SEX DIFFERENCES IN FETAL NEURAL STEM CELLS' RESPONSE TO ETHANOL

A Thesis

by

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASDs) can result from prenatal exposure to alcohol. Alcohol is a known teratogen and in utero exposure is the leading non-genetic cause of neurodevelopmental disabilities. Neural stem cells (NSCs) generate a majority of neurons in the adult brain during the mid-first to second trimester of gestation, a time at which pregnancy may not yet be recognized, resulting in a window of vulnerability for alcohol exposure. Our previous data shows that ethanol induces loss of stem cell capacity in NSCs but does not induce cell death. One cellular mechanism through which ethanol exerts its effects on NSCs is through actions on miR-9, a critical regulator of NSC differentiation. Ethanol exposure increases methylation at the miR-9 locus and subsequently reduces its expression in NSCs. Interestingly, previous studies have shown genetic sex-specific cellular behaviors within endogenous stem cells. Our study is the first to investigate sex-differences in the NSC response to ethanol. Using an ex vivo neurosphere culture of NSCs derived from mouse gestational day 12.5 fetal dorsal neuroepithelium, we assessed the effects of ethanol on neurosphere formation, cell cycle kinetics, as well as on NSC maturation in sex segregated cultures. Our data indicates that there is a sexually dimorphic response to ethanol in terms its effects on neurosphere formation, cell cycle kinetics, and NSC maturation. Further studies characterizing these sex differences in the NSC response to ethanol will help elucidate the pathophysiology of FASD.

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TABLE OF CONTENTS

Page

ABSTRACT……. ii

ACKNOWLEDGMENTS ……. iii

CONTRIBUTORS AND FUNDING SOURCE…….. iv

TABLE OF CONTENTS ……..……...…..…. v

LIST OF FIGURES ……..……..……..……..…. vii

LIST OF TABLES ……..……...…….….. viii

1. INTRODUCTION ……..……..……..…. 1

2. MATERIALS AND METHODS ……..……..…. 4

2.1 Neurosphere culture model…… ….…...……..……...…... 4
2.2 Ethanol treatment……...……...……....….. 4
2.3 Polymerase chain reaction (PCR) ……..……...…….. 5
2.4 Neurosphere- formation and MTT assays …….…..…..…. 7
2.5 Flow cytometry ..……...…….. 7
2.6 Quantitative reverse transcription
polymerase chain reaction (qRT-PCR) ……..……..….. 7
2.7 Statistical analysis ……..……...……...…….... 8

3. RESULTS ……...……..……...……..…..…. 9

3.1 Sex-dependent effects of ethanol on neurosphere formation as well as MTT ……..……...……. 9

3.2 Sex-dependent effects of ethanol on promoting cell cycle activity ……..……...……. 11

3.3 Sex-dependent effects of ethanol on alteration on the cell fate markers.……..……...……... 14

4. DISCUSSION ……..…..…….. 23

5. CONCLUSION ……...…….…… 26

REFERENCES……..…….…….…….. 27

LIST OF FIGURES

FIGURE		Page
1	The neurosphere culture model……..……...…….……..	5
2	An amplification plot of qPCR analysis ……....……	6
3	Neurosphere formation and MTT assays in responding to ethanol exposure after a 72 h culture period bar charts ……..…...	9
4	Sex and ethanol interactions in cell movement through the cell cycle …..…….…….....	13
5	Overall mRNA transcripts of neuronal development examined on treated and untreated neurosphere with ethanol ……..……..……..….	15
6	A comparison of nestin mRNA transcript responses to EtOH treatment between females and males using qRT-PC …….……..…....…..	16
7	A comparison of neurons mRNA transcript responses to EtOH treatment between females and males using qRT-PC ……..…..…….....…….	17
8	A comparison of astrocytes mRNA transcript responses to EtOH treatment between females and males using qRT-PC ……..…..…….	18
9	The measurement of the astrocytes	

markers of GFAP mRNA transcript control
group after normalizing to HPRT ….……..…. 19

10 A comparison of oligodendrocytes
mRNA transcript responses to EtOH treatment
between females and males using qRT-PC…….....……..... 20

LIST OF TABLES

TABLE Page

1 List of cell fate transcripts differentiation markers ……..... 20

2 Summary of normalize transcripts to detect
the less interaction of sex in response
to ethanol using two-way ANOVA analysis ……...……. 21

3 Female cells have a significant influences
by ethanol exposure on expression cell fate
markers using one-way ANOVA analysis ……...……...…. 21

4 Male cells have only a significant influences
by high ethanol dose exposure on expression cell
fate markers using one-way ANOVA analysis ……..... 22

5 Male and female control neurospheres have
a main effect of sex in early glial cells fate
marker using one-way ANOVA analysis ……..……..….. 22

1. INTRODUCTION

Fetal Alcohol Spectrum Disorders (FASDs) is the general term for combined conditions that are observed in children as a result of prenatal alcohol exposure (PAE) from a mother whose consumed alcohol during pregnancy [1]. This can result in learning and memory impairment, mental retardation, facial anomalies, psychological disorders, motor difficulties, hyperactivity disorders, and growth deficits [2-5]. The facial anomalies in FASD children include a smooth philtrum, shortened palpebral fissures, and a thin upper lip, whereas the growth deficits include reduce head circumference as well as weight or height under the 10^{th} percentile [6, 7]. The diagnosis of FASDs can range into fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorder (ARND), neurobehavioral disorder associated with prenatal alcohol exposure (ND-PAE), and alcohol-related birth defects (ARBD) [3]. FAS is considered the most severe condition of the FASDs [8-10] that was first repealed by Kenneth L. Jones and David W. Smith in the US in 1973 [8]. The leading, non-genetic cause of mental retardation is FASD which affects normal in fetal brain development [11]. A recent metanalysis has estimated that the worldwide prevalence of FASD at ~2.3% of the population [12]. Therefore, there is a need for research to understand the mechanism of alcohol's actions within the central nervous system to intervene to improve outcome in FASD patients.

Although there are public health initiatives which advise against alcohol use during pregnancy, alcohol exposure in early pregnancy still readily occurs due to the high rate of unplanned

pregnancies (45%) [9, 13-15]. Currently, there is no known safe level of alcohol intake during pregnancy [9]. During early pregnancy, most neurons of the adult brain are generated, making neurogenesis a particularly vulnerable period of development due to inadvertent alcohol exposure [4, 15].

Ethanol has the potential of producing irreversible damage to normal brain development [4] in targeting the developing cortex [5]. Ethanol's actions in the developing cortex result in widespread cortical thinning in FASD [16]. We have shown that within the cortex, while the developing neurons undergo cell death pathways in response to ethanol exposure [17, 18], the neural stem cells instead change their programming to increase cell cycle activity, indicative of differentiation of the more neural lineage committed neuroblasts, leading to a loss of the neural stem cell pool [4, 5]. Alcohol decreases the expression of particular ethanol-sensitive miRNAs, which normally act to prevent the translation of networks of mRNAs, affecting cell cycle and differentiation pathways [19].

