University of Alberta

The Effect of Caffeine on the Neurobehavioral and Neuropathological Outcome of the Newborn Rat

By

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Medical Sciences

Department of Paediatrics

Spring 2010
Edmonton, Alberta

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DEDICATION

I dedicate my thesis

to my parents who reared me to be a good person and encouraged me to be a good physician,

to my loving and devoted wife,

to my children who have made my life full with happiness and hope

and

to my teachers and colleagues.

THESIS ABSTRACT

Caffeine is used for the treatment of apnea of prematurity. The objective of this study was to determine the long term neuropathological and neurobehavioral effects of caffeine on the immature rat brain. Newborn rats were injected with either caffeine, or normal saline from postnatal days 3 to 7, equivalent to the human premature infant of 28-36 weeks. Behavioral tests revealed no abnormality in caffeine treated animals compared to controls. Fluro-Jade B stain of P4 rat brains showed that caffeine caused significant neuronal cell death in some areas of the brain, compared to controls, but this alteration was transient and not present at P8. Anti-NeuN stain at P21 showed significant neuronal cell loss in CA1 and hypothalamus regions in the caffeine group, but not at P160. Anti-Neurofilament M stain at P8, P21 and P160 showed no differences between the control and caffeine groups. We conclude that use of caffeine has no significant effect on the behavioral tests measured in our newborn rat pups. While caffeine caused neuronal cell death at P4, and neuronal cell loss in CA1 and hypothalamus regions at P21, there was no long-lasting effect on neuropathological outcome. However, given these latter findings, the use of caffeine in the premature infant must still be done with caution.

ACKNOWLEDGEMENTS

No research is a solo effort.

I would like to thank Dr. Jerome Y. Yager, who has been not only a valuable adviser through this research and thesis project, but also a great role model, a caring mentor and a truly wise supervisor. My special appreciation goes to Dr. Po-Yin Cheung for being a co-supervisor, for supervising me during the course “Directed Reading in Clinical Medicine”, and for his continuous support and advice. I would also like to thank Dr. Gregory D. Funk for being a member of the supervising committee and for many varied ways of demonstrating support and advice. My thanks go also to Arlene Figley for her excellent administrative help and to Dr. Richard Schulz for his chairing the examining committee.

To my colleagues in Dr.Yager's laboratory: Edward Armstrong, Dr. Alireza Karimi Pour, Keqin Shen, and Amy M. Black, I appreciate your patience, wisdom and your great contributions to my research and thesis. Thanks Ed for your advice, teaching and patience all through my research.

My heartfelt thanks are due to my parents for their continuous support and praying, and to all my brothers and sisters. To my lovely daughters - Mais, Lujein and Layan- thank you for all the joy and endless light and happiness you bring to my life.

Finally, my sincere thanks go to my love, my heart and my best friend: my wife; Nabeela. Thank you for the talking, listening, laughing and sharing your life with me. You are the best gift from GOD. I will continue to be grateful to you, my love, forever.

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LIST OF ABBREVIATIONS

AOP	Apnea of Prematurity
A_1	Adenosine Receptor Subtype A_1
A_{2A}	Adenosine Receptor Subtype A_{2A}
A_{2B}	Adenosine Receptor Subtype A_{2B}
A_3	Adenosine Receptor Subtype A_3
CAP trial	Caffeine for Apnea of Prematurity Trial
CYP1A2	Microsomal Cytochrome P450 Mono-Oxygenases
D_2	Dopamine Receptors Subtype D_2
DAB	Diaminobenzedine Tetrahydrochloride
FJB stain	Fluoro-Jade B stain
gm	Gram
GA	Gestation Age
GABA	γ-aminobutyric acid
GER	Gastroesophageal Reflux
Gs	'Stimulatory' G-proteins
Gi	'Inhibitory' G-proteins
h	Hour
kg	Kilogram
L	Liter
mg	Milligram
mL	Milliliter
MWM	Morris water maze
NeuN	Neuronal Nuclei
NS	Normal Saline
P1, P3	Postnatal Day 1, Postnatal Day 3
s.c	Subcutaneous
SEM	Standard Error of the Mean
µg	Microgram
µM	Micromole/ Litre

Introduction

Caffeine is currently one of the most commonly used drugs in neonatal intensive care units. The number of neonates who are treated with caffeine has increased significantly in the last decade (Du, Warrier et al. 2006). Caffeine and the other methylxanthines (aminophylline and theophylline) are used in the treatment of apnea of prematurity. In view of its lower toxicity, caffeine is the preferred drug (Comer, Perry et al. 2001; Henderson-Smart and Steer 2001). The main mechanism of action of caffeine in treatment of apnea of prematurity is through its competitive antagonism of adenosine at its receptors A_1 and A_{2A} in the central respiratory center, beside its competitive antagonism of adenosine at its receptors A_{2A} and A_{2B} in the peripheral respiratory center (peripheral chemoreceptors in the carotid body) (Comer, Perry et al. 2001; Bhatt-Mehta and Schumacher 2003).

Adenosine is released from all cells, including neurons and glia in the central nervous system. It is a neuromodulator that regulates the action of several neurotransmitters in the brain such as glutamate, dopamine, serotonin, γ-aminobutyric acid (GABA) and others. Adenosine receptors are widely distributed in the central nervous system. Adenosine is involved in the regulation of several functions of the brain: sleep and arousal, cognition and memory, stress and anxiety, susceptibility to seizures and analgesia. It has neuroprotective actions in experimental models of cerebral ischemia (Dunwiddie and Masino 2001; Ribeiro, Sebastiao et al. 2002).

Caffeine, a non-selective adenosine receptor blocker, can affect adenosine functions in the central nervous system. The long-term effect of caffeine therapy in preterm infants is not sufficiently known (Millar and Schmidt 2004). Despite recent studies indicating a benefit of caffeine for the premature infant regarding bronchopulmonary dysplasia and cerebral palsy, in the short term, these studies were done in human newborns with complicated medical backgrounds (Schmidt, Roberts et al. 2006; Schmidt, Roberts et al. 2007). Controversy remains about the long term and subtle effects of caffeine on the immature and still developing brain of the newborn. Previous work in our laboratory has shown that short term use of high dose caffeine increases cell death in selected regions of the brain (Black, Pandya et al. 2008).

The objective of my research is to determine the long term neurobehavioral and neuropathological effects of clinically relevant doses of caffeine, as used for apnea of prematurity. In this research, caffeine was administered in the rat pups in the neonatal age group in a dose and duration that is comparable to the practice in the neonatal intensive care. In the text of this thesis, in addition to providing the methodology, results and discussion of my research, I will review apnea of prematurity, which is the main reason for use of caffeine in neonatal intensive care units. I will also review the effects of caffeine on the developing brain in both human and animal research.

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Chapter 1: Apnea of Prematurity and Its Treatment

1.1. Apnea of Prematurity:

1.1.1. Background:

Advances in antenatal medicine and neonatal intensive care, including antenatal corticosteroid utilization, more aggressive delivery room resuscitation, the use of surfactant, and improved nutritional management have successfully resulted in enhanced survival rates of preterm infants (Jones, Karuri et al. 2005; Stephens and Vohr 2009). With progressive gestational age, survival rates increase from over 50% at 25 weeks' gestation to over 90% by 29 weeks' gestation (Jones, Karuri et al. 2005; Stephens and Vohr 2009). With better survival the incidence of problems of prematurity increase. One of the most common problems that face all neonatologists and paediatricians, who take care of these preterm infants, is apnea of prematurity (AOP). The importance of apnea of prematurity relates to its known association with increased developmental delay, and concerns regarding a predisposition to sudden infant death syndrome. As a result, AOP is treated aggressively in neonatal intensive care practise, with caffeine as the most common therapeutic intervention.

Prematurity is the most common reason for admission to neonatal intensive care units. Prematurity is defined as birth before 37 weeks gestational age, while extreme prematurity is defined as birth before 28 weeks (Meadow, Lee et al. 2004; Simhan and Caritis 2007).

Apnea of prematurity is a common problem in preterm infants. The incidence of apnea is inversely proportional to gestational age. It occurs in 35% of infants born at less than 32 weeks' gestation and more than 85% of infants born at less than 28 weeks' gestation (Comer, Perry et al. 2001; Bhatt-Mehta and Schumacher 2003).

