MECHANISMS OF ALCOHOL-INDUCED NEUROTERATOLOGY:

AN EXAMINATION OF THE ROLES OF

FETAL CEREBRAL BLOOD FLOW AND HYPOXIA

A Dissertation

by

SCOTT EDWARD PARNELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Medical Sciences

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Approved as to style and content by:

James R. West (Chair of Committee)	Timothy A. Cudd (Member)
Rajesh C. Miranda (Member)	Douglas P. Dohrman (Member)

James R. West
(Head of Department)

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ABSTRACT

Mechanisms of Alcohol-Induced Neuroteratology:
An Examination of the Roles of Fetal Cerebral
Blood Flow and Hypoxia. (December 2004)
Scott Edward Parnell, B.A., Texas A&M University
Chair of Advisory Committee: Dr. James R. West

Hypoxia (decreased tissue oxygen levels) has long been considered as a possible mechanism of alcohol-induced developmental deficits, yet research has not conclusively disproved this hypothesis, nor has it provided substantial evidence for a mechanism of developmental alcohol insults involving hypoxia. Previous research has shown that moderate acute doses of alcohol does not induce hypoxemia (decreased arterial oxygen levels), yet these same studies have shown that this same alcohol exposure does transiently decrease cerebral blood flow (CBF). This is significant because although developmental alcohol exposure did not result in hypoxemia, the decreases in CBF seen in these previous studies may induce hypoxia within the brain. Unfortunately, these experiments were only performed after acute doses of alcohol, so it is unknown if a more chronic or repeated alcohol exposure paradigm would have similar effects. The present study examined blood flow in the sheep fetus after repeated alcohol exposure in a binge-like paradigm throughout the third trimester. Additionally, this study examined the fetal neurovascular response to a subsequent infusion of alcohol after the repeated alcohol exposure. This latter experiment was designed to examine the hypothesis that alcohol exposure throughout the third trimester affects the normal responsiveness of the neurovasculature to alcohol (compared to previous research demonstrating acute alcohol-induced decreases in CBF). The results from the present experiments indicate that although few regions were significant, the majority of the regions (especially the brain regions) exhibited a trend for increases in blood flows after alcohol exposure. This phenomenon was especially prominent in the group receiving the lower dose of alcohol. Additionally, the data from this study demonstrated that after repeated alcohol exposures

the near-term sheep fetus did not respond to a subsequent dose of alcohol in a similar manner seen in previous experiments when the acute alcohol exposure was administered in alcohol naïve animals. After the final alcohol exposure the subjects in this study had either no effect in terms of blood flow or an increase in CBF. This is opposite to previous observations which demonstrated reduced blood flow in numerous brain regions. The present experiments suggest that alcohol does not induce fetal hypoxia, but does negatively affect the normal neurovascular response to alcohol. This latter phenomenon could have negative consequences on future development of the brain.

DEDICATION

To all those who were ecstatic upon being
admitted into graduate school…

Welcome to where time stands still
No one leaves and no one will
Moon is full, never seems to change
Just labelled mentally deranged
Dream the same thing every night
I see our freedom in my sight
No locked doors, No windows barred
No things to make my brain seem scarred…

-James Hetfield

ACKNOWLEDGMENTS

Just as it takes whole village to raise a child, it also takes an incredibly large number of people to "raise" a graduate student. My education and development, both as a scientist and as a person are due to more people than I will ever know. However, I will take this opportunity to express my gratitude to all those who made this possible…

I would most whole-heartedly like to thank:

Dr. James West for his constant guidance, for teaching me more about science and being a scientist than I thought possible, and for taking a chance.

Dr. Timothy Cudd, the driving force behind this research, for the constant physiology lessons.

The rest of my graduate committee, Dr. Rajesh Miranda and Dr. Douglas Dohrman for their advice, expertise and input, as well as their help in my classes.

Dr. Wei-Jung Chen for his seemingly infinite knowledge of statistics and for his advice.

Dr. Susan Maier for always taking care of me and ensuring that I always had what I needed.

The past and present members of the West lab for putting up with me for so long.

The faculty, students and staff of the Anatomy & Neurobiology Dept. and the rest of the alcohol and neuroscience researchers throughout Texas A&M University for their support, guidance and for my excellent education.

Debbie Marshall and the rest of the Cudd lab for all of their help and patience.

Cindy Reiter, my compatriot in many things, for her friendship, even after those not infrequent "discussions".

All of my friends for the occasional football and baseball games.

My parents, Joseph and Diane, for making me the person I am today.

and finally,

Lisa for her inspiration, unwavering support and infinite patience.

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CHAPTER I

INTRODUCTION

Alcohol use has been prevalent in our society for millennia, yet it was only about thirty-five years ago that alcohol was first formally identified as being teratogenic by a group of French scientists (Lemoine et al, 1968). Perhaps because this initial observation was published in an obscure French journal, it was not until five years later, when another group independently arrived at the same conclusion, that this phenomenon was widely publicized (Jones et al., 1973). A follow-up paper published by this same group labeled this phenomenon Fetal Alcohol Syndrome (FAS) and succinctly described the cardinal characteristics of FAS (Jones and Smith, 1973). These reports were the seminal papers in the FAS research field and were the impetus for thousands of subsequent human and animal studies.

FAS is currently diagnosed by the presence of three cardinal features, craniofacial abnormalities, pre- and post-natal somatic growth retardation and varying degrees of central nervous system (CNS) dysfunction associated with a confirmation of maternal alcohol abuse (Stratton et al., 1996). The most prominent abnormal facial features include an indistinct philtrum, thin upper lip, narrow eyes with skin folds at the corner, short nose and a low nasal bridge (Jones and Smith, 1973; Stratton et al., 1996). In general, these features can be described as an underdeveloped midface. Despite that most of the alcohol-induced craniofacial abnormalities are not often injurious (occasionally cleft lips and palates can occur) and tend to lessen over time, these characteristics are useful in diagnosing FAS.

Of the three major FAS deficits, the CNS disorders associated with prenatal alcohol exposure are the most prevalent and the most devastating. In addition, it has

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This dissertation follows the style and format of Alcoholism: Clinical and Experimental Research.

been amply demonstrated that the actual number of children affected by prenatal alcohol exposure is far more than the number that are actually diagnosed with FAS (Barr and Streissguth, 2001; Mattson et al., 1997). Children born to mothers that abuse alcohol can exhibit cognitive, motor and behavioral abnormalities even without the presence of accompanying craniofacial and growth deficits. For this reason, alcohol is considered the most common known cause of mental retardation in the U.S. (Abel and Sokol, 1986). Because prenatal alcohol exposure has such a wide spectrum of effects, and because it is quite often that some symptoms of FAS are present while others are absent, multiple terms are used to distinguish some of these effects (Stratton et al., 1996). A diagnosis is made of full FAS where all of the aforementioned symptoms are present, or partial FAS where some, but not all of the symptoms of full FAS are present. For example, the term alcohol-related neurodevelopmental disorders (ARND) is often used if behavioral, motor and/or cognitive deficits are present without any or all of the other common symptoms of FAS. Additionally, when other congenital defects related to alcohol exposure such as heart, kidney, skeletal, ocular or auditory problems are present, they are termed alcohol-related birth defects (ARBD). Finally, although not used diagnostically, fetal alcohol spectrum disorders (FASD) is a recent term used to encompass all of the deficits associated with developmental alcohol exposure regardless of their magnitude (Streissguth and O'Malley, 2000).

The most devastating results of prenatal alcohol exposure are the CNS deficits. These deficits include impairments in cognitive functions such as learning, memory and decision-making, as well as behavioral problems and motor difficulties, particularly problems with fine motor control (Mattson et al., 1999; Streissguth, 1986). Obviously, these deficits can pose significant problems both for the affected individuals and for society. There are currently no cures for FAS or ARND and the mechanisms of these alcohol-induced neurodevelopmental deficits are still not completely understood. Although preventing women from drinking during pregnancy would prevent FAS, this approach has proved less than successful at least in part because it has been extremely difficult to identify the women at greatest risk. An alternative approach is to find a way

to either prevent or repair the damage caused by heavy maternal drinking. One of the important steps towards these goals, and an area of a great deal of research, is to determine some of the neurobiological effects of prenatal alcohol exposure and then to identify the mechanism(s) of damage.

Many of the previously mentioned functional abnormalities due to developmental alcohol exposure are due to a wide spectrum of deficits occurring at the cellular level. These deficits range from transient disruptions in neurotransmission to alterations in gene expression to neuronal death (NIAAA, 2000). Any of these effects can have severe consequences. For example, alterations in neurotransmission can lead to disruptions in long-term potentiation (LTP) or the development of abnormal neuronal connections. Either of these effects can lead to long-term changes in cognitive functions, memory, behavior or fine motor control without actually inducing cellular death or dysmorphology.

Although the less physically obvious aspects of developmental alcohol exposure cause severe problems in the normal functioning of the affected individuals, these subtle effects of alcohol are difficult to quantify. For this reason, experiments examining the mechanisms of alcohol-induced deficits are often difficult to perform. However, morphological/anatomical defects, such as changes in cell number can be reliably quantified. Therefore, mechanistic studies often employ doses of alcohol that induce deficits such as cell death to ensure that neuronal damage is occurring on a readily quantifiable scale. This strategy aids a more accurate assessment of the possible mechanism under study or possible prevention strategies. For these reasons, and to necessarily limit the scope of this dissertation, the discussions and experiments in this study will focus on the morphological effects of alcohol such as cell death.

Deficits in Cell Number within the Central Nervous System

Proper development of the CNS is crucial for appropriate cognitive, behavioral and motor functions. Alterations in neuronal numbers can have severe consequences on these functions. Changes in cell number after developmental alcohol exposure within a

particular region of the brain can occur due to inadequate neurogenesis, deficits in cell migration or cell death (NIAAA, 2000). Alcohol has been shown to affect cell number in numerous regions throughout the brain via all three of these mechanisms. Due to the implications of developmental neuronal loss on future brain function, one of the best-studied aspects of fetal alcohol exposure is the loss of cells within the CNS.

As mentioned previously, there are numerous possible reasons for a lack of appropriate cell numbers in any given region of the brain after developmental alcohol exposure. Alcohol can have a direct effect by killing neurons, by preventing neurogenesis or impairing proper migration to a neuron's appropriate place. Alcohol can also kill neuroglia within the brain which are necessary for proper neuronal survival and function.

Deficits in Neurogenesis

In addition to killing post-mitotic neurons, alcohol is also capable of preventing neuronal progenitors from proliferating. Unless there is a post-alcohol exposure catch-up, the prevention of neurogenesis can be just as damaging as cell death. Alcohol has been shown to decrease neurogenesis in the cerebral cortex, hippocampus and cerebellum (Li, et al., 2002; Miller, 1992). Alcohol has also been shown to decrease neuronal proliferation in cell culture model systems (Cook et al., 1990a, b). These *in vitro* experiments are instrumental in determining the mechanisms of alcohol-induced changes in neuronal proliferation.

Alterations in Neuronal Migration

The vast majority of neurons of a particular type within the CNS are generated in one location and then migrate to their final position. This incredibly complex process is not completely understood, but it has been repeatedly established (especially since the widespread use of neuroimaging technology) that errors in neuroblast migration can have a profound effect on future neurological functioning. Non-alcohol related aberrant neuronal migration causes numerous congenital defects such as epilepsy, lissencephaly,

and schizencephaly, to name a few (Rakic, 1990). Although some of these diseases (and the underlying migration errors) are genetic in origin, many of them are due to insults during development. These insults can range from metabolic disorders to ischemia to neuroteratologic agents.

Alcohol is also capable of preventing neurons from migrating to their appropriate place. This idea was first introduced by Clarren et al. (1978) in a paper describing the brains of human autopsies that revealed migration errors in the neocortex and cerebellar cortex. Although the subjects in this study demonstrated ectopic neurons it was not observed that alcohol directly inhibited neuronal migration. However, subsequent studies in animals and *in vitro* have directly demonstrated that alcohol does indeed inhibit neuronal migration. Numerous studies in the neocortex using labeled neurons have demonstrated decreased neuronal migration due to prenatal alcohol exposure (Miller, 1992). Additionally, serotonergic midline raphe neuronal migration has also been shown to be inhibited by alcohol (Zhou et al., 2001).

Studies *in vitro* have also demonstrated decreased migration of cells on lamina from the cerebral cortex and granule cells from the cerebellum, as well as migration errors in cerebral cortex organotypic cultures (Hirai et al., 1999; Liesi 1997; Mooney et al., 2004). *In vitro* studies have also identified that one of the most likely possible mechanisms of alcohol-induced deficits in migration may involve an alcohol-induced interference with the L1 cell adhesion molecule (Bearer et al., 1999; Charness et al., 1994). Although alterations in cell migration are certainly not the only result of developmental alcohol exposure, these studies indicate that it is at least one of the causes of the behavioral, motor and cognitive deficits associated with ARND.

Cell Death

Cell death (at least for this discussion) is the loss of post-mitotic cells through either apoptosis or necrosis. Alcohol has been shown to induce neuronal death throughout the brain at various times throughout development. Interestingly, at alcohol doses that are physiologically relevant, alcohol does not induce cell loss equally in all

brain regions. In fact, some regions do not seem to even be affected by developmental alcohol exposure, at least in terms of neuronal loss. This phenomenon, termed regional vulnerability, is prevalent throughout the FAS literature. Alcohol has been shown to reduce the number of Purkinje and granule cells of the cerebellum, pyramidal cells of the hippocampal CA1 and CA3 regions, granule cells of the hippocampal dentate gyrus, mitral cells of the olfactory bulb, and pyramidal cells of the neocortex (Bonthius et al., 1992; Chen et al., 1998, 1999b; Livy et al., 2003; Miller and Potempa, 1990; Pierce et al., 1989; West et al., 1986; 2001). However, numerous brain regions do not seem to demonstrate neuronal death induced by developmental alcohol exposure, such as the locus coeruleus and the ventrolateral nucleus of the thalamus (Chen et al., 1999a; Livy et al., 2001). Even within the cerebellum there is regional vulnerability. Anterior aspects of the cerebellum (lobes I, X) exhibit neuronal loss after developmental alcohol exposure, but some of the posterior lobes, such as lobes VI and VII do not (Bonthius and West, 1990).

These data indicate that alcohol preferentially affects certain subgroups of cells, while leaving others intact. This phenomenon known as regional vulnerability raises an intriguing question. Why do some regions show deficits in cell numbers after alcohol exposure when others do not? The answer to this question underlies the search for some of the basic mechanisms of ARND. However, because many of the mechanisms involved in differential regional vulnerability are not well understood, this question remains unanswered. Determining the reason that some brain regions are vulnerable to developmental alcohol-induced cell death while others are not would provide a key to understanding how alcohol kills cells. This knowledge could provide potential therapeutic and preventive strategies for children exposed to alcohol during development.

Deficits in Glial Numbers and Functions

The majority of cells within the CNS are glial cells. Interactions between the glia and neurons are vital to the proper development and function of the CNS. Glia are

important for directing neuronal migration, metabolism, axonal and dendritic outgrowth and synaptic formation (Kandel et al., 2000). Glia exist as four main types within the CNS; oligodendrocytes, astrocytes, microglia and ependymal cells. Oligodendrocytes form myelin sheaths for the myelinated axons within the CNS. Astrocytes (including radial glia which direct neuronal migration in the cerebral and cerebellar cortices and then convert to astrocytes) are responsible for much of the support of the neurons around them as well as directing some of the neuronal development. Microglia are the macrophage cells of the CNS and ependymal cells line the ventricles of the brain. Abnormal glianeogenesis or any of these glial functions can have severe effects on both the development and the functions of the CNS.

Axonal myelination is critical for proper neurotransmission and alcohol has been shown to be able to inhibit the normal myelination of neurons. Alcohol has been shown to affect brainstem myelination during development (Lancaster et al., 1989). A paper by Riikonen et al. (1999) presented two out of eleven human patients with FAS with abnormal and delayed myelination of their white matter. Although not all of the FAS children in the study exhibited alterations in myelination, human subjects are very difficult to compare due to the usually wide variability in maternal drinking habits as well as the normal genetic variation among the human population. An additional complication in making observations in children with FAS is the hypothesis that some of the deficits observed after prenatal alcohol exposure may be exacerbated by genetic risk factors (Cavieres and Smith, 2000; Goodlett et al., 1989; Maier et al., 1994). However, experiments in rats (which are typically more genetically homogenous) have indirectly supported these findings. Studies of optic nerve development and myelination during developmental alcohol exposure have shown hypoplasia of the optic nerve with abnormal myelination of the axons present (Phillips and Krueger, 1992; Pinazo-Duran et al., 1997).

Additional potentially devastating CNS defects are the alcohol-induced deficits in astrocytes. Due to the number of astrocytes in the brain [about 10-50 times the number of neurons within the brain (Kandel et al., 2000)] and their wide range of

important functions (trophic, structural and neurotransmission support, migration and axonal guidance, partial control of cerebral blood flow, etc.) altering the normal functions of these cells can have a severe impact on CNS development and function. Alcohol can reduce the number of astrocytes within the cortex as well as alter the morphology and function of radial glial cells (Miller and Potempa, 1990; Miller and Robertson, 1993; Perez-Torrero, et al., 1997). Alcohol also alters the expression of certain genes within astrocytes, affecting how astrocytes interact with neurons (Aroor and Baker, 1997; Fletcher and Shain, 1993; Goodlett et al., 1993; Valles et al., 1996). Finally, alcohol can also interfere with certain astrocyte signal transduction pathways important for normal astrocyte function (Kotter and Klein, 1999; Luo and Miller, 1999). Together, these various effects of alcohol on glial cells represent an important possible mechanism of alcohol-induced alterations in CNS function.

Alcohol-Induced Alterations in Synaptic Formation

Normal CNS function is dependent on proper synaptic connections. Any alterations in either the number of synapses or the correct formation of those synapses can have severe repercussions on neurological functioning. Many of the behavioral, intellectual and motor deficits associated with prenatal alcohol exposure can possibly be attributed to synaptic changes. Numerous studies have shown that alcohol can decrease the number and alter the morphology of dendritic spines (Fabregues et al., 1985; Shetty et al., 1993; Tarelo-Acuna et al., 2000; Yanni and Lindsley, 2000). Determining the underlying mechanism(s) of alcohol-induced changes in synaptic formation, or developing therapeutic strategies to compensate for alterations in synapses would provide a much-needed alleviation of at least some of the detrimental effects of developmental alcohol exposure.

All of these data on the effects of alcohol exposure during brain development indicate that alcohol can affect numerous processes throughout the CNS. It would be a difficult task to determine which of these adverse effects are most devastating as all of them can have serious consequences. However, just as important, if not more so, than

determining the effects of developmental alcohol exposure, is determining the underlying mechanisms of these deficits in order to achieve the goals of preventing or repairing the deficits in FAS and ARND.

CHAPTER II

POTENTIAL MECHANISMS OF ALCOHOL-INDUCED NEUROTERATOLOGY

Alcohol has been shown to affect numerous developmental processes involved in cell division, migration, differentiation and communication as well as alterations within cells such as protein synthesis, metabolism, free radical formation and excitotoxicity. There are almost as many different hypothesized mechanisms for these effects as there are effects, partly because alcohol can exert its deleterious consequences through both direct and indirect mechanisms.

Direct Mechanisms

Alcohol can also act directly on developing cells to induce neuronal loss or damage. Ethanol is a small hydrophilic molecule that is capable of freely diffusing across cell and organelle membranes, interacting with proteins, DNA, RNA as well as other organic molecules. Due to this ubiquitous nature of ethanol, there are numerous sites of action of ethanol that may cause a wide variety of disruptions that alone can be deleterious or these disruptions may act in concert to induce either cell loss or cell dysfunction.

Oxidative Stress

Alcohol-induced oxidative stress as a possible mechanism of ARND is currently an area of intensive research. Although the formation of free radicals is a normal product of cellular metabolism, these free radicals are usually removed by free radical scavengers (antioxidants). However, either the excess formation of free radicals or the inhibition of free radical scavenging can lead to oxidative stress within a cell, which can initiate either apoptosis or necrosis (Han et al., 2001; Przedborski, et al., 1996; Ramachandran et al., 2003). It has been demonstrated that alcohol, possibly through

alcohol metabolism and most often in the form of reactive oxygen species (ROS), is capable of generating free radicals in both neuronal and non-neuronal cells (Brzezinski et al., 1999; Henderson et al., 1995; Montoliu et al., 1995; Davis et al., 1990). Alcohol can also decrease antioxidant levels, which would effectively raise free radical levels within a cell (Addolorato et al., 1997; Devi et al., 1996). Either generating free radical levels above normal production levels or inhibiting normal free radical scavenging abilities can result in excess free radicals within a cell which can then lead to cell death.

Excitotoxicity

Calcium influx in response to neuronal depolarization is a normal part of the neurotransmission process. Although this process is a necessary part of neuronal activity, excessive neuronal activation can lead to high cytoplasmic [Ca^{2+}], which can in turn lead to cell death. This excitotoxicity due to excessive calcium accumulation within neurons has been hypothesized to be a mechanism of ARND. Interestingly, excitotoxicity appears most likely to occur during alcohol withdrawal, rather than during the actual period of alcohol exposure and highest BACs. Excitotoxicity has been indirectly demonstrated after acute alcohol exposure in early postnatal rats through inhibition of N-methyl-D-aspartate (NMDA) receptors (Thomas et al., 1997, 2001). This inhibition of excitotoxicity led to reductions in the behavioral anomalies associated with developmental alcohol exposure. Chronic alcohol exposure in fetal guinea pigs has also been shown to possibly mediate excitotoxicity through upregulation of the NMDA receptors (Chiu et al., 1999). Excitoxicity in response to alcohol withdrawal has also been demonstrated in cultures of fetal rat neocortex and hippocampus, and this neurotoxicity was directly related to Ca^{2+} influx (al Qatari et al., 2001; Lindsley and Clarke, 2004; Mayer et al., 2002; Prendergast et al., 2000). Although the exact mechanisms by which high intracellular [Ca^{2+}] induces toxicity are not well understood, these studies indicate that alcohol can induce neurotoxicity, most likely as a direct result of neuronal hyperexcitability during alcohol withdrawal and that this may be a mechanism of at least some of the alcohol-induced developmental deficits.

Indirect Mechanisms

An environmental compound can affect development in multiple ways. A teratogenic drug does not necessarily need to directly interact with a target cell or organ in order to cause damage. There can be indirect interactions between a drug and a particular organ via metabolites, endocrine disruptions, uptake and utilization of nutrients (including alterations in metabolism) and hypoxia/ischemia. Many of the mechanisms proposed for alcohol-induced cell loss or disruption of function occur not as a direct effect of alcohol binding to certain proteins or biochemicals, but rather by the influence of alcohol on extraneous factors which in turn are the primary reason for the deficits seen after developmental alcohol exposure.

An interesting example of an hypothesized indirect mechanism of ARND is the proposal that the ethanol metabolite, acetaldehyde, may play at least some role in the induction of *in utero* effects of alcohol exposure. The first step in the metabolism of alcohol is the conversion of alcohol to acetaldehyde by the enzyme alcohol dehydrogenase (ADH). Acetaldehyde is then converted to acetate and water by aldehyde dehydrogenase (ALDH) (Goldstein, 1983). Although numerous studies have shown that alcohol can inflict deleterious consequences by itself, it has been proposed that at least some of the effects of developmental alcohol exposure *in vivo* may be due to the even more toxic compound acetaldehyde (Eriksson, 2001; Goldstein, 1983; Holownia et al., 1996). Although *in vitro* experiments demonstrating that neuronal death and malformations can occur even in the absence of acetaldehyde indicates that this compound is at least not the sole mechanism of alcohol-induced cell death during development, this illustrates an excellent example of the potential indirect actions of alcohol (Acosta et al., 1986; Chen and Sulik, 1996; Pantazis et al., 1993).

Deficits in Nutrient Supply

Normal fetal development is dependent on an adequate supply of nutrients from the mother, such as amino acids, carbohydrates, vitamins and oxygen. Alcohol has been hypothesized to interfere with fetal nutrient supply through maternal malnutrition.

Alcoholics are often malnourished and this malnutrition may interfere with fetal growth (Fisher and Karl, 1998). However, the majority of studies examining the effects of developmental alcohol exposure have used pairfed controls that receive similar levels of nutrient intake as the subjects receiving alcohol and have demonstrated that neuronal and growth deficits are not entirely a result of nutrient intake deficits (Chen et al., 1998, 1999b; Pierce and West, 1987; West et al., 1986, 2001). This does not imply that deficits in nutrient intake do not absolutely play any role in ARNDs or growth deficits, but does suggest that maternal malnutrition is not the sole mechanism of these defects.

An additional complication that may occur in women who abuse alcohol during pregnancy but are not malnourished and in animal model systems that expose the developing animal to alcohol *in utero* are potential alterations in the placental transfer of nutrients. The placenta is responsible for delivering nutrients to the fetus from the mother and alterations in the normal maternal-fetal transfer of these nutrients may interfere with fetal growth and development.

There are numerous studies demonstrating that alcohol inhibits normal placental uptake and delivery of nutrients. Alcohol has been shown to inhibit the transport of several amino acids, including two essential amino acids, tryptophan and histidine, which, in addition to alterations in placental transport, were also shown to be deficient in the alcohol-exposed rat fetus (Asai et al., 1985; Fisher et al., 1984; Gordon et al., 1985; Lin et al., 1990; Snyder et al., 1989). It has also been demonstrated that alcohol inhibits placental glucose transport (Falconer, 1990; Schenker et al., 1989; Snyder et al., 1986). Finally, alcohol has also been shown to alter placental uptake of folate, biotin and vitamin B6 (Fisher et al., 1985; Schenker et al., 1993, 1992). All of these nutrients are essential for normal development and alterations in the delivery of these nutrients may have severe repercussions on the developing fetus. Alcohol-induced changes in placental transport may play an important role in the deficits observed after developmental alcohol exposure.

Obviously, an intact and properly functioning placenta is vital to proper development. In addition to the necessity of proper transplacental transport of nutrients,

it is equally important that those nutrients are delivered to the various organs of the fetus. This is dependent on at least three major factors; 1) an adequate pool of resources, 2) blood flow to the various organs and 3) uptake of the nutrients by individual cells. Any or all of these actions of alcohol may act to hinder normal development and are discussed, at least in part, in later sections.

Hypercapnia and Acidemia

Similar to changes in PaO_2, changes in partial pressure of CO_2 ($PaCO_2$) or pH can have severe repercussions on the developing fetus. Increases in $PaCO_2$ (hypercapnia) has negative effects on the fetus such as alterations of the blood brain barrier (BBB) and increases in periventricular/intraventricular hemorrhage (Evans et al., 1976; Goddard et al., 1980; Van de Bor et al., 1986). Increases in blood pH (acidemia) or in tissue pH (acidosis) can also negatively affect development (Aleck et al., 1988; Ferrari et al., 1992; Ingemarsson et al., 1997). Only two experiments have demonstrated that alcohol can induce hypercapnia (Cudd et al., 2001; Mukherjee and Hodgen, 1982), while numerous others have not shown alcohol-induced hypercapnia (Ayromlooi et al., 1979; Gleason and Hotchkiss, 1992; Richardson et al., 1985). Similarly, some studies have shown that acidemia can develop after alcohol exposure (Cudd et al., 2001; Mann et al., 1975; Mukherjee and Hodgen, 1982), but other studies have not demonstrated this phenomenon (Ayromlooi et al., 1979; Richardson et al., 1985; Smith et al., 1989). These differences in results are most likely due to differences in alcohol exposure paradigms and doses. It may take higher doses of alcohol to induce hypercapnia and/or acidemia. Regardless, it seems clear that at least under certain circumstances, alcohol, even at moderate doses, is capable of altering $PaCO_2$ and pH. Excessive alterations in either of these factors could potentially be one of the mechanisms of alcohol-induced deficits.

Alterations in Growth Factors and Hormones

Growth factors and hormones are necessary for many aspects of cell proliferation and survival. Alterations in the production, delivery or efficacy of these factors can

either directly cause deficits or can exacerbate co-existing conditions. Alcohol has been shown to disrupt the efficacy of insulin-like growth factor-1 (IGF-1), a growth factor important for both cell division and the inhibition of apoptosis (Galli et al., 1995; Resnicoff, et al., 1993, 1996). Alcohol can also inhibit basic fibroblast growth factor (bFGF)-mediated glial cell proliferation, possibly through a down-regulation of the receptor for this protein (Luo et al., 1996; Luo and Miller, 1996). Two other growth factors important in the survival of differentiated neurons, glial-derived neurotrophic factor (GDNF) and nerve growth factor (NGF), can attenuate the neurotoxic effects of alcohol (Heaton et al., 1993; McAlhany et al., 1997). Changes in any of these growth factors from their normal levels or functions can have serious repercussions on the developing brain.

Although prenatal alcohol exposure has been shown to alter the level of numerous hormones, perhaps the best studied is the thyroid hormone. This hormone is important for development of the fetus, including the brain (Lauder, 1977; Porterfield and Hendrich, 1993; Rami et al., 1986a, b; Thorpe-Beeston et al., 1992). However, alcohol has been shown to decrease both thyroid hormone receptor expression in the brain and thyroid hormone concentrations during development (Cudd et al., 2002; Scott et al., 1998). These decreases in thyroid hormone concentration or action could contribute to the deficits observed after developmental alcohol exposure.

Hypoxia

Hypoxia was initially proposed as one of the possible mechanisms of alcohol-induced developmental deficits not long after FAS was first defined. This hypothesis is supported by the observation that both hypoxia and alcohol exposure during development induce similar craniofacial and neuronal deficits (Aitken and Schiff, 1986; Barnes and Walker, 1981; Bronsky et al., 1986; Goodlett et al., 1990; Hamre and West, 1993; Pierce et al., 1989). This hypothesis became especially compelling when it was discovered that populations of neurons that are exquisitely vulnerable to hypoxia (Purkinje cells of the cerebellum and CA1 pyramidal cells of the hippocampus) are also

extremely vulnerable to alcohol exposure during development (Aitken and Schiff, 1986; Barnes and Walker, 1981; Bonthius and West, 1990; Pierce et al., 1989). These comparisons led to the idea that hypoxia may be one of the key mechanisms of ARND and FAS.

In any discussion of hypoxia, it is important to differentiate between hypoxia and hypoxemia. Hypoxemia is defined as a deficient oxygenation of the blood, while hypoxia is defined as a deficiency in oxygenation of tissue. This concept is important, as it is possible to have an hypoxemic condition without inducing hypoxia through an increased efficiency in uptake of oxygen by the tissue or by increasing blood flow to a particular region (such as that which occurs in an embolic stroke). Likewise, it is feasible to have hypoxic tissues without hypoxemia by decreasing oxygen diffusion or blood flow. These issues are vital to designing and interpreting experiments regarding either the effects of hypoxia on physiological systems or when studying hypoxia as a mechanism of action of drugs such as alcohol.

One of the first studies examining the possibility of alcohol-induced fetal hypoxemia was performed in primates by Mukherjee and Hodgen (1982). They demonstrated that an intravenous bolus of ethanol constricted the umbilical vessels and caused fetal hypoxemia, hypercapnia (high arterial CO_2 levels) and acidemia (high arterial pH). However, this study has two problems that make conclusions difficult. First, the alcohol was administered intravenously over two minutes and caused a BAC of about 500 mg/dl. Although this BAC is within the realm of achievable BACs in humans, the vast majority of people do not reach this level. Second, this experiment was performed in anesthetized animals, which may affect the normal cardiovascular responses to insults. Given these complications, this study does not adequately address the hypothesis of alcohol-induced hypoxia.

Studies on placental blood flow and *in vitro* studies of umbilical arteries have also lent indirect evidence for the hypoxia hypothesis. Placental blood measured *in vivo* has been shown to be decreased by prenatal alcohol exposure (Falconer, 1990; Jones et al., 1981). Alcohol is also capable of inducing *in vitro* vasoconstriction of human

umbilical arteries (Altura et al., 1982; Savoy-Moore et al., 1989). Although these results do not directly demonstrate alcohol-induced hypoxia, it is possible that these reductions in placental and umbilical blood flow could inhibit oxygen (or other nutrient) delivery.

The sheep model system, which allows for accurate, repeated measurements of blood gases *in utero,* has been used to directly examine the possibility of alcohol-induced hypoxemia. Several studies have shown that acute exposure to alcohol in the near-term fetal sheep does not induce fetal hypoxemia (Falconer, 1990; Reynolds et al., 1996; Richardson et al., 1985; Smith et al., 1989). Results from studies exposing fetal sheep to multiple alcohol infusions during all or the last week of the third trimester equivalent have also not demonstrated hypoxemia except in one study where slight decreases in partial pressure of oxygen (PaO_2) levels were observed 24 hr after the beginning of the alcohol infusion (Cudd et al., 2001, Patrick et al., 1985; Richardson et al., 1987). With the exception of the Richardson et al. (1987) study, which had an unexplained increase in PaO_2 24 hours after the alcohol exposure, all of these fetal sheep studies demonstrated no changes in blood oxygen levels after alcohol exposure.

Although several of these studies indicate at least the possibility of alcohol-induced hypoxia, none of the studies in sheep consistently demonstrate hypoxemia. It is possible that the cardiovascular system in sheep does not respond to alcohol as in other species. However, it must be noted that the fetal sheep brain is vulnerable to the teratogenic effects of alcohol, which may indicate that hypoxia may not be a mechanism of alcohol-induced neuronal deficits (West et al., 2001).

In spite of the data, including the *in utero* studies, indicating that hypoxia may not be a mechanism of alcohol-induced developmental deficts, they do not entirely disprove the hypoxia hypothesis. It is possible that alcohol induces hypoxia through alternative mechanisms. In fact, it has been hypothesized that hypoxia may still occur on a regional level through reductions in regional blood flow (Cudd et al., 2001). A reduction in cerebral blood flow could induce an hypoxic environment through an ischemic mechanism (in either the entire brain or to particular regions) rather than

through an hypoxemic mechanism. Therefore, this is an area of study that certainly deserves further attention and will be discussed in the next chapter.