There are several factors associated with the severity of fetal outcomes as a result of PAE [13, 20]. These factors are differences in genetic susceptibility related to the metabolism of ethanol, maternal lifestyle such socioeconomic status, ethnicity, and maternal nutrition, as well as dose and timing of alcohol consumption during pregnancy [13, 20]. Additionally, prenatal alcohol exposure is able to affect stem cell populations throughout the body during early pregnancy, indicating that FASD may be categorized, in part, as part of a disease of stem cell biology [6].

Neural stem cells have two primary characteristics, namely the ability to self-renew and to

differentiate into neurons, astrocytes, and oligodendrocyte in CNS [21-24]. Previous studies in laboratory have shown that ethanol does not induce death of fetal NSCs [4], but cause the loss of NSCs by promoting early neurogenesis during neuroepithelial proliferation period [10]. In other words, within the embryonic cortical epithelium, ethanol promotes premature stem cells into blast cell maturation [19]. This not only prompts neuroepithelial differentiation but also changes the fate of neuronal and glial differentiation after long-term ethanol exposure [6, 25, 26].

Previous studies have shown that there are sex differences in stem cell biology. For example, Choi and colleagues reported that both embryonic stem cells and embryonic germ cells show hierarchical clustering grouped by sex rather than the location of stem cell origin indicating that sex chromosomes may play an important role in early stem cell fates [27]. Another study reported found that female stem cells reproduce more quickly than male stem cells in the drosophila gut [28]. Human induced pluripotent stem cell (hiPSC) function may be altered by genetic sex as female-derived hiPSCs may lose long non-coding RNA X-inactive specific transcript (XIST), increasing oncogenic potential in female hiPSCs [29]. Therefore, in this current study, we are the first to investigate the role of sex in the effects of ethanol on fetal neural stem cells.

2. MATERIALS AND METHODS

2.1 Neurosphere Culture Model

All animal procedures were approved by the Institutional Animal Care and Use Committee at TAMHSC. A detailed description of culturing and media preparing for neurospheres has been published formerly [10]. In short, female and male neurosphere cultures were derived from C57BL/6J and Ai14 mouse fetal telencephalon specifically dorsal neuroepithelium as of gestational day 12.5 and cultured as neurospheres. These neurospheres were maintained separately in media comprising GlutaMAX™ DMEM F-12 (Invitrogen, Carlsbad, CA. Cat# 11330-032), 20 ng/ml EGF (Invitrogen, Carlsbad, CA Cat# 53003-018), 20 ng/ml bFGF basic, 1x ITS-X (Invitrogen, Carlsbad, CA. Cat# 51500-056), 5 μg/ml heparin, 0.15 ng/ml LIF, and 20 nM progesterone (Sigma, St. Louis, MO. Cat#P6149) in T25 flasks. Depending on the level of confluence, the media was modified every two to three days and entirely incubated in a humidified environment at 37°C, 5% CO_2 for four to five days before passage. These cultured NSCs male and female were detached and used all at once for further experiments, Figure (1).

2.2 Ethanol Treatment

Concurrently, the neurospheres for each sex were cultured separately in T25 flasks and capped with a parafilm (VWR) sealed lid for ethanol evaporation prevention. Following the previous published studies [10], the ethanol dose was implied 60 mg/dL (13 mM), 120 mg/dL (26 mM),

320 mg/dL (70 mM) of ethanol (Sigma) as consumption level by social drinkers, binge drinkers [30] , and chronic alcoholics respectively [31]. Simultaneously, non-ethanol treated stem cells were cultured in the same culture conditions and experimental time course as control samples. Every two to three days, neurospheres received fresh medium comprising ethanol or control treatment, Figure (1). Last of all, cells were isolated and cellular extracts gathered on day five of treatment for RNA analysis [32].

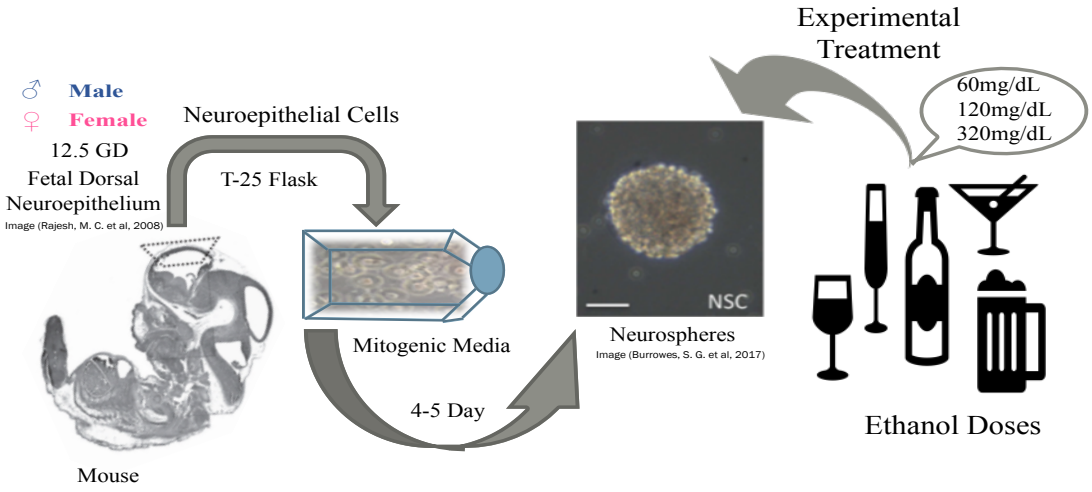

Figure 1. The Neurosphere Culture Model. It is a simplified model illustrates the neurosphere culture model to use with excremental ethanol treatment.

2.3 Polymerase Chain Reaction (PCR)

During culturing of neuroepithelium, cranial tissue was collected and DNA was rapidly isolated through alkaline lysis and incubation at 95°C for 15 minutes. The genetic sex was assessed using

a rapid qPCR protocol, using SYBR Fastmix (Quanta Biosciences) with previously published primers for repetitive sequences on the X [33] and Y [34] chromosomes. These primers were validated on postnatal day 1 pups, where sex could be determined by anogenital distance and showed that males had amplification of the Y chromosome repetitive sequence would occur at cycles prior to the X chromosome sequence while females showed an off-target product from the Y chromosome primers after the amplification of the X chromosome, allowing for an endogenous DNA loading control in female fetuses. This protocol allowed for the determination of genetic sex in 45 minutes and the pooling of same-sex tissue into the neurosphere cultures, as shown in Figure (2).