Apnea of prematurity is generally defined as the cessation of breathing for at least 20 seconds, or as a briefer episode of apnea accompanied by bradycardia, cyanosis, desaturation and pallor (Abu-Shaweesh 2004; Baird 2004; Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005). Periodic breathing is another term used in neonatal intensive care units. It is defined as recurrent pauses in respiration lasting 5 to 10 seconds followed by 10 to 15 seconds of rapid respiration. This irregularity of respiration is so common that it is considered normal in preterm infants (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Apnea of prematurity is classified into three categories based on the presence or absence of obstruction of the upper airways; central, obstructive, and mixed. Central apnea is characterized by a total cessation of inspiratory efforts with no evidence of obstruction. Obstructive apnea is breathing against an obstructed upper airway resulting in chest wall motion without airflow. The site of obstruction in the upper airways is mostly in the pharynx. It may also occur at the larynx, and possibly both sites. Mixed apnea is the most common type of apnea and consists of obstructed respiratory efforts usually following central pauses. Mixed apnea typically accounts for more than half of all apneic episodes,

followed in decreasing frequency by central and obstructive apnea (Baird 2004; Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Numerous conditions in the newborn may have apnea as a presenting sign. Before concluding that apnea is due to immature respiratory control, other potential causes should be considered. Central nervous system problems like intracranial hemorrhage, asphyxia, seizures, infections (sepsis, meningitis), and malformations of the brain all can produce apnea. Anemia may also present with an abnormal breathing pattern. Other conditions that sometimes precipitate apnea include metabolic disorders, such as hypoglycemia, acidosis, electrolyte imbalance and temperature instability. Medications such as opiates, magnesium sulphate and prostaglandin (PGE1) infusion all can cause apnea. Thus, before labeling a premature baby as having apnea of prematurity, a thorough consideration of other possible causes is always warranted, especially when there is an unexpected increase in the frequency of episodes of apnea and/or bradycardia (Baird 2004; Martin, Abu-Shaweesh et al. 2004).

Apnea of prematurity generally resolves by about 36-40 weeks post-conceptional age (PCA). In extremely premature infants (24 to 28 weeks' gestation), apnea may persist beyond 40 weeks PCA. The cardiorespiratory events in such infants return to the normal level at about 43 to 44 weeks PCA. Beyond 43-44 weeks, the incidence of cardiorespiratory events in preterm infants does not significantly exceed that in term babies. Many preterm infants have resolved their apnea and bradycardia by the time they are ready for hospital discharge as determined by maturation of temperature control and feeding pattern (Baird 2004;

Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005). An apnea-free observation period, usually ranging from three to seven days, is utilized as a criterion for determining discharge date. For a subset of infants, however, the persistence of cardiorespiratory events may delay discharge from the hospital. In these infants, apnea longer than 20 seconds is rare. Rather they exhibit frequent bradycardia to less than 70 or 80 beats per minute with short respiratory pauses (Baird 2004; Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

1.1.2. Pathophysiologic Mechanisms of Apnea of Prematurity:

Hypercapnia, hypoxia, and upper airway afferents are all thought to contribute to the pathophysiology of apnea of prematurity. The ventral surface of the medulla plays an essential function for central CO_2 chemosensitivity and modulation of afferent inputs from peripheral chemoreceptors and laryngeal afferents (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

It has been widely assumed that apnea of prematurity is caused by immaturity of brainstem respiratory rhythm generation that is in proximity to sites of central CO_2 chemosensitivity. The ventilatory response to CO_2 has been shown to increase with advancing postnatal and gestational age in preterm human infants. Therefore, the breathing response to CO_2 in preterm infants, especially those with apnea, is impaired when compared to term neonates or adults. Term neonates and adults increase their ventilation through an increase in both tidal volume and frequency, while preterm infants do not appear to increase frequency in response to CO_2. This response of respiratory timing during hypercapnic

exposure is associated with prolongation of expiratory duration (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Khan and colleagues found that the $PaCO_2$ apneic threshold in newborn infants is only slightly below the eupneic $PaCO_2$. This small difference is likely to make these infants more prone to respiratory oscillations, with frequent decreases in baseline $PaCO_2$ to levels below the apneic threshold, favoring the appearance of periodic breathing and apnea (Khan, Qurashi et al. 2005). Caffeine increases the sensitivity of the medullary respiratory centers to hypercapnia (Comer, Perry et al. 2001).

A decrease in arterial PaO_2 and oxygen saturation is the typical result of apnea in preterm infants, although the extent of that fall varies between infants. The decrease in oxygenation is directly related to the duration of apnea and the initial level of PaO2. A fall in oxygenation is reportedly greater in obstructive than central apnea (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005). With more severe bradycardia, both systolic and diastolic blood pressures may fall and this has been associated with a decline in cerebral blood flow velocity. Therefore, in infants without adequate cerebrovascular autoregulation, cerebral perfusion may decrease to very low levels during prolonged apnea and might potentially exacerbate hypoxic-ischemic brain injury in susceptible premature infants (Martin, Abu-Shaweesh et al. 2004).

During exposure to hypoxia, neonates exhibit a biphasic ventilatory response which consists of an initial increase in ventilation that lasts 1-2 minutes, followed by a decline in breathing, often to below baseline ventilation. This late

decline has been traditionally termed hypoxic ventilatory depression. The initial increase in ventilation is caused by stimulation of peripheral chemoreceptors, primarily in the carotid body. In preterm human infants, the hypoxic ventilatory depression is caused primarily by a decrease in respiratory frequency, as tidal volume remains relatively stable. The origin of the late depression is not well understood, but it may persist for several weeks postnatally in preterm infants (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Several theories have been postulated to explain hypoxic ventilatory depression, including a decrease in metabolism, a decrease in $PaCO_2$ secondary to the initial hyperventilation and accompanying decrease in cerebral blood flow, and hypoxia mediated central depression of ventilation, which represents the most likely primary mechanism. Multiple neurotransmitters have been implicated as mediators for hypoxic depression including adenosine, endorphins and GABA. The use of blockers for these neurotransmitters such as methylxanthines for adenosine, naloxone for endorphins, and bicuculline for GABA has been shown to be successful in preventing the late hypoxic depression and sustained the ventilatory response (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

In the fetal environment, where levels of PaO_2 are in the 20 mm Hg (2.6 kPa) range and gas exchange occurs at the placenta, hypoxic respiratory depression does not cause a problem because continuous breathing is not necessary. However, postnatally when pulmonary ventilation must be continuous, hypoxia-induced respiratory depression presents a problem. Hypoxic ventilatory

depression has been implicated as underlying apnea of prematurity, however, hypoxia does not appear to precede episodes of apnea and, in most occasions, the infants start with a normal PaO2 prior to the occurrence of apnea. Once hypoxia occurs it would appear to aggravate apnea and result in delayed recovery of the infant (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Nock and colleagues demonstrated that premature infants with more apnea had increased initial ventilation elicited by hypoxia compared with those with less or no apnea. Their finding reflects abnormal maturation of peripheral chemoreceptor responses manifested as an increase in peripheral chemoreceptor activity caused by previous intermittent hypoxic episodes (Nock, Difiore et al. 2004). This respiratory instability may predispose these infants to further episodes of apnea (Nock, Difiore et al. 2004; Cardot, Chardon et al. 2007).

Stimulation of the laryngeal mucosa, either chemically or mechanically, causes inhibition of breathing and apnea in humans and animals. This reflex-induced apnea is mediated through the superior laryngeal nerve, and has been shown to be associated with contraction of the thyroarytenoid muscle, causing closure of the glottis, signifying active stimulation of expiratory-related brainstem centers. Preterm infants have an exaggerated inhibitory reflex and they elicit prolonged apnea in response to instilling saline in the oropharynx. Hypercapnia increases, and hypocapnia decreases the threshold for superior laryngeal nerve stimulation-induced apnea. Theophylline, which stimulates respiratory neural output, blocks laryngeal-induced apnea (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Apnea and laryngeal adduction during laryngeal stimulation serve to protect the lungs from aspiration. While this response is assumed to be an essential protective reflex, an exaggerated response has been implicated as a cause of apnea of prematurity. This has also been implicated in gastroesophageal reflux (GER)-induced apnea in infants (Martin and Abu-Shaweesh 2005). The relation between gastroesophageal reflux (GER) and apnea in infants will be discussed later.

St-Hilaire et al found that laryngeal stimulation by water or HCl on day 7 of life (D7) of preterm lambs induced significant apneas, bradycardia, and desaturation. No significant apnea, bradycardia, or desaturation were observed on D14 (St-Hilaire, Samson et al. 2007). They concluded that laryngeal stimulation by liquids triggered potentially dangerous laryngeal chemoreflexes (LCR) in preterm lambs on D7, but not on D14. They proposed that maturation of the LCR between D7 and D14 was partly involved in the disappearance of apneas/bradycardias of prematurity with postnatal age (St-Hilaire, Samson et al. 2007).

Xanthines, which stimulate respiratory neural output, block laryngeal-induced apnea (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

1.1.3. Apnea of Prematurity and Neurodevelopmental Outcome in Very Low Birth Weight Infants:

Apnea episodes vary in severity, but there is no general agreement on what truly constitutes a more ‘‘severe’’ form of apnea, whether it is deeper

desaturation, more marked bradycardia or a combination of the two. The frequent hypoxic and bradycardic episodes may lead to significant decrements in brain oxygen delivery during critical phases of brain growth and development, which may be followed by re-oxygenation injury. Such hypoxia and re-oxygenation injury could generate reactive oxygen species and lead to cellular injury in the developing brain. (Janvier, Khairy et al. 2004).

Several retrospective studies showed an association between neurodevelopmental impairment and apnea of prematurity (Butcher-Puech, Henderson-Smart et al. 1985; Cheung, Barrington et al. 1999; Pichler, Urlesberger et al. 2003; Janvier, Khairy et al. 2004; Pillekamp, Hermann et al. 2007).