Any or all of these direct and indirect mechanisms may contribute to the deficits observed after developmental alcohol exposure. To make things more difficult, the mechanisms underlying ARND may vary depending on the brain region and the temporal pattern of exposure. In other words, a particular alcohol-induced mechanism may play a role in the cerebellar Purkinje cells during the third trimester equivalent, for example, but not be involved at all in other regions, or at different developmental time points. A further complication to this issue is the possibility that rather than alcohol directly causing some of the direct mechanisms, they may actually be a result of the indirect mechanisms. For example, alcohol may directly induce oxidative stress through inhibition of free radical scavengers, or alcohol may indirectly raise ROS levels by inducing hypoxia, which can also cause oxidative stress (Peng et al., 2003; Rogers et al., 2001; Tan et al., 1998; Xu et al., 2004). These complications make research on the underlying mechanisms of ARND difficult. However, with careful experimental design, these hypothesized mechanisms can be confirmed or disproven.

CHAPTER III

FETAL CEREBRAL BLOOD FLOW

Any comprehensive evaluation of hypoxia first requires an understanding of regional blood flow to and within the brain and therefore must necessarily take into consideration the anatomy of the arterial supply of the brain. Likewise, in studies determining the potential effects of drugs on CBF during development it is important to understand both the regulation of the cerebral arterial system and changes in this regulation during development.

Anatomy of the Ovine Cerebral Blood Supply

The anatomy of the neurovasculature is similar among many mammals, but the neurovascular anatomy of sheep is nearly identical to that of humans. There are only two major differences. The first and most obvious difference between humans and sheep is that of the course and distribution of the extra-cranial arteries giving rise to the arteries within the cranial vault (i.e. the cerebral and vertebral arteries). The other difference is that of the blood supply to the cerebellum.

In humans, the common carotid artery divides into the external and internal carotid arteries. The internal carotid artery passes into the cranial vault without major branches until it terminates in the anterior and middle cerebral arteries and the posterior communicating artery. The external carotid artery in humans gives off numerous branches supplying the head and neck until finally terminating in the superficial temporal artery and the maxillary artery. In addition, blood is also supplied to the human brain via the vertebral arteries (arising from the subclavian arteries), which coalesce to form the basilar artery. This basilar artery then divides to form the posterior cerebral arteries, which give off the posterior communicating arteries, forming, along with the arteries mentioned above, the Circle of Willis (Haines, 2002).

In contrast, the majority of blood to the brain of the adult sheep is delivered via branches of the external carotid arteries. The external carotid eventually gives rise to the

internal maxillary artery, which gives off the ramus anastomoticus and arteria anastomotica. These anastomoses branch out prior to the cranial vault to form the carotid rete mirabile. This carotid rete consists of numerous capillaries that diverge from the ramus anastomoticus and arteria anastomotica forming a capillary bed that then converges to form the internal carotid artery. This internal carotid artery then enters the cranial vault similar to that observed in humans. Interestingly, this formation of the carotid rete mirabile occurs after birth. Prior to birth, the internal carotid arises from the common carotid artery. However, within a few weeks after birth, the proximal segment of the internal carotid artery degenerates while the distal segment changes its source of blood from the common carotid artery to the carotid rete mirabile (Baldwin & Bell, 1963; Blackman et al., 1986; King, 1987).

Another difference between humans and sheep in their blood supplies into the cranial vault involves the vertebral arteries. In sheep, the vertebral arteries do not enter the cranial vault. Instead, they form anastomoses with the ventral spinal artery (the continuation of the basilar artery) and the common carotid arteries (just prior to the branching of the external and original internal carotids) via the occipito-vertebral anastomosis. Because of this unique pattern of anastomoses, blood flow within the basilar artery (under normal conditions) is in the caudal direction. This is opposite to that seen in humans, where the blood flow in the basilar artery is in the rostral direction.

The final major difference between sheep and humans is the difference in blood supply to the cerebellum. Humans have three arteries supplying the cerebellum, the superior cerebellar artery, anterior inferior cerebellar artery (AICA) and the posterior inferior cerebellar artery (PICA). Sheep, however, have only two arteries to the cerebellum, the caudal and rostral cerebellar arteries.

With the exception of the few key examples mentioned above, humans and sheep share many similarities in cerebral blood flow. The blood supply to the brains of both humans and sheep is supplied by three cerebral arteries (anterior, middle and posterior) and the basilar artery (Haines, 2002; Nanda, 1975). These arteries then give rise to pial arteries that, as the name implies, penetrate the pia mater and enter the brain. The pial

arteries gradually decrease in size as branches are given off until they then give rise to the arterioles and finally, the capillary bed, which actually supplies the parenchyma of the brain. Although there are small variations, as in the difference in blood supply between the human and sheep cerebella, this basic design of cerebral/basilar arteries to pial arteries to arterioles and then capillaries is preserved throughout mammals. The specific arterial names and the specific areas they supply are beyond the scope of this discussion, however, the similarity of the actual course of these arteries between humans and sheep provide the opportunity to confidently make comparisons between sheep and humans involving perturbations of CBF.

Regulation of Cerebral Blood Flow

The regulation of blood flow to the brain is an incredibly complex process and in spite of the enormous amount of research on the topic, it still is not entirely understood. There are many competing mechanisms that vie to maintain the appropriate blood flow throughout the many regions of the brain. These mechanisms must compensate for changes in blood pressure, blood gas content (O_2 and CO_2), pH, regional changes in metabolism, variations in intracranial pressure and changes in body posture. This compensation is vital in maintaining normal functions in the brain because the brain is intolerant of deficits in nutrients such as glucose and oxygen, as well as deficiencies in waste removal. These CBF regulation requirements necessitate a fine control of CBF on a global, as well as a regional basis.

Basic Principles of Blood Flow Regulation

Blood flow to the various regions of the body is regulated by changes in cardiac output and arterial diameter (vasodilation or vasoconstriction). These changes shunt blood to the places requiring it at any particular time. Unlike most of the rest of the body, however, the brain is not tolerant of sustained dramatic changes in blood flow or blood pressure. Therefore, the brain has developed mechanisms to isolate itself from the rest of the body. Blood flow to the majority of the organs is regulated by central

processes such as hormonal and neurogenic factors. However, with the exception of the heart, the regulation of CBF is unique in that it is primarily controlled by local factors (Berne and Levy, 2000; Blaustein and Walsh, 1996). This is necessary due to the large amount of blood flow required in the brain compared to most other organs, the unchanging volume of the cranial vault, as well as the need to isolate the brain from the normal changes in blood flow to other organs (i.e. "flushing" of the skin, decreases in the gastrointestinal tract, or increases in the skeletal musculature in response to sympathetic activation).

The neurovasculature must maintain a relatively static total CBF rate as well as be able to alter regional CBF based on differences in regional metabolism. In order to achieve these goals, the neurovasculature has developed very precise control mechanisms. As the arteries to the brain branch and decrease in size from the cerebral arteries/basilar artery to the pial arteries to the arterioles and finally to the capillary bed, the amount of regulation increases up to the point of the capillaries, which lack the smooth musculature necessary to adjust the diameter of arteries. Therefore, although some regulation of CBF is present in the larger arteries, most regulation occurs in the arterioles (Ursino, 1994). This is partly due to the physical inability of the larger arteries to change diameter as much as the smaller arteries, especially the arterioles. The arteriole regulation is also due to the necessity of regional adjustments in CBF (hence at the level of the arterioles) in order to compensate for local metabolic demands (activation of a specific region, i.e. Broca's area during speech).

The brain maintains its strict control over CBF in multiple ways by multiple regulatory inputs (Edvinsson and Krause, 2002; Hurn and Traystman, 2002; Vavilala et al., 2002). There is regulation based on blood pressure within the cranial arteries (autoregulation). The neurovasculature also responds to circulating chemical stimuli such as arterial O_2, CO_2, prostaglandins, catecholamines, vasopressin, oxytocin and angiotensin II. The CBF is also adjusted on a regional basis according to local metabolic demands. This level of control is probably the most extensive and exerts the most influence over CBF. Finally, there are neuronal inputs separate from those regulating the

above factors that are essential for tone regulation and pathological states such as ischemia or hypertension.

Autoregulation

The autoregulation of CBF is largely responsible for adjusting the CBF to compensate for changes in perfusion pressure (defined as arterial blood pressure minus intracranial pressure). Thus, autoregulation ensures that CBF remains constant over a range in adults of mean arterial pressures (MAP) between 50 to 60 mm Hg at the lower limit and 150 to 160 mm Hg at the higher limit. These range limit values are not precise, but the autoregulatory system is much less effective at adjusting CBF when the MAP falls outside of this range (Fig. 1). In spite of the sometimes dramatic changes in heart rate, blood pressure, blood flow to other organs and the constant changes in regional cerebral metabolism, and therefore changes in rCBF, the total blood flow to the brain is relatively constant under normal (nonpathological) conditions (Vavilala et al., 2002). For example, arterial blood pressure can change dramatically due to exercise, postural changes, etc; however, the total blood flow to the brain is maintained at a relatively constant rate in order to maintain constant overall nutrient delivery, metabolic and waste removal rates, as well as to prevent edema through excess CBF (Chillon and Baumbach, 2002).

The entire mechanism(s) of autoregulation are not entirely clear, although the majority of autoregulation seems to occur through a myogenic mechanism. This idea suggests that autoregulation occurs through the automatic adjustment of arterial smooth muscle cells (aSMC) by the transduction of vascular shear stress or changes in intravascular pressure (Bayliss, 1902). The constriction or relaxation of arteries by aSMCs most likely occurs via the transduction of pressure or stress by vascular endothelial cells (Bevan and Joyce, 1988a, b; Chillon and Baumbach, 1997; Wei and Kontos, 1982). The endothelial cells act as mechanoreceptors transmitting the changes in pressure or stress to the aSMC by releasing endothelium-derived relaxing factors

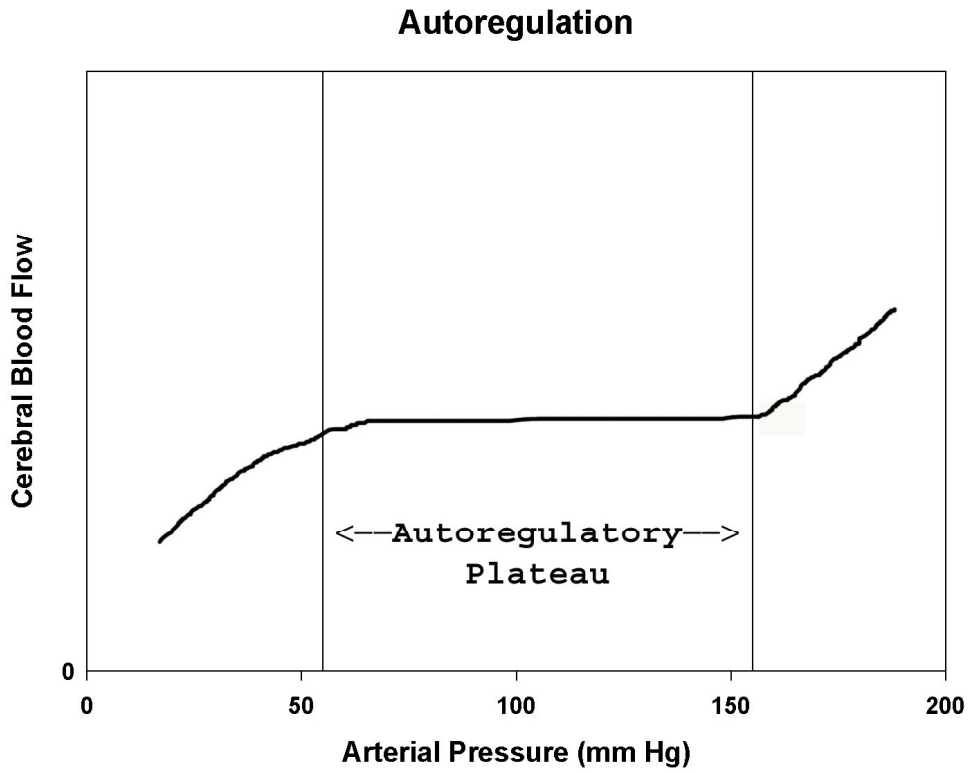

Figure 1: Autoregulation of Cerebral Blood Flow. CBF is kept constant over a range of arterial pressures from about 55 to 155 mm Hg in the adult through various autoregulatory mechanisms.

(EDRF) or endothelium-derived contracting factors (EDCF) (Rubanyi et al., 1986). Despite the extensive experimentation on the subject, little is known about these endothelium-derived factors or their downstream targets.

Another mechanism of autoregulation is the regulation of the neurovasculature in response to changes in perfusion pressure by metabolic factors. This hypothesis asserts that changes in perfusion pressure results in minor changes in metabolism (Vavilala et al., 2002). These changes in metabolism induce changes in local CBF. This hypothesis allows for local modulation of CBF on a more exact scale than that possible with myogenic modulation, although changes based on metabolic factors are not nearly as rapid as myogenic-induced changes (Kontos et al., 1978; Symon et al., 1973). Changes in CBF by metabolic factors can occur via either neurons or astrocytes (Kuschinsky and Wahl, 1978). As in the myogenic hypothesis, the exact mechanism(s) by which metabolic factors influence autoregulation are unclear, although numerous substances such as CO_2, O_2, numerous ions or other molecules known to influence CBF are not likely to play a role (Kuschinsky and Wahl, 1978; Phillis and DeLong, 1986). Kontos et al. (1987) suggested that a local reflex pathway might exist between the neurons in the local perturbed environment and the aSMC. They also suggested the possibility of the release of vasoactive substances by the astrocytes and/or neurons such as adenosine. Regardless, it is clear that at least some autoregulation is mediated by local metabolic factors.

Finally, although not strictly an aspect of autoregulation, changes in CBF in response to changes in perfusion pressure is also modulated by the sympathetic nervous system (SNS). This modulation, however, is slight and only seems to adjust the upper and lower autoregulatory limits and maintain vascular tone (Edvinsson et al., 1976; Gross et al., 1979; MacKenzie et al., 1979; Sadoshima et al., 1985). Sympathetic innervation via perivascular nerves from the superior cervical ganglion adjusts the upper and lower limits of autoregulation. This modulation of the autoregulatory limits serves two possible purposes, 1) to allow for autoregulation after either intense SNS activation or possibly during low sympathetic tone during sleep and 2) as a protective measure

during pathological states such as chronic hypotension (Bill and Linder, 1976; Edvinsson et al., 1976; Sadoshima et al., 1985). Although other neurotransmitters are likely to be involved, the most likely candidate for SNS-mediated neurovascular modulation is calcitonin gene related peptide (CGRP) through activation of ATP-sensitive K^+ channels (Hong et al., 1994; Kitazano et al., 1993). In spite of the fact that the SNS innervation of the neurovasculature is extensive, it is clear that the SNS merely plays a role in modulating CBF autoregulation rather than the more central roles of myogenic or metabolic factors.

It is widely believed that most autoregulation occurs at the level of the arterioles; however, at least some of the adjustments in response to perfusion pressure occur in the larger pial arteries and the cerebral arteries. The autoregulation resulting from metabolic factors occurs almost exclusively in arterioles. In contrast, most of the SNS-mediated modulation occurs in the cerebral and basilar arteries and the larger pial arteries, although some innervation of the smaller arteries is present. The myogenic aspects of autoregulation, meanwhile, mediate their effects throughout the neurovasculature ranging from the inflow vessels (i.e. cerebral arteries) to the arterioles.

These autoregulatory mechanisms are important in maintaining a constant perfusion pressure in the neurovasculature. Even short, transient episodes of hypotension can lead to ischemia, while equally brief bouts of hypertension can lead to edema, which, if prolonged can be as damaging as ischemia. Therefore, alterations of the autoregulatory system may have serious repercussions on the brain, especially the developing brain.

Oxygen and Carbon Dioxide

Cerebral blood flow is capable of being modified by endogenous chemical stimuli within the circulating blood such as oxygen and carbon dioxide. Exogenous drugs such as caffeine, cocaine, nicotine, etc are also capable of altering CBF. The endogenous vasoactive compounds play an important role in the regulation of CBF, while these exogenous compounds can either change blood flow inducing negative

effects, or in the case of medically useful drugs, can help to alleviate abnormal CBF due to pathological processes. However, under normal circumstances, the two most important modulators of CBF are O_2 and CO_2.

Perhaps the most potent endogenous chemical regulators of CBF are the gases O_2 and CO_2. The brain is very sensitive to changes in PaO_2 and $PaCO_2$, especially CO_2. This sensitivity is crucial due to the demand of the brain for stable PaO_2 and $PaCO_2$ levels. Decreases in PaO_2, resulting in hypoxemia, or abnormal increases or decreases in $PaCO_2$, hypercapnia or hypocapnia, respectively, all have rapid and dramatic effects on CBF. Therefore, it is not surprising that the responses of the neurovasculature to these molecules have been well studied, although there are still many questions left unresolved about the exact pathways.

Decreases in PaO_2 (hypoxemia) have been shown to be a potent vasodilator of the cerebral circulation. However, the neurovasculature does not seem to respond to small changes in PaO_2, but rather remains stable throughout a wide range of values until a lower threshold is reached, at which point hypoxemia-induced vasodilation occurs exponentially (Fig. 2) (Hurn and Traystman, 2002; James et al., 1969). This is probably due to the fact that there are other regulatory mechanisms, such as changes in erythrocyte perfusion patterns, that act to ensure adequate oxygen supply during small changes in PaO_2 (Krolo and Hudetz, 2000). It should also be noted that unlike the carefully controlled conditions of an experiment in which other vasoactive factors such as $PaCO_2$ are controlled, in normal physiological systems, changes in PaO_2 are often accompanied by changes in $PaCO_2$ and/or acidosis, which would assist or amplify changes in CBF after decreases in oxygen levels.

The precise mechanisms of hypoxemia-induced changes in CBF are unclear. Numerous chemoreceptor mechanisms have been proposed, but this is still inconclusive (Traystman et al., 1986). One area of the brain, the rostral ventrolateral medulla has been shown to play an important role in hypoxemia-induced vasodilation, but the exact mechanism is uncertain (Golanov and Reis, 1996; Sun and Reis, 1994a, b). The most likely candidates (or at least the best known) are factors released by either parenchymal

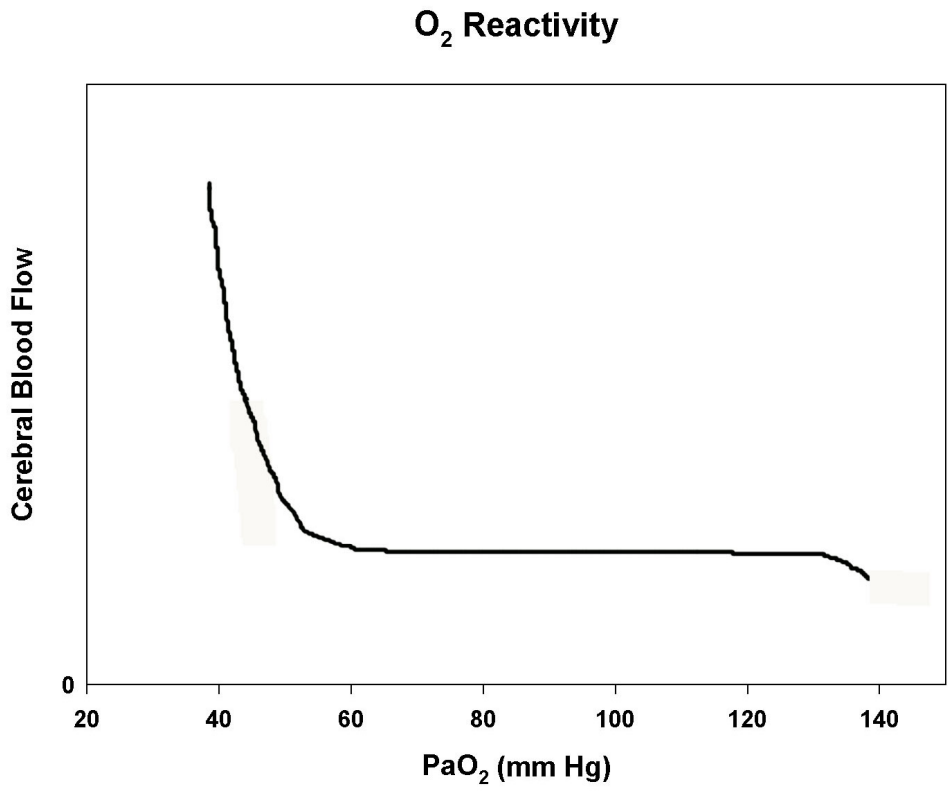

Figure 2: Cerebral Blood Flow in Response to Hypoxia. The neurovasculature responds to hypoxia by increasing CBF. This increase in CBF is limited to PaO_2 values lower than about 35 mm Hg.

or endothelial cells. The parenchymal cells, especially the neurons, can release adenosine, which is a well-known potent vasodilator, or to a lesser extent, nitric oxide (NO), in response to hypoxia (Meno et al., 1993; Morii et al., 1987; Winn et al., 1981). The endothelial cells of the neurovasculature also have several mechanisms to modulate cerebral blood flow. Two types of K^+ channels, ATP-sensitive and Ca^{2+}-activated K^+ channels, expressed on both the endothelium and aSMCs, attenuate hypoxemia-induced vasodilation by hyperpolarizing the aSMCs (Bonnet et al., 1991; Fredricks et al., 1994; Gebremedhin et al., 1994; Taguchi et al., 1994). There is also some evidence that hypoxemia may induce vasodilation by interfering with aSMC Ca^{2+} uptake, thereby inhibiting muscle tone (Hellstrand et al., 1977; Vinall and Simeone, 1986). Finally, the cytochrome P450 monooxygenase metabolites, epoxyeicosatrienoic acids (EETs) from the endothelium play a small role in vasodilation in response to decreased PaO_2 levels. This mechanism, however, has only been demonstrated *in vitro* and in the newborn piglet, so it is at least important during development, if not in the adult as well (Amruthesh et al., 1992; Leffler et al., 1997; Leffler and Fedinec, 1997).

Carbon dioxide has repeatedly been demonstrated to be a potent vasodilator of the neurovasculature. Even small changes in $PaCO_2$ dramatically change CBF. These changes are evident regardless of whether the $PaCO_2$ is increasing (hypercapnia) or decreasing (hypocapnia), resulting in increases or decreases in CBF, respectively (Fig. 3). Generally, increases in $PaCO_2$ by even one mm Hg causes a 6% increase in CBF (Hurn and Traystman, 2002; Reivich, 1964). These increases in CBF occur throughout the neurovasculature, but are most prominent in the arterioles (Auerr, 1978). These dramatic changes in CBF in response to hypercapnia are necessary because of the acidosis possible in hypercapnia and the limited ability of the brain to buffer hydrogen ions (H^+) and therefore pH. These factors make the adjustments of CBF in order to compensate for increases in $PaCO_2$, and therefore perivascular $[H^+]$, extremely important to the normal functions of the brain.

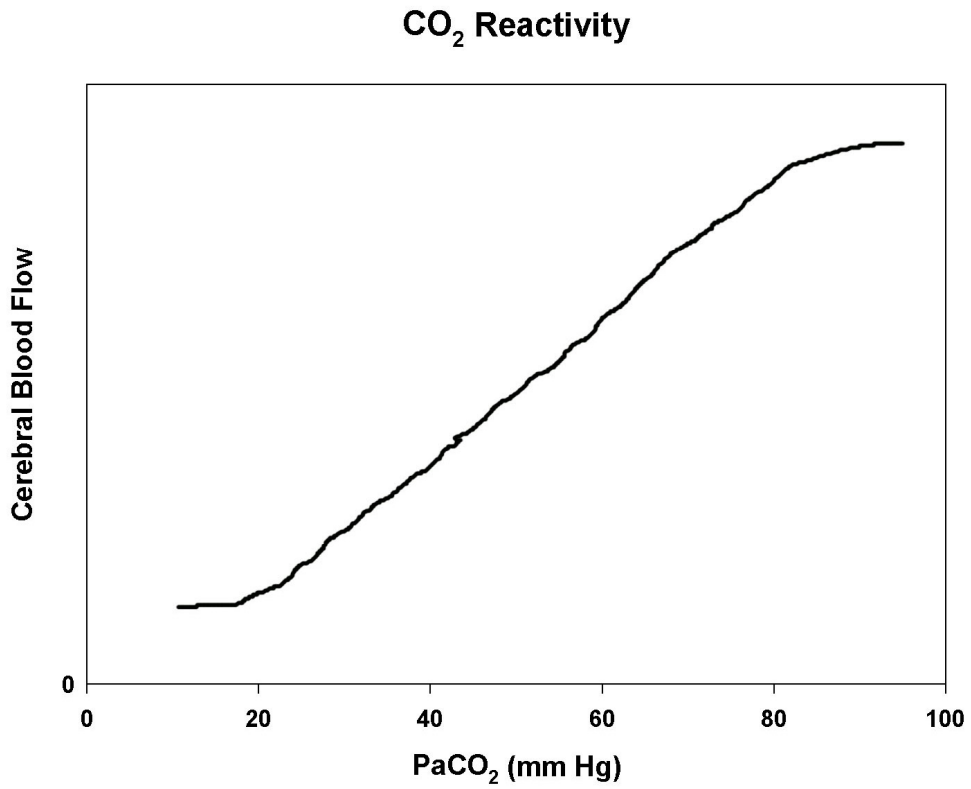

Figure 3: Cerebral Blood Flow in Response to Changes in $PaCO_2$. CBF changes over a wide range of $PaCO_2$ values. The neurovasculature is not capable of altering CBF when $PaCO_2$ becomes lower than about 20 or higher than 80 mm Hg $PaCO_2$.

The mechanisms of hypercapnia-induced increases in CBF are not as well understood as the mechanisms of hypoxemia-induced increases in CBF. Although some of the basic concepts of hypercapnia- and hypoxemia-induced increases in CBF are similar (i.e. endothelial, parenchymal and neurogenic affecters), the underlying mechanisms are quite different. The major mechanism of hypercapnia-induced vasodilation appears to be the accumulation of H^+ in the perivascular space (Gotoh et al., 1961; Koehler and Traystman, 1982; Kontos, 1977a, b). This vasodilation may occur through the activation of ATP-sensitive K+ channels (Santa, 2003). Although the precise mechanisms are uncertain, prostaglandins and possibly NO have been shown to be associated with hypercapnic-induced increases in CBF (Heinert, 1999; Leffler et al., 1994; Pickard and MacKenzie, 1973; Reid 1995; St Lawrence et al., 2002). Finally, there is also some evidence that brainstem vasomotor areas may play at least a small role in vasodilation associated with hypercapnia (Heinert et al., 1998; Pelligrino et al., 1999; Okamoto et al., 1997). Although multiple mechanisms are implicated in hypercapnia-induced increases in CBF, the most prominent vasodilator is perivascular $[H^+]$. The prostaglandins and neuronal innervation may play only a modulator role.

These data indicate that both oxygen and carbon dioxide are important regulators of CBF. While these gases may not affect CBF directly, they induce dramatic changes that are necessary to compensate for the negative effects that alterations in PaO_2 and $PaCO_2$ can induce.

Metabolic Regulation

Perhaps one of the most powerful modulators of CBF is the regulation that occurs to accommodate changes in metabolism (Fig. 4). The neurovasculature is not only capable of changing the total CBF rate in response to changes in metabolism, but is also able to alter CBF on a regional basis to adjust for regional variations in metabolism. Due to the fact that the brain has little ability to store glucose, the main source of energy in the brain, the CBF must constantly adjust to ensure an adequate nutrient supply to the regions that require it. The brain has a basal metabolic rate as a result of homeostatic

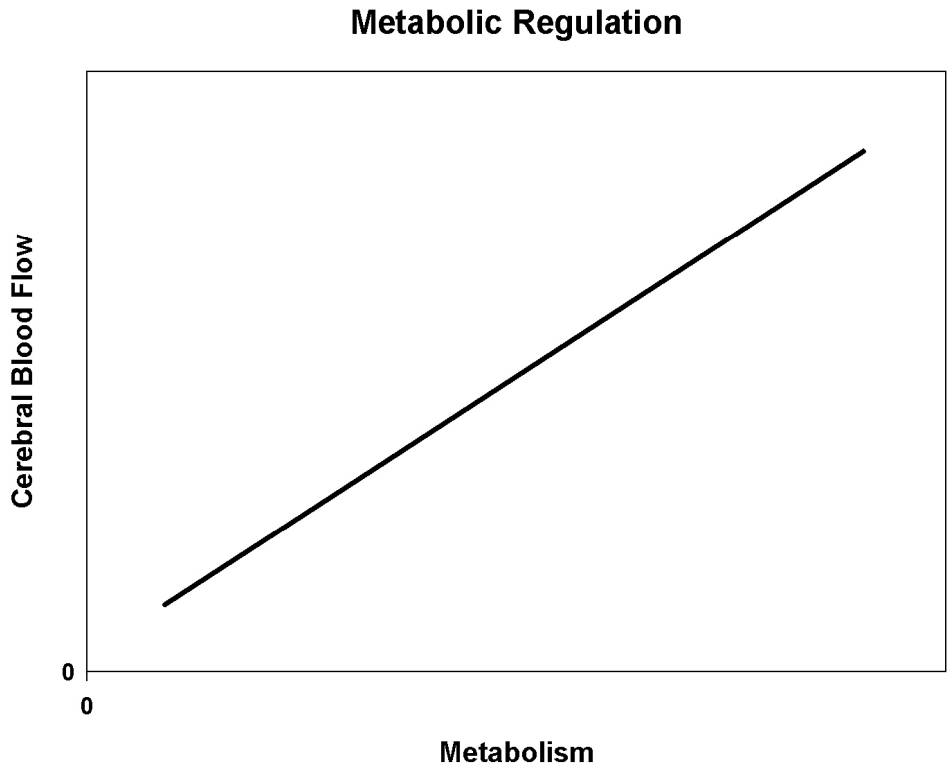

Figure 4: Regulation of Cerebral Blood Flow by Changes in Metabolism. CBF increases in a linear manner to accommodate increases in metabolism.

functions such as neurotransmitter and protein synthesis, glucose uptake, etc, that account for about 40% of the total energy used by the brain (Michenfelder, 1988). The additional 60% of total energy is used for the "functional" activity of the brain such as neurotransmission. This functional activity, except in specific regions, is transient in most regions of the brain. The regional adjustments in CBF coupled to these regional changes in metabolism, rather than a constant elevated CBF, is important considering that the brain, an organ that weighs about 2% of the total body, already requires about 15% of total cardiac output and about 20% of total oxygen consumption (Brust, 2000). Obviously, an additional constant CBF requirement would put an unnecessary stress on the cardiovascular system.

Regulation of CBF by metabolic factors occurs in two ways. First, there is the aforementioned regional CBF regulation to compensate for regional differences in metabolism. Second, the larger arteries (i.e. the cerebral or basilar arteries) are also capable of adjusting global CBF to ensure that an adequate blood supply is available for the entire brain and the associated transient changes in regional metabolism (Edvinsson and Krause, 2002). The global changes are minor, however, compared to regional changes in blood flow. It is the regional changes in CBF that are the driving impetus behind the delivery of required nutrients to activated brain regions.

The idea that CBF is coupled to metabolism was first proposed over one hundred years ago (Roy and Sherrington, 1890). Since those first experiments, this hypothesis has been tested using numerous techniques ranging from functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) to radioactive tracer molecules. These studies have clearly indicated a positive relationship between regional brain activation and rCBF (Cameron et al., 1990; Frostig et al., 1990; Hyder et al., 1994; Perlmutter, 1991). The most extreme examples of cerebral activity and metabolism are those of anesthesia and epileptic seizures. Most anesthetic agents induce decreases in electrophysiological activity with concomitant decreases in cerebral metabolic rate (CMR). These decreases in CMR are associated with decreases in CBF (Hansen et al., 1988; Maekawa et al., 1986; Newberg et al., 1984; Reinstrup et al., 1995). On the

opposite end of this spectrum is the increased cortical activity associated with seizures. Experiments in both patients and animals with epileptic seizures have demonstrated that CBF dramatically increases during seizures (Magistretti, 1997; Plum et al., 1968; Purves, 1981). Obviously, these examples are extreme and do not represent normal CMR/CBF coupling nor do they demonstrate the regulation of CBF on a regional basis, but they do demonstrate the range of cerebral activity/metabolism in which CBF regulation can occur.

Numerous studies in both humans and animals have demonstrated the intricate coupling between CMR and CBF (Fox and Raichle, 1986; Kim and Ugurbil, 1997; Villringer and Dirnagl, 1995). Although all of the mechanisms whereby CBF is altered by CMR are not completely understood, numerous mechanisms of metabolic regulation of rCBF have been discovered. These mechanisms fall into two categories; 1) alteration of CBF by molecules released into the extracellular/perivascular space as a direct result of metabolic changes that accompany neuronal activation and 2) neurogenic mechanisms that alter CBF parallel to neuronal activation. The first mechanism couples metabolism with cerebral blood by the release of factors (often end-products of metabolism) by either neurons or glia such as K^+, lactate, adenosine and H^+. The second mechanism involves the release of neurotransmitters onto receptors of the aSMCs. This often occurs in synapses on the aSMCs formed by the same neurons that are activated.

It is clear that the regulation of CBF is a very complex process. In spite of the fact that this area has been under intense scrutiny for over a hundred years, there is still much that is not known about CBF regulation. However, enough is known that we can use this information to make conclusions about factors, such as alcohol exposure, that can potentially disrupt CBF regulation.