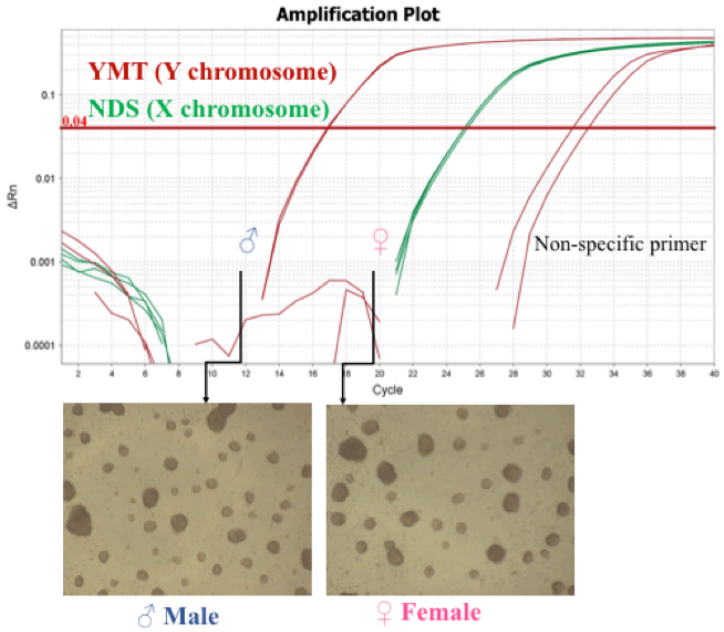

Figure 2. An Amplification Plot of qPCR Analysis. We isolated NSCs from the dorsal neuroepithelium of GD 12.5 and created sex segregated cultures based on the presence/absence of the YMT repetitive sequence on the Y chromosome using a sequence on the X chromosome as a control. NSCs were propagated and maintained as neurosphere cultures.

2.4 Neurosphere-Formation and MTT Assays

To measure the cell viability within the cell culture, we seeded 10,000 cells per well in 96-well cell culture plate. After 72 hours, neurospheres were counted and subsequently incubated for three hours with 1.2 mM of MTT (a tetrazole) reagent. MTT was subsequently solubilized with DMSO for 15 minutes. Absorbance at 570 nm was determined and provided a relative measure of viable cell count.

2.5 Flow Cytometry

This procedure was conducted according to Thermo Fisher Scientific protocol on evaluating the ability of cells to proliferate by measuring the DNA synthesis. For DNA synthesis activity, we used the Click-iT® EdU (5-ethynyl-2′-deoxyuridine) as incorporator into DNA. Flow Cytometry studies quantify the frequency of cells in the cell cycle from a sample of 200,000 cells, and data obtained from duplicate technical replicate and one biological replicate using Gallios software and Kaluza analysis software, Figure (4, F).

2.6 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was used to test primers for cell fate markers. The eight primers of interest were tested in triplicate. These primers are shown in the Table (1). The procedure is conducted according to Quanta Biosciences protocol by using isolated RNA from neurospheres via Nucleospin columns

from Qiagen and qScript™cDNA SuperMix to synthesize the cDNA. C_T values were determined using QuantStudio software (ABI/ThermoFisher). C_T values were normalized to an appropriate loading control (dCT) and subsequently normalize to the control values for each sex (ddCT). Since higher ddCTs indicate ~2-fold lower expression of mRNA, inverse values are shown (-ddCT) for ease of interpretation.

2.7 Statistical Analysis

Data were computed by analysis of variance (one- and two- way ANOVA) as well as post-hoc Fischer's Least Significant Difference (Fischer's LSD) tests to identify the statistical significant difference between groups via R-studio software. The statistical significant is set at $p<0.05$.

3. RESULTS

3.1 Sex-dependent effects of ethanol on on neurosphere formation as well as MTT

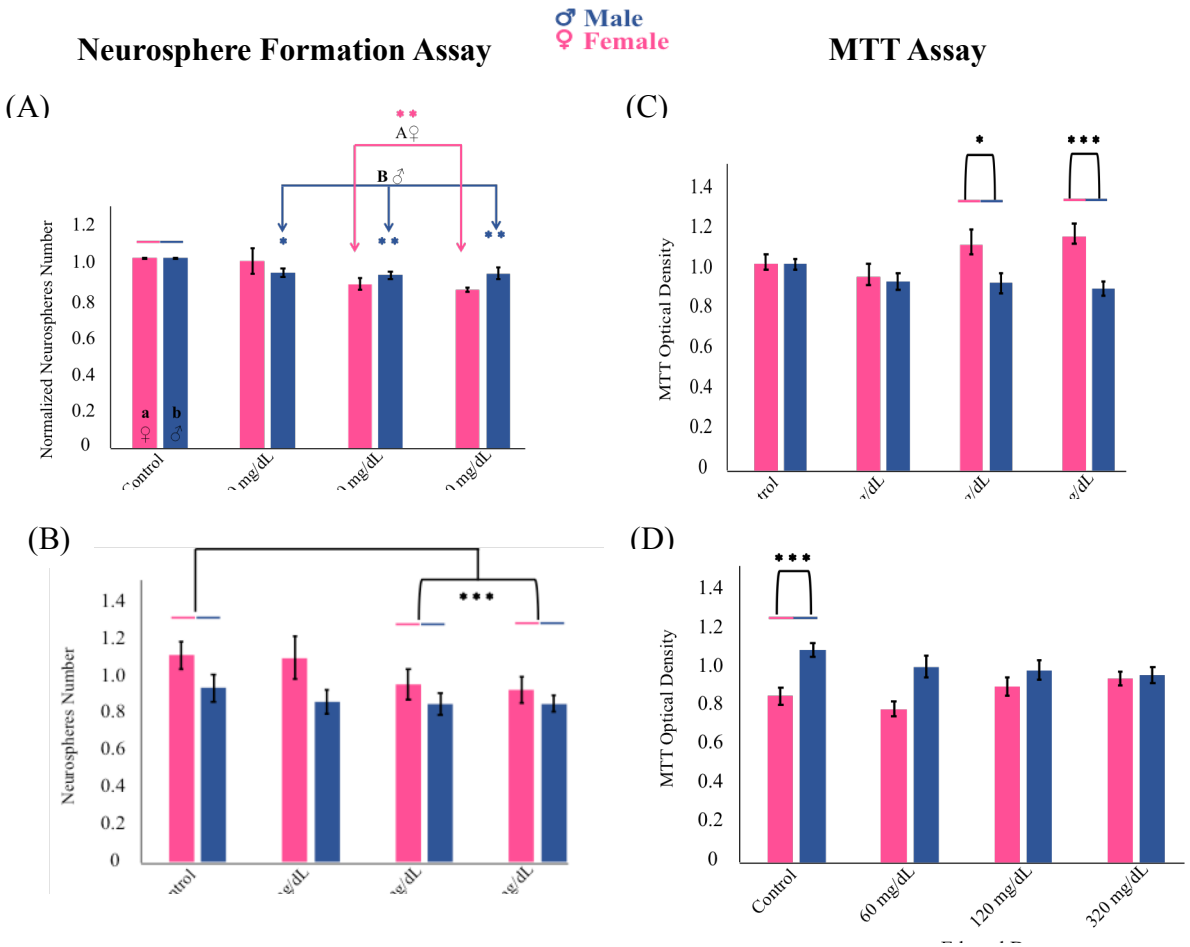

Figure 3. Neurosphere formation and MTT assays in responding to ethanol exposure after a 72 h culture period bar charts. (A) Normalized female NSCs to control shows decreased neurosphere formation after ethanol exposure while (B) showed within control group a sex differences and femalse NSCs decreases the neurosphere formation with doses > 60 mg/dL. (C) Ethanol exposure of 120 mg/dL and 320 mg/dL increase cell number in females about 30% compering to female non-treated with ethanol (control) while (D) shows that females have less initiative cells in the control group than males.