Janvier et al reviewed the charts of 175 preterm infants of less than <1250 g birth weight, or <32 weeks gestation and determined the number of days that apnea and bradycardia spells were noted by the nursing staff, during the initial hospitalization. She had demonstrated that there was a statistical association between neurodevelopmental impairment and the number of days of hospitalization during which apnea and bradycardia episodes were recorded by the nursing staff. That association persisted after correcting for multiple other known risk factors, including postnatal steroids, gender, and the duration of assisted ventilation (Janvier, Khairy et al. 2004). Butcher-Puech et al described a higher incidence of abnormal neurological development during the first year of life in infants who had obstructive apnea of more than 20 seconds duration (Butcher-Puech, Henderson-Smart et al. 1985). Cheung et al showed that there was a positive relationship between the severity of apnea spells recorded at the

time of planned discharge from the NICU, and neurodevelopmental impairment in premature infants, especially those with severe intraventricular hemorrhage (Cheung, Barrington et al. 1999). Pillekamp et al concluded that the more severe courses than expected of apnea and bradycardia of prematurity for postmenstrual age (PMA) or the resolution at a later PMA indicated an increased risk of neurodevelopmental disturbances at a corrected age of 13 months (Pillekamp, Hermann et al. 2007). Pichler et al demonstrated that bradycardia caused an aggravated decrease of cerebral hemoglobin oxygenation index (cHbD) and that there is a decrease of cerebral blood volume (CBV) during apnea with bradycardia. These effects on cHbD and CBV indicate a decrease of cerebral blood flow (CBF) during bradycardia. These findings raise the question whether bradycardia and its effects on cerebral hemodynamics have a significant long-term impact on neurological development (Pichler, Urlesberger et al. 2003).

On the other hand, other studies failed to show a relationship between apnea episodes and neurodevelopmental outcome (Levitt, Mushin et al. 1988; Koons, Mojica et al. 1993). Levitt et al monitored thirty infants born at less than 31 weeks gestational age for apnea, then examined them between 16 and 36 months looking for disabilities. She found a low incidence of disabilities associated with apneic attacks (Levitt, Mushin et al. 1988). Koons et al, in another retrospective study concluded that the presence of persistent neonatal apnea without additional adverse perinatal events did not appear to be associated with a higher incidence of significant developmental problems (Koons, Mojica et al. 1993).

1.1.4. Apnea of Prematurity and Gastroesophageal Reflux Disease in Very Low Birth Weight Infants:

Gastroesophageal reflux (GER) is a common problem for preterm infants (Molloy, Di Fiore et al. 2005). It is defined as the retrograde movement of gastric contents into the esophagus and above. The refluxate can rise to the upper esophagus, cross the upper esophageal sphincter and enter the pharynx, larynx and trachea (Miller and Kiatchoosakun 2004). GER is common in term and preterm infants and is diagnosed in about 7% of all infants during their first year of life to the extent that they are brought to medical attention. One of several factors contributing to GER in infants is their supine body position, resulting in the gastroesophageal junction being constantly submerged and a relatively large fluid intake. However, the major mechanism of GER in healthy preterm infants appears to be transient lower esophageal sphincter relaxation (Molloy, Di Fiore et al. 2005). The presence of acidic stomach contents in the area around the larynx excites chemoreceptors in the mucosa, which send afferent neural processes to the brainstem cardiorespiratory centers producing a rapid inhibition of breathing, hypertension, bradycardia and arousal. Intact function of this chemoreflex is important in the prevention of aspiration (Miller and Kiatchoosakun 2004). The presence of formula at the level of the hypopharynx and larynx may trigger apnea. Because both GER and apnea are familiar occurrences in the hospitalized premature infant, a cause and effect relationship was always a concern (Molloy, Di Fiore et al. 2005).

Several studies performed in the 1970s and 1980s linked apnea with GER. Herbst et al used esophageal motility and pH measurements to identify 14 infants aged 2–4 months in whom apnea and cyanosis appeared to be caused by GER (Herbst, Book et al. 1978). However, other investigations have failed to document that GER itself is a cause of the apnea of prematurity. Di Fiore et al found no evidence of a temporal relationship between acid-based GER and apnea in preterm infants. GER did not prolong apnea duration, nor did it exacerbate the decrease in heart rate and oxygen saturation (Di Fiore, Arko et al. 2005). A similar finding was shown by Barrington et al (Barrington, Tan et al. 2002).

1.1.5. Apnea of Prematurity and Feeding in Very Low Birth Weight Infants:

Well coordinated sucking and swallowing behavior in infants begins between 33 and 36 weeks post-conceptional age. During the time of feeding, the drive for feeding and respiration must be balanced, so that the infant can maintain normal ventilation and take adequate liquid in a reasonable period of time. This is accomplished by reciprocal reflex interconnections between the neuronal groups that serve feeding and respiration in the brainstem (Miller and Kiatchoosakun 2004). Infants born before 34 weeks gestation seldom succeed in coordinating oral feeding, due to general neurological immaturity. In such infants, ventilation is sacrificed to the demands of feeding (Shivpuri, Martin et al. 1983). Shivpuri et al showed that minute ventilation fell by 52% during continuous sucking in the preterm infant, and transcutaneous oxygen pressure decreased and CO_2 increased. Thus oral feeding impairs ventilation during continuous sucking in the premature infant. With increased post-conceptional age, the impairment disappears as

intermittent sucking develops (Shivpuri, Martin et al. 1983). The suppression of ventilatory drive during feeding may be accomplished in part by a reduction in CO_2 responsiveness at the level of the ventral medulla (Miller and Kiatchoosakun 2004). By term, coordination of breathing and feeding is more advanced, although not perfect (Shivpuri, Martin et al. 1983; Miller and Kiatchoosakun 2004).

1.1.6. Apnea of Prematurity and Sudden Infant Death Syndrome (SIDS) in Very Low Birth Weight Infants:

Apnea and SIDS remain linked epidemiologically because they both occur in preterm infants (Baird 2004). Early case reports seemed to indicate that children who had apnea were at risk to die of SIDS (Steinschneider 1972). Careful analysis of a large cohort of infants failed to find any relationship between apnea and later deaths, as Hoppenbrouwers et al found that apnea lasting >30 seconds was associated with immaturity and did not seem to be an immediate precursor of or causally related to SIDS (Hoppenbrouwers, Hodgman et al. 2008). No clinical evidence reliably links a ventilatory control abnormality to SIDS (Shivpuri, Martin et al. 1983). The apparent lack of a relationship between persistent apnea of prematurity and SIDS has become clearer in recent years. When age at SIDS is compared to the disappearance of pathological apnea, it is seen that apnea of prematurity resolves prior to the peak incidence of SIDS. This finding lends further support to the notion that apnea of prematurity is unrelated to SIDS (Baird 2004). See Figure 1-1.

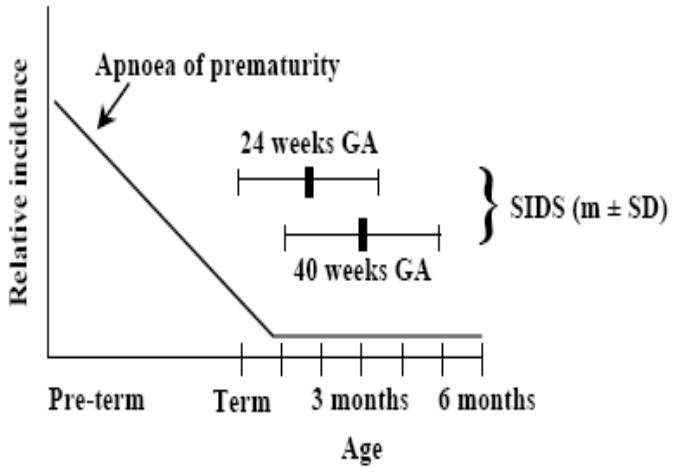

Figure 1.1: Relationship between the timing of peak incidence of SIDS to the (relative) incidence of apnea in infants with apnea of prematurity (Baird 2004).

1.2. Treatment of Apnea of Prematurity:

When a preterm infant develops apnea, it is very important to evaluate for secondary causes such as sepsis, seizures, and others. Treatment of apnea in these cases is related to treatment of the primary cause (Bhatt-Mehta and Schumacher 2003). Once a preterm infant is diagnosed to have apnea of prematurity, then specific treatment should be provided. In this section, options for treatment of apnea of prematurity will be discussed. Treatment of apnea of prematurity should be as non-invasive as possible, and should include both non-pharmacological and pharmacological approaches.

1.2.1. Non-Pharmacological Treatment of Apnea of Prematurity:

Continuous positive airway pressure (CPAP), high flow nasal cannulae, skin-to-skin nursing, olfactory stimulation, blood transfusion, positioning,

kinesthetic stimulation, oxygen, CO_2, and thermal drive are all used for treatment of AOP or have been examined in clinical research with variable results.