Developmental Changes in the Regulation of CBF

As the developing brain matures, it develops the regulatory mechanisms necessary for survival outside of the uterus. The effects of numerous cardiovascular stimuli are blunted in the *in utero* environment compared to the adult. This is possible

due to the protection afforded by the maternal compensation to these stimuli. This isolation is not complete, however, so the fetus must develop strategies to protect itself from external perturbations detrimental to normal development. Many of the normal neurovascular regulatory mechanisms present in the adult are also present in the fetus, except that the relative importance of each of these factors varies between adults and different developmental stages. These differences in the relative importance of the various regulatory mechanisms are due to both the immaturity of the fetal neurovascular system and the differences in fetal cardiovascular physiology. In order to enhance discussions, the development of the CBF regulation system in humans, primates and sheep is often divided into four periods, the immature fetus, the near-term fetus, the newborn and the adult.

The neurovascular responsiveness to hypercapnia changes during development of the brain. As mentioned previously, hypercapnia is a potent vasodilator of the neurovasculature. This phenomenon occurs at all developmental stages, but is blunted in the neonate and fetus, especially the immature fetus (Habgood et al., 1991; Helou et al., 1991; Rosenberg et al., 1982). The near-term fetus, however, is similar to the newborn in its relative response to hypercapnia (Purves and James, 1969; Rosenberg et al., 1982). Similar to $PaCO_2$, the response of the fetal neurovasculature to PaO_2 is different from that of the adult. Hypoxemia-induced vasodilation in the brain occurs at all developmental stages with an intact CNS, but similar to the relative developmental differences in hypercapnia-induced changes, the hypoxemia-induced changes are attenuated in the less mature brain, especially during mid-gestation (Gleason et al., 1990; Koehler et al., 1984; Jones et al., 1982). This phenomenon may be due to the underdevelopment of some of the CBF regulatory mechanisms (Busija, 1994; Toda and Hagashi, 1979: Wagerle et al., 1990). Alternatively, these differences may be due to the normal increases in $PaCO_2$ and decreases in PaO_2 observed *in utero* relative to the adult or newborn [newborn/adult $PaCO_2$; ~30-35 mm Hg vs. fetal $PaCO_2$; ~45-50 mm Hg, newborn/adult PaO_2; ~85-105 mm Hg vs. fetal PaO_2; ~15-25 mm Hg (Cudd et al., 2001; Davidson, 1987; Rosenberg et al., 1982).

The ability to autoregulate also changes during development. The preterm fetus (GD 92) has limited and inconsistent autoregulation abilities until approximately GD 120 (Helou et al., 1994; Papile et al., 1985). The autoregulatory curve in the near-term fetus and newborn animals is shifted to the left resulting in decreased lower and upper autoregulatory limits as well as a decreased range of autoregulation (Ashwal et al., 1984; Fletcher 1989; Leffler et al., 1986; Pasternak et al., 1985). This limitation in autoregulation may be due to either the underdevelopment of the arterial wall thickness and the associated myogenic tone or the inherent decreases in PaO_2 relative to more mature animals, which plays a role in autoregulation in fetal animals (Pearce et al., 1991; Sadoshima et al., 1985a; Tweed et al., 1983; 1986).

Brain metabolic rates vary according to age with metabolic rates [as measured by cerebral oxygen consumption ($CMRO_2$)] ranging from being the lowest in the immature fetus, to the mature fetus, then to the adult and is highest in the newborn (Gleason et al., 1990; Koehler et al., 1984; Rosenberg et al., 1982). Presumably, this change in cerebral metabolic rate over time is due at least in part to the intense development of the brain occurring during the perinatal period (Busija, 1994; Dobbing and Sands, 1979). The rate of CBF is reflected in these developmental differences in metabolic rate. However, it should be noted that some of the differences in CBF are due to other factors such as basal differences in PaO_2, $PaCO_2$, and oxygen extraction efficiency. Regardless, it has been demonstrated that the concept of CMR/CBF coupling has been confirmed within a single developmental stage at all four of the periods mentioned above (Busija and Leffler, 1987; Busija et al., 1988; Gleason et al., 1990, Koehler et al., 1984, Rosenberg et al., 1982). Although the slope of the CBF/metabolic coupling relationship may vary slightly between the different ages, these data still demonstrate that at any point from just past mid-gestation (the earliest time point measured) to adult CBF changes to compensate for cerebral metabolic rates.

Taken together, these studies indicate that although the degree of CBF alteration may be somewhat blunted, the neurovasculature of developing animals, especially the newborn and near-term, responds to acute changes in environment in a similar manner to

adults. This similarity allows for inferences regarding the regulation of the fetal neurovasculature from adult studies, when necessary, which are far more numerous than those during development.

Fetal Cerebral Blood Flow and Alcohol

The effects of alcohol on CBF have been mostly studied in the adult. Numerous reports indicate that acute alcohol exposure in adult humans increases CBF to most regions (Mathew and Wilson, 1986; Newlin et al., 1982; Sano et al., 1993; Schwartz et al., 1993; Tiihonen et al., 1994; Volkow, et al., 1988). Similar results have been reported in rats, at least for low to moderate doses of alcohol, while higher doses have been shown to decrease CBF (Hemmingsen & Barry, 1979; Hoffman et al., 1986; Lyons et al., 1998). However, conflicting studies have shown that acute moderate alcohol exposure reduces CBF and is a vasoconstrictor (Altura and Altura, 1987; Friedman et al., 1981; Goldman et al., 1973; Gordon et al., 1995). Chronic doses of alcohol have similar effects as acute high doses of alcohol in that alcoholics have shown to have decreases in CBF (Dally et al., 1988; Erbas et al., 1992; Melgaard et al., 1990; Rogers et al., 1983). The changes in CBF could be due to the pathological alterations associated with alcohol abuse, especially in the long-term alcoholics; however, it is also possible that at least some of the alcohol-induced pathologies are a result of changes in CBF. Although some of these studies, particularly the studies in humans, vary in their alcohol doses, blood flow measurement techniques, and the neurodegeneration associated with chronic alcohol use, it is clear that alcohol alters CBF in adults.

In spite of the large amount of literature regarding the effects of alcohol on CBF in adults, very little has been done to study this same phenomenon during development. The few studies in this field have been performed in pregnant sheep, which lends itself well to this research. The initial examination of potential alcohol-induced effects on fetal CBF was done by Richardson et al. (1985) who determined that an acute exposure to a moderate dose of alcohol (1 g/kg) late in gestation [third trimester equivalent (0.88 of gestation)] reduced blood flow to the cerebral cortex, subcortex, pons and medulla. They

also showed a trend for decreases in the cerebellum, however, this was not significant. Another study performed closer to the middle of gestation [second trimester equivalent (0.62 of gestation)] with a similar acute dose of alcohol failed to demonstrate any changes in fetal CBF (Gleason and Hotchkiss, 1992). Finally, the third and final examination of potential alcohol-induced changes in CBF demonstrated that a longer alcohol exposure of three weeks during the first trimester equivalent resulted in changes in the responsiveness of the newborn neurovasculature to an hypoxic episodic insult (Gleason et al., 1997). Taken together, these studies indicate several important points, 1) an acute alcohol exposure is capable of reducing fetal CBF during the brain growth spurt, which if repeated often enough, may lead to a cumulative hypoxic/ischemic effect on the fetal brain. 2) Alcohol exposure during early gestation leads to functional changes in the neurovasculature which may have negative effects later in development if the brain is no longer able to compensate for challenges that it would normally be able to handle (Gleason et al., 1997). Both of these phenomena may play a role in altering normal neurovascular development, which could then impact normal CNS development and function.

CHAPTER IV

METHODS

The sheep model system was used for these experiments. This species was used for several reasons. 1) The approximate relative period of brain development in sheep (termed the third trimester equivalent) is similar to the human third trimester. The equivalent period of brain development is postnatal in rodents but is prenatal in sheep (similar to humans), allowing for *in utero* experimentation. This fact is important in determining or controlling for possible maternal-fetal interactions during development or for possible ways in which alcohol can interact with the maternal-fetal unit. This is also important as the third trimester equivalent forms part of the brain growth spurt, an intense increase in neurodevelopment (Dobbing and Sands, 1979). The brain growth spurt begins *in utero* approximately during the third trimester and continues postnatally. 2) The size of the sheep fetus is similar to that of humans. This allows for more accurate comparisons between these experimental data and humans. The size advantage in sheep also allows for more extensive experimentation in sheep compared to smaller species. For instance, due to the size of the third trimester ovine fetus, it is possible to take large, multiple blood samples or to perform chronic instrumentations. The size of the fetal sheep brain towards the end of gestation (about 50 g) is another advantage, which allows for more extensive and more accurate regional dissections compared to the much smaller neonatal rodent brain at the same relative period of development (about 0.5 g).

These methods are divided into four separate experiments. 1) Blood flow after repeated alcohol exposure. 2) Blood flow during a final alcohol exposure after repeated alcohol exposure. 3) Cardiovascular measurements (PaO_2, $PaCO_2$, pH, hematocrit, heart rate, arterial blood pressure and BAC). 4) Neuronal cell counts after repeated alcohol exposure during the third trimester equivalent. The first three experiments were performed in the same set of animals, while the fourth experiment was a separate set. The alcohol exposure paradigms were identical between both sets of subjects with the exception that the fourth experiment did not have a 0.75 EtOH group. Blood flow

measurement protocols were similar between the first and second experiments. All methodology that is identical among experiments is only presented once.

Experiment #1: Blood Flow after Repeated Alcohol Exposure

This experiment examined the effects of repeated alcohol exposure during the third trimester equivalent on blood flow. Blood flow was measured just prior to a final alcohol exposure and 24 hours after the second to last alcohol exposure. The purpose of this experiment was to determine if repeated alcohol exposure could affect blood flow in the fetus without alcohol actually being present at the time of measurement.

Subjects

Suffolk ewes (aged 2-6) years were used in these studies. The ewes were induced to ovulate using CIDR progesterone inserts and then bred to rams fitted with a marking harness to determine time of conception. After conception, the ewes were kept outside until gestational day (GD) 90. At that point, ewes were brought inside and housed in individual cages at a constant temperature of 22°C on a 12:12 light/dark cycle. The ewes were always kept in sight of at least one other ewe to prevent separation anxiety.

Treatment Groups

This experiment consisted of four treatment groups; two alcohol groups and two control groups. The first alcohol group (1.75 EtOH) was administered a 1.75 g/kg dose of alcohol while the second alcohol group (0.75 EtOH) was given alcohol at a lower 0.75 g/kg dose. These alcohol dose were chosen because the high dose has previously been demonstrated to induce cerebellar Purkinje cell loss and the low dose is similar to the dose previously shown to acutely decrease fetal regional cerebral blood flow (Richardson et al., 1985; West et al., 2001) The control to these two groups was the saline control (SC). This control was administered 0.9% saline at a volume comparable to those of the alcohol groups (taking into account differences in maternal body weight). The final group was a normal control group (NC). This group underwent the surgical procedures similar to the other groups in order to infuse the microspheres, but did not

receive any infusions. This group was included to control for any possible changes in fetal blood flow associated with the maternal infusion of saline.

Prenatal Alcohol Exposure in Sheep

Pregnant sheep were exposed to alcohol from gestational days (GD) 90-133 (the third trimester equivalent). The ewes were exposed in a binge-like pattern with alcohol infusions on three consecutive days with four days between exposures (Fig. 5). The alcohol solution [40% ethanol (1.75 EtOH group) or 17% ethanol (0.75 EtOH) in 0.9% saline solution] was administered over a one-hour period via a peristaltic pump (Cole-Parmer) into a catheter placed in one of the jugular veins. These alcohol doses produced peak blood alcohol concentrations (BAC) of about 190 mg/dl in the 1.75 EtOH group and 85 mg/dl in the 0.75 EtOH group. The SC group received saline in a similar fashion to the alcohol administered to the EtOH groups.

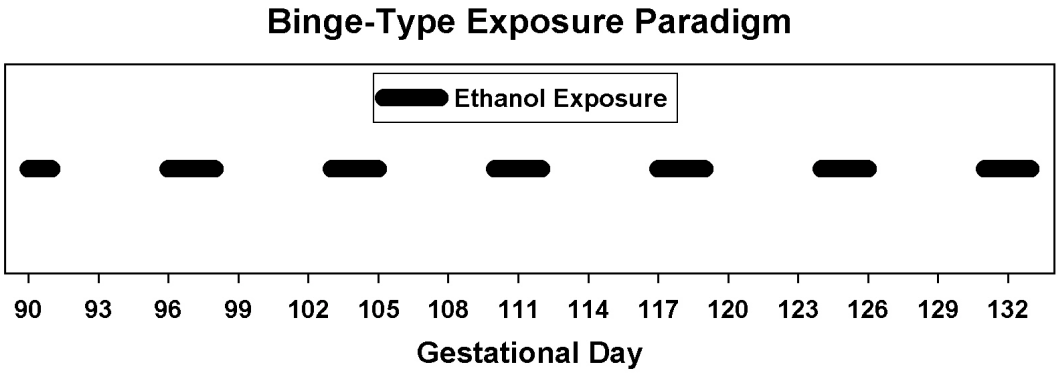

Figure 5: Binge-Type Exposure Paradigm. The subjects were exposed to alcohol for three consecutive days with four days between binges.

Fetal Surgery

On about GD 120 the pregnant sheep underwent surgery to implant permanent indwelling catheters into the fetus. The ewe was administered an injectable anesthetic

consisting of ketamine and diazepam, intubated and maintained under anesthesia via isofluorane, an inhaled anesthetic. Once anesthetized, the ewe was taken into the surgery room where sterile surgical techniques were observed throughout the surgery. The ewe's abdominal cavity was opened with a six-inch midline incision caudal to the umbilicus. A small part of the uterus was excised and a small incision was made in it in order to gain access to the fetus. The left posterior leg of the fetus was then exteriorized in order to insert catheters into the femoral artery and saphenous vein. The saphenous vein catheter was inserted into the vein up to the point where the end of the catheter was at the caudal extent of the inferior vena cava. The incision used to insert the catheters was then sutured shut and a third catheter external to the fetus was bundled with the femoral and saphenous catheters in order to measure amniotic pressure (used to gain a more accurate analysis of fetal blood pressure). A fourth catheter was then placed into the brachial artery through the left anterior leg. The uterus was then closed and returned to the maternal abdominal cavity and the catheters, which exited the uterus, were then externalized through the maternal abdominal wall and placed into a pouch sewn onto the side of the ewe until needed for experimentation. The maternal abdominal cavity was then closed and the ewe was removed from anesthesia.

Radiolabelled Microsphere Infusion

On GD 133, the last day of alcohol administration, blood flow was measured using 15 μm radiolabelled microspheres just prior to the last alcohol infusion (0 hr) using Cobalt (^{57}Co)-labeled microspheres (Perkin-Elmer). The microspheres were injected into the inferior vena cava (in fetal animals 90% of venous return is shunted away from the lungs through the foramen ovale and ductus arteriosus). The microspheres were then distributed throughout the body via the cardiovascular system in direct proportion to the blood flow of each target organ. Due to the fact that the microspheres are 15 μm in diameter (about twice the diameter of a mature red blood cell), the microspheres become lodged in the capillary bed of the organ to which they flow. The low number of microspheres injected into the fetus is miniscule relative to the number of

red blood cells and capillaries in the fetus, therefore these microspheres do not affect overall blood flow or tissue perfusion.

Simultaneously during the microsphere injection, blood is withdrawn from the brachial and femoral arteries by a syringe pump (Harvard Apparatus) for two minutes beginning at the point of microsphere injection. Because these samples from the femoral and brachial arteries were withdrawn at a known rate for a known amount of time, the "blood flow" from these arteries was known. These reference withdrawals were used in part of the calculations to determine the blood flow from the various regions of the fetus.

Fetal Tissue Dissection

After the last microsphere injection the ewe was euthanized by injection of 75 mg/kg sodium pentobarbital into the jugular vein followed by a thoracotomy to ensure effective euthanasia. A midline incision was made along the abdomen of the ewe and the entire uterus containing the fetus was removed from the ewe. Samples were taken from the myometrium and the caruncles and the fetus was removed from the uterus for further dissection. The samples taken are listed in Table 1.

Radioisotope Counting and Blood Flow Calculations

The tissue samples were analyzed using a multi-window gamma counter (Packard Cobra II Auto-Camera). The multi-window function of the gamma counter allows for the differentiation among the γ-emission energies of each isotope (different isotopes were used for each of the 0, 1, 2 and 6 hr measurements; ^{57}Co, ^{46}Sc, ^{85}Sr and ^{95}Nb, respectively) and obtains the counts per minute (CPM) of each tissue sample. Because one batch of microspheres was used for multiple subjects and each set of samples were not analyzed at the same time, the CPM of each tissue sample and standards were corrected for radioisotope decay (Eq. 1). These corrections ensure that equivalent counts are obtained among all of the subjects.

Table 1: Dissected Regions

Brain

Frontal Cortex	Parietal Cortex	Temporal Cortex	Occipital Cortex
Medial/Dorsal Lateral/Dorsal Medial/Ventral Lateral/Ventral	Medial Anterior/Lateral Posterior/Lateral Cingulate Cortex	Anterior/Dorsal Posterior/Dorsal Anterior/Ventral Posterior/Ventral	Medial/Dorsal Lateral/Dorsal Medial/Ventral Lateral/Ventral

Subcortex	Cerebellum	White Matter/Misc.
Thalamus Hypothalamus Superior Colliculus Inferior Colliculus Caudate Nucleus -Head Hippocampus	Lateral Hemispheres Superior Vermal Region Anterior/Inferior Region Posterior/Inferior Region	Corpus Callosum Optic Chiasm Spinal Cord -Rostral Cervical Olfactory Bulb

Midbrain	Pons	Medulla
Rostral Caudal	Rostral Caudal	Rostral Caudal

Organs

Triceps Quadriceps Skin (overlying) -Triceps -Quadriceps	Heart Lungs Liver Kidneys Spleen	Adrenal Thyroid Thymus Gonads

Eq. 1

$$\mathbf{CPM_c = \frac{CPM_u}{0.5^{\frac{t}{T}}}}$$

where CPM_c is the corrected counts, CPM_u is the uncorrected counts, t is time (in days) from the initial measurement of radioactivity and T is the half-life (in days) of the specific isotope being measured.

These corrected CPMs of the samples and standard [20 µl of microspheres (25,000 microspheres/µl)] were then used to calculate the number of microspheres (of each isotope) in each gram of tissue (Eq. 2).

Eq. 2

$$\mathbf{N_s = \frac{(CPM_s / CPM_{st}) \times N_i}{WT_s}}$$

where N_s is the number of microspheres in the tissue sample, CPM_s and CPM_{st} are the corrected CPMs of the sample and standard, respectively, N_i is the number of injected microspheres (500,000) and WT_s is the sample weight in grams.

Then, the known flow rate of the reference withdraw and the corrected counts of the reference withdraw are used in order to determine the number of microspheres per unit of flow rate (Eq. 3).

Eq. 3

$$\mathbf{N_f = \frac{(CPM_{rw} / CPM_{st}) \times N_i}{F_{rw}}}$$

where N_f is the number of microspheres per minute, CPM_{rw} is the CPM of the reference withdraw and F_{rw} is set flow rate of the reference withdraw.

From these calculated values, the flow rate to each tissue sample can be calculated (Eq. 4).

Eq. 4

$$F_t = \frac{N_f}{N_s}$$

where F_t is the tissue sample flow rate in ml/min/g. These calculations were repeated for each isotope in each animal to obtain blood flow rates at each of the four time points measured.

Statistical Analyses

The data from the 0 hr time point blood flow was analyzed using a one-way analysis of variance (ANOVA) with treatment (1.75 EtOH, 0.75 EtOH and SC) as between factors. Each region was analyzed separately. The data from the two control groups was analyzed using an unpaired *t*-test for each region. All *post hoc* analyses were performed were appropriate using Fisher's protected least significant difference (PLSD).

The brain data from both Experiment #1 and #2 were also analyzed according to the major arterial supply (anterior, middle and posterior cerebral and basilar artery). For example, the regions supplied by the anterior cerebral artery were combined to determine if overall blood flow through this artery was affected by alcohol exposure. The division of regions by arterial supply is listed in Table 2. The pooled data for each artery was then analyzed as each of the individual regions.

Experiment #2: Blood Flow during the Final Alcohol Exposure

This experiment examined the effects of a final alcohol exposure on blood flow in the fetus. Blood flow was measured at 1 hr, 2 hr and 6 hr after the beginning of the alcohol infusion. The purpose of this experiment was to measure the responsiveness of the neurovasculature to a final alcohol infusion after repeated exposures during the third trimester equivalent. The subjects and methodology in this experiment were similar to

Table 2: Arterial Regions

Anterior Cerebral Artery

Frontal Cortex - Medial/Dorsal - Lateral/Dorsal - Medial/Ventral - Lateral/Ventral	Parietal Cortex - Medial Cingulate Cortex	Olfactory Bulb	Corpus Callosum

Middle Cerebral Artery

Parietal Cortex - Anterior/Lateral - Posterior/Lateral	Temporal Cortex - Anterior/Dorsal - Posterior/Dorsal - Anterior/Ventral - Posterior/Ventral	Caudate Nucleus - Head Hippocampus

Posterior Cerebral Artery

Subcortex - Thalamus - Superior Colliculus - Inferior Colliculus	Occipital Cortex - Medial/Dorsal - Lateral/Dorsal - Medial/Ventral - Lateral/Ventral	Cerebellum - Superior Vermis

Basilar Artery

Brainstem - Rostral Midbrain - Caudal Midbrain - Rostral Pons - Caudal Pons - Rostral Medulla - Caudal Medulla	Cerebellum - Lateral Hemispheres - Anterior/Inferior Vermis - Posterior/Inferior Vermis

Experiment #1 with the exception of the timing of the microsphere injections and data analyses.

Radiolabelled Microsphere Infusion

Radiolabelled microspheres were injected as in Experiment #1 except that they were injected one (1 hr), two (2 hr) and six (6 hr) hours after the beginning of the final one hour alcohol infusion. In order to measure four different time points within the same fetus (including the microspheres from Experiment #1 at 0hr), the microspheres injected at each time point were labeled with different isotopes. The isotopes used were Cobalt (^{57}Co), Scandium (^{46}Sc), Strontium (^{85}Sr) and Niobium (^{95}Nb) (Perkin-Elmer) for the 0, 1, 2 and 6 hr time points, respectively.

Statistical Analyses

Due to the variability among the treatment group means at the 0 hr time point, the data from the 1, 2 and 6 hr time points were normalized to the 0 hr time point. This was done in order to examine the relative response of the neurovasculature in each treatment group to the final alcohol exposure independent of the baseline (0 hr) blood flow. This conversion was necessary because analyses of the 1, 2 and 6 hr time point data without the z-score conversion would be confounded by the altered baseline blood flow among the treatment groups. The resultant z-scores from the 1.75 EtOH, 0.75 EtOH and SC groups were then analyzed using a two-way ANOVA with treatment and time as between factors. The z-scores from the control groups were analyzed using separate two-way ANOVAs. All pair-wise comparisons were performed when appropriate using one-way ANOVAs (1.75 vs. 0.75 vs. SC comparisons) or Fisher's PLSD (SC vs. NC).

Experiment #3: Cardiovascular Measurements

This experiment measured BACs, PaO_2, $PaCO_2$, pH, hematocrit, heart rate and arterial blood pressure in the same subjects used in Experiments #1 and 2. These

dependent measures were valuable in verifying previous data, but mostly to verify that the microsphere infusion did not alter these cardiovascular parameters.

Blood Alcohol Concentrations

BACs were measured 0, 1, 2 and 6 hr relative to the beginning of the final alcohol infusion by taking 20 µl from a femoral artery blood sample and placing it into a vial containing 200 µl 0.6 N perchloric acid and 4 mM 1-propanol (internal standard). The sample was then analyzed by head-space gas chromatography (Varian, model #3900). The results were expressed in mg/dl.

Blood Gas Analyses

Blood gas was measured at the 0, 1, 2 and 6 hr time points coinciding with the microsphere infusions. Arterial blood (1.5 ml) was slowly withdrawn (without bubbles) using a syringe from the catheter in the fetal femoral artery. The blood was then analyzed for PaO_2 $PaCO_2$ and pH levels using a blood gas analyzer (ABL 5; Radiometer, Westlake, OH)

Hematocrit Measurements

Hematocrit was measured at the same time points as the blood gas measurements. Blood was drawn half-way up into a 200 µl capillary tube and then centrifuged to separate the red blood cells and the plasma. The hematocrits were expressed as the percentage of cells to plasma.

Heart rate and Blood Pressure Measurements

Heart rate and mean arterial pressure were measured in the femoral artery beginning at the start of the final alcohol infusion until one hour after the end of the infusion (two hours total). These parameters were measured using a strain gauge transducer (Isotec, Quest Medical, Allen, TX) and recorded by analog-to-digital recorder (DAQ Card-AI-16XE-50, National Instruments, Austin, TX) and a computer. The data

from these dependent variables were measured and analyzed using 60 second averages, however the data are presented graphically as 5 minute averages in order to reduce graph clutter.

Statistical Analyses

Each of the dependent measures in this study was analyzed by repeated measures ANOVAs with treatment (SC, 0.75 EtOH and 1.75 EtOH or SC and NC) as a between factor and time as a within factor. All pairwise comparisons were performed using one-way ANOVAs.

Experiment #4: Neuronal Cell Counts

This experiment was designed to determine the effect of alcohol exposure during the third trimester equivalent on the number of olfactory bulb mitral cells, hippocampal CA1 and CA2/3 pyramidal cells and hippocampal dentate gyrus granule cells. This experiment was designed to determine if alcohol can affect neuronal cell counts in these regions similar to that observed in the cerebellar Purkinje cells (West et al., 2001). These olfactory bulb and hippocampus data, combined with the data obtained previously in the cerebellum will also be used to determine if any possible changes in CBF due to alcohol exposure correlate with the pattern of neuronal loss. The subjects in this experiment were exposed to alcohol in an identical pattern to the previous three experiments, however, this experiment did not have a 0.75 EtOH group.

Subjects

Fifteen Suffolk ewes were used in this experiment. The ewes were bred, housed and exposed to alcohol in a similar manner to the other experiments.

Fetal Brain Dissection

On the day after the final alcohol infusion, GD 134, the sheep were euthanized with an overdose of sodium pentobarbital. The fetus was removed from the ewe and

perfused via the descending aorta with a phosphate-buffered saline (PBS) solution followed by a 1% (w/v) paraformaldehyde/1.25% (v/v) gluteraldehyde solution. Once the lower aspect of the fetus was well perfused, the perfusion catheter was placed in the common carotid trunk to further perfuse the brain in order to ensure adequate brain perfusion. The brain was then removed from the skull and further dissected. The olfactory bulbs were removed at the junction of the caudal olfactory bulb and olfactory tract. The brain was then divided parasagittally and the thalamus, subthalamic structures and brainstem were removed from the overlying cortical structures by cutting through the internal capsule. The hippocampus was then removed from the rest of the brain.

Tissue Processing

The olfactory bulbs and hippocampi were dehydrated through a series of ethanol solutions and then infiltrated with historesin (methyl methacrylate; Leica) by immersion in a series of historesin/ethanol solutions with gradually increasing historesin concentrations. When the regions were fully infiltrated with historesin, they were embedded in a 100% historesin/historesin hardener solution to polymerize the historesin. Care was taken to initially keep the embedded brains cool because the historesin polymerization is an exothermic reaction and large volumes of polymerizing historesin rapidly overheat and form bubbles around the embedded tissue. The embedded blocks were then allowed to fully harden. Once the blocks hardened, both the olfactory bulb and hippocampus were sectioned coronally into 30 μm serial sections using a microtome (Leica) equipped with a steel carbide knife. The sections were immersed in water and then mounted onto glass slides and allowed to dry. The sections were then stained in cresyl violet (a nucleic acid stain) and coverslipped.

Stereological Cell Counting

The mitral cells of the olfactory bulb and the CA1 and CA2/CA3 pyramidal and dentate gyrus cells of the hippocampus were counted. The mitral cells were only counted

in the anterior olfactory bulb where the mitral cell line is a complete circle (as viewed coronally).

In order to obtain the total estimated cell number in each region, the volume of each region was obtained using Cavalieri's Principle (Gundersen et al., 1988) by drawing a line around the region being counted and then placing a series of crosses (+) on the computer screen and counting the number of crosses overlying the region. Each of these crosses has a known area associated with it. The number of crosses counted was multiplied by the area associated with it as well as the distance between counted sections to obtain the reference volume (Vref) of each region (Eq. 5).

Eq. 5 $$\mathbf{V_{ref} = \Sigma pi \times A(pi) \times t}$$

where V_{ref} is the volume of the region being counted, Σpi is the sum of the number of points (pi) counted, *A*(pi) is the area associated with each point and *t* is the distance between counted sections.

The estimation of cell density was obtained by following the optical disector method (Gundersen and Jensen, 1987; West and Gundersen, 1990). The number of cells (using the nucleoli as a counting reference) were counted within a voxel (a two-dimensional frame with the z-axis as the third dimension) of known volume. The voxel was initially placed at a random spot outside of the tissue and then moved through the section at set intervals. Cells within each of these voxels were counted to obtain an estimate of cell density (Eq. 6)

Eq. 6 $$\mathbf{N_v = \frac{\Sigma Q}{\Sigma disector \times A(fr) \times h}}$$

where Nv is the numerical density of the counted cell type within the V_{ref}, Σ*Q* is the sum of the cells counted, Σdisector is the sum of the number of frames counted, A(fr) is the area associated with each disector frame and *h* is the known distance between the

top and bottom of the disector frame in the z-axis. A(fr) x *h* is the volume associated with the voxel in which the cells were counted.

The total estimated number of cells within the region is then calculated by multiply the reference volume by the cell density (Eq. 7).

Eq. 7 $$\mathbf{n = V_{ref} \times N_v}$$

where *n* is the total estimated number of cells.

Statistical Analyses

The data from these experiments were analyzed using an unpaired *t*-test to compare the EtOH and SC groups and another unpaired *t*-test to compare the SC and NC groups. This was repeated for each of the regions counted and for each dependent measure (cell number, region volume and cell density).

CHAPTER V

RESULTS

Experiment #1: Blood Flow after Repeated Alcohol Exposure

1.75 EtOH, 0.75 EtOH and Saline Control Groups

There were six brain regions and four non-neuronal organs that had significantly different blood flows among the treatment groups at the 0 hr time point (Figs. 6-16). The 0.75 EtOH group was significantly higher than the SC group in the anterior/lateral parietal cortex [$F(2, 22) = 3.815$, $p < 0.05$] (Fig. 7), posterior/dorsal temporal cortex [$F(2, 22) = 3.587$, $p < 0.05$] (Fig. 8), lateral/dorsal occipital cortex [$F(2, 22) = 3.863$, $p < 0.05$] (Fig. 9) and anterior/inferior cerebellum [$F(2, 21) = 3.644$, $p < 0.05$] (Fig. 11). The 0.75 EtOH group was also significantly higher than both the 1.75 EtOH and SC groups in the cingulate cortex [$F(2, 22) = 4.422$, $p < 0.05$] (Fig. 7) and head of the caudate nucleus [$F(2, 22) = 3.777$, $p < 0.05$] (Fig. 10). None of the other brain regions were significantly different among any of the groups. However, throughout most of the rest of the brain, there was a trend for the 0.75 EtOH group to be higher than both the 1.75 EtOH and SC groups and to a lesser extent for the 1.75 EtOH group to be slightly higher than the SC group (Figs. 6-16).

Analyses of the brain regions based on arterial supply (anterior, middle and posterior cerebral and basilar arteries) at the 0 hr time point did reveal any significant differences in blood flow among any of the treatment groups (Fig. 17). As in the individual brain regions, there was a small trend in the 1.75 EtOH group and a larger trend in the 0.75 EtOH group to have higher blood flows than the controls.

There were also four non-neuronal organs that showed statistically significant differences in blood flow among the treatment groups (Figs. 18-20). The 0.75 EtOH group was significantly higher than the SC group in the thyroids [$F(2, 20) = 4.542$, $p < 0.05$] and thymus [$F(2, 20) = 4.019$, $p < 0.05$], while there were no differences among the 1.75 EtOH group and the other groups in these regions (Fig. 18). The lung showed a significantly higher blood flow in the 0.75 EtOH group versus both the 1.75 EtOH and SC groups [$F(2, 21) = 5.114$, $p < 0.05$] (Fig. 19). The 0.75 EtOH group was significantly higher than the 1.75 EtOH group in the gonads with no differences among any of the groups from the SC group [$F(2, 20) = 3.834$, $p < 0.05$] (Fig. 18). Similar to the brain regions, the 0.75 group showed a trend towards higher blood flows in the organs versus the other groups, however, this trend was not as consistent among all organs. This trend was not evident in the muscle, skin (except for the skin over the triceps) or liver samples. The spleen showed a trend towards higher blood flows in both EtOH groups versus the SC group.

Saline Control vs. Normal Control Groups

There were no differences between the SC and NC groups in any of the brain regions or other organs measured. There was a small trend for some of the non-neuronal organs and most of the cortical regions in the NC group to be higher than the SC group.