The two primary functions of stem cells are to self-renew and differentiate. To test the ability of neural stem cells to self-renew, we examine the neurosphere formation (NS-formation). Our result for four trials after normalized to each individual sex's control shows that there is a significant dose-dependent effect of ethanol on the formation of neurospheres ($F(3,152) = 14.686$; $p < 0.001$). While there is no effect of sex on the neurosphere formation, there is an interaction effect between sex and treatment ($F(3,152) = 4.293$; $p < 0.01$). Since we observed that over the ranges of ethanol doses females show a larger decrease in neurosphere forming capabilities than the male-derived cultures Figure (3, A).

Moreover, when normalizing to the average of all controls, instead of sex specific normalization, when performing a two-way ANOVA, we find that no interaction effects but an effect of sex ($F(1,152)= 36.376$; p-values <0.001) and ethanol on the neurosphere cultures ($F(3,152)= 6.622$; p-values <0.001). Overall, females form significantly more neurospheres than males. Ethanol decreases neurosphere formation at doses greater than 60 mg/dL Figure (3, B).

Using an MTT assay in measuring the mitochondria function through viable cells absorbance to MTT, we determined a significant effect of sex after normalizing to each individual sex's control ($F(1,152)= 9.851$; $p < 0.01$) on NSC progeny number after culturing cells for 72 hours. Additionally, there was a significant interaction effect $F(3,152) = 2.727$; $p < 0.05$) between sex and ethanol exposure. Post-hoc testing revealed that ethanol exposure selectively increased NSC progeny number in females as shown in Figure (3, C).

Likewise, we found a significant interaction effect between sex and ethanol ($F(3,152) = 2.872$;

p <0.05) and a significant main effect of sex (F(1,152) = 19.810; p <0.01) when normalizing to the average of all controls on absorbing MTT by viable cells, as shown in Figure (3, D). Overall females absorbed significantly less MTT than males. Females have shown significantly increase and males have significantly a slight decrease in NSC progeny absorbance. Overall, this indicates there are sex-dependent effects of ethanol in measuring the mitochondria function that increase selectively in females.

3.2 Sex-dependent effects of ethanol on promoting cell cycle activity

The aim of the cell cycle assessment that measured by flow cytometry was to determine if changes in the rate of cell proliferation underlined the sex-specific effects of ethanol on neurosphere growth. Our results for three replicated after normalizing to each individual control shows that there is a significant interactive effect between sex and ethanol exposure in cell cycle activities after 3 days of ethanol exposure. The proportion of cells in G0/G1-phase shows a significant interaction effect of sex and ethanol exposure (F(3,102) = 9.181; p <0.01). and a significant effect of sex (F(1,102) = 9.356; p <0.01). While cells from females exhibit a significantly decreased proportion in G0/G1-phase, males have significantly slight increased proportion of the G0/G1-phase with elevated doses, as shown in Figure (4, A).

Interestingly, the proportion of cells in S-phase not only shows a significant interaction effect between sex and ethanol exposure (F(3,102) = 2.736); p <0.05) and a significant effect of sex (F(1,102) = 6.186; p <0.05) but also a significant main effect of ethanol (F(3,102)= 4.428; p <0.01). Females have a higher proportion of cells in S phase in response to 120 mg/dL ethanol Figure (4,

B). This indicates that females have the capability to replicate the DNA synthesis with doses of ethanol up to 120 mg/dL.

Similarly to the proportion in S-phase, the proportion of cells in G2/M-phase has showed a significant interaction effect between sex and ethanol exposure ($F(3,102) = 3.603$; $p < 0.05$) as well as both a significant effect of sex ($F(1,102) = 8.824$; $p < 0.01$) and a significant effect of ethanol ($F(3,102) = 2.90$; $p < 0.05$). Notwithstanding the above, females surprisingly have approximately 30 % a significant high proportion of cells in G2/M-phase at the highest dose of ethanol (320 mg/dL), while males have significantly decreased in G2/M phase cells at lower dose of ethanol (120 mg/dL), as illustrated in Figure (4, C).

The median of the fluorescent intensity of EdU is an indicator of the rate of DNA synthesis, and thereby an indication of how quickly cells are moving through the S phase. The rate of EdU incorporation showed a significant interaction effect between sex and ethanol exposure ($F(3,102) = 2.836$; $p < 0.05$), a significant effect of sex ($F(1,102) = 5.545$; $p < 0.05$), and a significant effect of ethanol ($F(3,102) = 2.924$; $p < 0.05$). Specifically, females have a significantly greater alcohol dose-dependent increase in the rate DNA synthesis than males, as shown in Figure (4, D). These data suggest that in response to ethanol, female stem cells move more rapidly through the cell cycle, Figure (4, E). Given that transit amplifying cells move through the cell cycle more quickly than their parent stem cells, these data also indicate that ethanol-treated female neurospheres may

contain a higher proportion of transit amplifying cells (neural progenitor cells).