Nasal CPAP is widely used in the treatment of apnea of prematurity, usually at 5–6 cm H_2O. It is used to treat the obstructive part of apnea of prematurity, so it works in mixed and obstructive apneas, but does not reduce the incidence of central apnea (Miller, Carlo et al. 1985; Martin, Abu-Shaweesh et al. 2004). CPAP appears to be effective by splinting the upper airway with positive pressure and decreasing the risk of pharyngeal or laryngeal obstruction. CPAP probably also benefits apnea by increasing functional residual capacity (FRC) and so improving oxygenation status. At higher FRC, time from cessation of breathing to desaturation and resultant bradycardia is prolonged (Martin, Abu-Shaweesh et al. 2004; Milner and Greenough 2004; Martin and Abu-Shaweesh 2005). High-flow nasal cannula therapy had been suggested as an equivalent treatment modality to CPAP that may allow CPAP delivery while enhancing mobility of the infant for parents and caretakers (Sreenan, Lemke et al. 2001). However, the drawbacks of this study are that it had a small sample size and the observational period was only for 6 hours. For severe or refractory episodes, endotracheal intubation and artificial ventilation may be needed (Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005).

Kangaroo care, or skin-to-skin nursing, has achieved widespread acceptance for stable infants, and provides an opportunity for greater parental involvement (Martin and Abu-Shaweesh 2005). Although advocates of this

approach have suggested a decrease in apnea rates, Bohnhorst et al's study has not supported this impression (Bohnhorst, Gill et al. 2004).

Marlier et al introduced a pleasant odor in the incubators of 14 preterm infants born at 24 to 28 weeks gestational age who had recurrent apneas despite caffeine and doxapram therapy and found it of therapeutic value (Marlier, Gaugler et al. 2005). However, this study had no randomization, no control group, and had a small number of cases.

The oxygen carrying capacity can be increased by transfusions of red blood cells. The effect of blood transfusion on breathing patterns or apnea in premature infants was evaluated by several clinical trials. Blank et al in his randomized, controlled trial designed to study the benefits of a booster transfusion in a group of 56 premature infants, found no difference (65 vs 63%) in the incidence of apnea between the two groups (Blank, Sheagren et al. 1984). In another study, Westkamp et al concluded that blood transfusions significantly reduced heart and respiratory rates in anemic infants, but had little effect on apnea of prematurity (Westkamp, Soditt et al. 2002).

AOP has been shown to respond to changes in posture. Very premature infants studied before neonatal unit discharge, were found more efficiently with fewer arousals in the prone position; however, they had more central apneas (Bhat, Hannam et al. 2006). Reher et al found no significant advantage of the 15° head-up tilt position over a standard horizontal position (Reher, Kuny et al. 2008).

The effect of kinesthetic stimulation in the form of oscillating or rocking waterbeds for the treatment or prevention of apnea of prematurity has been

summarized in the Cochrane database of systematic reviews. It did not show significant effectiveness in reducing the frequency of apnea (Osborn and Henderson-Smart 2000; Henderson-Smart and Osborn 2002).

The simple provision of supplemental oxygen to relieve hypoxemia has not been systematically studied. Few observational studies suggest that periodic breathing and apnea were reduced when oxygen was administered (Weintraub, Alvaro et al. 1992).

Al-Saif et al, in a novel study, examined the effect of inhalation of low concentration of CO_2 (0.5% -1.5%) as a potential treatment for apnea of prematurity. The findings of this study suggested that inhalation of low concentrations of CO_2 in preterm infants with apnea decreased the number and time of apneas, improved oxygenation, and increased ventilation (Al-Aif, Alvaro et al. 2001).

Tourneux et al in a study suggested that breathing instability during mild thermal challenges in near-term neonates is unrelated to levels of body temperature changes but is controlled by processes involved in body heat loss (BHL). These findings suggest that manipulating the thermal environment with a view to increasing BHL may be an additional or even alternative means of clinical apnea treatment that may hasten the resolution of apnea of prematurity (Tourneux, Cardot et al. 2008)

1.2.2. Pharmacological Treatment of Apnea of Prematurity:

Several drugs have been used to treat or prevent apnea of prematurity, most of which have been evaluated by systemic reviews.

1.2.2.1. Methylxanthines:

Methylxanthines have been the mainstay of pharmacologic treatment of apnea of prematurity. There is now a four decade long history of their use in preterm infants. Xanthine therapy increases minute ventilation, improves CO_2 sensitivity, decreases hypoxic depression of breathing, enhances diaphragmatic activity, and decreases periodic breathing (Bhatt-Mehta and Schumacher 2003; Martin, Abu-Shaweesh et al. 2004).

Both theophylline and caffeine are effective in treatment and prevention of apnea of prematurity, (Henderson-Smart and Davis 2000; Henderson-Smart and Steer 2001) and in increasing the chance of successful extubation of extremely preterm infants (Henderson-Smart and Davis 2000). Caffeine can also be used to prevent postoperative apnea in preterm infants (Henderson-Smart and Steer 2001).

Caffeine and theophylline appear to have similar short term effects on apnea/bradycardia, however, caffeine has certain therapeutic advantages over theophylline. Caffeine has a larger therapeutic window (Steer and Henderson-Smart 2000; Bhatt-Mehta and Schumacher 2003). Caffeine is more easily absorbed and has a longer half-life that allows once daily doses. It also has fewer side effects, as indicated by tachycardia or feeding intolerance leading to change in dosing (Steer and Henderson-Smart 2000; Bhatt-Mehta and Schumacher 2003).

For the above reasons, caffeine is currently widely used as first-line drug therapy in apnea of prematurity (Steer and Henderson-Smart 2000; Bhatt-Mehta and Schumacher 2003). Caffeine, as a treatment of apnea of prematurity, will be discussed in the next chapter.

1.2.2.2. Doxapram:

Doxapram is a potent respiratory stimulant that has predominantly peripheral chemoreceptor effects. It is used when a methylxanthine does not significantly reduce the frequency of apnea episodes. It also increases minute ventilation and tidal volume (Steer and Henderson-Smart 2000; Bhatt-Mehta and Schumacher 2003). At higher doses, it appears to exert a central effect. The half-life is reported to be about 10 hours in the first few days of life, and about eight hours at 10 days of age. The recommended dosage is 1 to 2.5 mg/kg/h given by continuous IV infusion. That dosage can be decreased to 0.5 to 0.8 mg/kg/h when apnea episodes become less frequent (Bhatia 2000; Bhatt-Mehta and Schumacher 2003). Henderson-Smart et al, in a Cochrane review, concluded that doxapram might reduce apnea within the first 48 hours of treatment; however, there are insufficient data to evaluate the precision of this result or to assess potential adverse effects. No long term outcomes have been measured. The review found significant short term side effects of doxapram such as hypertension, excessive central nervous system stimulation, gastrointestinal disturbances and heart block (Henderson-Smart and Steer 2004). Sreenan et al (2001) suggested an association between the total dose and duration of doxapram treatment and isolated mental developmental delay in infants weighing less than 1250 grams at birth.

1.2.2.3. Carnitine:

Carnitine, a quaternary amine synthesized from the amino acid lysine, is essential for the transport of fatty acids across mitochondrial membranes for beta-oxidation metabolism and ketone generation. Deficiency of carnitine leads to a decrease in long chain fatty acids that are available for beta-oxidation, resulting in a decrease in energy production at the muscular level. Kumar et al in their Cochrane review mentioned that in a case series of infants with apnea and periodic breathing, a decrease in such episodes was noted following 48 hours of treatment with oral carnitine. In his review, he found no trials of the use of carnitine in treatment of apnea of prematurity, however, he found two trials where carnitine was used as prophylaxis for apnea. Both trials did not show a statistically significant difference in the frequency of episodes of apnea, the length of ventilation, or the duration of hospital stay. These trials enrolled a small number of patients, with very few developing recurrent apnea in either of the groups (Kumar, Kabra et al. 2004).

1.2.2.4. Creatine:

Bohnhorst et al randomized 38 preterm infants with significant apnea of prematurity into 2 groups; 19 received 200 mg/kg per day of oral creatine supplementation for two weeks, and 19 received placebo. The researchers' rationale for the use of creatine was that it is a substrate in the regeneration of adenosine diphosphate to adenosine triphosphate and it is important for the transport of high-energy phosphates in the muscle from the site of production (mitochondria) to the site of use (myofibers). In the human, the neonate is

regarded as creatine-deficient. However, creatine supplementation did not improve symptoms of apnea of prematurity (Bohnhorst, Geuting et al. 2004).

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Chapter Two: Caffeine as Treatment of AOP

Introduction:

The WHO Pocketbook of Hospital Care for Children recommends caffeine citrate and aminophylline to prevent apnea in premature babies (McCallum and Duke 2007) . The use of caffeine in neonatal intensive care units has increased significantly in the last years, as it became the drug of choice for the treatment of apnea of prematurity (Comer, Perry et al. 2001; Du, Warrier et al. 2006).

2.1. Structure & Pharmacodynamic Properties of Caffeine:

Caffeine is a 1, 3, 7-trimethylxanthine. It occurs naturally in the fruit of Coffea arabica and related species. Two other methylated xanthines, theophylline and theobromine, also occur naturally. These methylated xanthenes: caffeine, theophylline and theobromine are structurally related to a number of important endogenous metabolites including adenosine, purines, xanthine and uric acid (Comer, Perry et al. 2001). See Figure 2-1.

