4 g/kg ETOH). Open arm visits were elevated in both groups exposed to 4 g/kg ETOH postnatally, but not those exposed to 3 g/kg ETOH. Therefore, dose-dependent increases in total activity in the EPM appeared to be due to changes in open arm exploration. Alterations in open arm exploration have similarly been shown in previous rodent models of FASD with rats (Brocardo et al., 2012, Osborn et al., 1998).

The present findings from the OF and EPM are interesting and suggest multiple potential effects of ETOH, including hyperactivity and increases in the choice to
enter an open arm and/or to travel within the open arms. Postnatal exposure to 4 g/kg ETOH was generally associated with hyperactivity in the OF, regardless of prenatal treatment. Postnatal 4 g/kg ETOH also increased visits to the open arms in the EPM. The combination of maternal DID and postnatal 4 g/kg ETOH mirrored these effects, while also producing increased open arm distance. It is intriguing that those with 4 g/kg ETOH alone tended to make more entries into open arms, but failed to show increased distances traveled in the open arms. This suggests that these subjects exited the open arms having explored the arms less thoroughly than those with combined maternal and postnatal 4 g/kg ETOH exposure. It may be concluded that postnatal ETOH exposure at the 4 g/kg dose may reduce the inhibitory control of entries into an open arm, while the combination of maternal DID and postnatal ETOH intubation may reduce the inhibitory control of further exploration of the open arm after an entry. The present phenotypes of hyperactivity and inhibitory failures are an important feature of the present mouse models, as those with FASD are typically hyperactive and may have difficulty avoiding risky behaviors or aversive consequences, leading to various adverse consequences in childhood and adolescence (Green et al., 2007) into adulthood (Moore & Riley, 2015).

Additional deficits were identified in the Morris Water Maze. Offspring exposed to neonatal 4 g/kg ETOH, with or without maternal DID, performed as well as controls on the first trial in the maze, but quickly developed a pattern of longer latencies and distances traveled to the platform relative to controls. This impairment was observed across four days of testing and into the probe test, where these offspring failed to
persistently return to the platform location. These subjects also tended to take longer to locate the novel platform in the reversal component, although they reached the novel platform without longer distances traveled. These animals did not appear to have motor deficits, as they performed as well as controls on the visible platform test. Taken together, it is concluded that the poorer performance resulted from neonatal ETOH-induced learning deficits rather than motivational or motor effects. These effects were not affected by maternal ETOH consumption and were not reliably produced by neonatal exposure to the lower dose of 3 g/kg ETOH. Offspring with exposure to 3 g/kg ETOH tended to show longer escape latencies, although this did not reach significance in combined DID with this treatment and did not result in increased distances to escape. Taken together, these data suggest that higher neonatal doses may be necessary to produce spatial learning deficits in these mice, as has been described in analogous 3rd-trimester rat models (Cronise et al., 2001). Robust learning deficits suggest face validity for the neonatal exposure model, as poor learning outcomes are commonly reported among children and adolescents with FASD (Green, 2007).

The data generated in this study display ETOH-induced alterations in at least three domains: activity regulation, inhibitory control, and spatial learning. These phenotypes were expressed following neonatal exposure to higher doses of ETOH, specifically the 4 g/kg dose which resulted in longer exposures to BECs above 200 mg/dl. These findings are in line with the broader literature on critical windows in the third trimester of brain development. More uniquely though, these data suggest that
the present 3-trimester model, combining maternal ETOH treatment with postnatal intubations, can model interactions between ETOH exposures prior to and during the third-trimester equivalent of brain development. Although with the current paradigm, maternal DID alone did not alter behavior due likely to a lower BEC, it led to a qualitatively distinct inhibitory deficit when combined with neonatal treatment. The present data do not suggest a clear mechanism for such effects but do suggest that maternal DID and neonatal intubations may be suitable methods for investigating how early, sub-threshold exposures to ETOH could contribute to FASD. Based on the present findings, future research should apply the present models to investigate how early, moderate ETOH exposures may impact inhibitory pathways and/or alter their sensitivity to later insults.

In summary, the current study examined behavioral characteristics in adolescent mice with early ETOH exposure. By manipulating the dosage, timing, and methods of ETOH delivery, our results provided an inclusive aspect on how behavioral changes induced by fetal alcohol exposure are affected by these factors. It is a useful reference for the future investigation on the neurogenetic mechanism underlying the FASD.