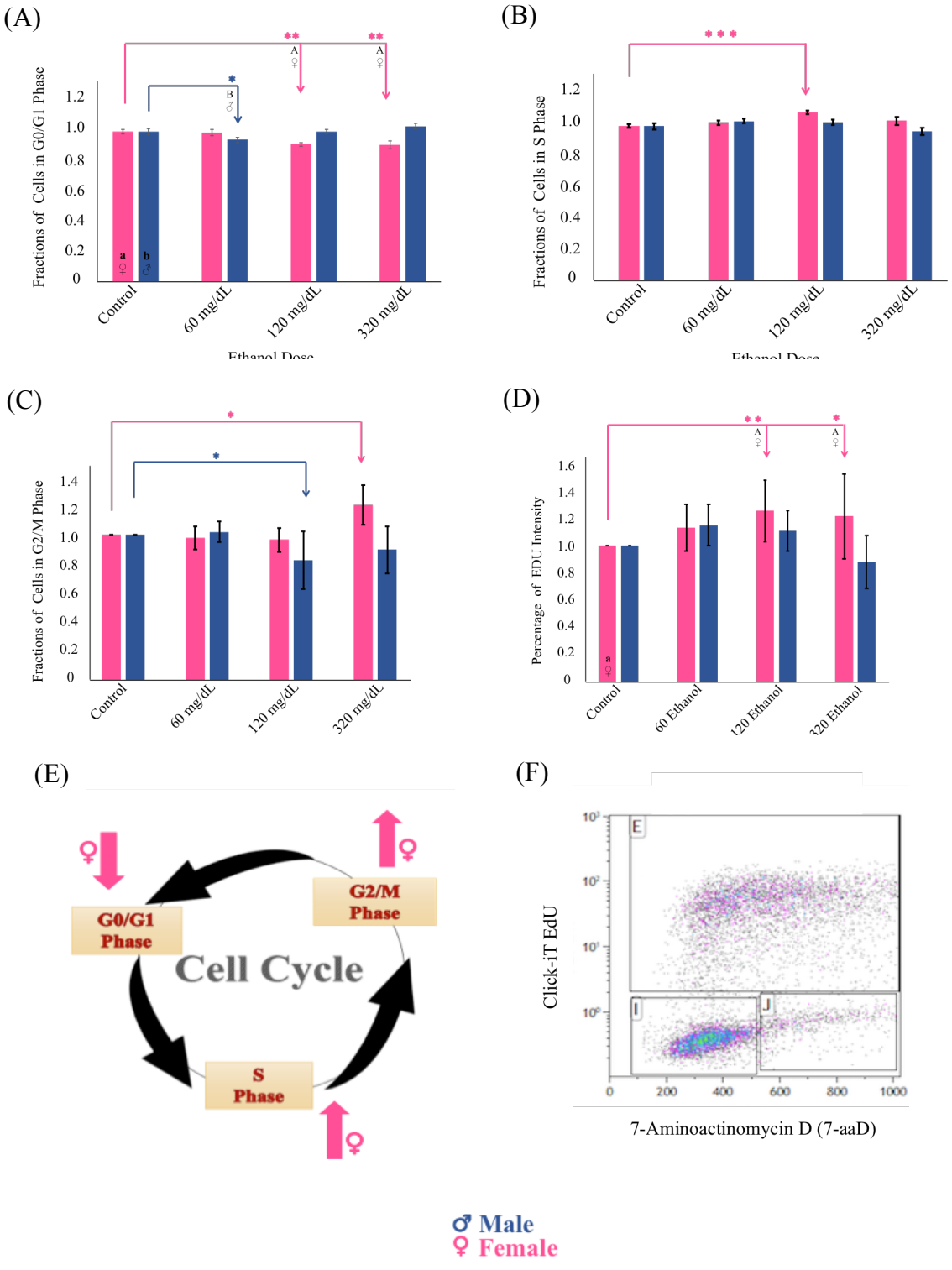

Figure 4. Sex and ethanol interactions in cell movement through the cell cycle.

Figure 4 Continued. Sex and ethanol interactions in cell movement through the cell cycle. (A) Flow cytometry revealed that in the Go/G1 phase of the cell cycle, males have fewer cells in response to 60 mg/dL ethanol while females only have decreased cells in response to 120 mg/dL and 320 mg/dL ethanol. (B) However, flow cytometry revealed also that in the S phase of the cell cycle, females have increased the cells at 120 mg/dL responding of ethanol. (C) Flow cytometry revealed also that in the G2/M phase of the cell cycle, females have increased the cells at 320 mg/dL responding of ethanol. (D) The median percentage EdU intensity at differerent ethanol concentration with a significant difference seen in females at 120 mg/dL and 320 mg/dL ethanol. (E) A diagrammatic summary that females cells move quickly through the cell cycle in responding to ethanol. (F) Shows the distribution of cells within the cell cycle as [I insert] is the distribution of cells in G0/G1 phase fraction, [E insert] is the distribution of cells in S phase fraction, and [J insert] is the distribution of cells in G2/M phase fraction after after 1 hour of cells incubation with EdU.

3.3 Sex-dependent effects of ethanol on alteration on the cell fate markers

In order to clarify and further understand the effects of ethanol on the cell cycle, we used qRT-PCR to examine differences in gene expression of specific cell fate markers, Figure (5). We investigated 4 different genes including (HPRT, ACTB, GAPDH, PGK1) as normalizers. While these loading control transcripts have been shown to be unchanged across treatment groups in previous studies, here we found that particularly in the female cells that both expression of individual controls, as well as the geometric mean of the controls, was significantly altered between treatment groups Table (2). HPRT was unchanged in male cells between ethanol treatments, therefore was used as a loading control for one-way ANOVAs of the effect of ethanol dose on male cells. Since all controls were significant for the females, we chose GAPDH as a control as it had the lowest range of CT values, therefore it was the most robust to ethanol treatment in the females. For sex differences in mRNA expression in the control male and female cells, HPRT

was used as the control.

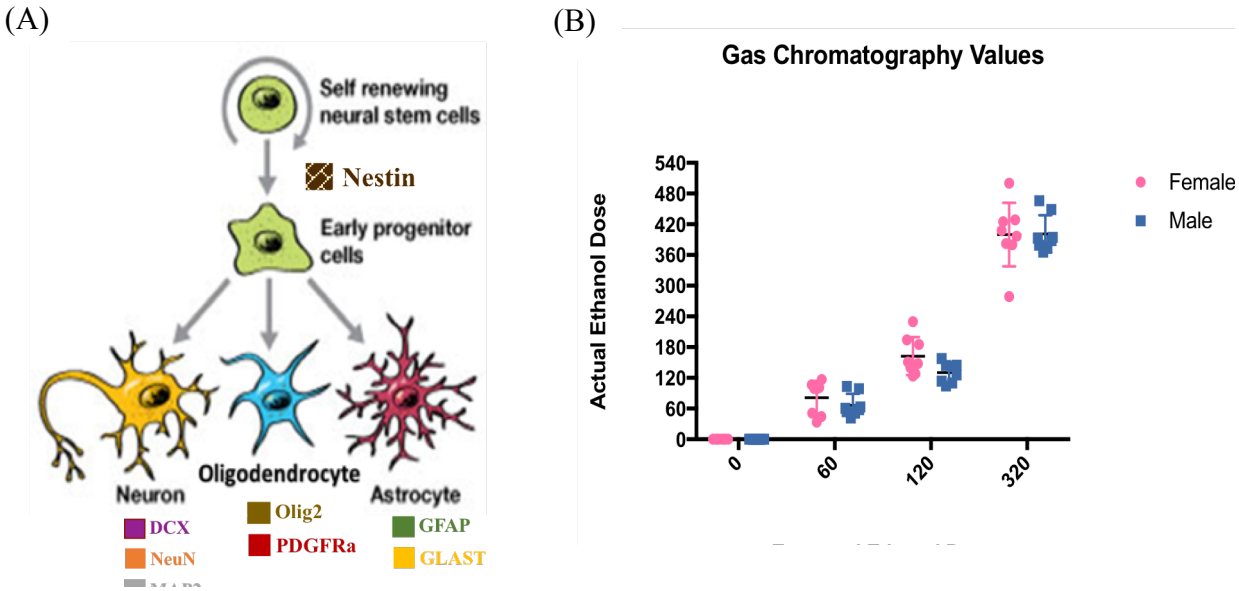

Figure 5. Overall mRNA transcripts of neuronal development examined on treated and untreated neurosphere with ethanol. (A) Generally, ethanol's effects on the expression of maturation-related mRNA transcripts is dependent on genetic sex. (B) Gas chromatography values shows the average of actual values of ethanol within 5 days.

Nestin is a commonly used neural stem/progenitor cell molecule marker in the central nervous system (CNS) [35]. We observed a significant effect of ethanol on the expression of nestin mRNA in female neurosphere ($F(3,14) = 52.52$; $p < 0.001$), as shown in Figure (6, A). Male neurosphere nestin levels were not affected by ethanol ($F(3,15) = 2.868$; $p = 0.07$), as shown in Figure (6, B), nor were there any differences in the levels of nestin transcript in male and female control neurospheres, Table (4) and Table (5) respectively. In the female cells, nestin mRNA was increased in the 60mg/dL ethanol exposed neurospheres (1.50 -ddCT) and decreased in the cells exposed to 320 mg/dL ethanol, Table (3). This indicates that females' neurospheres produce more of neural

stem/progenitor cells at the lower doses but produce fewer neural stem/progenitor cells at 320 mg/dL, indicates a loss of stemness or an increase in differentiation.