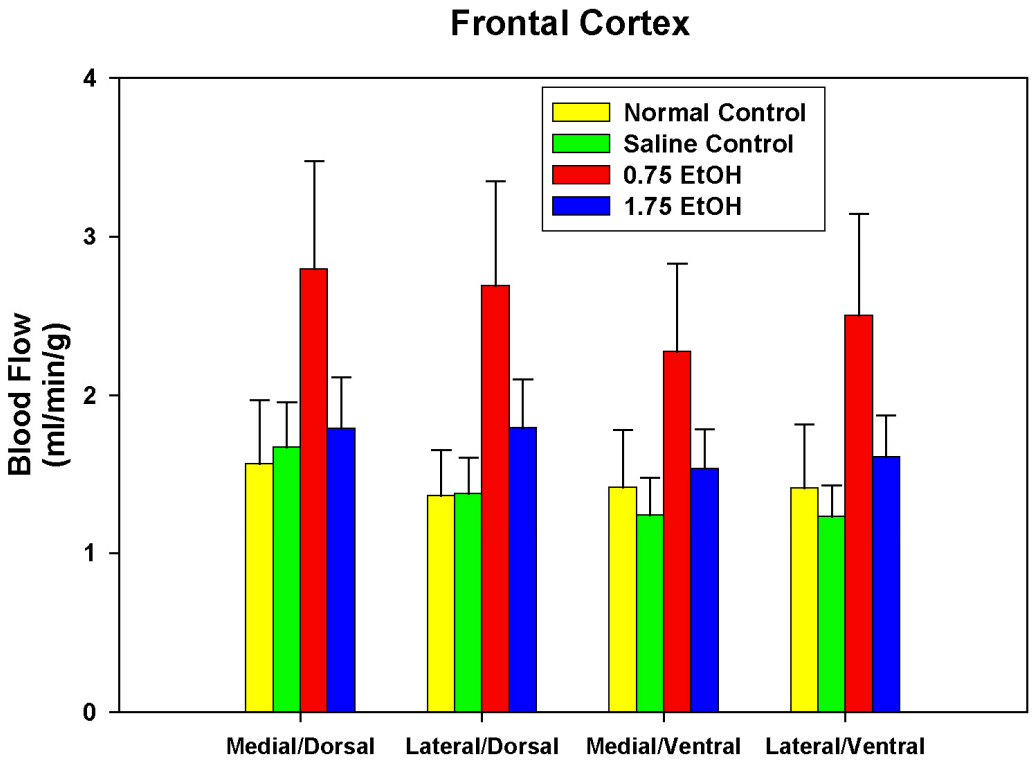

Figure 6: Blood Flow in the Frontal Cortex at the 0 hr Time Point. There were no significant differences among any of the treatment groups in any of the regions of the frontal cortex.

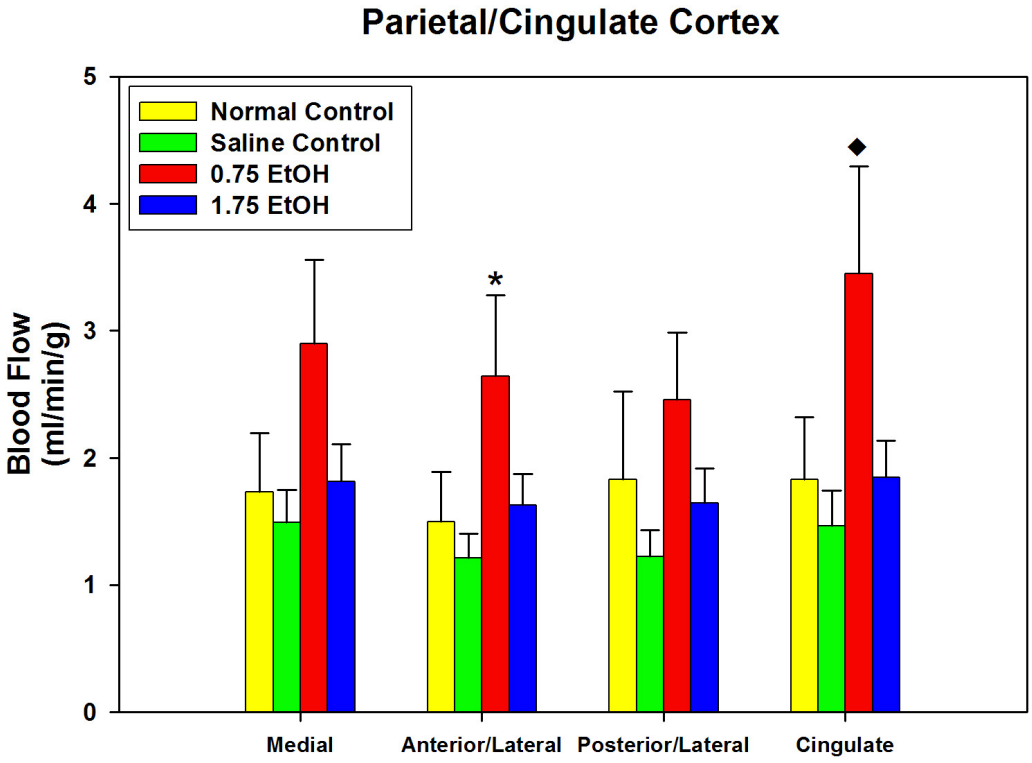

Figure 7: Blood Flow in the Parietal Cortex and Cingulate Cortex at the 0 hr Time Point. Blood flow was significantly higher (*: $p < 0.05$) in the anterior/lateral parietal cortex in the 0.75 EtOH group compared to the SC group. The 0.75 group was significantly higher (◆: $p < 0.05$) in the cingulate cortex compared to the 1.75 EtOH and SC groups. There were no differences among of the treatment groups in either the medial or posterior/lateral parietal cortex.

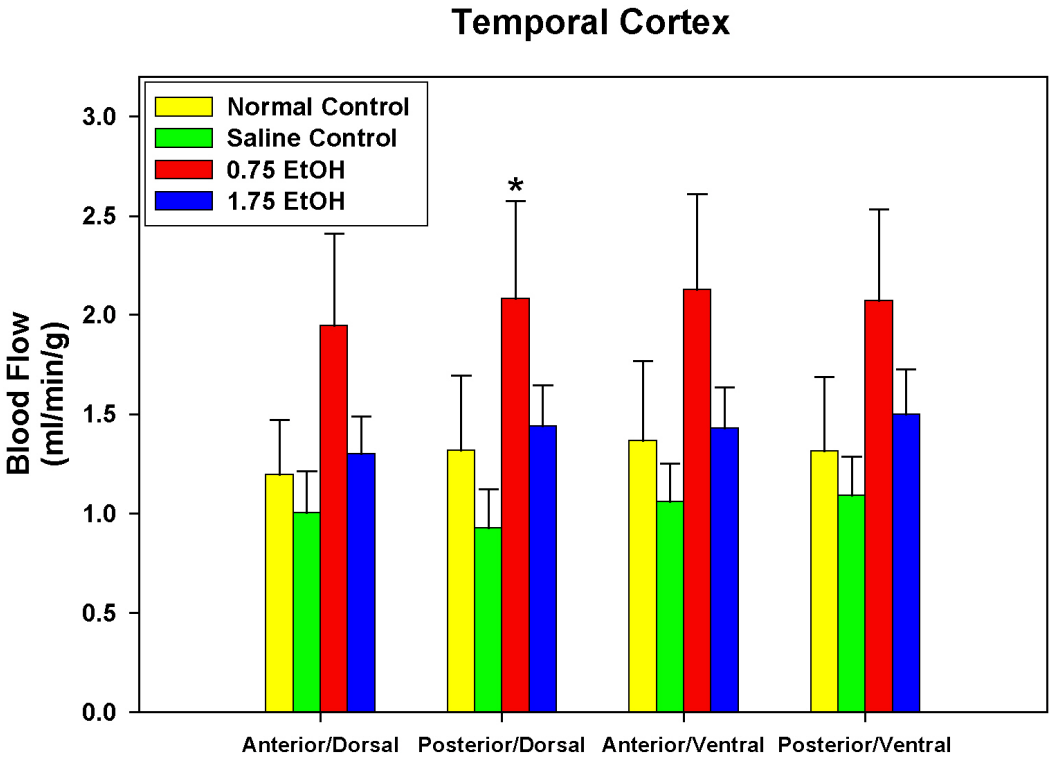

Figure 8: Blood Flow in the Temporal Cortex at the 0 hr Time Point. Blood flow was significantly increased (*: $p < 0.05$) in the posterior/dorsal temporal cortex in the 0.75 EtOH group compared to the 1.75 EtOH and SC groups. There were no significant differences among any of the treatment groups in the other areas of the temporal cortex.

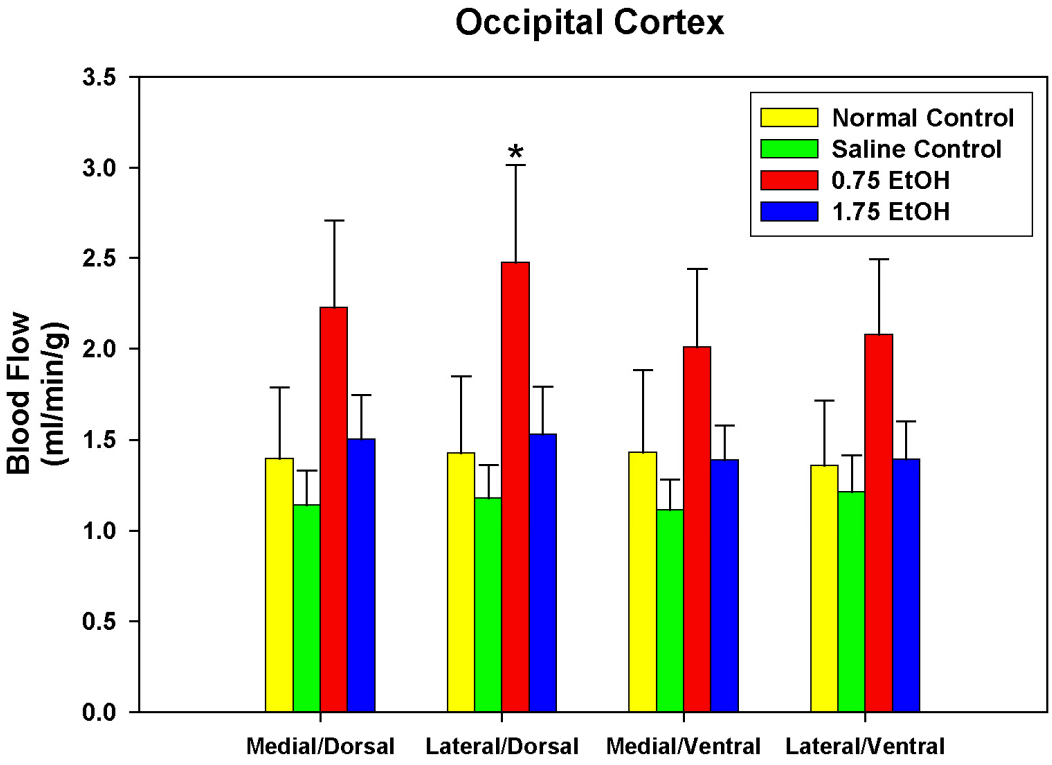

Figure 9: Blood Flow in the Occipital Cortex at the 0 hr Time Point. Blood flow in the lateral/dorsal occipital cortex was significantly higher (*: $p < 0.05$) in the 0.75 EtOH group compared to the SC group. There were no significant differences among any of the other treatment groups in the other regions of the occipital cortex.

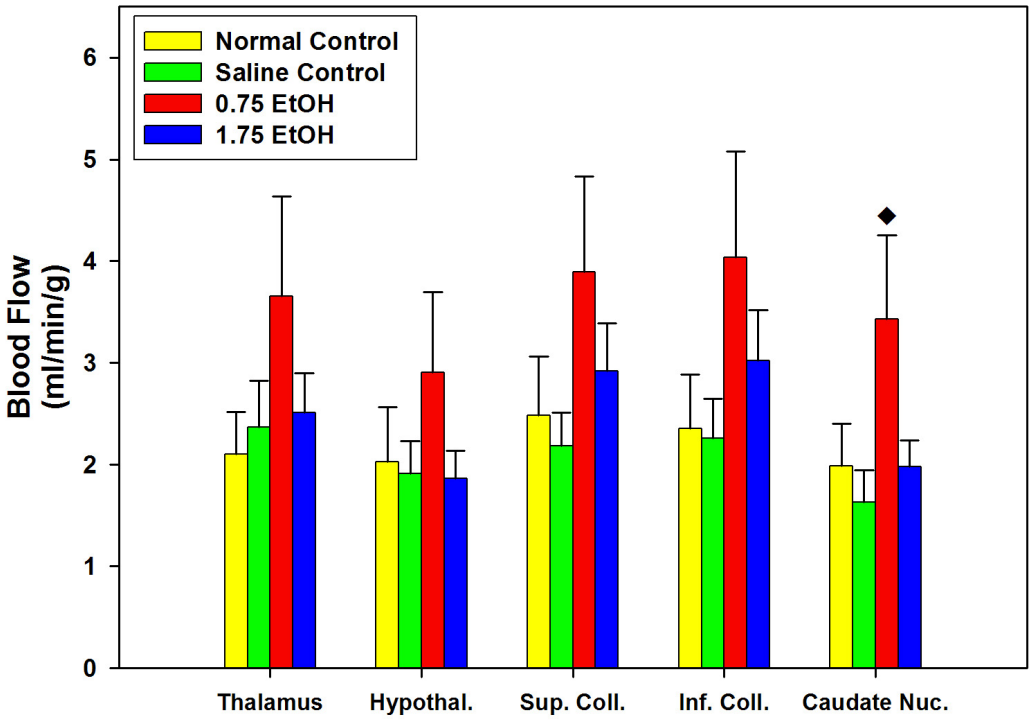

Figure 10: Blood Flow in the Subcortical Regions at the 0 hr Time Point. Blood flow was significantly increased (◆: $p < 0.05$) in the head of the caudate nucleus in the 0.75 EtOH group compared to the 1.75 EtOH and SC groups. There were no significant differences among any of the treatment groups in the thalamus, hypothalamus or superior and inferior colliculi.

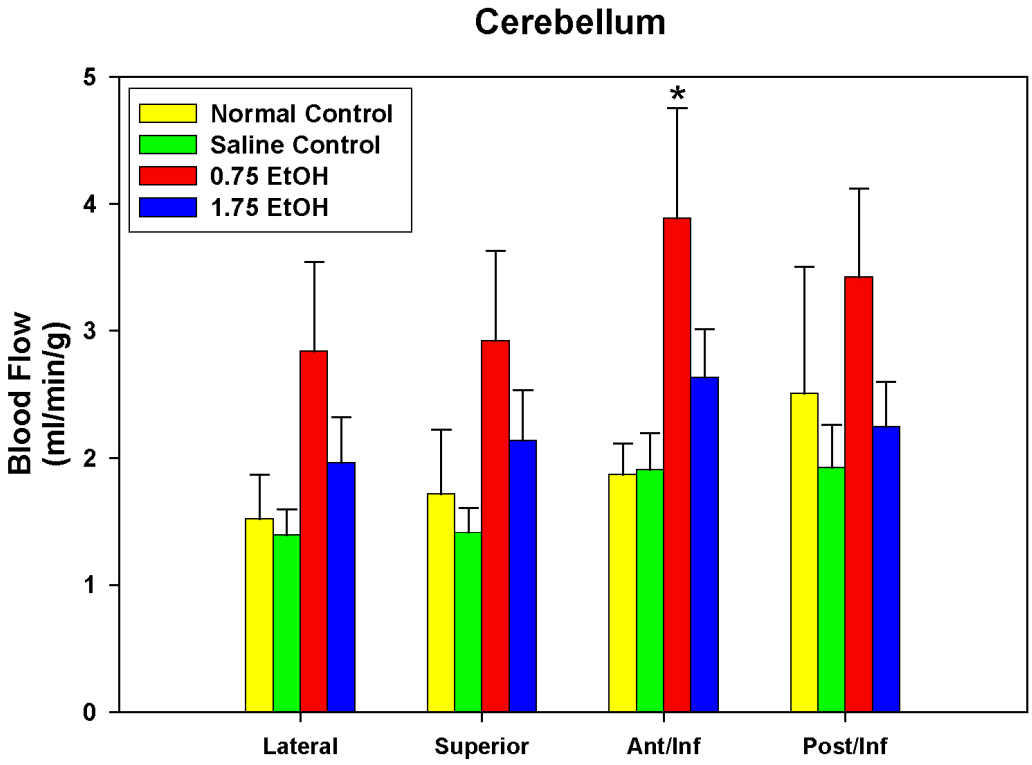

Figure 11: Blood Flow in the Cerebellum at the 0 hr Time Point. Blood flow in anterior/inferior cerebellum was significantly higher (*: $p < 0.05$) in the 0.75 EtOH group compared to the SC group. There were no differences among any of the treatment groups in the other regions of the cerebellum.

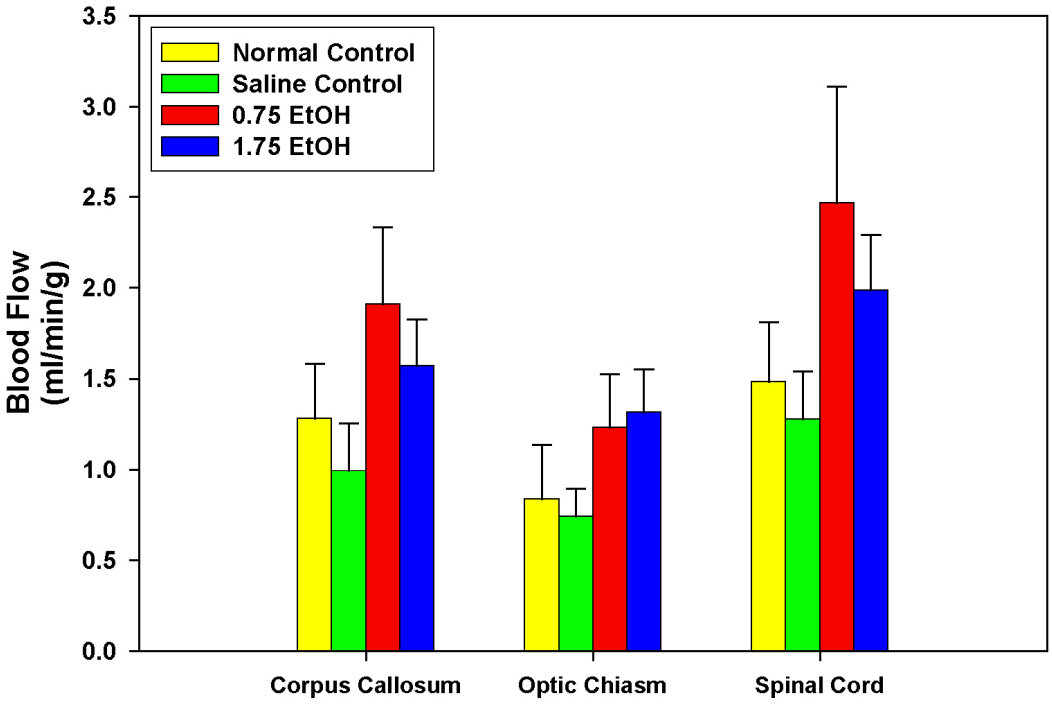

Figure 12: Blood Flow in the Corpus Callosum, Optic Chiasm and Rostral Cervical Spinal Cord at the 0 hr Time Point. There were no significant differences among any of the treatment groups in any of these regions.

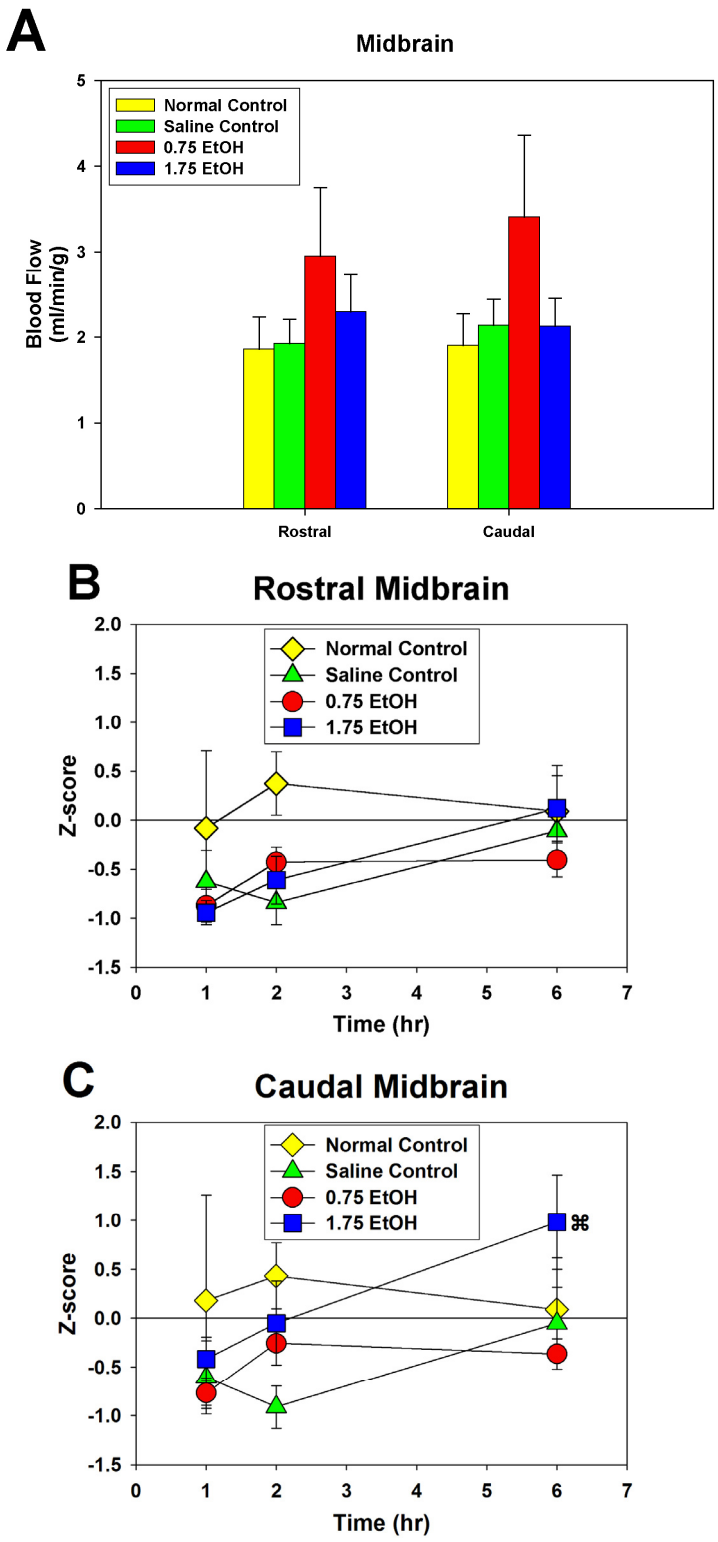

Figure 13: Blood Flow in the Midbrain. The 1.75 EtOH group was significantly higher (⌘: $p < 0.05$) than the 0.75 group in the caudal midbrain after the final alcohol exposure (C).

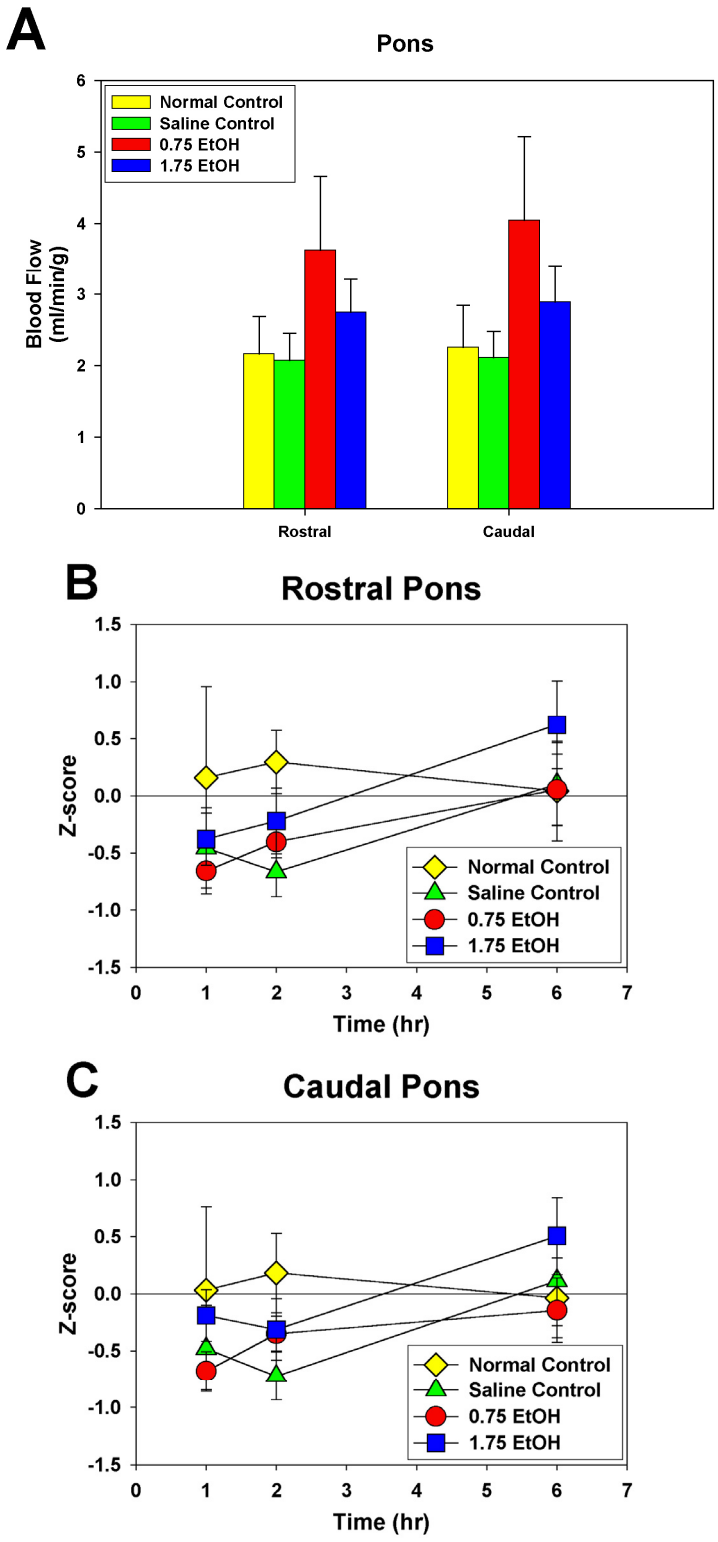

Figure 14: Blood Flow in the Pons. There were no differences among any of the groups at any of the times measured.

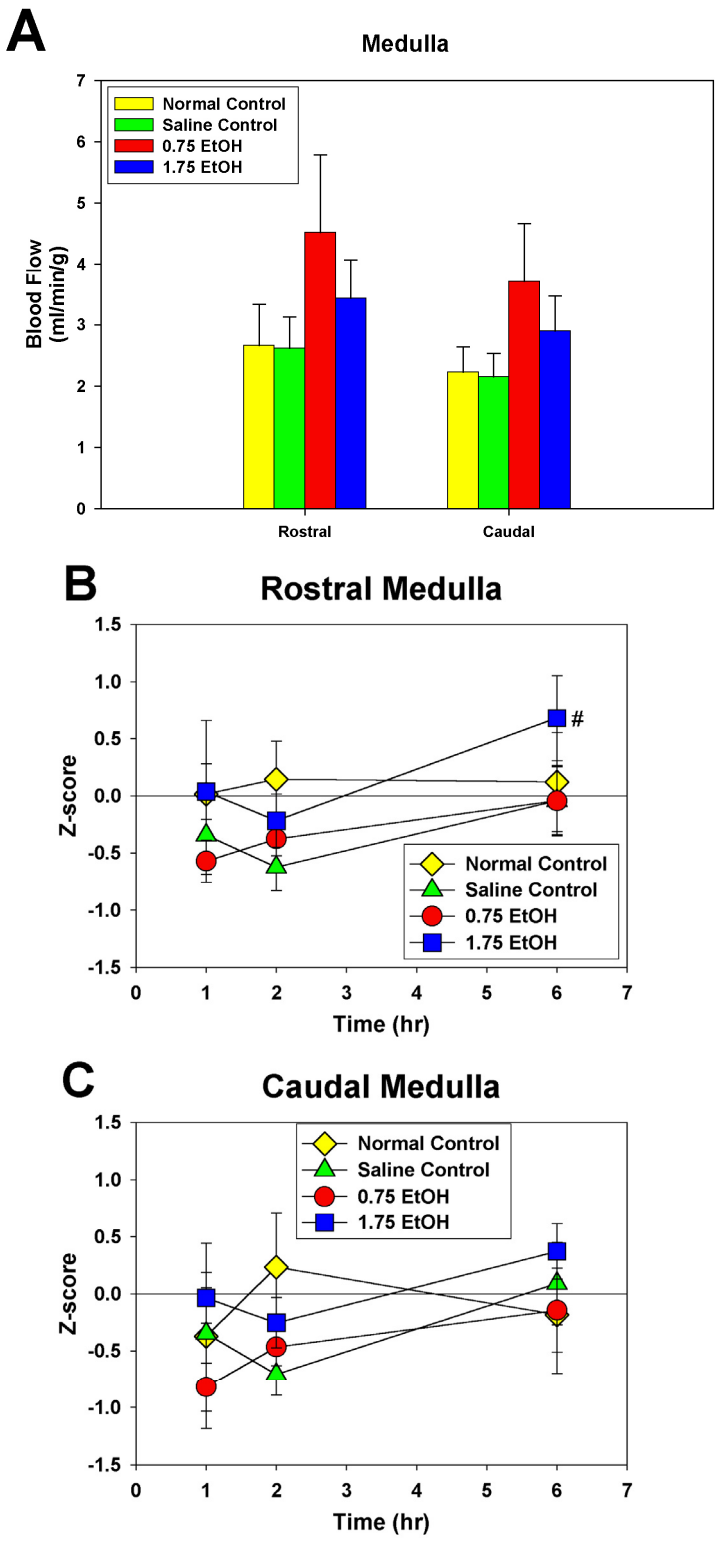

Figure 15: Blood Flow in the Medulla. Blood flow was significantly increased (#: $p < 0.05$) in the 1.75 EtOH group relative to the 0.75 and SC groups after the final alcohol exposure (B).

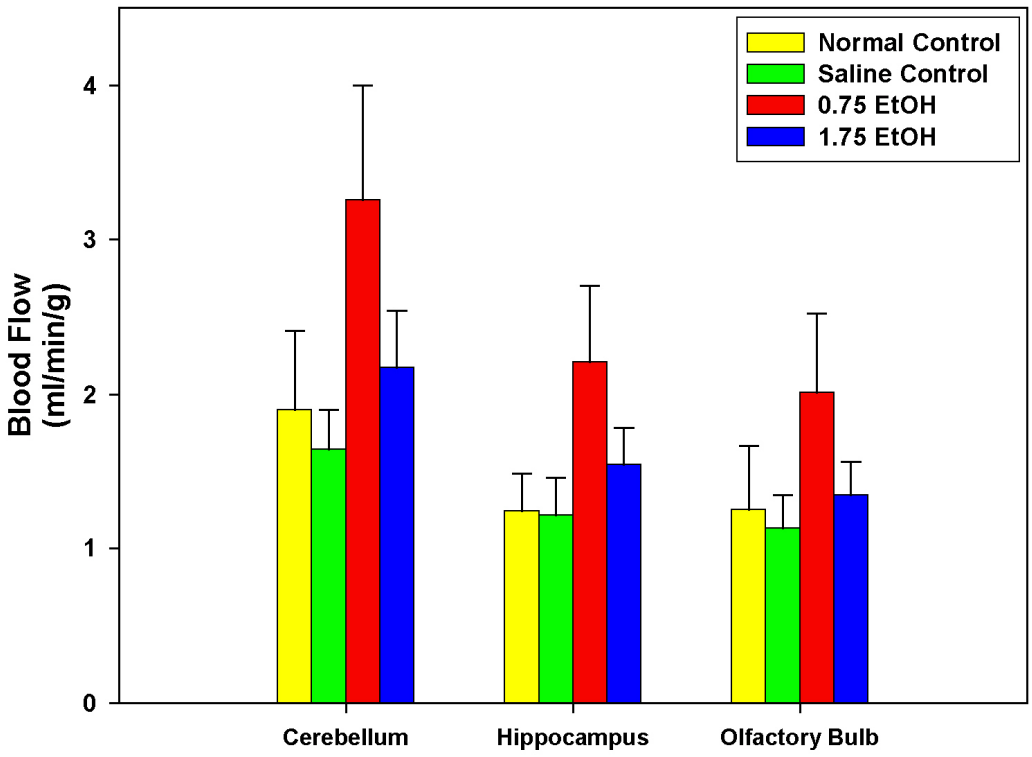

Figure 16: Blood Flow in the Cerebellum, Hippocampus and Olfactory Bulb at the 0 hr Time Point. There were no significant differences among any of the treatment groups in any of these brain regions.