Caffeine has three basic cellular mechanisms of action: antagonism of adenosine receptors, inhibition of phosphodiesterase, and mobilization of intracellular calcium.

Most of the observed pharmacological effects of caffeine (increased metabolic activity, relaxation of smooth muscle and the stimulation of cardiac, respiratory and central nervous systems) are mediated by its antagonism of the actions of adenosine at cell surface receptors: A_1 and A_{2A} (Comer, Perry et al.

2001). Caffeine binds to adenosine A_1 and A_{2A} receptors with inhibition constant (IC_{50}) values of about 40-50 µM (Ukena, Schudt et al. 1993; Comer, Perry et al. 2001). The IC_{50} is defined as the concentration of a drug required to reduce activity for a defined substrate (e.g.; enzyme) by 50% (Bachmann and Lewis 2005).

Caffeine is also a weak inhibitor of phosphodiesterase activity, so the concentration of caffeine required for 50% inhibition (IC_{50}) is in the millimolar range (Ukena, Schudt et al. 1993; Comer, Perry et al. 2001). At millimolar concentrations, caffeine is likely to be toxic in vivo. Clinical doses are well below the IC_{50} of caffeine for phosphodiesterases, thus it is very unlikely that inhibition of phosphodiesterase activity mediates the therapeutic actions of caffeine (Comer, Perry et al. 2001).

The third mechanism of action of caffeine is mobilization of calcium from intracellular storage sites and inhibition of voltage-sensitive calcium channels. High concentrations of caffeine (≥250 µM) are required for calcium mobilization. This action may therefore contribute more to the toxic effects of caffeine (Comer, Perry et al. 2001).

2.2. Systemic Effects of Caffeine:

Caffeine stimulates central respiratory centers, increases the sensitivity of central medullary areas to hypercapnia, increases mean respiratory rate and minute volume, and increases pulmonary blood flow. In preterm infants, caffeine improves the compliance of the respiratory system (Comer, Perry et al. 2001).

Adenosine can produce both contraction and relaxation in the pulmonary artery. Adenosine induces contraction (vasoconstriction) via the activation of A_1 receptors and vasodilatation through the activation of adenosine A_2 receptors. Adenosine receptors in the large pulmonary artery has been reported to be of the A_{2B} subtype and those in small arteries appear to be of the A_{2A} subtype (Tabrizchi and Bedi 2001).

Caffeine can directly stimulate the myocardium in preterm neonates. Caffeine has a positive inotropic and chronotropic effect on the heart as it increases ventricular output, stroke volume and mean arterial blood pressure (Comer, Perry et al. 2001). These effects of caffeine on the CVS are through its antagonistic effect on adenosine receptors. Cardiac myocytes and vascular cells readily synthesize, transport, and metabolize the endogenous adenosine and act to regulate both interstitial and plasma adenosine concentrations. Adenosine slows heart rate, reduces atrial contractility, slows AV conduction, antagonizes the stimulatory effects of catecholamines, and reduces activity of pacemaking cells. Adenosine is one of the most commonly used drugs in the diagnosis and treatment of supraventricular tachycardia (Shryock and Belardinelli 1997). Yilmaz et al showed that intravenously injected nucleosides adenosine, uridine and cytidine decreased arterial pressure by activating peripheral adenosine receptors in a rodent model. Adenosine and uridine decreased heart rate as well. These effects were attenuated by the use of adenosine receptor antagonists (Yilmaz, Coskun et al. 2008).

Adenosine is cardioprotective during episodes of cardiac hypoxia/ ischemia. Rork et al demonstrated that the administration of an A_{2A} adenosine receptor selective agonist during reperfusion is cardioprotective in the isolated, perfused mouse heart via inhibition of the cardiac mast cell degranulation leading to a decrease in tryptase release (Rork, Wallace et al. 2008).

Tikh et al. found that stimulation of A_{2A} adenosine receptors enhances the contractile response to adrenergic stimulation in the murine heart directly through an effect on contractility and indirectly by an attenuation of the anti-adrenergic actions of the A_1 adenosine receptors. In addition, A_{2A} activation supports contractile function during low-flow ischemia, resulting in an increased contractile function at the given reduced perfusion pressure (Tikh, Fenton et al. 2006).

Activation of A_{2A} and A_{2B} adenosine receptors causes vasodilatation, while activation of A_1 receptors causes vasoconstriction (Shryock and Belardinelli 1997). This vasodilatation effect of adenosine through A_2 receptors is attenuated by theophylline (El-Kashef, Elmazar et al. 1999).

Hoecker et al showed that oral administration of a high loading dose of caffeine base (25 mg/kg), in preterm infants, resulted in a marked reduction of cerebral and intestinal blood flow velocity (BFV), but there was no change in left ventricular output (LVO), blood pressure and heart rate (Hoecker, Nelle et al. 2002). When she divided this high loading dose into two doses given four hours apart, there was a decrease in BFV in cerebral arteries after the second dose, whereas BFV in intestinal arteries and LVO were not affected (Hoecker, Nelle et

al. 2006). Soraisham et al showed that a single 10 mg/kg loading dose of caffeine did not cause a significant reduction in superior mesenteric artery BFV, and no significant changes were observed in heart rate, blood pressure and incidence of necrotising enterocolitis (Soraisham, Elliott et al. 2008).

The Caffeine for Apnea of Prematurity (CAP) trial showed a statistically significant decrease in the incidence of patent ductus arteriosus (30% versus 40%) and in the rates of surgical ligation (4.5% versus 12.6%) in the group treated with caffeine citrate (Schmidt, Roberts et al. 2006). The assumed explanation of this finding is that caffeine is a diuretic and a vasoconstrictor, related to adenosine antagonism and may have prostaglandin antagonistic activity. As a response to this finding, Clyman et al conducted a study on preterm sheep to examine the effect of caffeine on the ductus arteriosus and he found no evidence of a direct effect of therapeutic caffeine concentrations on ductus contractility (Clyman and Roman 2007).

Many of the renal effects of caffeine are presumed to be due to its antagonism of adenosine signaling in the kidney, but caffeine may also act via prostaglandin synthesis. In the kidney, responses to adenosine include afferent arteriolar constriction, efferent arteriolar dilation (Lai, Patzak et al. 2006), a decrease in glomerular filtration rate and solute delivery to the proximal tubule, and a decrease in renin release (Shryock and Belardinelli 1997). Kulick et al confirmed that A_1 adenosine receptors in the proximal tubules are increased in response to low salt intake, so solute and fluid reabsorption increased. He showed that A_1 adenosine receptor antagonists increased proximal tubule and urine flow

and decreased absolute fluid reabsorption leading to a diuretic effect (Kulick, Panico et al. 2008). Dobrowolski et al showed that intramedullary infusion of adenosine at 5µg/kg/h did not alter local blood flow or sodium excretion, whereas urine flow increased and urine osmolality decreased (Dobrowolski and Sadowski 2004).

Caffeine causes an increase in glomerular filtration rate and blocks sodium re-absorption, leading to diuretic effects. Caffeine increases urinary flow rate, creatine clearance and water input/output ratio over pretreatment levels. Renal calcium excretion in preterm infants is increased by treatment with caffeine or theophylline. Caffeine has no effect on serum sodium, potassium, calcium, and phosphorus concentrations. However, at clinically used doses, the effects of caffeine on renal function in neonates is minimal (Zanardo, Dani et al. 1995; Comer, Perry et al. 2001).

Caffeine reduces lower gastroesophageal sphincter pressure and increases gastric secretion. This effect resolves in two weeks after stopping therapy. The increase in episodes of reflux is independent of plasma caffeine concentrations or drug efficacy. The mechanism of lower esophageal sphincter relaxation may be through enhancement of cyclic AMP levels (Comer, Perry et al. 2001).

2.3. Physiologic Effects of Caffeine:

In the neonate, caffeine may increase blood glucose concentrations acutely due to increased glycogenolysis; however, this is seen infrequently in the usual clinical situation (Comer, Perry et al. 2001).

An increase in oxygen consumption and energy expenditure has been reported in preterm neonates after 48 hours of caffeine therapy in comparison to the control group, which persisted through 4 weeks. The daily weight gain in the caffeine group was significantly lower than the control group despite the similar caloric intake in both groups during the study period (Bauer, Maier et al. 2001). In this study caffeine-treated infants required lower environmental temperatures to maintain normothermia (Bauer, Maier et al. 2001). The CAP trial validated a reduced weight gain in the caffeine cohort, with the greatest difference noted after two weeks, but the difference resolved at 4 weeks of treatment (Schmidt, Roberts et al. 2006). The study's follow up to the corrected age of 18 to 21 months showed that the mean percentiles for height, weight, and head circumference did not differ significantly between the two groups (Schmidt, Roberts et al. 2007).