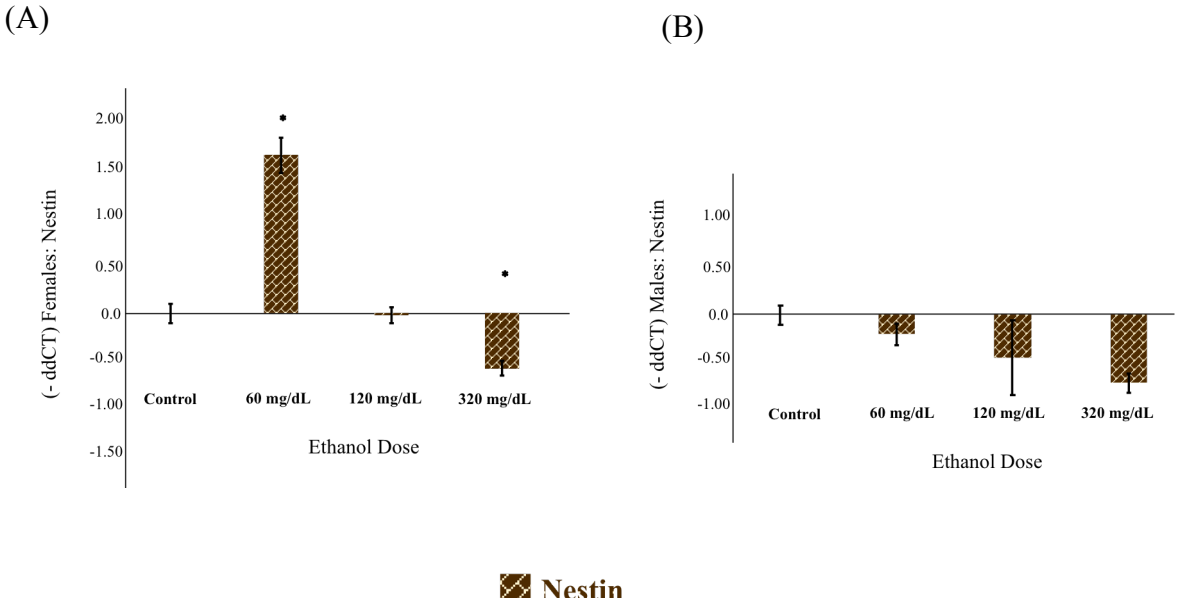

Figure 6. A comparison of nestin mRNA transcript responses to EtOH treatment between females and males using qRT-PC. The neural stem / progenitor cells (Nestin) mRNA transcript has induced significantly at low dose of ethanol exposure about 2-fold in female-derived cells, while males' cells have not affected by ethanol treatment.

To determine whether the neurosphere differentiate into immature neurons or neurons, we examined DCX, NeuN, and MAP2 mRNA expression [36, 37]. We detected a significant effect of ethanol on the expression of DCX mRNA in female neurosphere ($F(3,14) = 42.4$; $p < 0.001$), but neither NeuN nor MAP2 were significantly affected by ethanol, as shown in Figure (7, A). In female cells, DCX mRNA was decreased in the 60 mg/dL ethanol exposed neurospheres (-1.50 - ddCT) Table (3). This indicates that females' neurospheres produce fewer immature neurons cells at lower doses. Similar to that observed for nestin levels, males neurosphere NeuN and MAP2

were not affected by ethanol as shown in Figure (7, B), nor were there any differences in the levels of NeuN and MAP2 transcripts in male and female control neurospheres, Table (4) and Table (5) respectively.

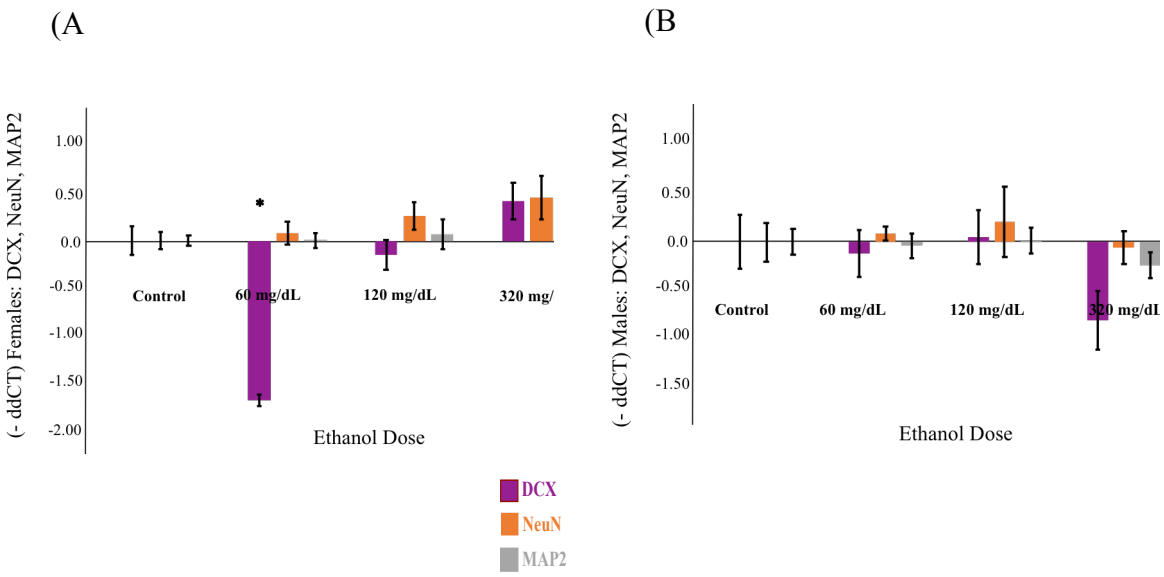

Figure 7. A comparison of neurons mRNA transcript responses to EtOH treatment between females and males using qRT-PC. The measurement of the neurons mRNA transcripts show at the low dose of ethanol exposure decreases significantly about 2-fold the immature neuronal marker DCX, only in females' cells, while males' cells have not affected by ethanol treatment.

Additionally, to define whether the neurosphere transition from neurogenic to gliogenic cells, we evaluated marker for both astrocytes and oligodendrocytes. We used GFAP and GLAST expression to identify the differentiation of neurospheres into astrocytes [38, 39]. We observed a significant effect of ethanol on the expression of GFAP and GLAST mRNAs in female neurospheres Table (3), and in male neurospheres Table (4). In female cells, both GFAP and

GLAST mRNAs were decreased at the 60 mg/dL ethanol exposed neurospheres (~ -1.60-ddCT) and (~ -0.98-ddCT) respectively as shown in Figure (8, A) whereas in the males cells, both GFAP and GLAST mRNAs were also decreased but only at the 320 mg/dL dose of ethanol (~ -2.50-ddCT) and (~ -0.98-ddCT) respectively, as shown in Figure (8, B) and Table 3 and 4. On the other hand, in male and female control neurospheres, there was a 2-fold (1 ddCT) difference in expression GFAP transcript between males and females, with the males showing higher expression Figure (9), but there was not an effect of sex in GLAST transcript levels, (Table 5). This indicates that females' neurospheres are more sensitive at lower dose at 60 mg/dL and produce fewer astrocytes while males' neurospheres produce fewer astrocytes at high ethanol doses at (320 mg/dL).