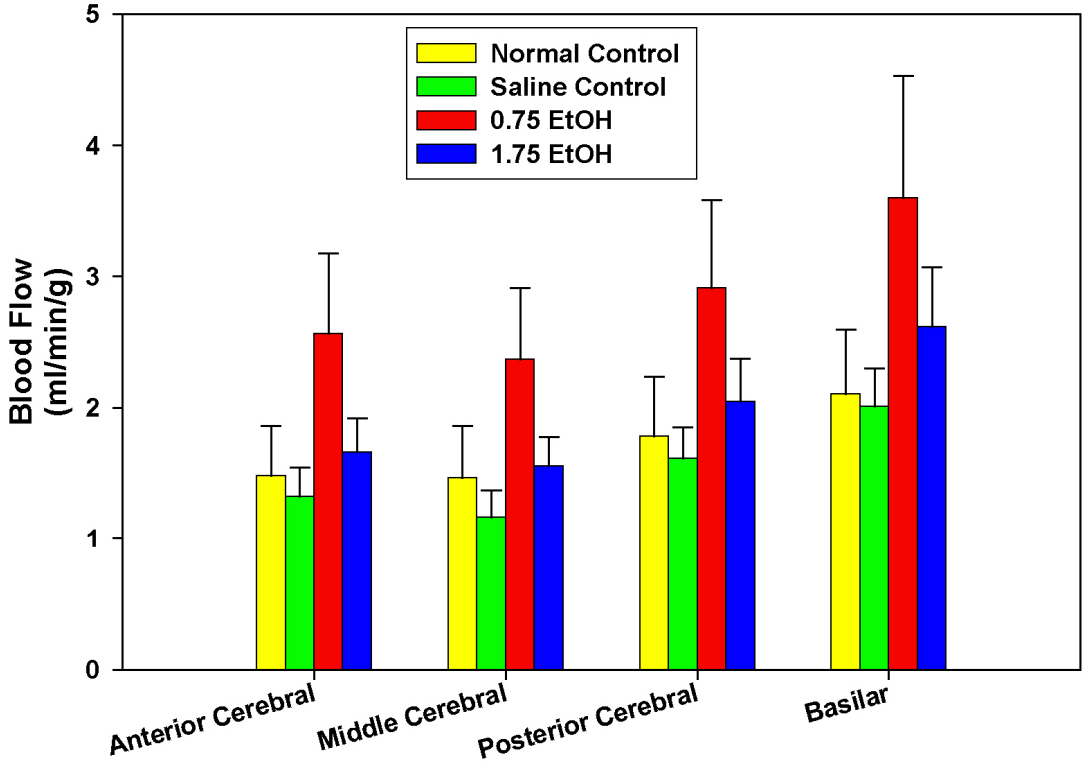

Figure 17: Blood Flow in the Major Arteries of the Brain at the 0 hr Time Point. There were no significant differences among any of the treatment groups in any of these arteries.

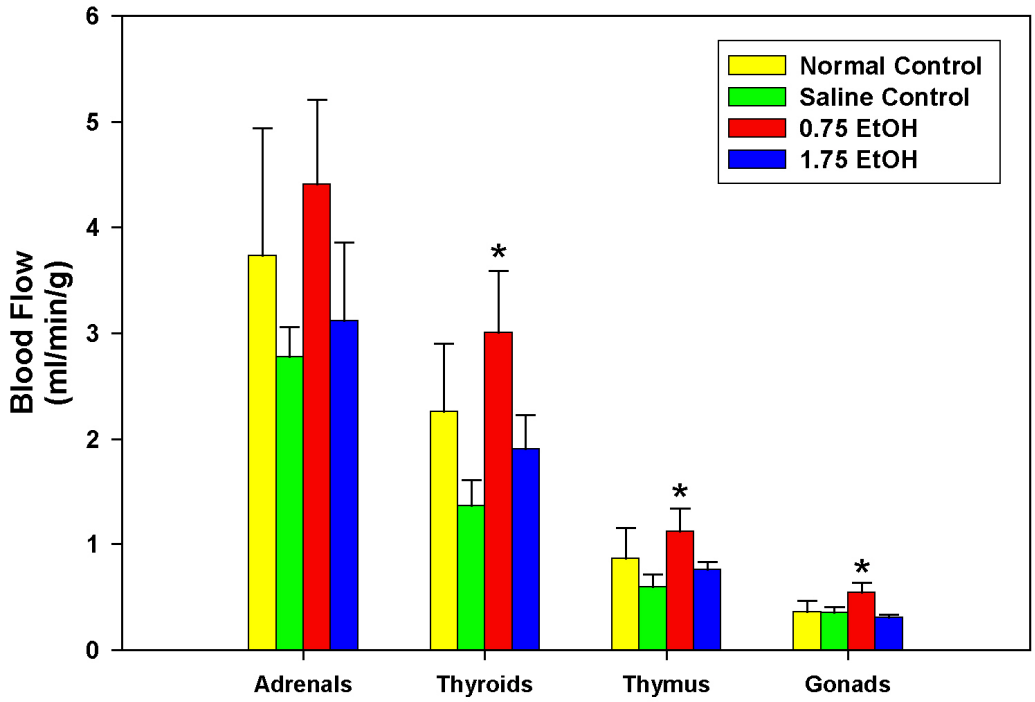

Figure 18: Blood Flow in the Adrenals, Thyroids, Thymus and Gonads at the 0hr Time point. The thyroids, thymus and gonads had significantly higher (*: $p < 0.05$) blood flows in the 0.75 EtOH group compared to the SC group. There were no significant differences among any of the treatment groups in the adrenals.

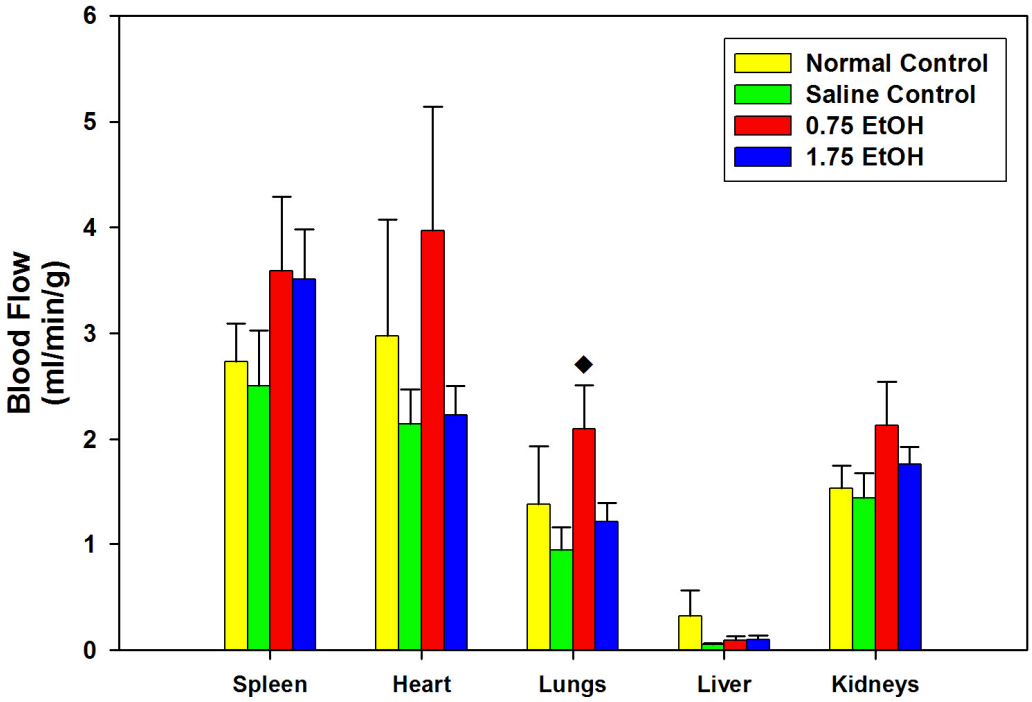

Figure 19: Blood Flow in the Spleen, Heart, Lungs, Liver and Kidneys at the 0 hr Time Point. Lung blood flow in the 0.75 EtOH group was significantly higher (◆: $p < 0.05$) than both the 1.75 EtOH and SC groups. There were no differences among any of the treatment groups in any of these other organs.

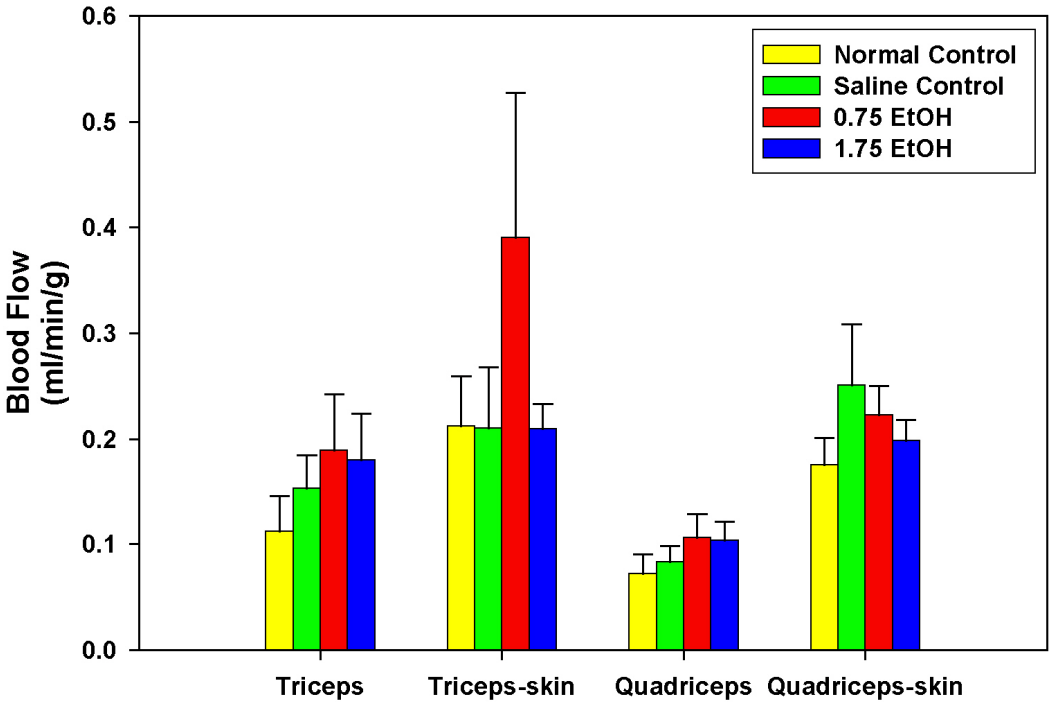

Figure 20: Blood Flow in the Triceps and Quadriceps Muscles and the Overlying Skin. There were no significant differences among any of the treatment groups in any of these areas.

Experiment #2: Blood Flow during the Final Alcohol Exposure

The data for the 1, 2 and 6 hr time points were normalized to the 0 hr time point due to the fact that some of the brain regions and organs were quite different among the treatment groups at the 0 hr time point and the majority of the rest showed a trend toward increases in the 0.75 EtOH group. Therefore, these analyses are based on changes in blood flow relative to the 0 hr time point. This normalization allows for comparisons among groups independent of the 0 hr baseline value in order to more accurately determine the possible changes in blood flow due to the final, acute alcohol exposure.

1.75 EtOH, 0.75 EtOH and Saline Control Groups

There were four brain regions and five non-neuronal organs that showed differences in blood flow in response to a final alcohol exposure among the treatment groups (Figs. 13-15, 21-34). The 1.75 EtOH group was significantly higher than both the 0.75 EtOH and SC groups in the hypothalamus [$F(2, 62) = 5.237$, $p < 0.01$] (Fig. 26a), posterior/inferior cerebellum [$F(2, 61) = 3.175$, $p < 0.05$] (Fig. 27d) and rostral medulla [$F(2, 62) = 3.483$, $p < 0.05$] (Fig. 15b). The caudal midbrain had a significantly higher blood flow in the 1.75 EtOH group relative to the 0.75 EtOH group but not the SC group [$F(2, 60) = 4.29$, $p < 0.05$] (Fig. 13c).

The analyses from the brain regions based on arterial supply (anterior, middle and posterior cerebral and basilar arteries) demonstrated that there were no significant differences among any of the treatment groups at the 1, 2 or 6 hr time points (Fig. 30).

The non-neuronal organs showed a similar pattern of change in blood flow compared to the brain, where the 1.75 EtOH group had increased blood flows (Figs. 31-34). The thyroid was the only region measured that had decreases in blood flow due to alcohol exposure. In this organ the SC group was significantly higher than the 1.75 EtOH group, but there were no differences between the 0.75 group and the 1.75 EtOH and SC groups [$F(2, 57) = 3.25$, $p < 0.05$] (Fig. 31b). The thymus demonstrated a higher blood flow in the 1.75 group versus the 0.75 group, while the SC group was not significantly different from the others [$F(2, 59) = 3.395$, $p < 0.05$] (Fig. 31c). The 1.75 EtOH group had a higher blood flow in the liver compared to the SC but not the 0.75 group [$F(2, 60) = 3.554$, $p < 0.05$] (Fig. 33c). The quadriceps muscles [$F(2, 57) = 7.342$, $p < 0.005$] and gonads [$F(2, 59) = 5.461$, $p < 0.01$] (Fig. 31d had higher blood flows compared to both the 0.75 EtOH and SC groups (Fig. 34b).

Saline Control vs. Normal Control

There was only one difference between the SC and NC groups, with the NC group having a higher blood flow in the thalamus than the SC group [$F(1, 32) = 4.515$, $p < 0.05$] (Fig. 25a).

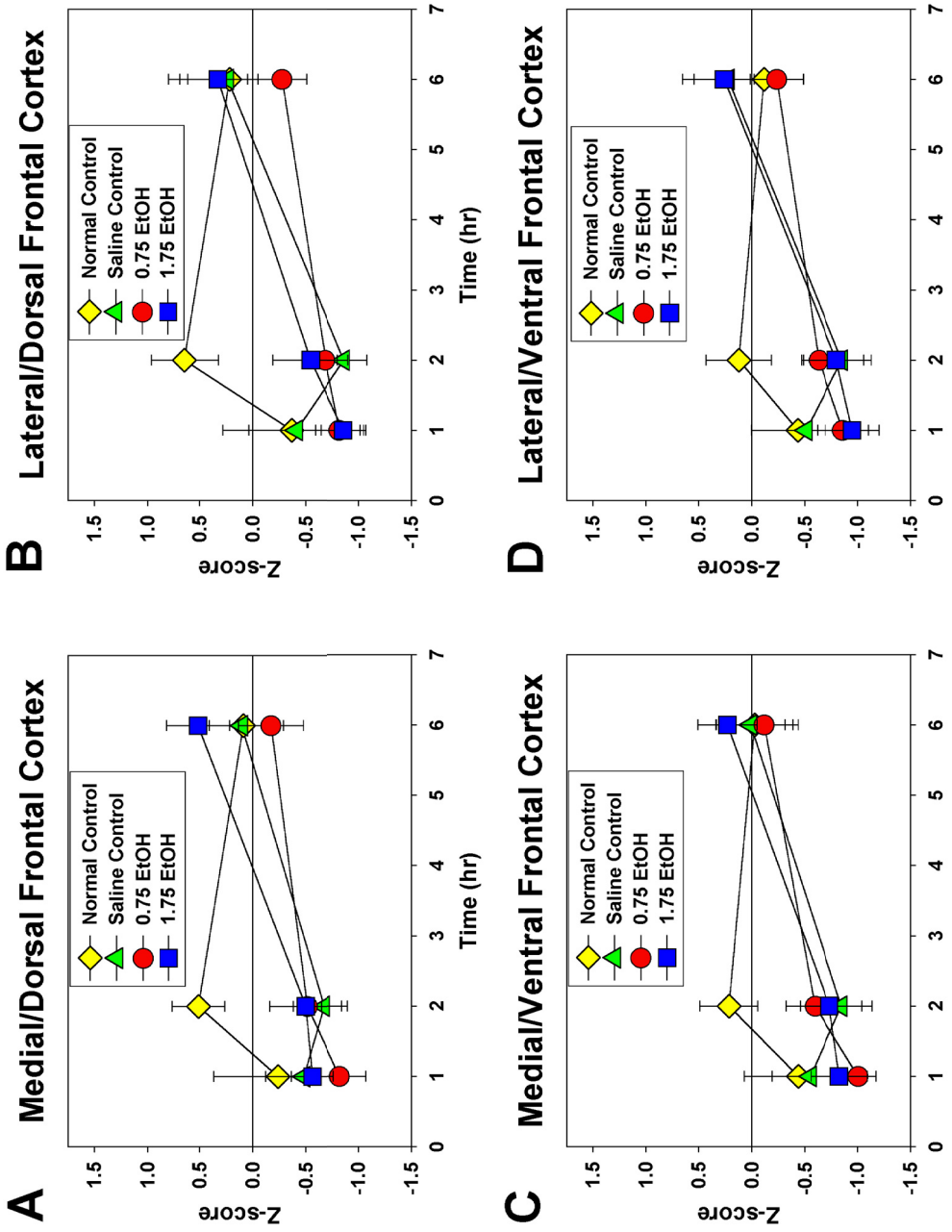

Figure 21: Blood Flow in the Frontal Cortex at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in the frontal cortex regions.

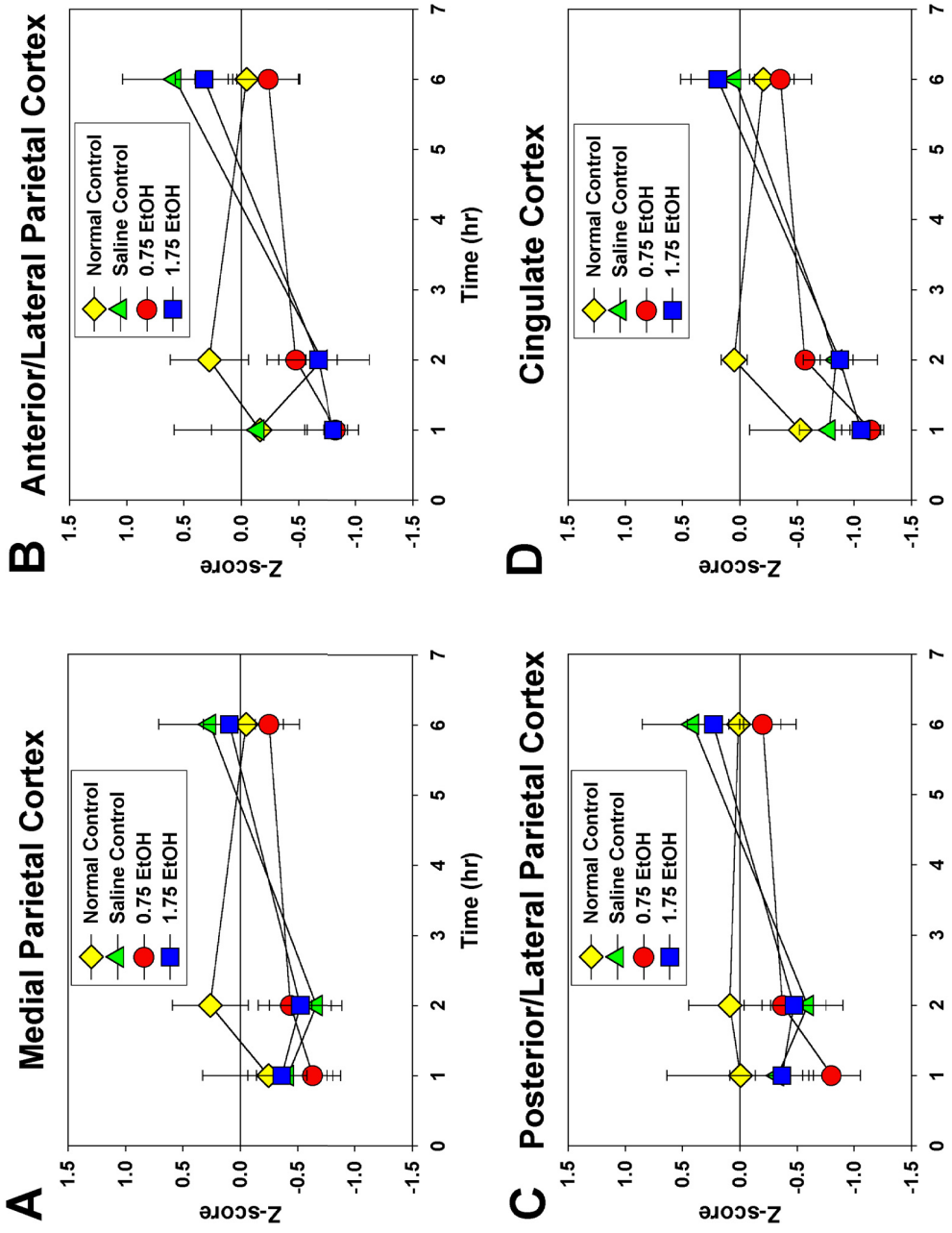

Figure 22: Blood Flow in the Parietal and Cingulate Cortex at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these brain regions.

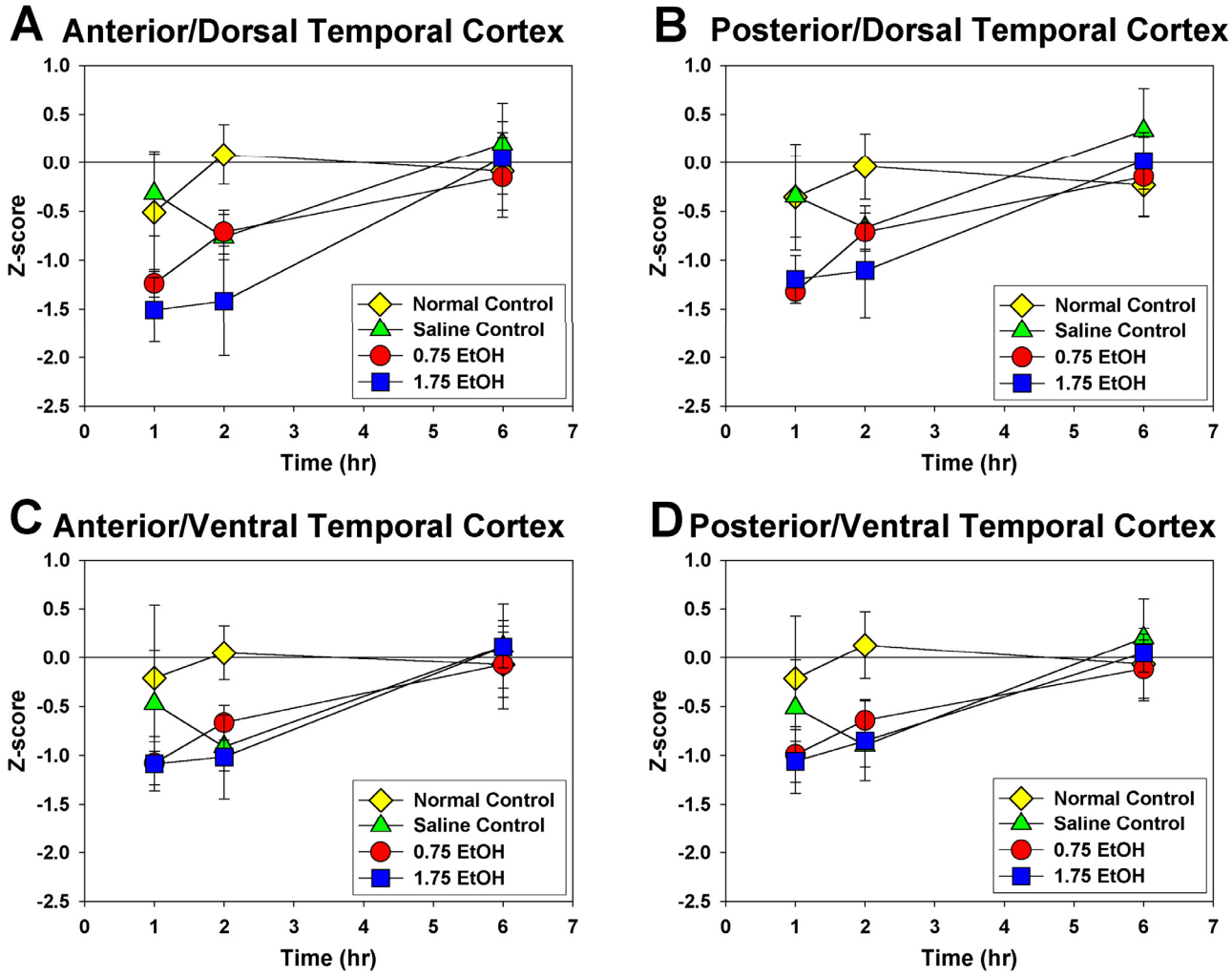

Figure 23: Blood Flow in the Temporal Cortex at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these brain regions.

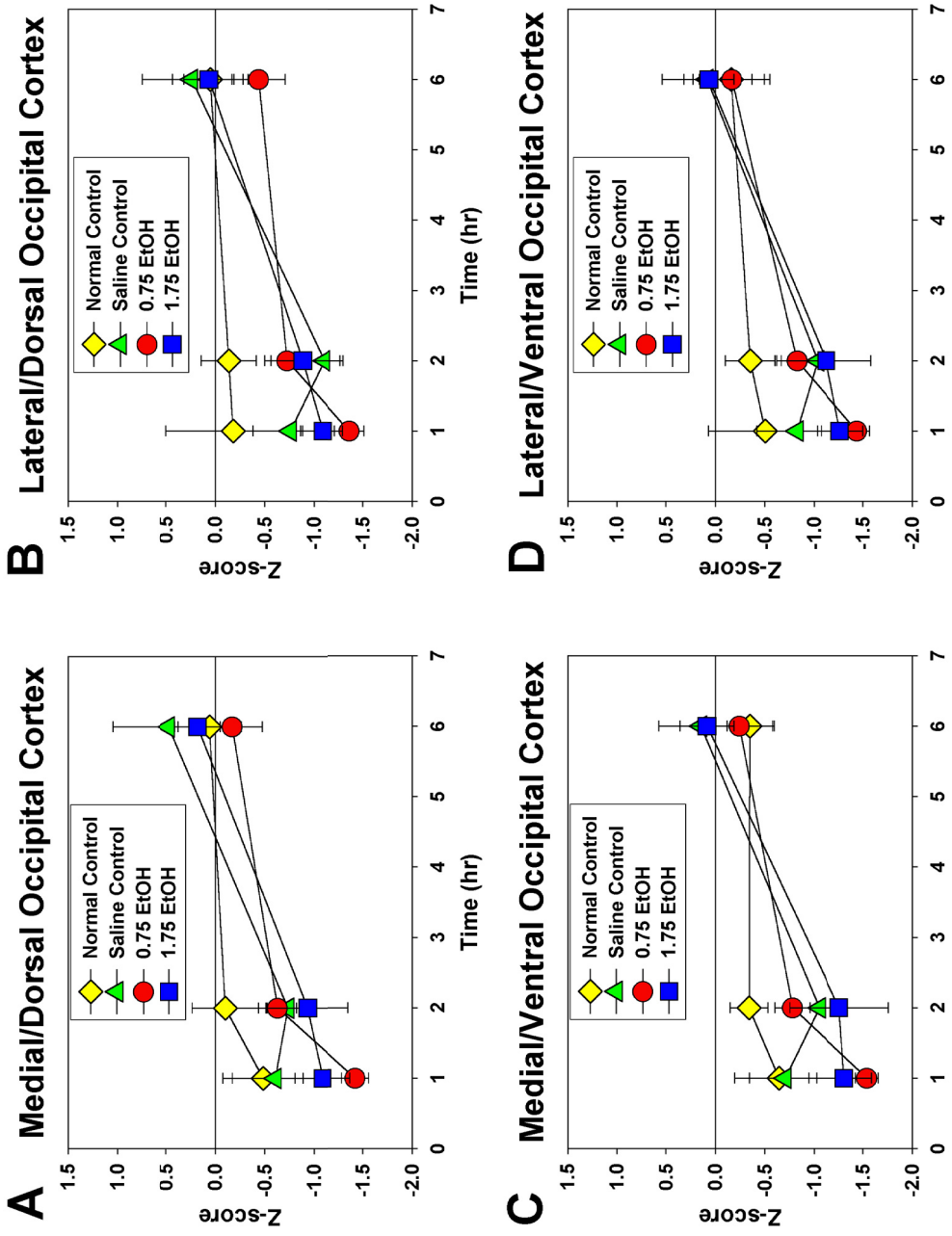

Figure 24: Blood Flow in the Occipital Cortex at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these brain regions.

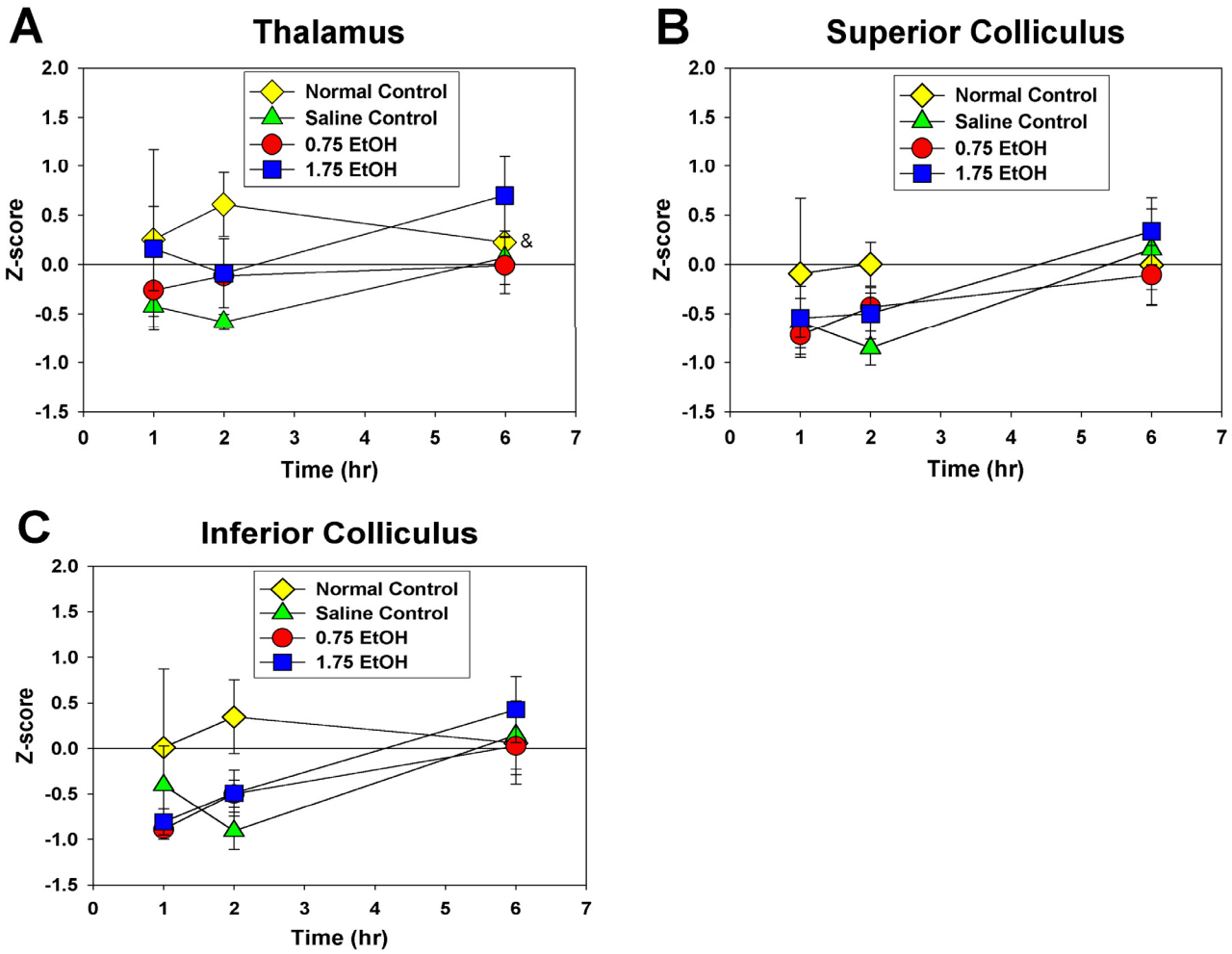

Figure 25: Blood Flow in the Thalamus, Superior Colliculus and Inferior Colliculus at the 1, 2 and 6 hr Time Points. There was a significant difference (&: $p < 0.05$) between the NC and SC groups in the thalamus (A). There were no significant differences among any of the treatment groups in any of the other regions.

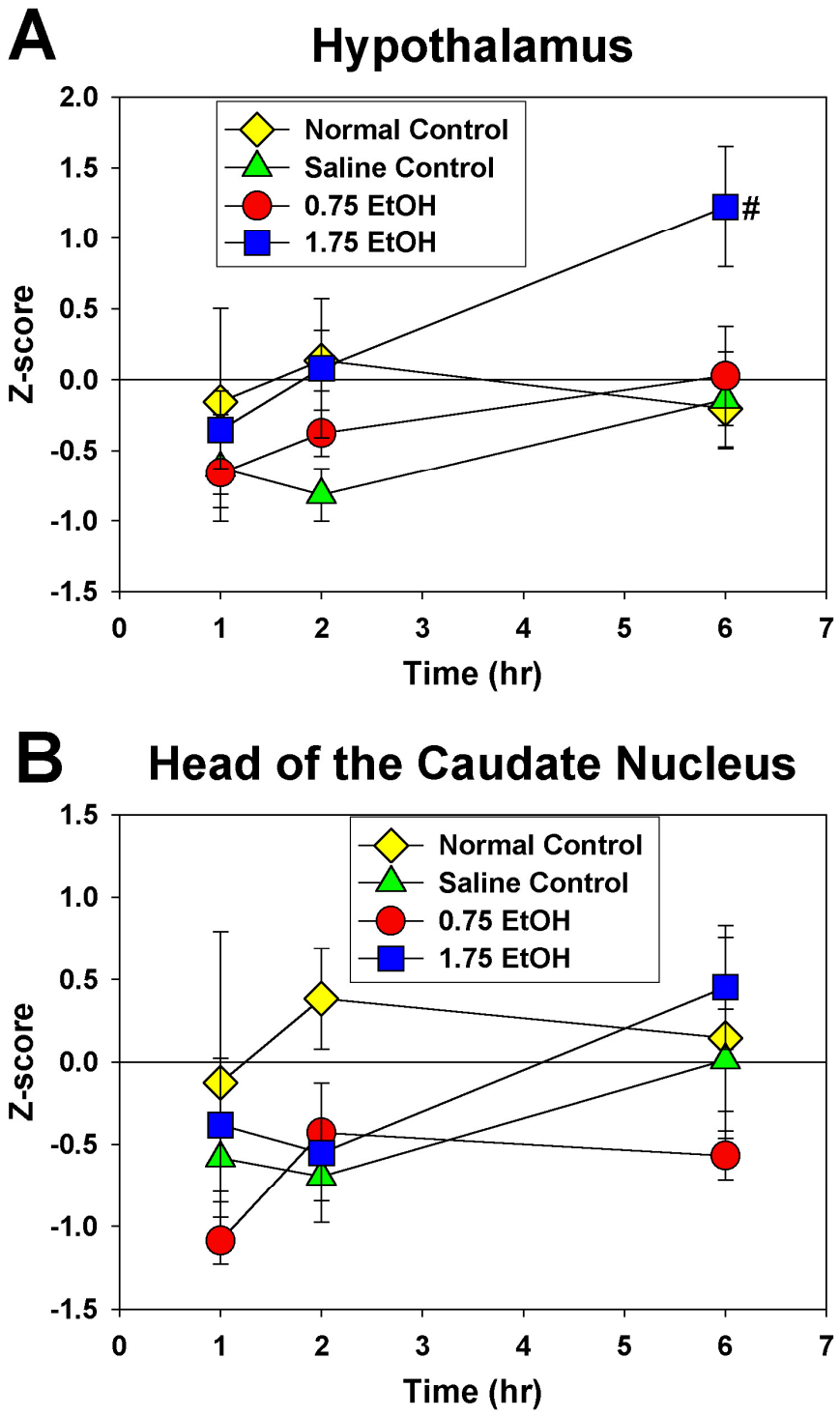

Figure 26: Blood Flow in the Hypothalamus and Head of the Caudate Nucleus at the 1, 2 and 6 hr Time Points. The 1.75 EtOH group was significantly higher (#: $p < 0.01$) than the 0.75 EtOH and SC groups (A).