2.4. Pharmacokinetic Properties of Caffeine:

The pharmacokinetic properties of caffeine in neonates, particularly those born prematurely, are markedly different from those observed in adults or children. This is largely because neonates have a limited ability to metabolize caffeine as they have an immature hepatic system. Little information is available on the effects of hepatic or renal diseases on caffeine pharmacokinetics in neonates (Comer, Perry et al. 2001).

The pharmacokinetics of caffeine are largely independent of the route of administration and there is practically no first-pass metabolism of caffeine. Administration of caffeine citrate via oral and intravenous routes results in similar

peak plasma concentrations of the drug, indicating almost complete bioavailability of caffeine following oral doses. Concomitant administration of caffeine citrate with feeds does not affect bioavailability (Comer, Perry et al. 2001; Charles, Townsend et al. 2008).

Caffeine is lipophilic and readily passes through most membranes in the body without accumulating in the tissues. The apparent volume of distribution of caffeine is approximately 0.85 L/kg (Micallef, Amzal et al. 2007; Charles, Townsend et al. 2008). Biotransformation of caffeine occurs in the liver via microsomal cytochrome P450 mono-oxygenases (CYP1A2) and via the soluble enzyme xanthine oxidase. The predominant process of caffeine metabolism in the preterm infant is N7-demethylation, which matures at about 4 months of age. N3- and N7-demethylation increase exponentially with postnatal age, regardless of birth weight or gestational age (al-Alaiyan, al-Rawithi et al. 2001; Comer, Perry et al. 2001). The female neonate demonstrates a higher rate of caffeine metabolism than the male (al-Alaiyan, al-Rawithi et al. 2001). In neonates, caffeine is eliminated predominantly via renal excretion according to first-order kinetics; approximately 86% of the drug is excreted unchanged in the urine (within 6 days), compared with less than 4% in adults (Comer, Perry et al. 2001). Metabolism of caffeine is limited in neonates due to their immature hepatic enzyme system. Demethylation increases with postnatal age until a plateau is reached at 120 days of age. The elimination half-life of caffeine in infants decreases from birth until it reaches adult values at approximately 60 weeks post conceptional age (Comer, Perry et al. 2001). The elimination half-life of caffeine in premature neonates is

prolonged to 100-120 hours, which is significantly longer than in neonates born at term (Comer, Perry et al. 2001; Charles, Townsend et al. 2008). Caffeine half-life may be prolonged further in infants who have cholestatic jaundice and in infants that are exclusively breastfed (Comer, Perry et al. 2001).

2.5. Caffeine Dosage and Interactions:

Cytochrome P450 1A2 (CYP1A2) is the major enzyme involved in metabolism of caffeine, so caffeine has the potential to interact with drugs that are substrates for CYP1A2. Cimetidine and ketoconazole can inhibit caffeine metabolism, necessitating lower doses of caffeine, while phenytoin and phenobarbital can increase caffeine elimination, possibly requiring higher doses (Comer, Perry et al. 2001).

Caffeine has normally been formulated as caffeine citrate for administration as a slow intravenous infusion (over 20 to 30 minutes) or orally to premature neonates (Comer, Perry et al. 2001; Leon, Michienzi et al. 2007).

Another formulation of caffeine mentioned in earlier literature is caffeine benzoate for intramuscular administration (Cattarossi, Colacino et al. 1988).

Percutaneous application of caffeine to treat AOP, although it was shown to be effective, is not used in clinical practice due to the difficulty in the accurate administration of this type of dosage form and the lack of a standardized, commercially available drug preparation (Amato, Isenschmid et al. 1991).

The recommended loading dose of caffeine citrate is 20mg/kg given orally or infused intravenously over half hour, in neonates not treated previously.

Twenty four hours after the loading, maintenance treatment with caffeine citrate 5 mg/kg once daily should be given orally or by intravenous infusion over 10 minutes (Leon, Michienzi et al. 2007). With this regimen, Leon et al reported the therapeutic range to be 8 to 20 mg/L in the plasma (Leon, Michienzi et al. 2007). In another study the therapeutic range was reported to be 5 to 20 mg/L (Natarajan, Botica et al. 2007). There are no data concerning the use of caffeine citrate in infants with renal or hepatic disease and hence it should be administered with caution in such patients (Comer, Perry et al. 2001; Leon, Michienzi et al. 2007).

Leon et al in a retrospective review, determined serum caffeine concentrations at 7 days after starting therapy with a 20 or 25 mg/kg loading dose and a 6 mg/kg/d maintenance dose in 154 infants with a mean gestational age of 29 weeks. With this regimen, Leon et al reported the therapeutic range to be 8 to 20 mg/L in the plasma. The conclusion was that routine measurement of steady-state serum caffeine concentrations in infants 24 to 35 weeks gestational age is not required in the absence of ongoing apnea/hypopnea or signs compatible with toxicity (Leon, Michienzi et al. 2007). In another review a total of 231 caffeine blood levels were obtained from 101 preterm infants with a median gestation of 28 weeks and birth weight of 1030 g. The caffeine citrate dose used ranged from 2.5 to 10.9 mg/kg (median: 5 mg/kg), and the levels ranged from 3.0 to 23.8 mg/L. Levels were between 5.1 and 20 mg/L in 94.8% of plasma caffeine levels. The authors concluded that therapeutic drug monitoring is not necessary when caffeine is used for the treatment of apnea of prematurity in neonates (Natarajan, Botica et al. 2007).

Saliva and urine samples were suggested in the literature as noninvasive methods to monitor caffeine levels, as alternatives to the plasma (de Wildt, Kerkvliet et al. 2001; Cattarossi, Violino et al. 2006).

2.6. Therapeutic Efficacy of Caffeine:

Caffeine currently is considered the pharmacologic treatment of choice for apnea of prematurity. A Cochrane review has shown that caffeine is an effective therapy in reducing the number of apneic spells and the use of mechanical ventilation in the 2 to 7 days after starting treatment (Steer and Henderson-Smart 2000; Henderson-Smart and Steer 2001).

The risk of postoperative apnea following general anesthesia, which is predominantly central, persists for about 60 weeks after conception. Henderson-Smart and Steer concluded in their Cochrane review that caffeine can be used to prevent postoperative apnea/bradycardia and episodes of oxygen desaturation in growing preterm infants if this is clinically indicated (Henderson-Smart and Steer 2001).

Methylxanthines also increase the chances of successful extubation of preterm infants within one week of the time of extubation as was shown in a Cochrane review (Henderson-Smart and Davis 2000).

2.7. Adenosine:

As mentioned before, the pharmacological effects of caffeine are mediated by its antagonism of adenosine at cell surface A1 and A2 receptors (Comer, Perry et al. 2001). Adenosine is released from all cells, including neurons and glia

(Ribeiro, Sebastiao et al. 2002). It is a very important substance in the homeostasis of the cells of the nervous system, and is involved in cell death namely in the prevention or induction of apoptosis (Di Iorio, Kleywegt et al. 2002).

Adenosine receptors (ARs) are members of the G protein-coupled receptor family and mediate the multiple physiological effects of adenosine. They are widely distributed in most species and mediate a diverse range of physiological and pathological effects. Adenosine receptors have been classified into four subtypes on the basis of molecular, biochemical and pharmacological characteristics: A_1, A_{2A}, A_{2B} and A_3 receptors (Rees, Lewis et al. 2002; Ribeiro, Sebastiao et al. 2002). All adenosine receptor subtypes are glycoproteins. A_1 and A_3 receptors usually couple to 'inhibitory' G-proteins (Gi and Go) whereas the A_{2A} and A_{2B} receptors couple to 'stimulatory' G-proteins (Gs). Accordingly, A_{2A} receptors stimulate adenylyl cyclase, while A_1 receptors inhibit this enzyme. Adenosine, through the receptor A_1, acts pre-synaptically throughout the nervous system to inhibit neurotransmitter release (Ribeiro, Sebastiao et al. 2002).

Neuromodulation by adenosine is exerted through activation of the high affinity receptors (A_1 and the A_{2A}), which are important in physiological status, and of the low affinity receptor (A_{2B}), which might be relevant in pathological conditions. The A_3 receptor is a high affinity receptor in humans; however, it has a low density in most tissues. The adenosine A_1 receptor is highly expressed in brain cortex, cerebellum, hippocampus, and dorsal horn of spinal cord. The A_{2A} receptor is highly expressed in the striato-pallidal GABAergic neurons and

olfactory bulb; it is expressed in lower levels in other brain regions. A_{2B} possesses low levels of expression in the brain. A_3 has apparently intermediate levels of expression in the human cerebellum and hippocampus and low levels in most of the brain (Ribeiro, Sebastiao et al. 2002). See Figure 2-2.

Adenosine receptors are also present in the peripheral autonomic and somatic nervous systems (Ribeiro, Sebastiao et al. 2002).

2.8. The Effects of Caffeine and Adenosine on the Developing Brain:

Caffeine is a somnolytic agent and central stimulant. The effects of caffeine on the CNS are mediated through its blockage of adenosine receptors. Inhibition of adenosine receptors affects the release, turnover and levels of several other neurotransmitters including dopamine, noradrenaline, serotonin, acetylcholine, and other excitatory and inhibitory amino acids. At higher (potentially toxic) concentrations, caffeine can also bind to benzodiazepine sites on γ-aminobutyric acid (GABA) receptors (Comer, Perry et al. 2001).