(A) (B)

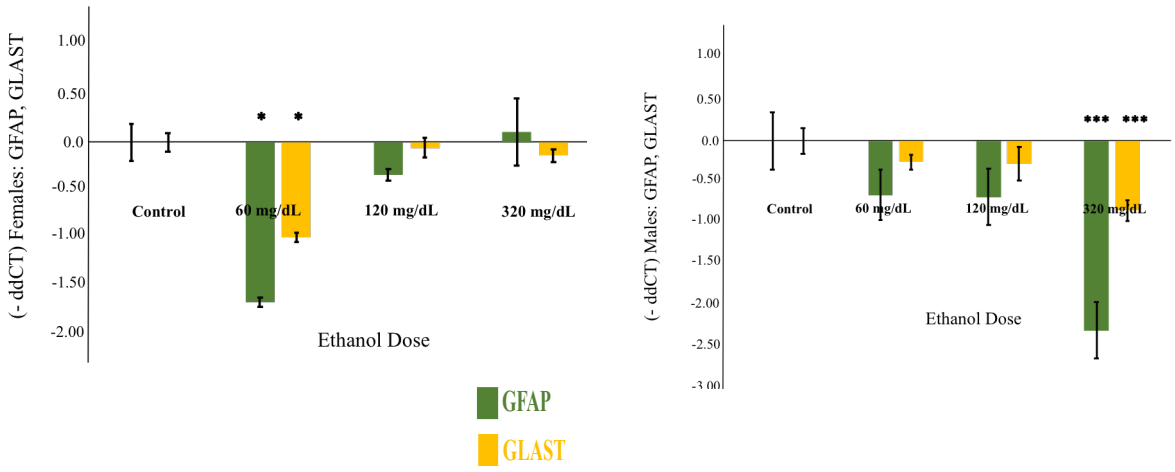

Figure 9. The measurement of the astrocytes markers of GFAP mRNA transcript control group after normalizing to HPRT. It showed significantly sex differences of similar magnitude in opposite direction between both females' and males' cells

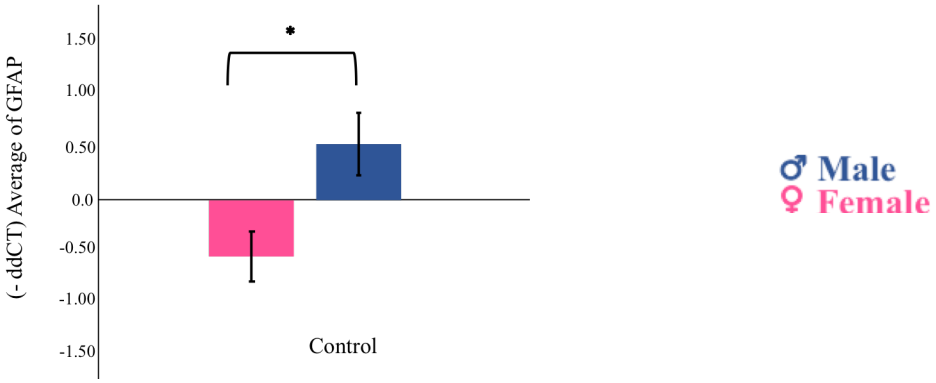

Figure 8. A comparison of astrocytes mRNA transcript responses to EtOH treatment between females and males using qRT-PC. The measurement of the astrocytes markers shows at the low dose (60 mg/dL) of ethanol exposure decreases significantly both mRNA transcripts in females' cells while males' cells significantly decreased at high dose of ethanol (320 mg/dL).

We also examined expression of Olig2 and PDGFRa, markers of oligodendrocytes differentiation [40, 41]. We observed a significant effect of ethanol in the male and female neurospheres on the expression of Olig2 mRNA, but not PDGFRa Table (3 and 4). In the male and female control neurospheres, there was no difference in Olig2 and PDGFRa expression Table (5). In both female and male cells, Olig2 mRNA levels were decreased in response to 320mg/dL ethanol Figures (10, A and B) and Table (3 and 4). The reduction of Olig2 indicates that both females and males have a reduced capability to commit to the oligodendrocyte lineage in response to high levels of ethanol.

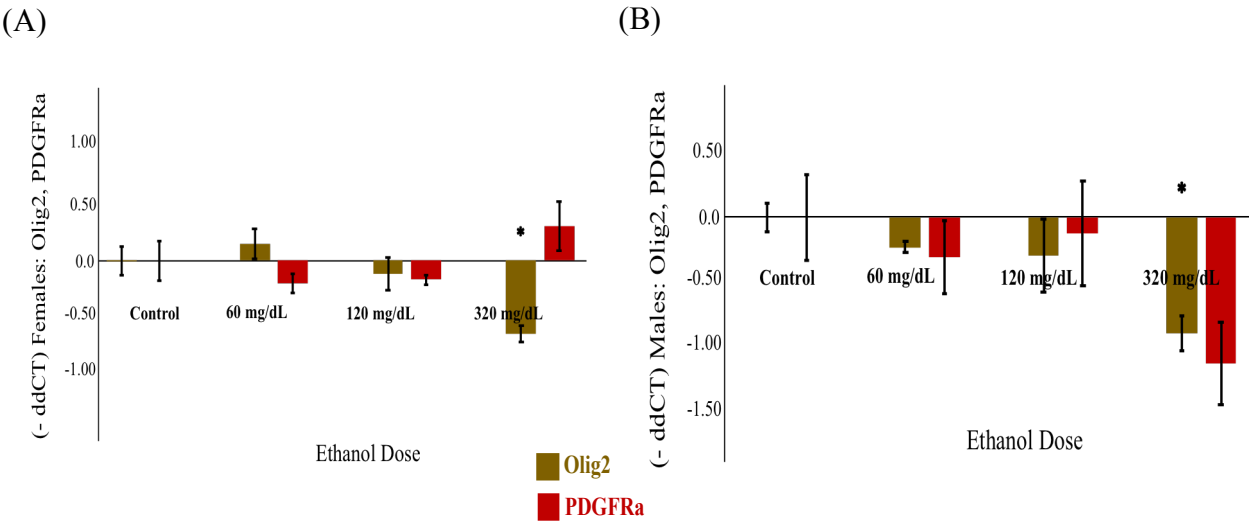

Figure 10. A comparison of oligodendrocytes mRNA transcript responses to EtOH treatment between females and males using qRT-PC. The measurement of the oligodendrocytes markers show both females' and males' cells has decreased significantly at high dose of ethanol exposure (320 mg/dL) only on Olig2 mRNA transcripts. Females' cells have a smaller effect on olig2 about 0.50-fold decreased while males' cells have about 1-fold decreased.

Table (1) List of cell fate transcripts differentiation markers. Cell fate transcripts are used to detect the diffrentiation of neuroshere in both sex in response to ethanol.

mRNA transcript's name	Marker of
* Nestin	Neural stem/progenitor cell
* doublecortin (DCX)	Neuronal lineage committed progenitor cell/ immature neurons
* Neuronal nuclear(NeuN) * Microtubule-associated protein-2 (MAP2)	Neurons
* Glial fibrillary acidic protein (GFAP) * Glutamate aspartate transporter (GLAST)	Astrocytes
* Platelet-derived growth factor receptor alpha (PDGFRa) * oligodendrocyte transcription factor 2 (Olig2)	Oligodendrocytes

Table (2) Summary of normalize transcripts to detecte the less intraction of sex in response to ethanol using two-way ANOVA Analysis.