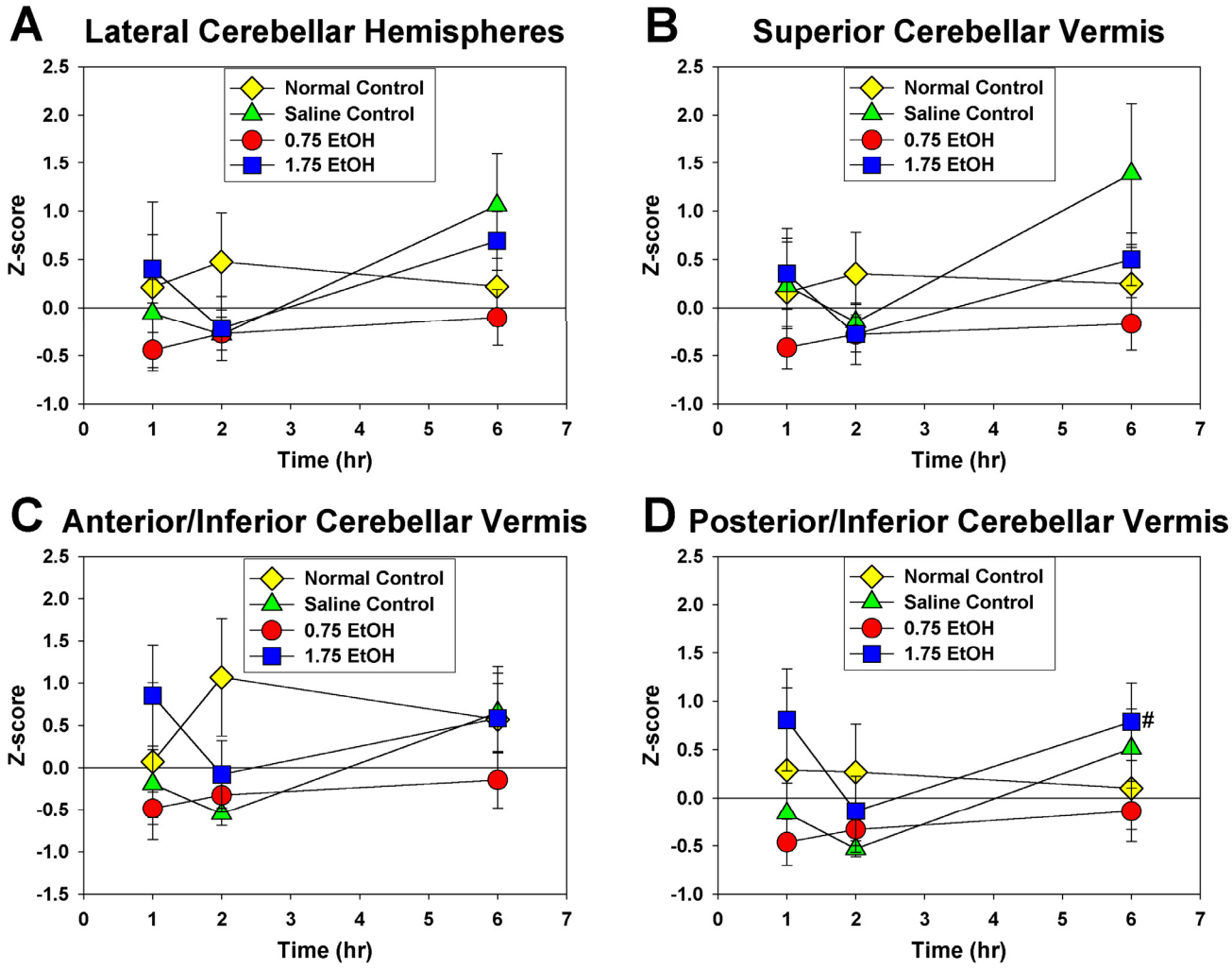

Figure 27: Blood Flow in the Cerebellum at the 1, 2 and 6 hr Time Points. The 1.75 EtOH group was significantly higher (#: $p < 0.05$) than the 0.75 EtOH and SC groups in the posterior/inferior cerebellar vermis (D).

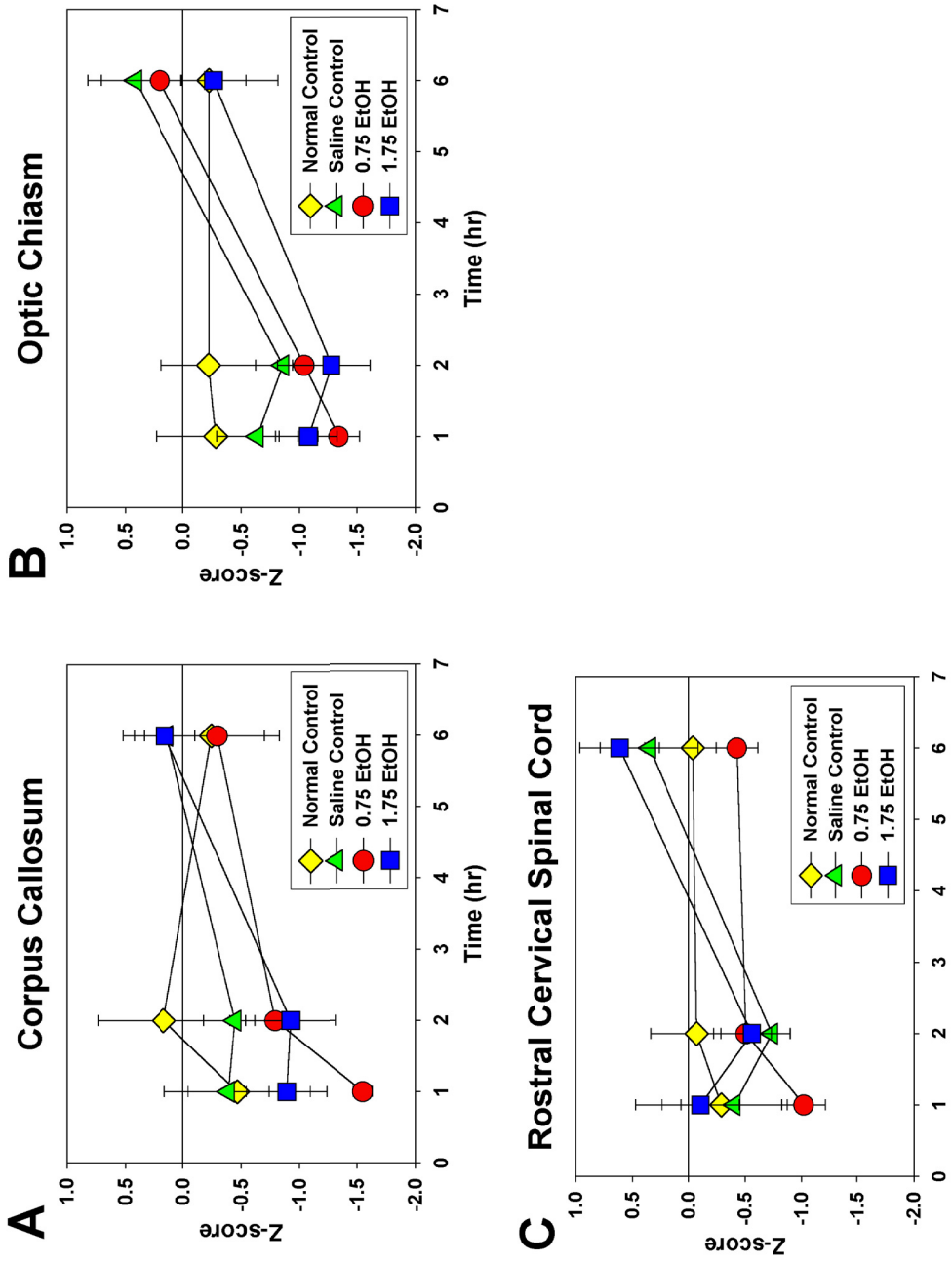

Figure 28: Blood Flow in the Corpus Callosum, Optic Chiasm and Rostral Cervical Spinal Cord at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these regions.

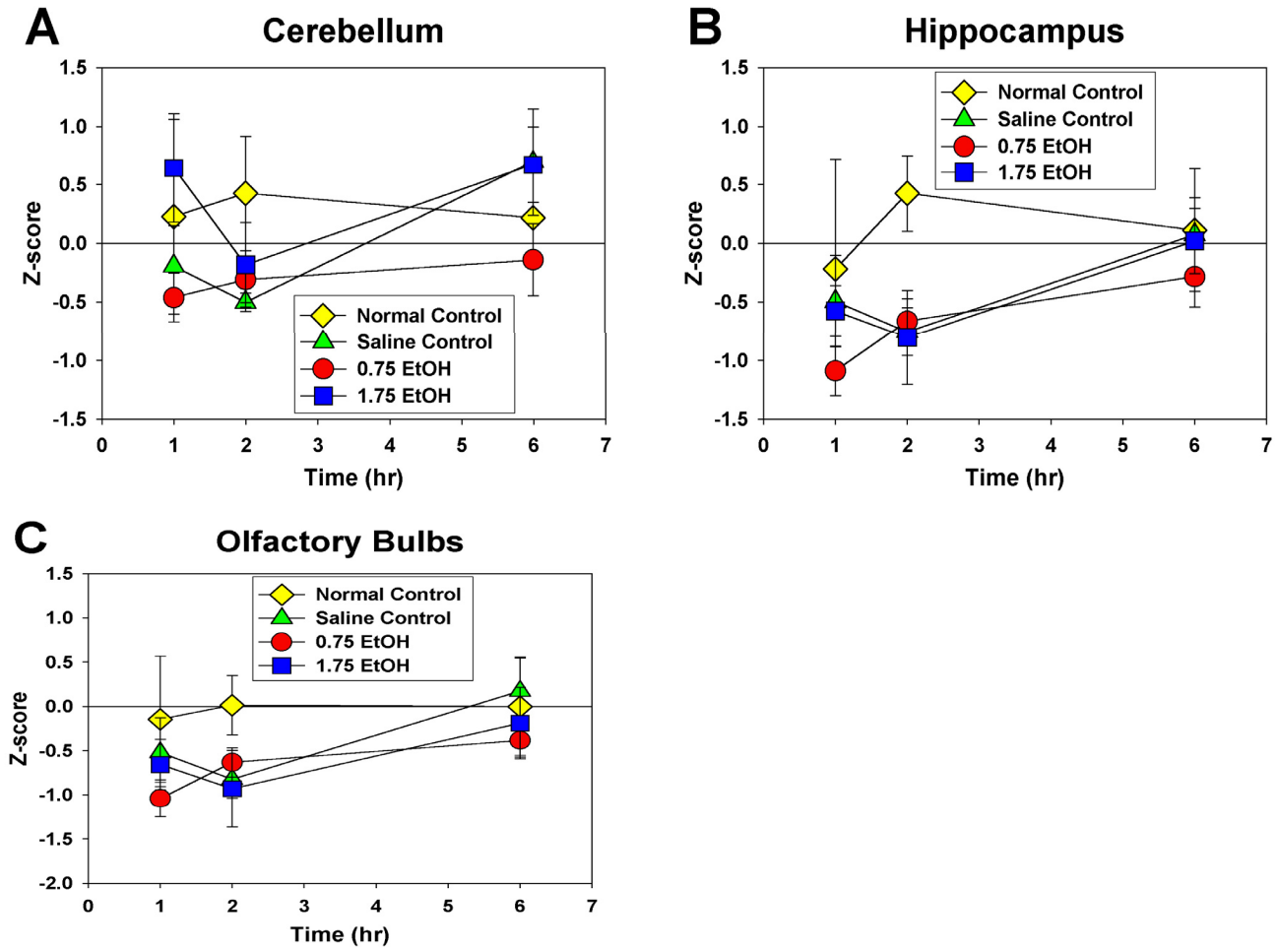

Figure 29: Blood Flow in the Cerebellum, Hippocampus and Olfactory Bulb at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these regions.

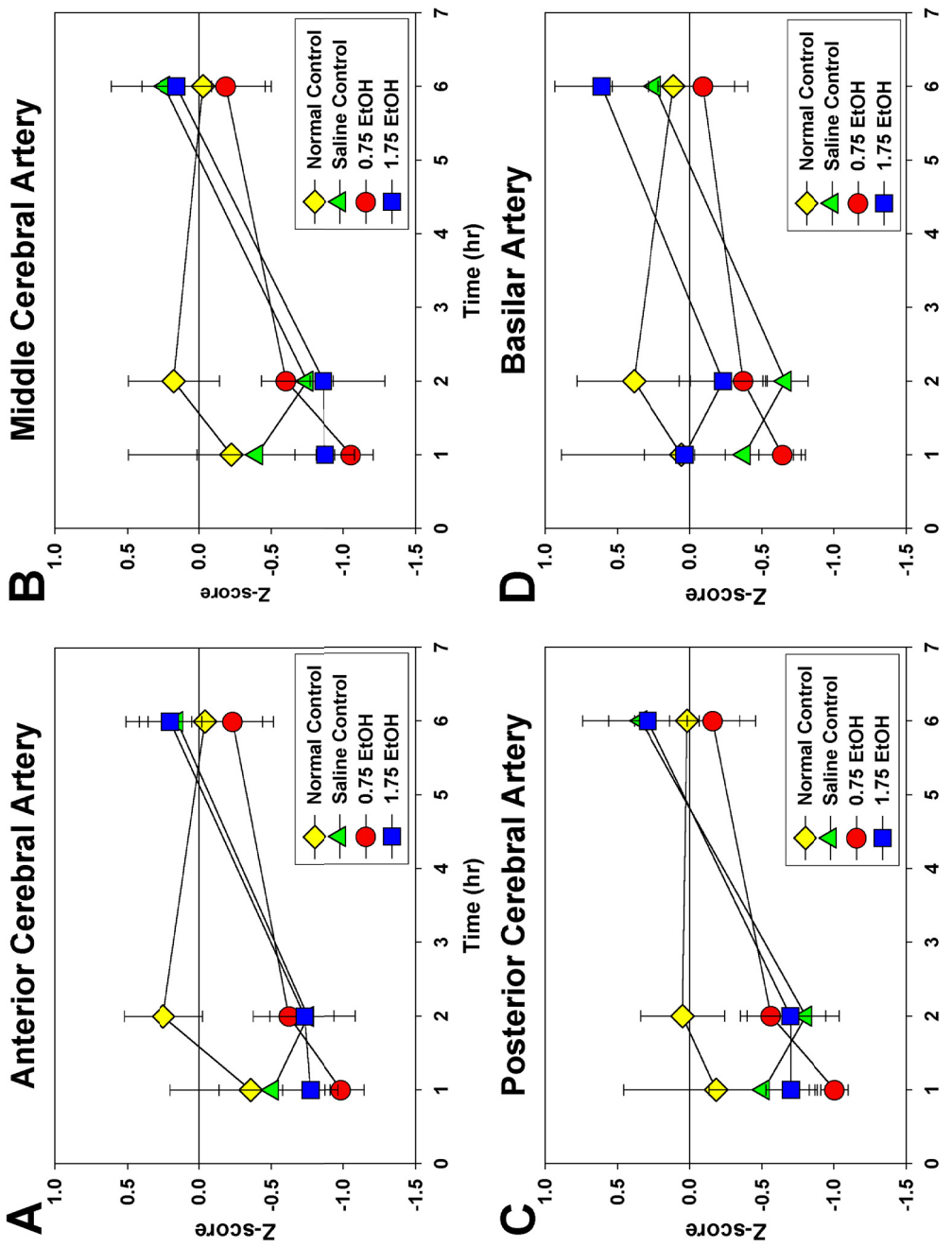

Figure 30: Blood Flow in the Major Arteries of the Brain at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in any of these arteries.

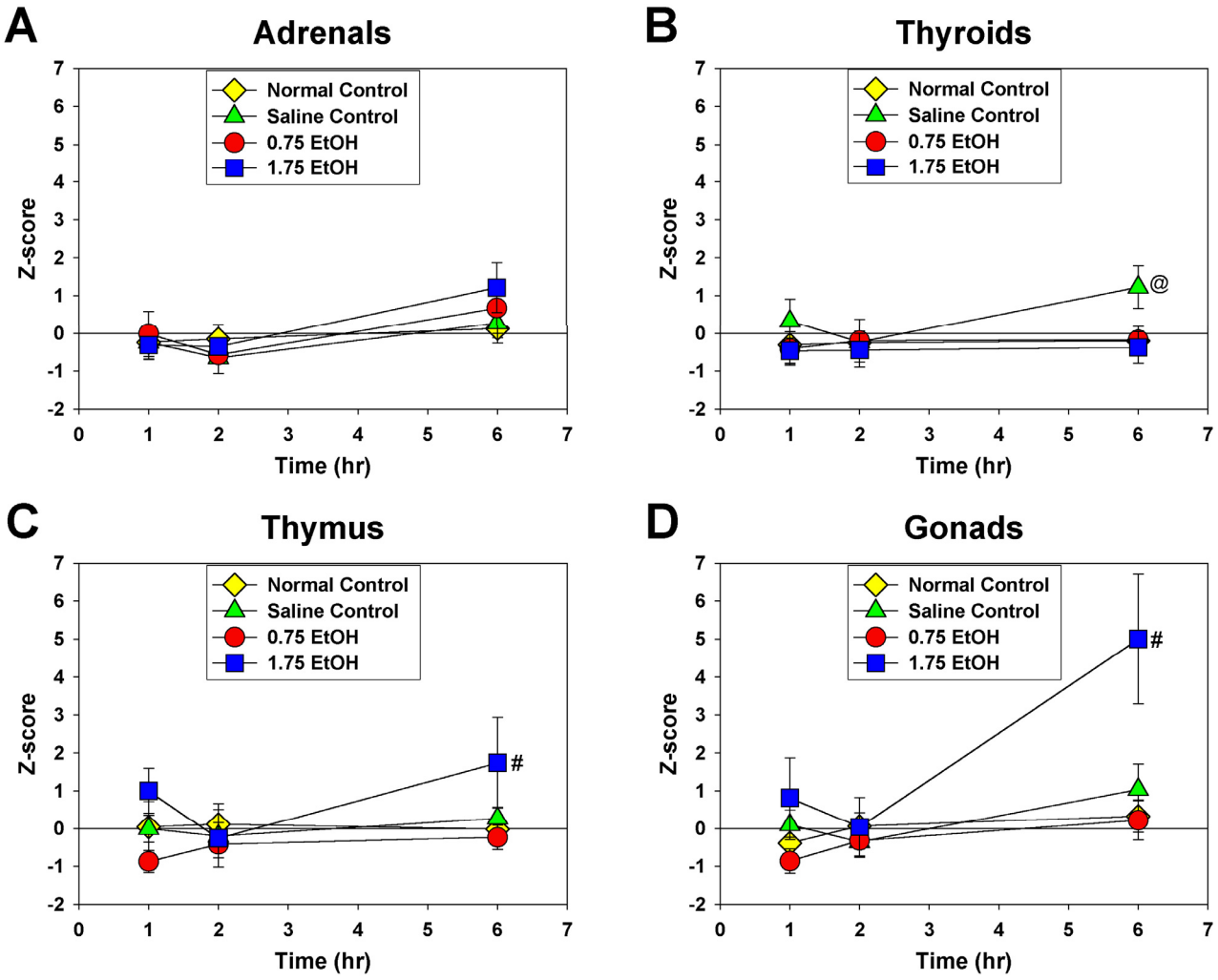

Figure 31: Blood Flow in the Adrenals, Thyroids, Thymus and Gonads at the 1, 2 and 6 hr Time Points. SC was higher (@) than 1.75 EtOH (B) in the thyroids while 1.75 EtOH was higher (#: $p < 0.05$) than 0.75 EtOH and SC in the thymus (C) and gonads (D).

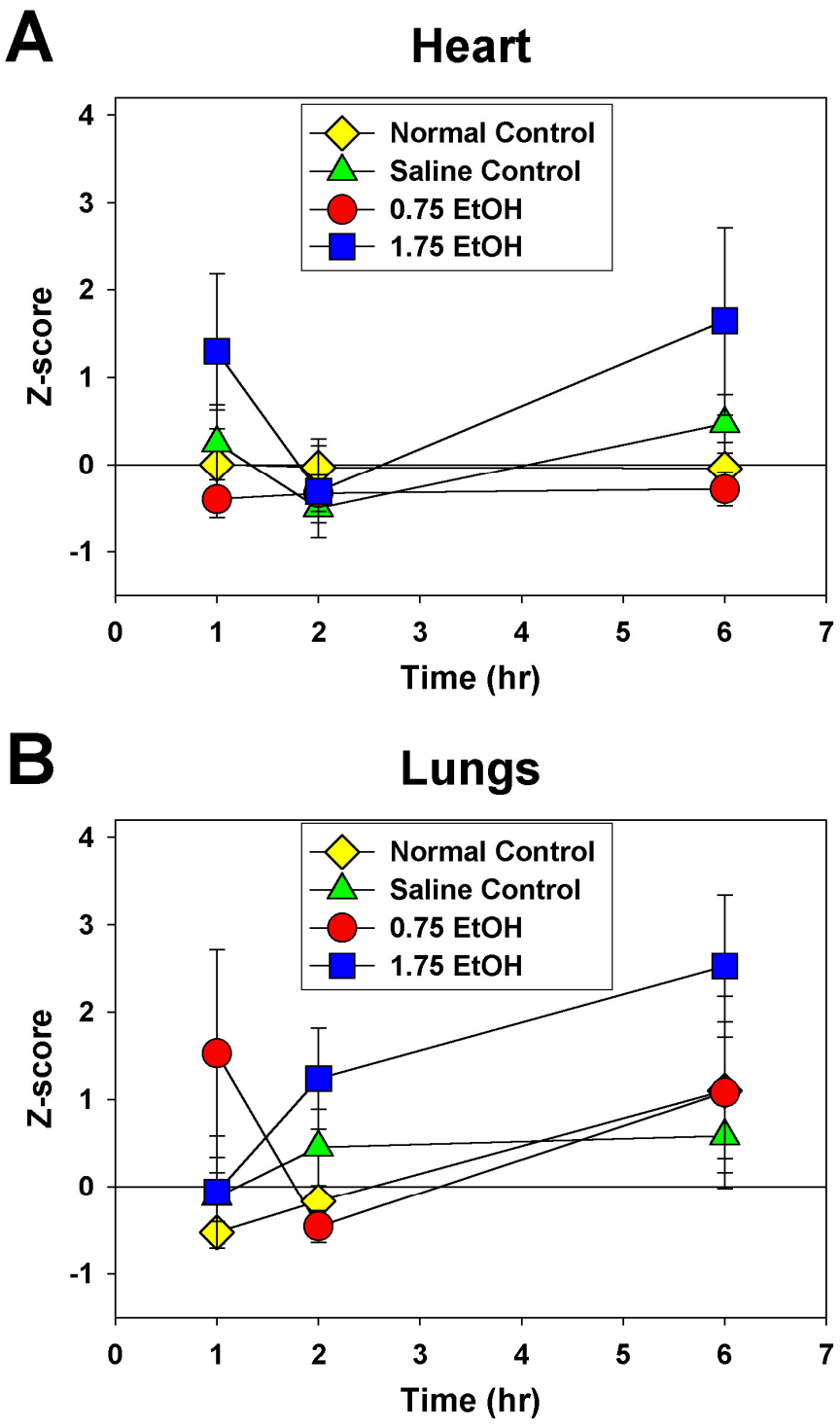

Figure 32: Blood Flow in the Heart and Lungs at the 1, 2 and 6 hr Time Points. There were no significant differences among any of the treatment groups in either of these organs.

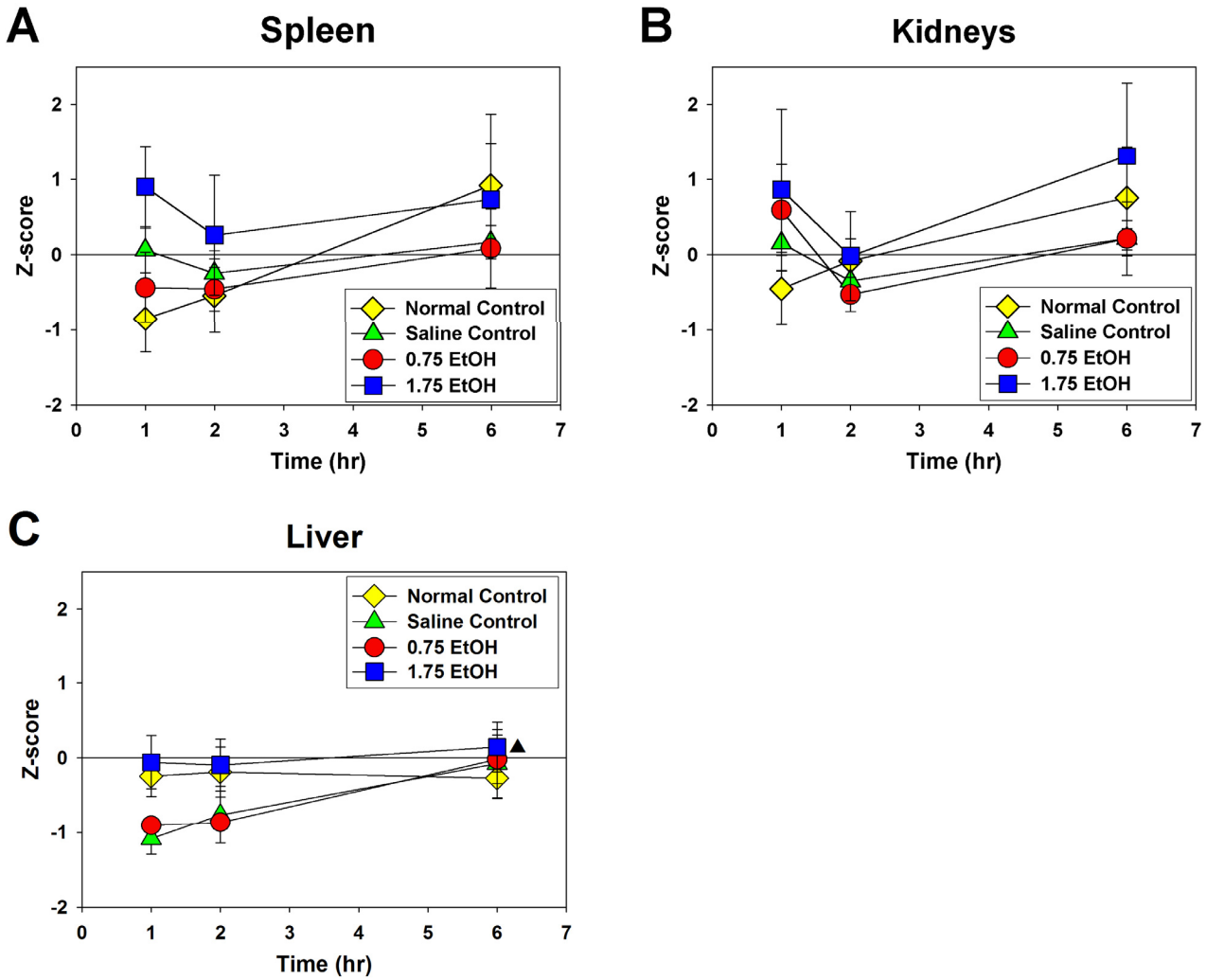

Figure 33: Blood Flow in the Spleen, Kidneys and Liver at the 1, 2 and 6 hr Time Points. The 1.75 EtOH group was significantly higher (▲: $p < 0.05$) than the SC group in the liver (C).

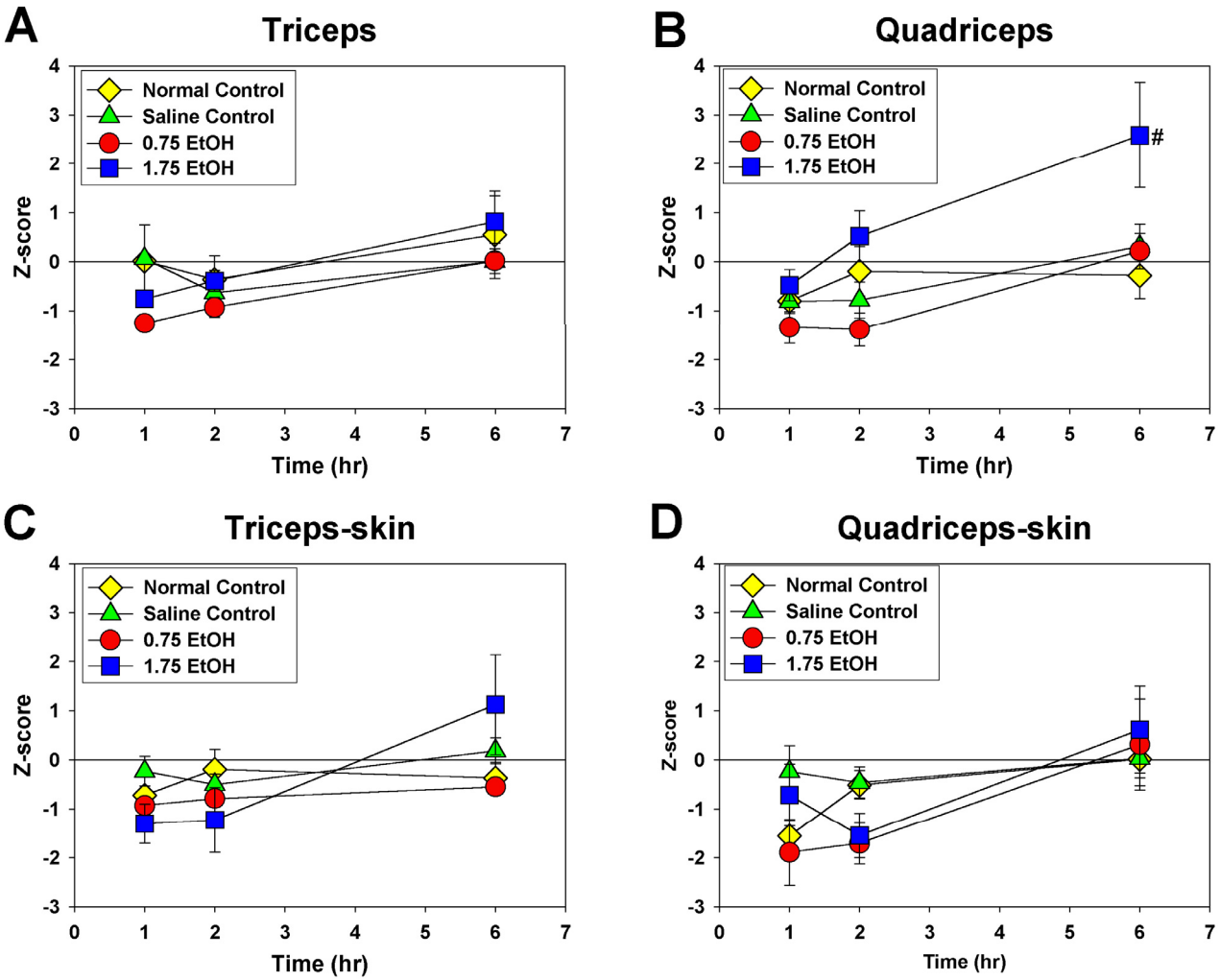

Figure 34: Blood Flow in the Triceps and Quadriceps Muscles and the Overlying Skin at the 1, 2 and 6 hr Time Points. The 1.75 EtOH group was significantly higher (#: $p < 0.005$) than the 0.75 EtOH and SC groups in the quadriceps muscles (B).

Experiment #3: Cardiovascular Measurements

1.75 EtOH, 0.75 EtOH and Saline Control Groups

There were no significant differences among these groups in terms of PaO_2, hematocrit and arterial pH, although the 1.75 EtOH group demonstrated a trend to have a lower pH compared to the other two groups (Figs. 35-36). $PaCO_2$ was significantly lower [$F(2, 69) = 6.194$, $p < 0.005$] in the 1.75 EtOH group compared to the 0.75 EtOH and SC groups (Fig. 36). Heart rate was significantly increased in the 1.75 EtOH group compared to both the 0.75 EtOH and SC groups, while the 0.75 EtOH group was also significantly higher than the SC group [$F(2, 2879) = 247.026$, $p < 0.001$] (Fig. 37a). Mean arterial pressure was significantly decreased in the 1.75 EtOH group compared to the SC group [$F(2, 2879) = 33.011$, $p < 0.001$] (Fig. 37b).