2.8.1. Breathing:

Adenosine receptors play a crucial role in control of breathing. Adenosine A_1 receptors are the main adenosine receptors in the brainstem. Gaytan et al showed the importance of the rostral pons in respiratory modulation through adenosine A_1 receptor system. This study showed that postnatal exposure to caffeine modulates the ontogeny of the adenosine A_1 receptor network. This could imply that the role of caffeine to decrease the incidence of neonatal respiratory disturbances may be due to the earlier than normal development of the

adenosinergic system in the brain (Gaytan, Saadani-Makki et al. 2006). Herlenius et al examined the perinatal development of respiratory rhythm generation and the role of adenosine receptor A_1 in its modulation, in a rat model, from embryonic day 18 to postnatal day 3 using an in vitro brain stem preparation. They concluded that respiration is modulated by adenosine A1 receptors at the level of the medulla oblongata in the fetal period that is dependent on the age of the fetus (Herlenius, Aden et al. 2002).

A_{2A} and A_{2B} are the main adenosine receptors in carotid body peripheral chemoreceptors (Gauda, Northington et al. 2000; Conde, Obeso et al. 2006). Conde et al demonstrated the role of endogenous adenosine, via presynaptic A_{2B} and postsynaptic A_{2A} receptors in the carotid body chemoreceptors, in causing an excitatory response to hypoxia manifested by catecholamine release. This response to hypoxia explains the hyperventilation phase of the preterm infant's breathing response to hypoxia, which is followed by respiratory depression leading to apnea. Conde's findings provide an explanation of the observed inhibitory action of caffeine on hyperventilation produced by hypoxia (Conde, Obeso et al. 2006).

Carotid sinus nerve activity is augmented by adenosine binding to A_{2A} adenosine receptors and attenuated by dopamine binding to D_2 dopamine receptors (Gauda, Northington et al. 2000). Gauda et al demonstrated in rats that the level of A_{2A} adenosine receptor mRNA expression is significantly decreased by postnatal day 14, while D_2 dopamine receptor mRNA levels are significantly

increased by day 3, possibly contributing to the maturation of hypoxic chemo-sensitivity with advancing age (Gauda, Northington et al. 2000).

The overall effect of caffeine on breathing is that it increases mean respiratory rate and minute volume, stimulates central respiratory centers and increases sensitivity to hypercapnia, increases pulmonary blood flow and improves the compliance of the respiratory system (Laubscher, Greenough et al. 1998; Comer, Perry et al. 2001).

2.8.2. Sleep and the Level of Arousal:

Adenosine works as a natural sleep-promoting agent; it accumulates during periods of sustained wakefulness and decreases during sleep (Ribeiro, Sebastiao et al. 2002; Fredholm, Chen et al. 2005). The sleep inducing properties of adenosine are in line with its A_1 receptor-mediated inhibitory action and may involve multiple neuronal populations in the CNS (Ribeiro, Sebastiao et al. 2002). Thakkar et al suggested that adenosine acting via A_1 receptors in the orexin-rich lateral hypothalamus may have a key role in the regulation of sleep -wakefulness by reducing the activity of the orexin neurons in the lateral hypothalamus, in addition to its actions in other brain areas, which reinforces the role of adenosine as a homeostatic regulator of sleep (Thakkar, Engemann et al. 2008).

Curzi-Dascalova et al compared the effect of caffeine on sleep organization in 10 neurologically normal and clinically stable preterm neonates to 5 matched controls not on caffeine treatment. She found no significant differences in sleep organization between the 2 groups (Curzi-Dascalova, Aujard et al. 2002). Her finding wasn't consistent with Hayes et al study, which showed that in apneic

preterm infants; chronic methylxanthine treatment appears to produce sleep deprivation secondary to its stimulatory action on arousal and on motor systems (Hayes, Akilesh et al. 2007).

2.8.3. Anxiety and Pain:

The involvement of adenosine A_1 receptors in anxiety was confirmed in several animal studies. Johansson et al showed that the mice knocked out for adenosine A_1 receptors displayed increased anxiety-related behavior as well as thermal hyperalgesia (Johansson, Halldner et al. 2001). Hong-Zhen Pan et al had demonstrated that neonatal caffeine exposure to rat pups results in remarkable changes in adenosine receptor regulated functions including thermal hyperalgesia in the hot plate test, lower anxiety than controls in the dark-light transition and the elevated plus-maze tests in both male and female rats. These results suggest that the preterm infants, who are treated with caffeine for apnea of prematurity, are at risk of adenosine receptor-related behavioral dysfunctions that may exist for a long period (Pan and Chen 2007).

2.8.4. Cognition and Memory:

Endogenous adenosine, through A_1 and A_{2A} receptors, plays a role in both cognition and memory. The cognitive effects of caffeine are mostly due to its ability to antagonize adenosine A_1 and A_{2A} receptors in the hippocampus and cortex, the brain areas mostly involved in cognition (Ribeiro, Sebastiao et al. 2002). Dall'Igna et al demonstrated that the blockade of adenosine A_{2A} receptors prevents β-amyloid-induced impairment of cognitive performance in the inhibitory avoidance and spontaneous alternation tasks, an effect mimicked by the

sub-chronic administration of the non-selective adenosine receptor antagonist caffeine (Dall'Igna, Fett et al. 2007). Hong-Zhen Pan tested the retention of avoidance learning in rats exposed to caffeine in the neonatal age. The test was done at postnatal age 35-37 (P35-37). The study demonstrated that neonatal caffeine exposure produced significant memory impairment in the step-through avoidance learning task in both male and female rats (Pan and Chen 2007). In a previous study, caffeine-treated neonatal rats were trained to avoid an electrified grid and tested for retention 24h, 72h, and 7 days later at ages of P28 or P70-90. At P28, caffeine-treated rats required more trials to avoid the electrified grid than did control rats in both males and females. At P70-90 there was no effect of caffeine treatment on learning; however, at 72 h test, the effect of caffeine on retention differed between genders. Neonatal caffeine treatment decreased retention in males while it improved retention in females significantly (Fisher and Guillet 1997). Although the results of these studies are not homogenous, they do indicate that caffeine has a role in cognition and memory brain functions.

2.8.5. Neuronal Protection, Injury and Maturation:

Tonic activation of A_{2A} receptors may cause noxious actions during injury, while tonic activation of A_1 receptors may cause neuroprotection (Ribeiro, Sebastiao et al. 2002).

Li et al used a mouse model to test the effects of caffeine on traumatic brain injury. In this study it was found that neurological deficits, cerebral edema and inflammatory cell infiltration that occurred after traumatic brain injury were all significantly attenuated in mice pretreated chronically for 3 weeks with

caffeine in drinking water compared with the mice not pretreated with caffeine. Furthermore, in this study, it was found that chronic caffeine treatment attenuated glutamate release and inflammatory cytokine production, effects that correlated with an up-regulation of the neuronal A_1 receptor mRNA (Li, Dai et al. 2008). By contrast, acute treatment with caffeine (intraperitonial injection, 30 min before traumatic brain injury) was not effective in protecting against traumatic induced brain injury. These results suggest that chronic, but not acute, caffeine treatment attenuates brain injury, possibly by A_1 receptor-mediated suppression of glutamate release and inhibition of excessive inflammatory cytokine production (Li, Dai et al. 2008). Back et al generated ventriculomegaly and reduced cerebral myelination in mice reared in hypoxia (10% oxygen) from postnatal days 3 (P3) through 12. He found that myelination was enhanced and ventriculomegaly reduced in hypoxia-exposed neonatal pups treated with caffeine from P3 to P12 (Back, Craig et al. 2006). Bona et al demonstrated that low dose caffeine exposure, in the first 7 days of life, decreased hypoxic ischemic brain damage by 30% in the 7-day-old rats (Bona, Aden et al. 1995).

Kang et al studied the effect of administration of high dose caffeine on neuronal death in neonatal rats. Three doses of intra-peritoneal caffeine at 50 mg/kg per dose were given at 5h interval on postnatal day 7. It was found that pro-apoptotic protease caspase-3 was activated to mediate neuronal death following exposure to caffeine, indicating that caffeine may cause caspase-3-dependent neuronal cell death (Kang, Lee et al. 2002). In a study done at University of Alberta by Black et al, it was found that, in a rat model, caffeine

citrate at a dose of 100 mg/kg given subcutaneously at P7 caused significant cell death measured by Fluoro-jade B and activated caspase-3, at 12 and 24 hour post-caffeine injection in several areas of the brain (Black, Pandya et al. 2008).

A_1 receptors also may play a role in neuronal maturation since, in the forming central nervous system, A_1 receptor activation inhibits the development of axons and can lead to leukomalacia. Turner et al showed, in a rat model, that A_1 adenosine receptor activation induced ventriculomegaly and white matter loss (Turner, Yan et al. 2002).