Two Way ANOVA			
Transcript	Sex (F(1,29); p)	EtOH (F(3,29);p)	Sex:EtOH (F(3,29);p)
HPRT	15.10; <0.001	10.20; <0.001	15.17; <0.001
ACTB	30.93; <0.001	41.58; <0.001	24.16; <0.001
GAPDH	14.535; <0.001	14.716; <0.001	6.488; <0.01
PGK1	0.714; >0.05	20.704; <0.001	5.67; <0.01
Geometric Mean	17.92; <0.001	26.36; <0.001	16.43; <0.001

Table (3) Female cells have a significant influences by ethanol exposure on expression cell fate markers using one-way ANOVA Analysis.

Sex	Transcript's Name	(F(3,14) = ; p)	Ethanol Treatment	(- ddCT)
Female	Nestin*	F= 52.52 ; p <0.001	60 mg/dL	↑ 1.50
			320 mg/dL	↓ -0.50
	DCX*	F= 42.4 ; p <0.001	60 mg/dL	↓ -1.50
	NeuN	F= 1.881 ; p = 0.179	Ø	Ø
	MAP2	F= 3.141 ; p = 0.059	Ø	Ø
	GFAP*	F= 19.32 ; p <0.001	60 mg/dL	↓ ~ -1.60
	GLAST*	F= 34.55 ; p <0.001	60 mg/dL	↓ ~ -0.98
	Olig2*	F= 7.359 ; p <0.01	320 mg/dL	↓ ~ -0.70
	PDGFRa	F= 2.438 ; p = 0.108	Ø	Ø

Table (4) Male cells have only a significant influences by high ethanol dose exposure on expression cell fate markers using one-way ANOVA Analysis.

Sex	Transcript's Name	(F(3,15) = ; p)	Ethanol Treatment	(- ddCT)
Male	Nestin	F= 2.868 ; p= 0.07	Ø	Ø
	DCX	F= 2.129; p= 0.139	Ø	Ø
	NeuN	F= 0.284 ; p= 0.836	Ø	Ø
	MAP2	F= 0.84 ; p= 0.493	Ø	Ø
	GFAP*	F= 8.854 ; p = 0.001	320 mg/dL	↓ ~ -2.50
	GLAST*	F= 6.367 ; p <0.01	320 mg/dL	↓~ -1.00
	Olig2*	F= 6.875 ; p <0.01	320 mg/dL	↓ ~ -0.90
	PDGFRa	F= 2.419 ; p = 0.107	Ø	Ø

Table (5) Male and female control neurospheres have a main effect of sex in early glial cells fate marker using one-way ANOVA Analysis.

Treatment	Transcript's Name	(F(1,8) = ; p)	Sex	(- ddCT)
Control	Nestin	F= 1.666 ; p = 0.233	Both Sex	Ø
	DCX	F= 0.332 ; p = 0.581		Ø
	NeuN	F= 0.00 ; p = 0.984		Ø
	MAP2	F= 2.038 ; p = 0.191		Ø
	GFAP*	F= 7.986 ; p <0.05	Female	↓ ~ - 0.60
			Male	↑ ~ 0.60
	GLAST	F= 0.176 ; p = 0.686	Both Sex	Ø
	Olig2	F= 0.035 ; p = 0.855		Ø
	PDGFRa	F= 1.402 ; p = 0.27		Ø

4. DISCUSSION

Ethanol is well known as a teratogen [42-46]. Previous research indicates that ethanol exposure results in the thinning of the developing cerebral cortex by increasing premature maturation and decreasing the self-renewal of NSCs [4, 5, 10, 11]. Interestingly, other studies have been shown that there are sex differences in the stem cell biology and in the FASDs children outcomes. Moreover, A reported study by Streissguth has shown sex differences in FASDs children such FAS females showed a decreased in cerebellar surface area compared to FAS males and control [47], indicating a role for sex differences in the thinning/volume loss of brain structures in response to ethanol. We are the first to investigate genetic sex affects NSC biology both in the presence and absence of ethanol.

Our data suggests that cells within female neurospheres move more quickly through the cell cycle. This is shown in a number of ways including by increased rates of EdU incorporation, indicating faster DNA synthesis, alongside increased proportion of cells in S-phase. These data could explain the increase seen in the MTT assay for female cells, with the more rapid pace of the female cells moving through the cell cycle resulting in additional cells within the neurospheres. These data agree with the work by Hudry which showed that female stem cells reproduce more quickly in the drosophila gut [28]. These data together indicate quicker cell division may be a feature of female stem cells.

Our data also shows sex differences in respond to ethanol on neural developmental mRNA

transcripts. Overall, the mRNA transcripts on females' cells was reduced at the lower dose of ethanol (60 mg/dL) than males' cells. This data suggests that mRNA transcripts on females' cells are more sensitive to lower dose of ethanol than males' cells. Interestingly, also at the lower dose of ethanol, nestin was induced specifically in females' cells. These data come along with Streissguth report that women who consumed alcohol during pregnancies had increased the ratio of males' to females' offspring in all three trimesters and that may indicate females are more vulnerable to ethanol than males [47]. It is also not clear yet if this increase on nestin mRNA transcript expression is an attempted for females c' cells to recover their stemness.

Chromosomes themselves, particular since the X chromosome does not fully condense during barr body formation so that there are some regions of the X-chromosome that are more autosomal like and therefore will have twice as high dose as males [48, 49]. During the fetal development, female fetus brain protected from masculinizing through the α-fetoprotein binding to maternal estradiol in the bloodstream that sequester the estrogen for masculinizing the brain [50]. However, ethanol exposure can cause an alteration on α-fetoprotein production which may exhibit sex differences in FASD [51]. Also, Nuñez and McCarthy reported that GABA receptor shows sex differences in maturation that happened early in females than males [52]. Since sex differences implied on GABA receptor and ethanol well known to get access GABA receptor [53, 54], this may indicate a locus of interaction.

The outcomes from this research reveal sex-dependent effects of PAE on the mid first- second trimester of NSCs where they at this time-window generate the most neurons of the adult brain

rapidly [55]. To understand the changes in the cellular level, we will investigate the neurosphere transcriptome induced by ethanol exposure as well as by genetic sex using RNA sequencing examination. Also, we will assess the cross-sectional area of neurospheres using ImageJ as another measure of cellular growth. These data will help to validate the MTT assay as there may be fewer viable cells due to decreases in cell cycle progression, which would be reflected in smaller neurospheres.

5. CONCLUSION

Taking all the above into account, we are the first to examine the effects of ethanol on fetal neural stem cells in the role of sex differences. In the fetal alcohol prenatal exposure, ethanol affects both sexes differently. We found generally that ethanol exposure influences the growth of neurospheres and their maturation during the neural development. Additionally, ethanol exposure induced females' cells to move quickly through the cell cycle. Female-derived NSCs are more sensitive to lower doses of ethanol. These data suggest that females may be more vulnerable to ethanol expusre at lower dose.

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