Blood alcohol concentrations peaked at the 1 hr time point (coinciding with the end of the alcohol infusion). Peak maternal BAC was 185 mg/dl and peak fetal BAC was 106 mg/dl in the 1.75 EtOH group (Fig. 37). In the 0.75 EtOH group, peak maternal and fetal BACs were 85 and 55 mg/dl, respectively (Fig. 38). The BACs were significantly higher in the 1.75 EtOH group compared to 0.75 EtOH group in both the ewe and fetus [$F(1, 24) = 19.296$, $p < 0.005$] and [$F(1, 18) = 20.144$, $p < 0.005$], respectively.

Saline Control vs. Normal Control

There were no differences between the SC and NC groups in any of the cardiovascular dependent measures.

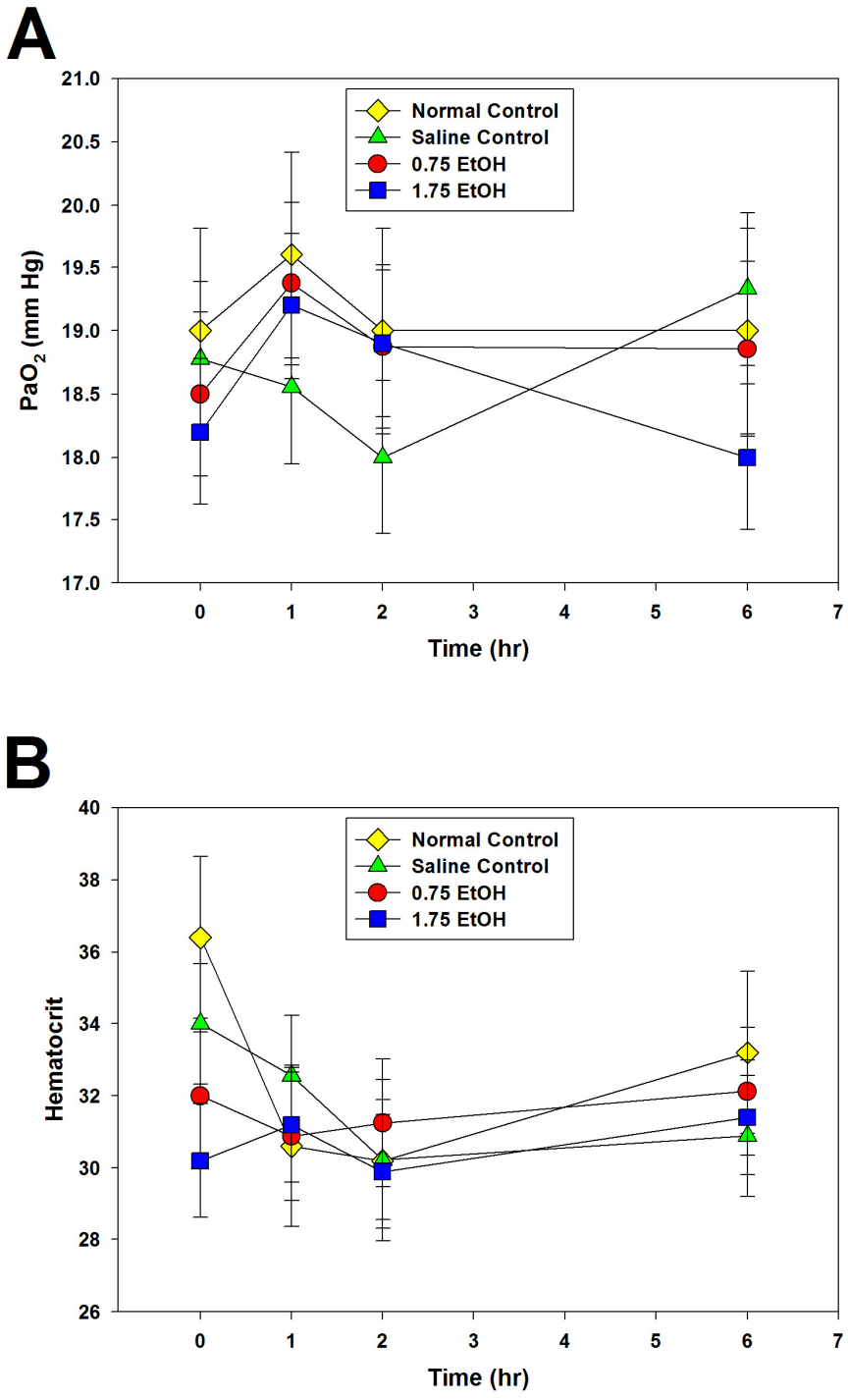

Figure 35: PaO_2 and Hematocrit. There were no significant differences among any of the treatment groups in either PaO_2 or hematocrit.

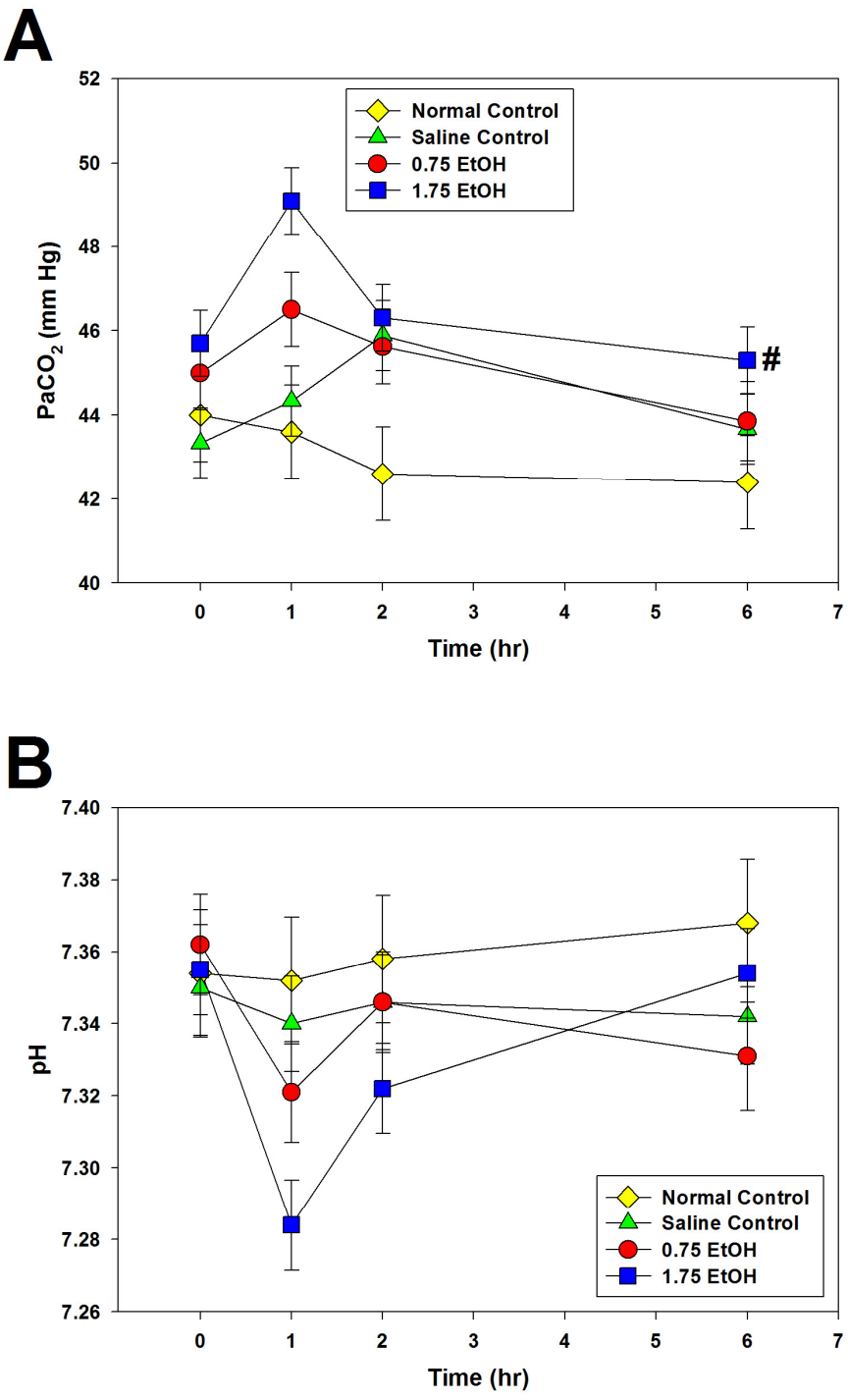

Figure 36: $PaCO_2$ and pH. PaCO2 was significantly higher (#: $p < 0.005$) in the 1.75 EtOH group compared to the 0.75 EtOH and SC groups (A). There were no significant differences among any of the treatment groups in pH.

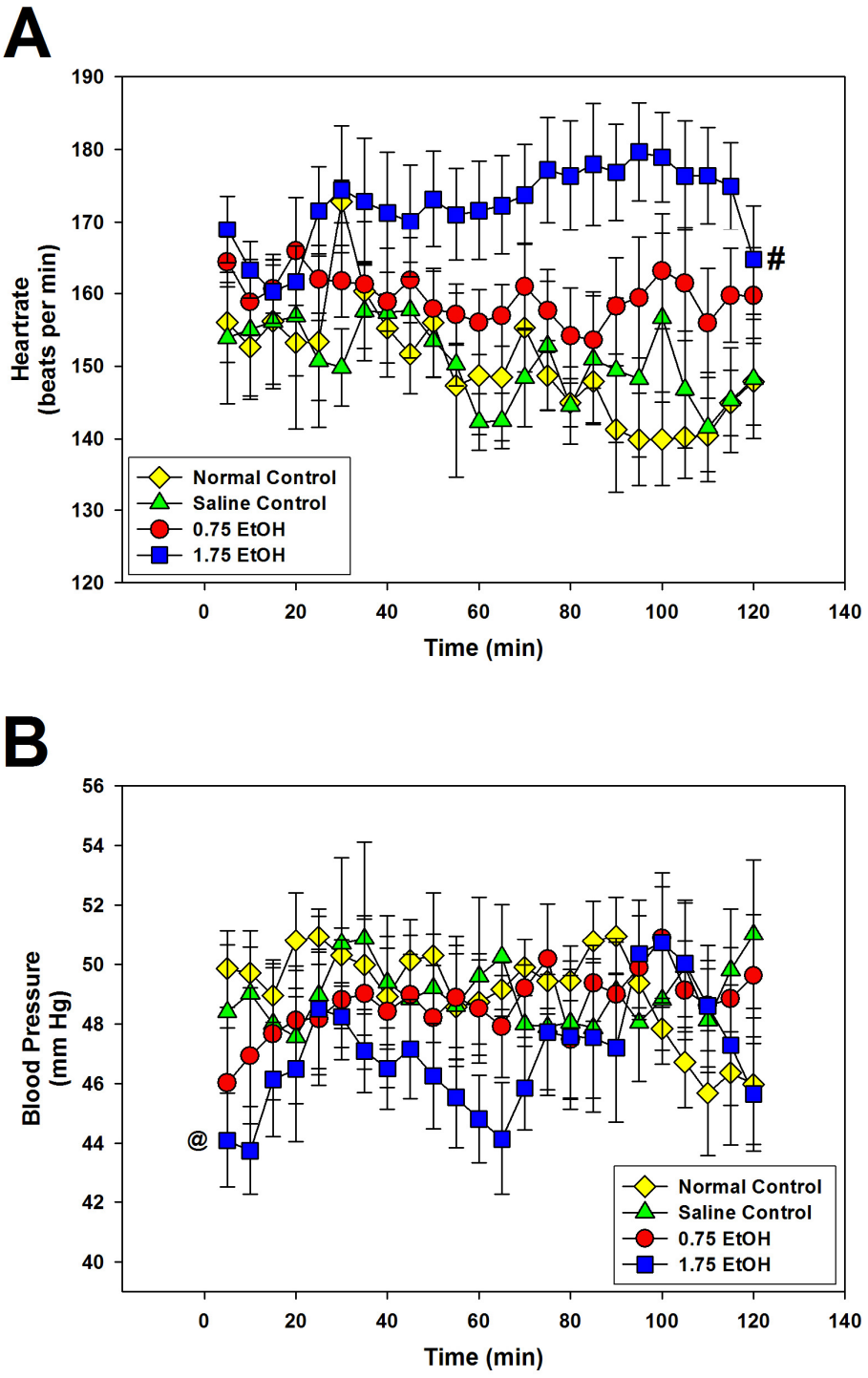

Figure 37: Fetal Heart Rate and Blood Pressure. Heartrate in both the 1.75 EtOH and 0.75 groups was significantly higher (#: $p < 0.001$) than the SC group. Mean arterial pressure was significantly lower (@: $p < 0.001$) in the 1.75 EtOH group compared to the 0.75 and SC groups.

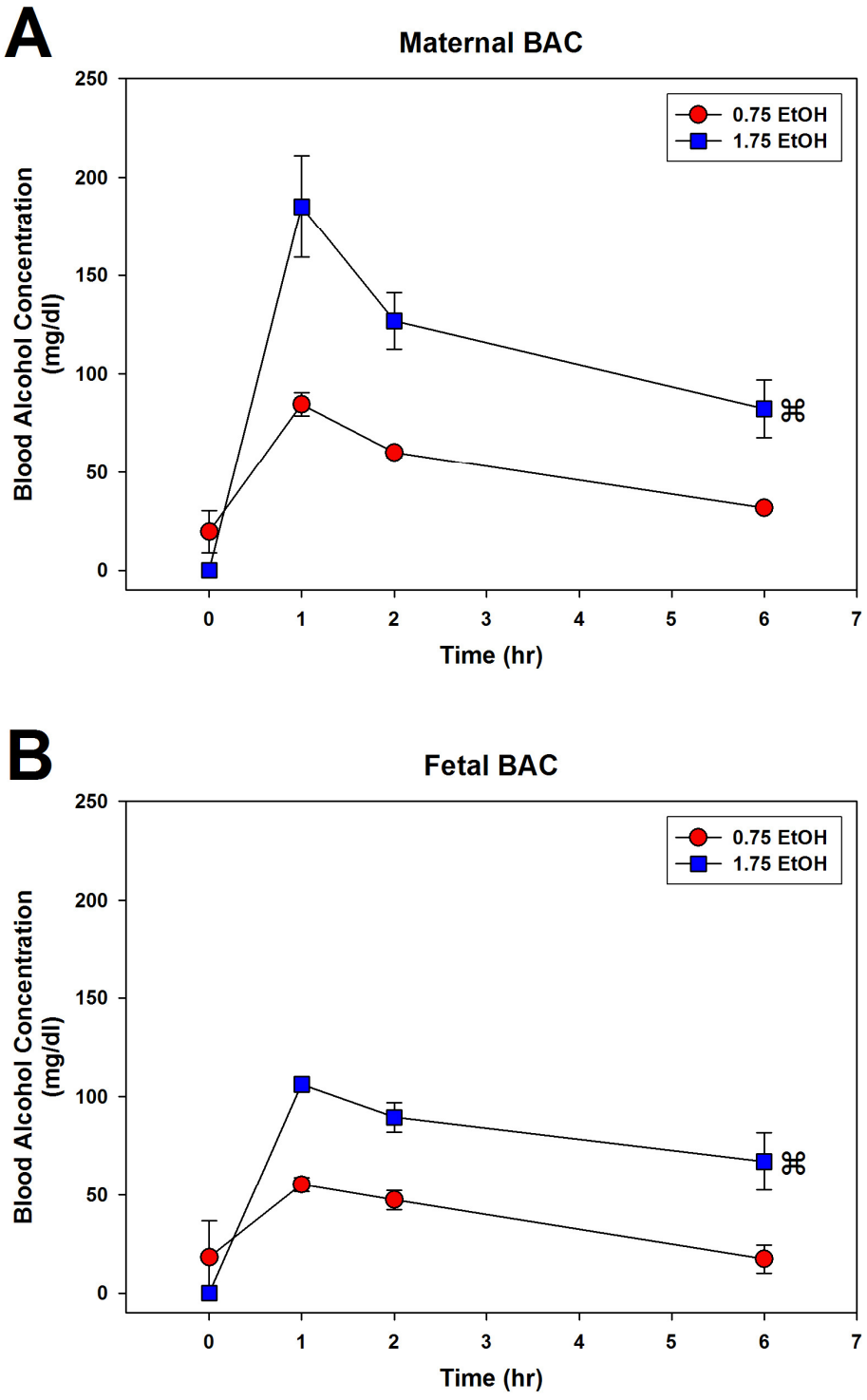

Figure 38: Maternal and Fetal BAC. The BAC was significantly higher (⌘: $p < 0.005$) in the 1.75 EtOH group than the 0.75 EtOH group in both the ewes (A) and fetuses (B).

Experiment #4: Neuronal Cell Counts

In this experiment, mitral cells in the olfactory bulb, pyramidal cells in the CA1 and CA2/3 regions of the hippocampus proper and granule cells in the hippocampal dentate gyrus were counted after alcohol exposure during the third trimester equivalent. These data were used to determine the regional vulnerability of the fetal sheep brain to alcohol and to compare with the CBF data to determine if the pattern of CBF after alcohol exposure correlates with the pattern of cell loss.

The results from this experiment did not reveal any significant differences in cell number, volume or cell density among any of the groups in the CA1 or CA2/3 regions (Figs. 39-40) of the hippocampus proper as well as the dentate gyrus (Fig. 41). There were no significant differences in olfactory bulb mitral cell number, volume or cell density between the 1.75 EtOH and SC groups (Fig. 42). The NC group, however, had a significantly larger olfactory bulb volume and number of mitral cells in the olfactory bulb compared to the SC group (Fig. 42a, c).

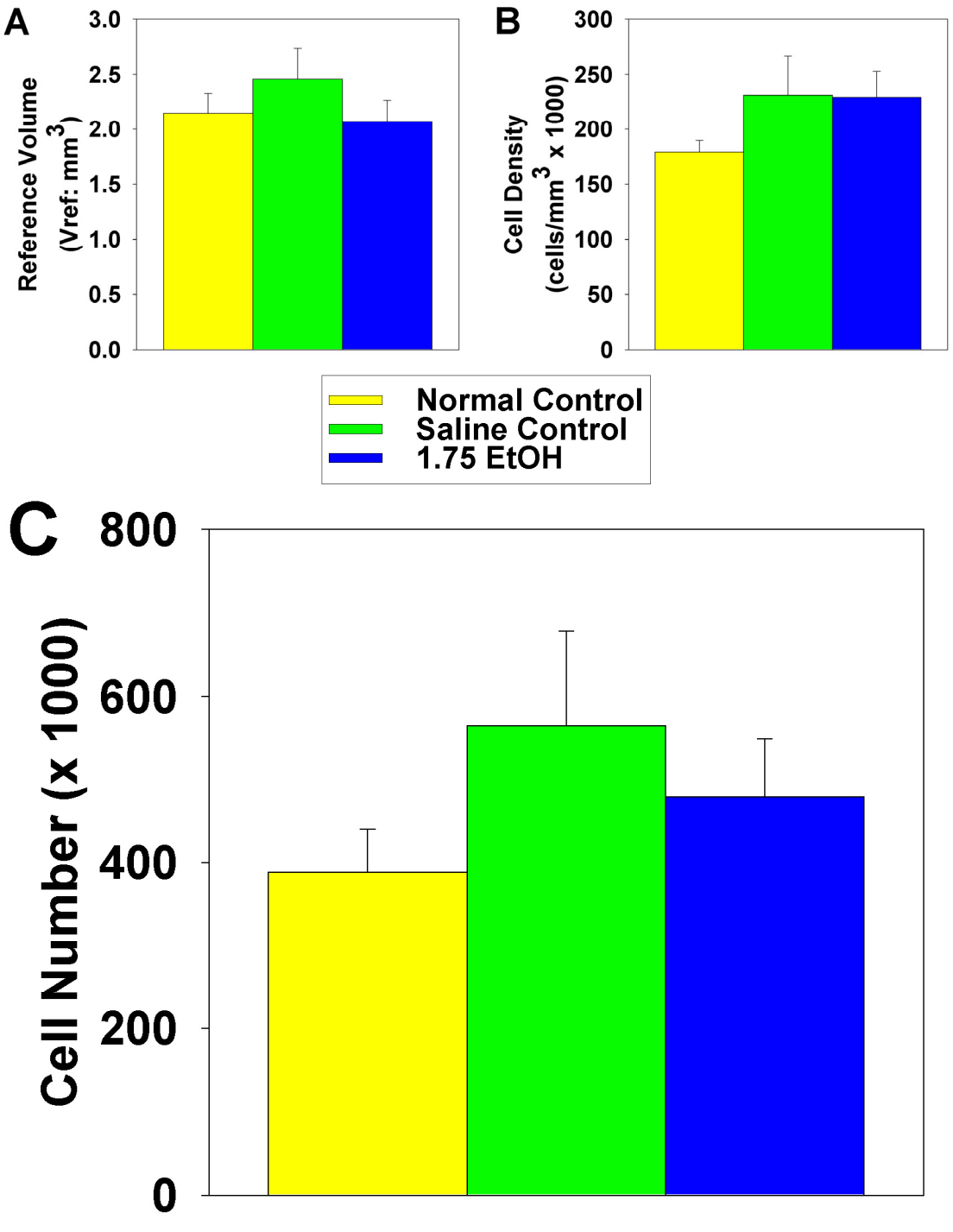

Figure 39: Cell Counts in the Hippocampal CA1 Region. There were no significant differences among any of the treatment groups in any of the dependent variables.

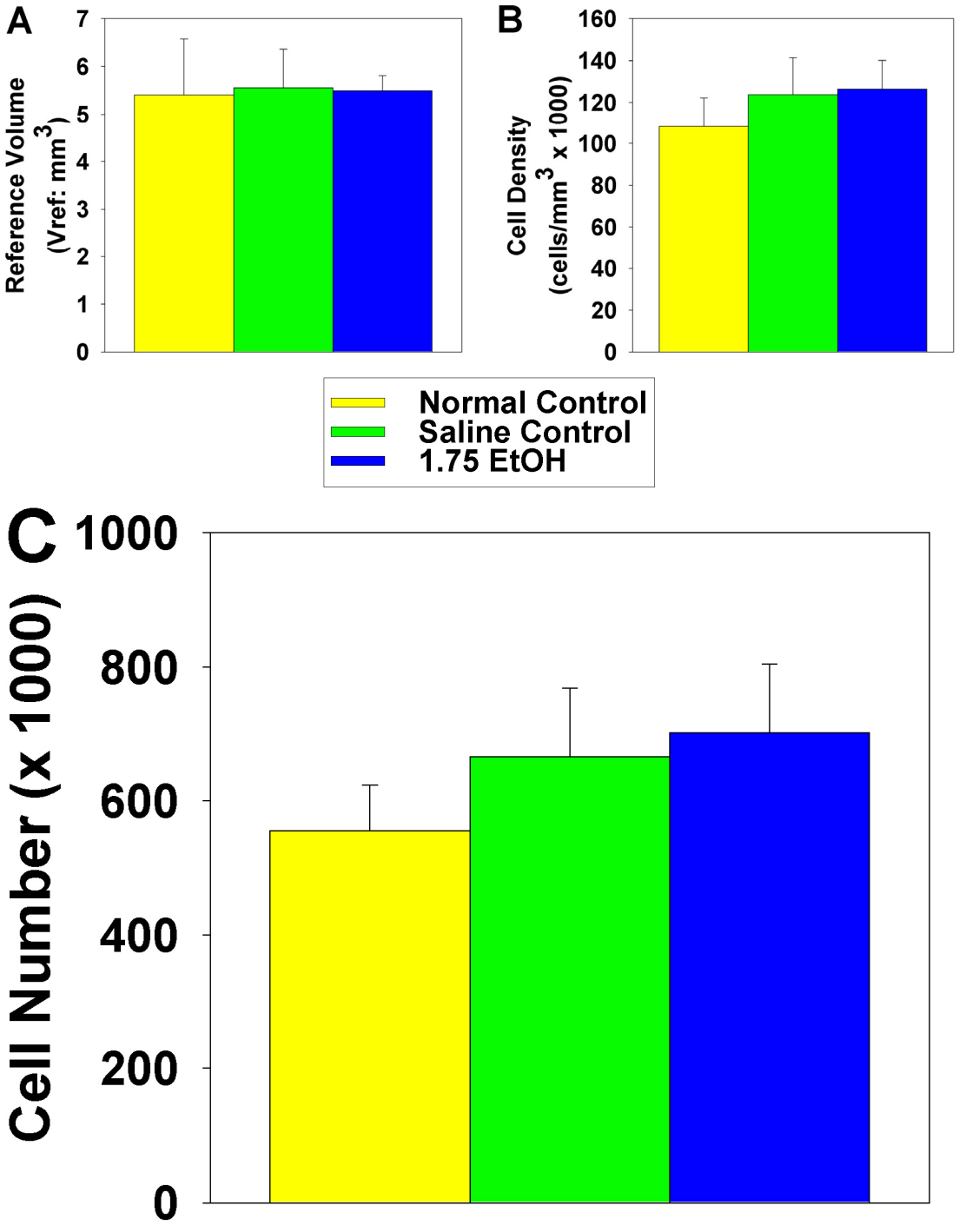

Figure 40: Cell Counts in the Hippocampal CA3 Region. There were no significant differences among any of the treatment groups in any of the dependent variables.

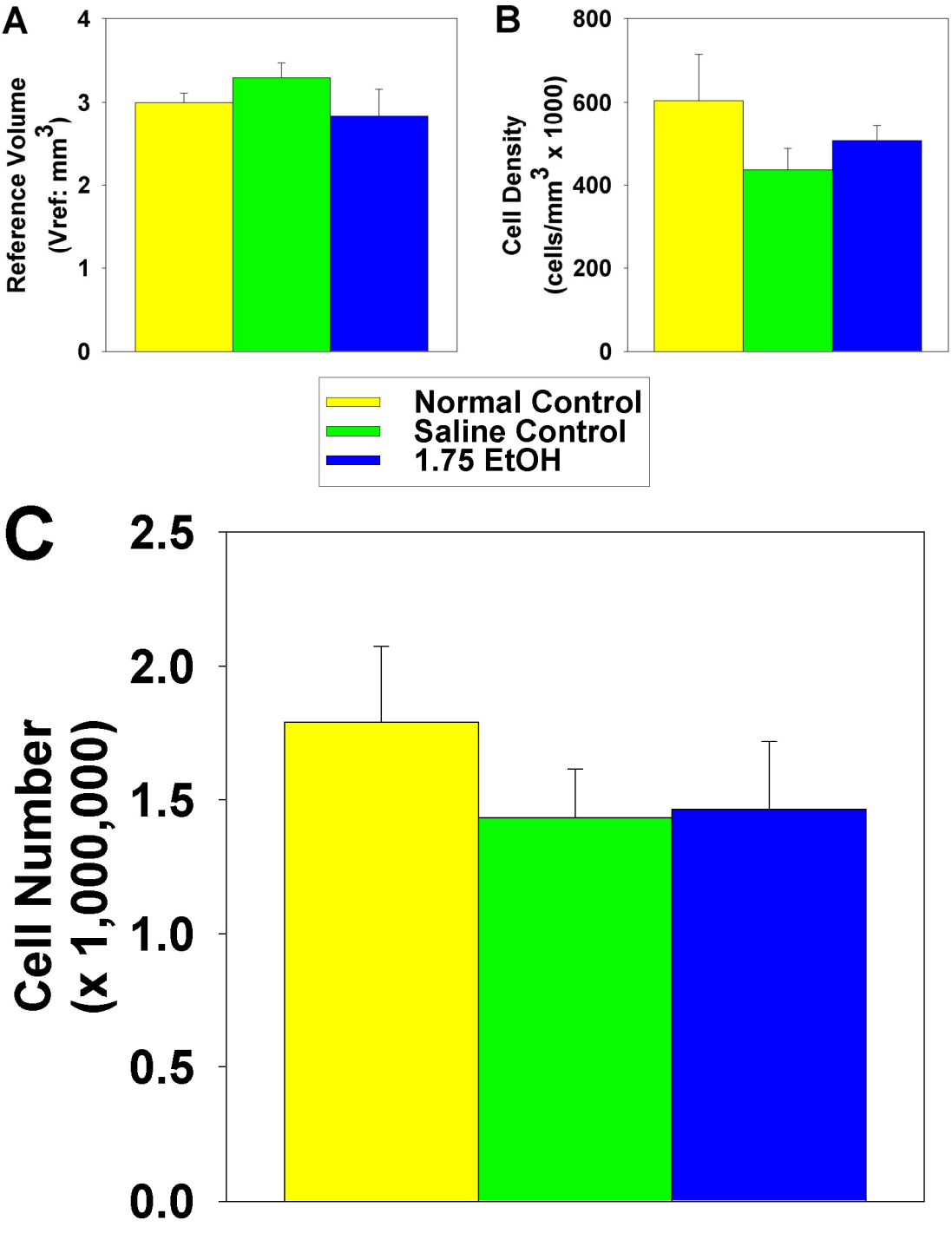

Figure 41: Cell Counts in the Hippocampal Dentate Gyrus. There were no significant differences among any of the treatment groups in any of the dependent variables.

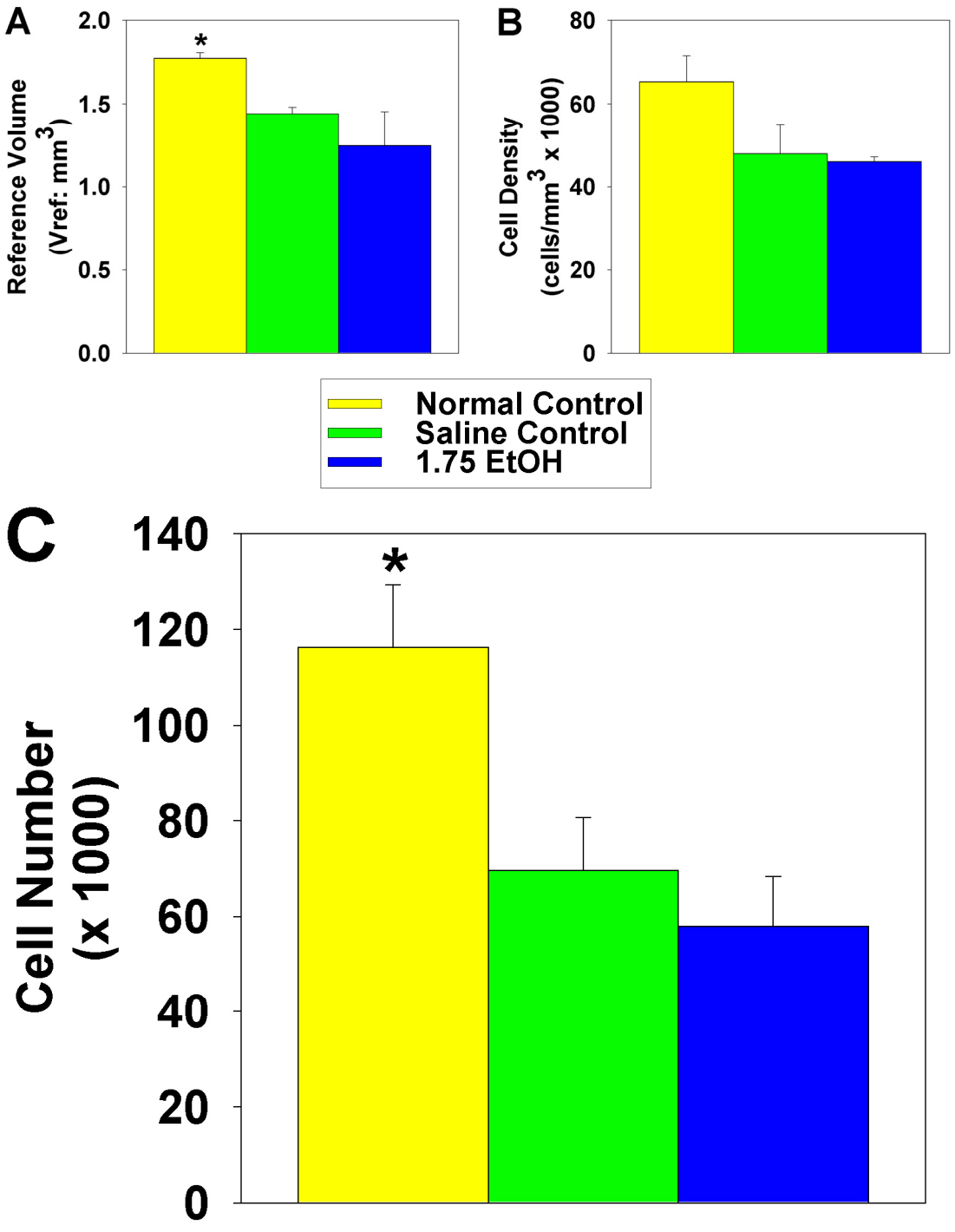

Figure 42: Cell Counts in the Olfactory Bulb. There were no significant differences between the 1.75 EtOH and SC groups. The NC group was significantly higher (*: $p < 0.05$) than the SC group in Vref (A) and mitral cell number (C).

CHAPTER VI

SUMMARY AND DISCUSSION

This study is the first to examine cerebral blood flow in the fetus after repeated alcohol exposure during the third trimester equivalent. The goal of this study was to examine the hypothesis that hypoxia is a mechanism of cell death due to developmental alcohol exposure. Specifically, this study tested the possibility that although low to moderate doses of alcohol do not appear to induce fetal hypoxemia, alcohol can still cause hypoxia by reducing blood flow to the brain.