Acknowledgement

First authorship of this manuscript is shared between Lu Dai, Wenhua Xu, Andrew Hawkey, and Hui Li.
Chapter 4: Summary and Future Direction

Chromium & T cell immunity

Chromium (Cr) is a heavy metal widely used in the various industrial process. Since the first cancer case associated with chromium was reported in Scotland a century ago (Langard, 1990), a myriad of studies have been conducted in an attempt to elucidate the relationship between exposure to chromium compounds and increased incidence of certain cancers. Given that the immune system defends the host body against mutated/tumor cells, any factor that affects components of the immune system may sabotage the host immunity, cause immunosuppression and therefore, nurture the development of cancer. Due to the natural capacity of T cells in mediating immune response and in directing cytolysis of the mutated/tumor cells, T cells play a central role in host anti-tumor immunity.

As the first step to understand the effect of Cr(VI) on the T cells mediated anti-tumor immunity as well as its role in Cr(VI) carcinogenesis, in current study, we used primary cultured mouse splenic T cells as an *in vitro* model and tested whether and how T cells are affected by Cr(VI) at concentrations relevant to occupational and environmental exposure. Results obtained in this study provide a reference for the future *in vivo* study and lay a groundwork for the further investigation on the mechanism of the immunosuppression caused by Cr(VI). Our results showed that Cr(VI) inhibited the activation and function of T cells. The regulation of T cells activation and response is a complex process consisting of both stimulatory and inhibitory cell intrinsic signaling pathway, known as immune checkpoints (Sharma...
and Allison, 2015). The inhibition of the stimulatory pathway or the activation of the inhibitory pathway prevents T cells response against mutated/tumor cells and contributes to tumorigenesis. The immune checkpoint protein, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1), can inhibit the T cell effector function (Rudd et al., 2009, Dong et al., 2017). Therefore, in order to fully understand the process of how Cr(VI) disrupts T cells immunity and facilitates the immune escape of mutated/tumor cells, it will be interesting to investigate the role of Cr(VI) on the immune checkpoint pathways.

**Early ethanol exposure & behavioral deficits in young mouse**

Behavioral anomalies such as hyperactivity and impulsive behaviors as well as deficits in learning and memory are observed in children with FASD. Project 2 and 3 characterized behavioral phenotypes resulted from ETOH exposure during the early neurodevelopmental period in pre-weaning and adolescent mice. Our results showed that offspring exposed to 4 g/kg ETOH per day from PD 4 to PD 10 (third trimester), regardless of prenatal treatment (first- and second trimester) displayed a series of behavioral deficits. The results are in line with previous studies showing that binge-like alcohol exposure during the early postnatal period in rats and mice causes deficits in spatial learning and memory (Wozniak et al., 2004, Furumiya and Hashimoto, 2011).

Given the advantages provided by mouse model in studies on the etiology of a variety of neurological disorders, as well as results presented in current studies, there are a number of future investigations needed to be done to explore the neurogenetic
mechanisms beneath the observed behavioral changes. First of all, it will be important to evaluate morphological changes in brain structures that correspond to the behavioral deficits outlined in our results. Structures, such as prefrontal cortex, hippocampus, amygdala, and thalamus, are of particular interest due to their roles in cognitive regulation, spatial learning, information retention, and anxiety, fear conditioning (Pourtois et al., 2013, Duarte-Guterman et al., 2015, Frith and Dolan, 1996). In addition, the decreased number of neurons was observed in rodents subjected to acute postnatal ETOH treatment (Bonthius and West, 1990, Dikranian et al., 2005, Wozniak et al., 2004). Although apoptotic neurodegeneration has been linked to reduced brain volume and neuron losses (Saito et al., 2016, Wozniak et al., 2004), changes in neurogenesis, especially neurogenesis during the third trimester are worth investigating. The previous study showed that the suppression of neurogenesis during the third trimester may lead to abnormal neuro-behavioral manifestations prevalent in preterm infants (Malik et al., 2013). Therefore, studies on how early ETOH exposure affects neurogenesis and its potential genetic targets will serve as a valuable groundwork for developing and screening therapeutic compounds against FASD. Moreover, accumulating evidence suggests that ethanol-induced behavior defects, such as hyperactivity, may be associated with hypomyelination caused by ethanol-induced damage to oligodendrocytes and consequently agenesis of the corpus callosum (CC). The effects of ethanol on CC development may also provide valuable information towards better understanding ethanol neurotoxicity.
Reference


JONES, A. W. & HARDING, P. 2013. Driving under the influence with blood alcohol concentrations over 0.4 g%. *Forensic Sci Int*, 231, 349-53.