These present results are important for two reasons. First, these low to moderate doses of alcohol can induce behavioral, cognitive and/or motor deficits in spite of the absence of hypoxemia, inferring that hypoxemia, if not necessarily hypoxia, is probably not a mechanism of fetal alcohol-induced deficits. Therefore, if alcohol does not induce hypoxemia, but does reduce CBF, hypoxia could still occur. Second, hypoxia as a mechanism of alcohol-induced deficits was proposed over twenty years ago, yet has neither been shown to be a tenable mechanism of alcohol-induced deficits nor has it been entirely disproven.

Although this present study did not attempt to answer this question fully, it was designed to examine one possible cause of alcohol-induced fetal hypoxia. In order to answer this question, these experiments employed two alcohol doses (0.75 and 1.75 g/kg) and measured blood flow prior to and after a final alcohol exposure (0, 1, 2 and 6 hrs) after repeated alcohol exposure throughout the third trimester.

It is important to note that although both blood flow experiments (Experiments #1 and #2) were performed in the same subjects, they actually measured two distinct events. Experiment #1 measured the effects of repeated alcohol exposure on blood flow when little or no alcohol was present (a few subjects had not completely cleared the alcohol and exhibited very low BACs at the 0 hr time point). The possible permanence (long term effects) of repeated alcohol exposure was not measured, but Experiment #1 still determined blood flow at a point in development when the brain is vulnerable to

alcohol insults. Experiment #2 measured the responsiveness of the neurovasculature to a subsequent alcohol exposure after repeated alcohol exposures throughout the third trimester equivalent. Previous studies on CBF after a single acute alcohol exposure (Richardson et al., 1985) allow comparisons between an acute exposure and the acute exposure after a binge-like exposure throughout the third trimester in this study.

Summary

Experiment #1 indicated there were few regions that exhibited statistically significant alterations in blood flow, although there was a strong trend for alcohol, especially the low dose (0.75 g/kg), to increase CBF throughout the brain. The head of the caudate nucleus and cingulate cortex showed significant increases in blood flow in the low dose group compared to the high dose group and saline controls. CBF in the low dose alcohol group was also significantly higher than in the saline control group in the anterior/lateral parietal cortex, posterior/dorsal temporal cortex, lateral/dorsal occipital cortex and the anterior/inferior cerebellum.

There were similar low dose alcohol-associated increases at the 0 hr time point in some of the other organs measured. The lungs had significantly higher blood flows after the low dose alcohol exposure than both the high dose alcohol and saline control groups. Blood flow in the thyroids, thymus and gonads was also significantly higher in the low dose group compared to the saline control group. The trend seen in the brain for the low alcohol dose to induce higher blood flow rates than the other groups was also present in many of the non-neuronal organs.

As in Experiment #1, there were few regions that demonstrated statistically significant differences in blood flows in the second experiment. The high dose alcohol group was significantly higher than both the low dose and saline controls in the hypothalamus, rostral medulla and posterior/inferior cerebellum, while the high dose group was also higher in the caudal midbrain compared to the low dose group. Except for these four regions, there were no other areas of the brain that were significantly different among the treatment groups. The blood flows in the normal control group were

not significantly different from those of the saline control group. Additionally, except for a slight trend for a small percentage of the regions to have a trend for higher blood flows in the normal controls, there were no consistent trends among any of the groups.

Interestingly, there were some significant differences in blood flow in some of the non-neuronal organs examined. The gonads and quadriceps muscles had significantly higher blood flows due to the high dose alcohol exposure compared to the low dose and saline control groups. The high dose of alcohol also produced higher blood flows compared to the low alcohol dose group in the thymus and the saline control group in the liver. The thyroid was the only organ to demonstrate significantly lower blood flows in response to alcohol exposure with the high alcohol dose group being significantly lower than the saline controls.

Peripheral Organs

Thyroid hormone is essential for proper development of the CNS and decreased hormone amounts induce deficits in cortical and cerebellar development, neuronal proliferation, synaptic formation and myelination (Genuth, 2000). However, developmental alcohol exposure has been shown to reduce thyroid hormone levels, including a study in fetal sheep exposed to an identical alcohol exposure regimen as in the present study (Cudd et al., 2002; Hannigan and Bellisario, 1990; Hernandez et al., 1992). In addition, prenatal alcohol exposure in rats has been demonstrated to alter adult thyroid function, indicating that the detrimental effects of alcohol exposure on the thyroid gland during development may be permanent (Wilcoxen and Redei, 2004). The decrease in blood flow to the thyroid gland after a similar dose of alcohol may help explain these alcohol-induced decreases in thyroid hormone. The decreases in blood flow to the thyroids shown in the present study may influence circulating hormone levels by altering the development of the thyroid gland, or alternatively, by altering the synthesis of thyroxine.

Interestingly, blood flow in the majority of the non-neuronal organs exhibited similar changes in blood flow as in the brain, indicating that there is not a general

differential effect of alcohol on blood flow between the brain and other organs of the fetus. The major exceptions to this were in the thyroid gland after the final alcohol exposure and the muscles and skin at the 0 hr time point which, except for the skin over the triceps, was not increased in the alcohol groups. The reasons for these increases in blood flow to the non-neuronal organs are unclear. One possibility that must be considered is alcohol-induced alterations in the development of the sympathetic nervous system. Although local factors do play a small role in the regulation of the peripheral (non-neuronal) circulation, the sympathetic nervous system (SNS) is by far the most important factor (Berne and Levy, 2000; Blaustein and Walsh, 1996). Sympathetic neuronal activity acts to constrict blood vessels and substantial evidence indicates that prenatal alcohol exposure in rats blunts normal sympathetic activity (Gottesfeld et al., 1990; 1997; 1998). Unfortunately, this effect has not been tested during the third trimester equivalent. If alcohol exposure during this period also inhibits normal sympathetic activity, then this inhibition or the abnormal development of the sympathetic nervous system may account for the increases in blood flow in many of the organs after developmental alcohol exposure. This idea is supported by the increased blood flow from the 0 hr time point after the final alcohol exposure.

This hypothesis may explain the increased blood flow in the high dose alcohol group, but due to the fact that blood flow in the low alcohol group was substantially higher than the high alcohol group, this hypothesis is less satisfactory for the low dose alcohol group. One might assume that a higher dose of alcohol would affect the sympathetic system more than the low dose. If this assumption is correct, an additional or alternative mechanism must be present to explain the enhanced blood flow after the low alcohol exposure. It is possible that the low dose of alcohol induced the production and/or secretion of vasodilatory factors, such as adenosine or NO (both of which have been demonstrated to be affected by alcohol), but this would have to be a relatively long term (hours) effect of alcohol as the blood flow was increased even after almost 24 hours after the alcohol exposure (Baraona et al., 2002; Dizon et al., 2004; Nagata et al., 1996). Alternatively, the low dose of alcohol may inhibit the SNS, while the high dose of

alcohol may either not affect the SNS or may activate an antagonistic system in addition to inhibiting the SNS. Unfortunately, at present, these hypotheses have not been tested.

Cardiovascular Findings

The cardiovascular data gathered in this study were in agreement with those published previously in Cudd et al. (2001). There were no significant differences among the treatment groups in fetal PaO_2, pH and hematocrit, although there was a trend for pH to be lower after the high dose alcohol exposure. Fetal arterial blood pressure was significantly decreased after the final high dose alcohol exposure compared to the low dose alcohol and saline control groups. Conversely, fetal heart rate was increased after the final alcohol exposure in both alcohol groups compared to the saline control group. The increase in fetal heart rate is most likely due to decreases in arterial pressure. The blood alcohol concentrations (BACs) in the high dose alcohol group in the first three experiments were lower than those from Experiment #4 [previously published in Cudd et al., (2001)]. These differences may be due to strain differences as Experiment #4 used Rambouillet ewes and Experiments #1, #2 and #3 used the Suffolk strain of sheep. It is quite possible that these different strains of sheep metabolize alcohol at different rates as has been reported in rats and in different human ethnic groups (Agarwal, 2001; Agarwal and Goedde, 1992; Foroud and Li, 1999; Pastino et al., 2000; Spuhler and Deitrich, 1984). Although similar data has previously been published, there are several interesting findings from these data that are worth reiterating. The first is the lack of alcohol-induced differences in arterial oxygen tension (PaO_2), which indicates that none of the subjects in any of the groups were hypoxemic. Second, the high dose alcohol-induced increase in arterial carbon dioxide tension ($PaCO_2$) compared to the other groups indicates that this group is hypercapnic. This is important because hypercapnia may affect neuronal development (Lehmenkuhler et al., 1989). Finally, the finding that alcohol did not affect hematocrit levels is interesting because it indicates a lack of change in the level of red blood cells (RBC) which is important because these cells carry oxygen. Reductions in RBC quantity could impair oxygen delivery, but these data

indicate that the present alcohol exposure paradigm does not affect hematocrits. Additionally, the neurovasculature can alter CBF in order to compensate for changes in viscosity, which can occur during increases or decreases in RBC levels (Harrison, 1989; Holzman et al., 1986). The lack of change in RBC levels indicates that changes in blood flow are not a result of alcohol effects on hematocrit.

Cell Counts

The cell count experiments demonstrated that there were no significant differences between the alcohol exposed subjects (1.75 g/kg) and the saline controls in any of the regions examined (olfactory bulb and hippocampal CA1, CA3 and dentate gyrus). Likewise, there were no differences between the normal control and saline control groups with the exception of the olfactory bulb. The volume of the olfactory bulb and the estimated mitral cell number was significantly higher in the normal controls compared to the saline controls. The lack of differences between the alcohol and saline control groups are contrary to those performed in neonatal rats demonstrating that alcohol exposure during the third trimester equivalent reduces cell numbers in the olfactory bulb and the CA1 region of the hippocampus (Chen et al., 1999b; West et al., 1986). However, the BACs achieved in these studies were quite different as the rat pups in the previous studies reached BACs of about 300-380 mg/dl while the sheep in the present study reached about 230 mg/dl in the fetus and 260 mg/dl in the ewe. These differences in BACs may be the reason for the discrepancies among these studies.

When these data are combined with the cerebellar Purkinje cell loss observed by West et al. (2001), they demonstrate that changes in CBF are not correlative with the pattern of neuronal loss. This suggests that the increases in CBF after alcohol exposure are not by themselves responsible for the neuronal loss seen in the cerebellum. It is possible, however, that certain regions, such as the cerebellum, may be more susceptible to the alcohol-induced changes in CBF. Another possibility is that the increases in CBF may attenuate the negative effects of alcohol. These two factors may act together to induce neuronal deficits. This hypothesis would be relatively simple to test by

administering alcohol while artificially limiting global CBF to a similar rate as in the controls.

Biphasic Effects of Alcohol

The 1.75 g/kg dose of alcohol produced a trend for higher blood flows throughout the brain after the repeated alcohol exposure, but this small increase was not nearly as dramatic as in the low dose. The increases in blood flow in the low alcohol dose group at the 0 hr time point was not statistically different in most of the brain regions due to the variability among the subjects. However, this increase in CBF compared to the saline control group was almost 50% in some regions and was fairly consistent throughout the brain.

This study is the first to demonstrate an increase in CBF after fetal alcohol exposure. The finding that the low dose of alcohol increased (albeit non-significantly) CBF compared to the high alcohol dose and saline control groups is interesting in that it suggests a biphasic effect of alcohol on blood flow. Numerous other studies have also shown a similar biphasic effect of alcohol. Alcohol can exert biphasic effects on neuronal activity and the activity of numerous enzymes. However, these effects have been mostly demonstrated in adult subjects and *in vitro*. One of the few experiments examining developing neurons was from the fetal serotonergic dorsal raphe nucleus, which were transplanted to the anterior chamber of the adult eye (Backman and Granholm, 1992). In that study, the developing neurons increased their basal firing rate after exposure to lower doses of alcohol (0.5-3.0 mM), while higher doses of alcohol (10-100 mM) inhibited the basal firing rate. Studies in adults have also observed similar biphasic effects in the dorsal hippocampus and locus coeruleus (Grupp, 1980; Verbanck et al., 1990).

Alcohol also has biphasic effects on the activities of several enzymes. Low doses of alcohol have been shown to increase microtubule associated protein 2 (MAP2) phosphorylation (which modulates microtubule assembly) in rat brain, while high doses decreased phosphorylation (Ahluwalia et al., 2000a). Endoplasmic reticulum Ca^{2+}-

transport ATPases also demonstrated a biphasic effect in that low alcohol doses activated these Ca^{2+} pumps, but were inhibited by higher doses of alcohol (Mitidieri and de Meis, 1995). Similar results have been observed in synaptosomal Na^{+}/K^{+} ATPases where lower alcohol doses increased activity, but were inhibited by higher doses (Foley and Rhoads, 1992; Marques and Guerri, 1988). All of these increases in enzymatic activity, as well as the previously mentioned possible increases in neuronal activity, by low doses of alcohol are energy-intensive process which, if sustained, could contribute to increases in CBF through a mechanism involving cerebral metabolic rate (CMR)/CBF coupling.

The phenomenon of CMR/CBF coupling becomes more complex when alcohol withdrawal is considered. As mentioned earlier, alcohol withdrawal during development can induce cell death through an excitotoxic mechanism (Thomas et al., 1997, 2001). In fact, alcohol withdrawal can lead to an overall increase in neural activity (Bonthius et al., 2001), which may lead to a concomitant increase in CBF. However, there are two factors that argue against this conclusion. First, it would be expected that the higher dose of alcohol would induce more profound withdrawal effects, yet CBF was increased more dramatically in the low dose alcohol group. It is difficult to fully explain this apparent discrepancy due to the limited time frame in which blood flow was measured. Because the animals receiving the different doses would fully metabolize their alcohol at different time points, it would be assumed that they would exhibit different temporal periods of alcohol withdrawal. The second problem with a conclusion concerning withdrawal-induced increases in CBF is that until actual evidence of withdrawal (e.g. increasing EEG activity during decreasing BACs) in this animal model system is confirmed, attributing physiological phenomena to withdrawal would be merely speculation.

An additional possible reason for the increase in CBF observed in the alcohol groups is the increased cell proliferation, and therefore the increases in metabolism associated with cell proliferation, observed in certain cell types in the brain after low to moderate doses of alcohol. Studies by Miller (1989; 1996) have shown that prenatal alcohol exposure in rats late in gestation during the period of subventricular zone

proliferation (GD 18-21) increases proliferation of these precursor cells. Prenatal alcohol exposure has also been shown to increase corticospinal neurons in rats (Miller, 1987). Similar increases in cell number have been demonstrated in the hippocampal formation dentate gyrus where a postnatal alcohol exposure in rats increased the granule cells in this region by 10% (West et al., 1986). Furthermore, although they are not associated with the CNS, dose-dependent biphasic effects have been demonstrated in chick tibia bone cells *in vitro* and in a leukemic cell line, K-562, where a lower dose of alcohol increased proliferation, while a higher dose decreased it (Dvilansky et al., 1984; Farley et al., 1985). Considering that the numerous studies mentioned earlier regarding alcohol-induced cell loss did not observe either increased proliferation or these biphasic effects, it is difficult to conclude that alcohol, even at lower doses, consistently increases proliferation. However, even increased proliferation in a subset of cells within the brain could contribute in a small part to the increases in CBF observed in this study. Unfortunately, it unknown to what extent these relatively small increases in cell proliferation in certain areas of the brain would affect CBF, especially considering that it has been repeatedly demonstrated that alcohol reduces cell numbers thoughout much of the brain.

A final possible explanation for the more substantial increases in CBF after exposure to the low dose of alcohol compared to the other groups is the possibility that the low dose of alcohol is enhancing angiogenesis or vascular modeling. Unfortunately, little research has been devoted to this topic. The single study on alcohol-induced angiogenesis in the fetus demonstrated that moderate alcohol exposure (10-20 mM) upregulates vascular endothelial growth factor (VEGF) mRNA expression and stimulates angiogenesis in chick chorioallantoic membrane (Gu et al., 2001). Additionally, Kelly et al., (1990) demonstrated that high doses of alcohol administered to postnatal rat pups increases capillary diameter in the cerebellum and hippocampus. Although the functional significance of this latter study is uncertain, this area of alcohol-induced changes in angiogenesis or vascular remodeling certainly deserves further examination.

Together, these data and hypotheses provide a possible mechanism for the biphasic effects of alcohol on CBF seen in the present study. However, because no studies have provided a definitive link between alcohol exposure, increased neuronal activity or proliferation and increased CBF, this idea will require further research.

Increases in Fetal CBF

Although the increase in CBF in the high dose alcohol group was not as dramatic as in the low dose alcohol group, it still demonstrated a consistent increase in CBF compared to the saline control group throughout the brain at the 0 hr time point, as well as an increase in several regions after the final alcohol exposure. There are multiple possible reasons for these increases in CBF. One possibility for the small global CBF increase after the high dose alcohol exposure is the hypercapnia seen in this group. This possibility is unlikely, however, for two reasons; 1) hypercapnia-induced vasodilation is typically a global phenomenon, yet only a few brain regions were higher in the high dose alcohol group during the time course experiment (when hypercapnia was occurring) and, 2) changes in CBF usually occur rapidly in response to changes in $PaCO_2$, yet the CBF increases in this group was only a global effect at the 0 hr time point, when $PaCO_2$ was not different from the controls.

The increase in CBF in the 1.75 EtOH group may be due to vasodilation induced by inflammatory processes. The neurovascular surface has numerous receptors for the wide variety of cytokines (Pearce, 2002). These receptors mediate immune responses to a wide variety of factors such as infection, ischemia, stroke, trauma (mechanical injury), hypertension and growth factors such as NGF and BDNF (Introna et al., 1994; Lebel et al., 2000; Szaflarski et al., 1995). In many of these inflammatory processes, CBF is increased through the action of cytokines (Bonmann et al., 1997; Botchkina et al., 1997; Fassbender et al., 1996; Osuka et al., 1998). Additionally, very heavy prenatal alcohol exposure (defined by the authors as 60 or more drinks per week) in humans has been shown to be associated with increased levels of several interleukins (IL-1α, IL-1β and IL-6) as well as tumor necrosis factor-α (TNFα) in newborn cord blood (Ahluwalia et

al., 2000b). All of these cytokines have been shown to mediate increases in CBF, although more moderate levels of alcohol in this study was not associated with changes in cytokine levels. Because this experiment was performed in humans, the only ethically viable time point for measurement was at birth, so it is possible that cytokine levels were increased at earlier time points, especially during or immediately after an alcohol exposure episode. Unfortunately, the cytokine levels after developmental alcohol exposure have not been well studied. It would be interesting to determine if cytokines are released in response to neuronal damage within the brain (i.e. an inflammatory response), or if the cytokines can mediate alcohol-induced cellular damage as has been established in the liver and in *in vitro* neurons and glia (Davis et al., 2002; Spinozzi et al., 1991; Soeda et al., 2001). If the former hypothesis is correct during development, then it is possible that the CBF increases in the 1.75 EtOH group in the present study is simply a side effect of the alcohol-induced interleukin and TNFα release.

Neurovascular Responsiveness

Perhaps one of the most significant findings in this study are the results regarding neurovascular responsiveness after repeated alcohol exposures. These data suggest that alcohol exposure, especially the higher dose, during the third trimester equivalent alters the normal responsiveness of the neurovasculature to a subsequent alcohol exposure. This conclusion is based on previous experiments demonstrating that an acute 1 g/kg dose of alcohol in the near-term sheep fetus reduces CBF and the data from the current experiments indicating that after repeated alcohol exposures, the sheep fetus at the same point in gestation no longer responds in a similar manner to alcohol (Richardson et al., 1985). This finding is important because it implies that alcohol alters the normal neurovascular response to alcohol. It is possible that if the neurovascular response to alcohol changes after repeated exposures, the response to other insults, such as hypoxia, hypercapnia, acidemia or changes in perfusion pressure may be altered as well. For example, Gleason et al. (1997) provided an indirect corollary to the present study by demonstrating that alcohol exposure in early gestation fetal sheep alters the

neurovascular responsiveness of the newborn lamb to hypoxia. These possible changes in neurovascular responsiveness to vascular insults are clinically important as many of these insults occur as a normal aspect of life (i.e. transient hypoxia during birth and the subsequent changes in cardiopulmonary physiology). The possible inability of the neurovasculature to adjust CBF in order to compensate for these or other factors may expose the developing brain to an environment detrimental to normal growth at periods when the brain is vulnerable to developmental insults.

The mechanism(s) underlying these changes in neurovascular responsiveness are beyond the scope of this study, but several possibilities exist that deserve examination. First, it is possible that alcohol is affecting the neurogenic control of CBF. Although most neuronal innervation of the neurovasculature is believed to modulate basal vascular tone and to aid the more dominant regulators of CBF, activating or severing these neuronal connections does have a negative effect on the responsiveness of the neurovascular system to alterations in factors such as PaO_2, perfusion pressure and possibly $PaCO_2$ (Edvinsson et al., 1976; Golanov and Reis, 1996; James et al., 1969; Morita et al., 1994; Paulson et al., 1990; Sun and Reis, 1994b). Additionally, the neurogenic modulation system is still developing during the third trimester equivalent. It is possible that the repeated alcohol exposure in this study altered the normal development of the perivascular innervation and therefore altered the ability of the nervous system to modulate CBF or neurovascular tone.

A second possibility to explain the changes in neurovascular responsiveness to alcohol is the hypothesis that either the neurogenic and/or myogenic mechanisms that adjust CBF developed tolerance to the effects of alcohol. It has been well established that chronic (repeated) exposures to alcohol induces cellular and behavioral tolerance to alcohol (Hoffman and Tabakoff, 1985). Several studies in adult rat non-neuronal vasculature have demonstrated that the vasodilatory effects of acute alcohol administration are lessened after repeated exposures (Altura et al., 1980; Knych et al., 1984; Strickland and Wooles, 1988). Further studies have demonstrated that the alcohol-induced vasodilation of cerebral vessels is most likely due to a reduction in intracellular

[Mg^{2+}], which modulates cytosolic [Ca^{2+}] and transmembrane Ca^{2+} currents (Ema et al., 1991; Zhang et al., 1993). Another study in *in vitro* arterial smooth muscles cells and in the intact rat brain suggested that the tolerance observed previously after repeated or prolonged exposure to alcohol was due to an attenuation of the alcohol-induced reductions in intracellular Mg^{2+} levels (Li et al., 2001). Unfortunately, this study did not actually evaluate the presence of tolerance to alcohol-induced neurovascular dilation, so it is still unclear if the neurovasculature develops tolerance to the effects of alcohol. This area deserves further study, especially considering the results of the present study which implies that repeated alcohol exposure may induce neurovascular tolerance.

Another important aspect of increased CBF that should be considered is the effect that increases in fetal CBF may have later in life. For example, in adults, a reduction in CBF (often of unknown etiology) is a risk factor for the development of Alzheimer's disease (AD), although reduced CBF alone does not appear to induce AD (Crawford, 1998). Although the current study demonstrated increases in CBF, the long-term effects are unknown. It would be interesting and beneficial to both the FAS and AD research fields to examine this issue. The ability of the neurovasculature to properly regulate itself is also important in the prevention of stroke. The neurovascular abnormalities seen in this data, if permanent, may increase the possibility of strokes in aging subjects.

Hypoxia

These present data provide further evidence against the hypothesis that alcohol exposure induces deficits in the developing brain via a mechanism involving hypoxia. This evidence consists of the lack of hypoxemia after fetal alcohol exposure coupled with the findings in this study that alcohol did not decrease CBF. As mentioned previously, hypoxia can occur as a consequence of decreased PaO_2 and/or blood flow. Considering that neither of these phenomena occurred in this study, the probability that hypoxia is occurring as a result of prenatal alcohol exposure decreases dramatically. This is significant as hypoxia is often cited as a possible mechanism of ARND and further

proof of the unlikeliness of this mechanism allows for the focus of research on other, more probable mechanisms.

Although this present study severely limits the possibility of alcohol-induced hypoxia as a mechanism of ARND, it does not absolutely disprove this hypothesis. Alcohol could still induce hypoxia through mechanisms involving underlying causes other than hypoxemia or decreases in CBF. Additionally, alcohol still might induce hypoxia at doses or exposure regimens not examined in the current study or in the other studies providing proof against the hypoxia hypothesis.

The assertion that a binge-like alcohol exposure during development does not appear to induce hypoxia in the fetus must necessarily be qualified because other possible alcohol exposure paradigms exist that these experiments did not examine. First, this study examined the effects of alcohol only in a binge-like pattern. Although this is a common mode of alcohol abuse, and one that can induce developmental deficits, it is certainly not the only drinking pattern in humans. Many alcoholics consume large amounts of alcohol on a much more regular basis, often daily, and quite often reach higher BACs than that achieved in this study. It is certainly possible that a more chronic-type alcohol exposure could induce hypoxia in the fetal brain. Second, in the present experiments, alcohol was only administered during the third trimester equivalent. This is the period of greatest brain growth, which includes ongoing vasogenesis and neurovascular maturation necessary for life outside of the maternal environment. However, neurovascular development prior to this point has been shown to be affected by exposure to alcohol, as well as other drugs and insults and is absolutely critical to both neuronal development and neurovascular physiology later in development (Gleason et al., 1997). This study suggests that an alcohol exposure paradigm that included earlier periods of development may have very different results from those presented here. Furthermore, the implications are that it may not be possible to predict how longer-term alcohol exposure would affect neurovascular development. This is an important point given that many women abuse alcohol throughout pregnancy and hardly any of them drink exclusively during the third trimester as was modeled in the present studies.

In spite of the aforementioned hypoxic possibilities and qualifications, it must be pointed out that an identical binge-like exposure to the present experiments was sufficient to induce an approximate 25% loss in Purkinje cells in the developing cerebellum (West et al., 2001). Therefore, although alcohol is hypothetically capable of inducing hypoxia in the fetal brain after longer exposure periods, during chronic exposure or with higher BACs, the current studies indicate that this Purkinje cell loss is most likely due to mechanisms other than hypoxia. Considering that the third trimester equivalent has been shown to be the period most vulnerable to alcohol-induced cell loss and that, at least in rats, the peak BAC achieved (as opposed to total alcohol consumption) is the key indicator of cell loss, the probability that hypoxia is involved in the majority of ARND cases is further decreased (Bonthius and West, 1990). This idea becomes especially important upon the realization that although cell loss is a severe result of alcohol exposure during development, numerous other, possibly equally damaging effects can occur, such as abnormal synaptic connections at lower BACs than that required for neuronal loss (Hoff, 1988; Lancaster and Samorajski, 1987; Sutherland et al., 1997). If the alcohol doses required for cell loss do not appear to induce hypoxia, the negative effects induced by lower doses of alcohol are even more likely to occur in the absence of hypoxia. This is supported, at least in part, by the data from the 0.75 g/kg alcohol group in this study that did not exhibit hypoxemia or decreases in CBF.

Further proof against hypoxia as a mechanism of ARND is the study by Cudd et al., (2000) demonstrating that a binge-like alcohol exposure in postnatal rat pups did not reduce global brain intracellular high-energy phosphate concentrations. Although these measures were global, rather than regional, and were performed in postnatal animals, the authors concluded that alcohol exposure did not diminish whole brain oxygen levels. The data from most of these experiments indicate that hypoxia is an unlikely mechanism, but definitive proof of this is still lacking. Although technically difficult, *in vivo* microdialysis or cerebral oxygen consumption experiments could aid in answering this question.

Alternative Mechanisms

Although hypoxia may not be a mechanism of alcohol-induced neuronal deficits at any but the highest doses that can be tolerated by mother and fetus, there are still numerous possible mechanisms to account for the various deficits observed after developmental alcohol exposure. Making the search for these possible mechanisms more difficult is the probability that different doses of alcohol exert their differential effects through different mechanisms. In other words, a mechanism by which lower doses of alcohol decreases a process such as synaptogenesis may not necessarily be the same as the mechanism of alcohol-induced cell death at higher doses of alcohol.

One possibility that may arise due to the increases in CBF is that alcohol may induce neuronal damage or death through a mechanism involving edema. The fetal brain is exquisitely vulnerable to dramatic changes in perfusion pressure, hence the complex mechanisms designed to prevent them. Hypoxic-ischemic insults often result in brain edema usually as a consequence of hyperreperfusion (De Haan et al., 1997; Mallard et al., 1993; Tan et al., 1994; 1996; Tan et al., 1999). So far, only one published study has examined the possibility of alcohol-induced edema during development. That study found that prenatal alcohol exposure in rats induced edema in some of the offspring (Church et al., 1995). Another study found edema in the offspring of pregnant rats exposed to acetaldehyde, the major metabolite of alcohol (Sreenathan et al., 1982). Additionally, the adult brain is also susceptible to the effects of alcohol in terms of increasing extracellular fluid. A relatively high dose (~5 g/kg) of alcohol administered daily for five days induced neurodegeneration in the entorhinal cortex, dentate gyrus and olfactory bulbs (Collins et al., 1998). Interestingly, this neurodegeneration was accompanied by edema and electrolyte accumulation that when prevented with furosemide (an electrolyte transport inhibitor and diuretic), the neurodegeneration in the cortex and dentate gyrus was also reduced by 75-85%. The lack of experimentation on the possibility of alcohol-induced edema during development, makes drawing conclusions difficult. This is also confounded by the finding that hypertension (~40% increase in MAP over controls) induced by dopamine administration in fetal sheep failed

to result in cerebral edema even in the immature fetus, which has a decreased autoregulatory capability compared to the mature fetus (Harris et al., 2001). Given these results, if alcohol does induce edema, it is probably not solely a result of increased blood flow, but either a blood flow-independent mechanism or a result of increased blood flow combined with other mechanisms related to developmental alcohol exposure. Regardless, this is an area that warrants further investigation.

Other possible mechanisms of alcohol-induced neuronal deficits related to changes in CBF involve hypothyroidism and hypercapnia. Both of these conditions were discussed earlier as possible mechanisms underlying fetal alcohol-induced brain damage. It is quite probable that either of these mechanisms may induce cell loss, especially at higher alcohol doses (i.e. 1.75 g/kg). Although lower doses of alcohol may not induce neuronal damage through these mechanisms, it is probable that other neuropathological processes are occurring, such as alterations in normal neurovasculature regulatory mechanisms, that could induce not only immediate deficits such as alterations in neuronal activity, but long-term deficits that may not be exhibited either during the period immediately after the alcohol exposure (i.e. days to months) or with gross anatomical measurements.

Finally, it is also possible that these increases in blood flow, regardless of their underlying cause(s), may expose the developing brain to abnormal levels of hormones or growth factors. These factors, if increased in the neuronal environment (due to the increases in CBF), during key periods of development could induce aberrant neuronal morphology, migration, function or connectivity. Any of these deficits would lead to alterations in normal cognitive, behavioral or motor functions.

The search for the mechanisms of ARND and FAS is an ongoing goal of research on a very complex problem. Obviously, these problems are entirely preventable by the cessation of alcohol use during pregnancy. However, this solution is not necessarily an easy one. First, alcoholism is a difficult disease to cure and second, even social drinkers who are not alcoholics may not entirely understand the risk associated with even moderate alcohol use during pregnancy. This is further complicated by the fact that not

all women who drink during pregnancy, even heavy drinkers, have offspring with at least some of the more obvious effects of prenatal alcohol exposure. A useful, although difficult, approach to combat this problem would be to identify the myriad risk factors for alcohol-induced developmental deficits. Rather than attempting the global education and intervention strategies currently in practice, more focused efforts could be applied to the women at the greatest risk for delivering children affected by alcohol. This may not be the ideal solution, but given limited resources, research and time, it may be the most practical one at present.

Unfortunately, the sociological prevention of alcohol abuse during pregnancy does not seem likely in the immediate future. Therefore, until women stop abusing alcohol during pregnancy, there will be a need for an increased focus on determining the mechanisms of fetal alcohol-induced brain damage in order to tailor preventive or therapeutic strategies designed to ameliorate the detrimental effects of developmental alcohol exposure. As in research on various risk factors, the search for the mechanisms of alcohol-induced developmental deficits is also a complex problem. It is clear that alcohol can affect the developing brain in numerous ways depending on the specific brain region, the amount of alcohol consumed, and the developmental timing of the alcohol exposure. In addition, there are numerous other factors that may possibly play a role in the development of ARND such as genetics, preconceptional alcohol exposure, nutrition, poly-drug use, as well as other diseases that could interact with alcohol to affect brain development. Clearly, with these complex interactions and confounding variables, there is not going to be a single "magic potion" that will prevent or cure every case of ARND. However, research on the underlying mechanisms of ARND may elucidate possible preventive or therapeutic measures of at least the more severe effects of alcohol exposure during development.

It is likely that the most effective approach to prevent or ameliorate the effects of alcohol exposure during development will incorporate aspects of both sociological prevention strategies and pharmacological measures. The latter is important not only until effective preventive strategies are developed, but also for those who will inevitably

slip through the cracks of such a system. Hopefully, given time, resources, persistence and creativity, these preventions and/or therapies will be possible.

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VITA

Scott Edward Parnell

Permanent Mailing Address:
3603 Sunningdale Way
Durham, NC 27707
Phone: (919) 401-9277
E-mail: sparnell@med.unc.edu

Education:

Ph.D., Medical Sciences/Neuroscience, Texas A&M University, 2004
B.A., Biology, Texas A&M University, College Station, Texas, 1998

Research Experience:

1/2004-12/2004	NIH Predoctoral Fellow (NIAAA)
8/1999-12/2004	Graduate Research Assistant, TAMUSHSC
8/1998-8/1999	Research Assistant, TAMU COM

Funding:

National Institute on Alcohol Abuse and Alcoholism: F31 AA014451 Predoctoral Fellowship "The Effects of Alcohol on Fetal Cerebral Blood Flow." (2004-2006).

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