# Vita

## Education

<table>
<thead>
<tr>
<th>Date</th>
<th>Degree</th>
<th>Institution</th>
<th>Location</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>08/2012-present</td>
<td>Ph.D. student</td>
<td>Department of Toxicology and Cancer Biology, College of Medicine, University of Kentucky, USA</td>
<td>USA</td>
<td>Mentor: Gang Chen, Ph.D.</td>
</tr>
<tr>
<td>09/2007-06/2012</td>
<td>Bachelor of Medicine</td>
<td>Xiangya School of Medicine, Central South University, China</td>
<td>China</td>
<td></td>
</tr>
</tbody>
</table>

## Experience

<table>
<thead>
<tr>
<th>Date</th>
<th>Position</th>
<th>Institution</th>
<th>Location</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>03/2013-present</td>
<td>Research assistant</td>
<td>Gang Chen Lab, Molecular and Biomedical Pharmacology, University of Kentucky, USA</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>01/2012-06/2012</td>
<td>Preventive Medical Internship</td>
<td>Changsha City Center for Disease Control and Prevention.</td>
<td>China</td>
<td></td>
</tr>
<tr>
<td>07/2011-09/2011</td>
<td>Research assistant</td>
<td>Shanghai Cancer Institute-Zonghai Li Experimental Group (national key laboratory), China</td>
<td>China</td>
<td></td>
</tr>
<tr>
<td>02/2011-08/2011</td>
<td>Clinical internship</td>
<td>Changsha No.8 hospital, China</td>
<td>China</td>
<td></td>
</tr>
<tr>
<td>01/2011-02/2011</td>
<td>Research assistant</td>
<td>Molecular Immunopharmacology Laboratory, Kunming Institute of Zoology, CAS, China</td>
<td>China</td>
<td></td>
</tr>
<tr>
<td>05/2009-06/2012</td>
<td>Research assistant</td>
<td>Health Toxicology Laboratory, School of Public Health, Central South University, China</td>
<td>China</td>
<td></td>
</tr>
</tbody>
</table>

## Honors and Awards

<table>
<thead>
<tr>
<th>Date</th>
<th>Award</th>
</tr>
</thead>
<tbody>
<tr>
<td>07/2014-06/2015</td>
<td>Kentucky Opportunity Fellowship</td>
</tr>
<tr>
<td>12/2013-06/2014</td>
<td>Kentucky Opportunity Fellowship</td>
</tr>
<tr>
<td>09/2011-05/2012</td>
<td>Second-class Scholarship, Outstanding Student, CSU</td>
</tr>
<tr>
<td>09/2010-07/2011</td>
<td>Third-class Scholarship, CSU</td>
</tr>
<tr>
<td>09/2009-07/2010</td>
<td>Second-class Scholarship, Outstanding Student, CSU</td>
</tr>
<tr>
<td>09/2008-07/2009</td>
<td>Second-class Scholarship, Outstanding Student, CSU</td>
</tr>
</tbody>
</table>

## Presentations and Posters

<table>
<thead>
<tr>
<th>Date</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>02/06/2017</td>
<td>Seminar Speaker at the Department of Toxicology and Cancer Biology, University of Kentucky</td>
</tr>
<tr>
<td>10/17/2016</td>
<td>Poster presentation, 9th Conference on Metal Toxicity and Carcinogenesis, Lexington, KY</td>
</tr>
<tr>
<td>12/03/2015</td>
<td>Neuropharmacology Journal Club, University of Kentucky</td>
</tr>
<tr>
<td>09/16/2014</td>
<td>Toxicology Student Forum Journal Club, University of Kentucky</td>
</tr>
</tbody>
</table>

## Peer-reviewed publication

88