Exposure to caffeine within the neonatal age in rat pups increased the subsequent expression of adenosine A_1 receptors in the thalamus and the cerebellum (Etzel and Guillet 1994) and also in cortex and hippocampus (Guillet and Kellogg 1991). Gaytan et al showed that in neonatally treated pups with caffeine, a significant increase in the number of adenosine A_1 receptors was found in several respiration-related nuclei of rat brainstem at P6 but not at P8 or P21. This indicates that caffeine may decrease the incidence of AOP due to the earlier than normal development of adenosinergic system in the brains of preterm infants (Gaytan, Saadani-Makki et al. 2006).

The caffeine for apnea of prematurity (CAP) trial was done to study the short and the long term effects of caffeine on preterm infants (Schmidt, Roberts et al. 2006). The primary outcome of the CAP trial was a composite of death, cerebral palsy, cognitive delay, deafness or blindness at a corrected age of 18 to 21 months. The results of the 18 to 21 months follow up of the CAP trial showed that treatment with caffeine as compared with placebo reduced the incidence of

cerebral palsy and cognitive delay. The rates of other outcomes did not differ significantly between the two groups (Schmidt, Roberts et al. 2007). The possible explanations of the CAP trial findings are that caffeine could lead to stabilization of respiratory status, decrease the episodes of hypoxia/hyperoxia, and decrease the incidence of bronchopulmonary dysplasia in the caffeine group. Furthermore, the chronic use of caffeine for weeks in the extreme preterm infants could have a neuroprotective role as it has been shown in animal models.

2.8.6. Seizures:

One of the physiological roles of adenosine is an endogenous anti-epileptic function. The anti-epileptic properties of adenosine are mostly due to the inhibitory actions of A_1 receptors upon synaptic transmission in the hippocampus (Ribeiro, Sebastiao et al. 2002; Fredholm, Chen et al. 2005). Roseti et al demonstrated that blocking the adenosine receptors in the human brain improves the stability of GABA-ergic neurotransmission (Roseti, Martinello et al. 2008). This can explain why caffeine, in toxic dose, can cause seizure activity.

Li et al, in his study about the effects of caffeine on traumatic brain injury, found that the group of mice which were given high intra-peritoneal doses of caffeine citrate at 50mg/Kg developed seizure activity (Li, Dai et al. 2008). Guillet et al showed that early exposure to caffeine in neonatal rats resulted in an altered sensitivity to chemo-convulsants at 28 and 42 days of age (Guillet 1995). Some of these effects were gender specific, as she found that thresholds for seizures produced by some of the chemo-convulsants were higher in females than males (Guillet and Dunham 1995).

Banner et al reported three full-term infants who manifested CNS toxicity after parenteral administration of large doses of caffeine benzoate injection. The infants received caffeine benzoate in doses that ranged from 36 to 136 mg/kg. They exhibited the following symptoms: tachypnea, fine tremor of the extremities, opisthotonus, tonic-clonic movements and non-purposeful jaw and lip movements. The overdose of caffeine produced a clinical picture that suggested neonatal seizures and prompted therapy with anticonvulsants (Banner and Czajka 1980). Anderson et al reported a 31-week gestation neonate, following an accidental administration of a 160 mg/Kg of caffeine, whose CNS manifestation was hypertonia. His symptoms resolved in 7 days (Anderson, Gunn et al. 1999). In another case report a 30-day-old preterm infant born at 28-weeks gestational age was exposed to 300 mg/kg of caffeine base by mouth accidentally. The patient exhibited agitations, irritability, beside other symptoms. No seizure activity was observed. The effects of intoxication lasted for 96 hours and then completely resolved (Ergenekon, Dalgic et al. 2001).

2.8.7. Cerebral Blood Flow:

Hoecker et al found that with a high loading dose of pure caffeine (25 mg/kg), there was a marked reduction in the blood flow velocity (BFV) of the cerebral and intestinal arteries, without changing left ventricular output (LVO) (Hoecker, Nelle et al. 2002). When they divided the loading dose of caffeine to two doses, they found a decrease in BFV in cerebral arteries after the second dose, whereas BFV in intestinal arteries and LVO were not affected (Hoecker, Nelle et al. 2006). It was found, in a previous study, that the standard loading dose of

caffeine which is 20 mg/kg of caffeine citrate had no effect on cerebral BFV (Saliba, Autret et al. 1989). This finding was confirmed by Dani et al. They studied the brain hemodynamics using near-infrared spectroscopy (NIRS) and cerebral doppler ultrasonography after three days of either a loading dose of pure caffeine of 10 mg/kg, followed by maintenance dose of 2.5 mg/kg per day or loading dose of aminophylline of 5 mg/kg followed by maintenance dose of 1.25mg/kg, twice a day. They found that caffeine did not significantly affect the cerebral BFV while aminophylline induced a significant, but transient increase in cerebral BFV (Dani, Bertini et al. 2000).

El-Kashef et al demonstrated that adenosine is capable of producing relaxation in blood vessels through the activation of adenosine A_2 receptors. This vasodilatation effect of adenosine through A_2 receptors is attenuated by theophylline (El-Kashef, Elmazar et al. 1999).

These studies indicate that caffeine, at the usual therapeutic doses used in treatment of apnea of prematurity, has no significant effect on the cerebral blood vessels; however, at high and toxic doses, caffeine can cause vasoconstriction in these vessels through adenosine antagonism at A_2 receptors.

2.8.8. Pituitary Gland:

A_1 and A_{2A} receptors are expressed in pituitary cells. A_{2B} adenosine receptor is the physiologically dominant receptor subtype in the pituitary, while A_1 receptor expression in the cell lines is present in a low affinity conformation. Adenosine was shown to stimulate growth hormone (GH) secretion. The effects of xanthines, such as caffeine, theophylline and theobromine, which act as

adenosine receptor antagonists, have also been examined in the pituitary gland. These compounds have been shown to increase ACTH and corticosterone, while lowering TSH and GH concentrations (Rees, Lewis et al. 2002).

2.9. The Effect of Caffeine on Pregnancy:

Several studies have been done to explore the effect of caffeine on pregnancy in human and animal species. Savitz et al found little indication of harmful effects of caffeine on miscarriage risk (Fetal loss before 20 weeks of pregnancy) within the range of caffeine consumption reported in his study which was around 200 mg per day (Savitz, Chan et al. 2008). However, Weng et al demonstrated that high doses of caffeine intake of 200 mg per day or more during pregnancy increased the risk of miscarriage, independent of pregnancy-related symptoms (Weng, Odouli et al. 2008). These epidemiological studies were contaminated by recall bias of the interviewed women, which may explain the difference in results between the two studies.

The results of the studies included in a systemic review suggested that a large increase in the risk of congenital anomalies is unlikely to result from consumption of caffeinated beverages during pregnancy. The 10% to 20% excess risk associated with coffee intake suggested by the summary estimates for cardiovascular malformations and oral clefts might be explained by recall bias or confounding (Browne 2006). In another study, no evidence was found for a teratogenic effect of caffeine consumption of 300 mg and above during pregnancy with regard to cardiovascular malformations (Browne, Bell et al. 2007). Christian

et al reviewed the teratogenic, developmental and reproductive effect of caffeine during pregnancy in both human and animal research. In animal studies he found that the amount of caffeine intake needed to manifest the above effects during pregnancy should be very high and comparable to a toxic level of caffeine in human. He found that the developmental NOEL (No Observable Effect Levels) in rodents is approximately 30 mg/kg per day (The required dose to cause changes in the behavior of the offsprings), the teratogenic NOEL (The required dose to cause teratogenic effects in the fetuses) is more than 250 mg/kg per day (Christian and Brent 2001).

In a large prospective observational study, caffeine consumption of more than 300 mg per day during pregnancy was associated with an increased risk of fetal growth restriction (odds ratio of 1.4) and this association continued throughout pregnancy (CARE Study Group 2008). However, in a randomized controlled trial, women were divided into two groups in the second half of pregnancy; the caffeinated instant coffee group (median daily of caffeine intake was 317 mg) and the decaffeinated instant coffee group (median daily of caffeine intake was 117 mg). The study showed that there was no difference in birth weight or length of gestation between the two groups (Bech, Obel et al. 2007). Smith et al demonstrated a decrease in fetal weight, placental weight and crown-rump length in the animal groups that received either a single daily dose of caffeine at 100mg/kg per day or four divided doses. The retardation of skeletal ossification and the major fetal abnormalities, mainly ectrodactyly, were seen only in the group given I00 mg caffeine/kg in a single daily dose. The groups

which received lower doses of caffeine or received it in drinking water didn't show any of the above abnormalities (Smith, McElhatton et al. 1987). Christian et al mentioned that in animal models the reproductive NOEL (The required dose that can cause miscarriages or fetal growth restriction) is approximately 80-120 mg/kg per day (Christian and Brent 2001). These animal studies indicate that for caffeine to have a significant effect on pregnancy in human, the mother should consume large amount of caffeinated beverages. Caffeine to be teratogenic, the pregnant woman needs to consume around 80-100 mg/kg per day of caffeine, which will be a toxic dose and even if it is taken as a single dose, it can be fatal (Holmgren, Norden-Pettersson et al. 2004).

2.10. Caffeine and Lactation:

Several studies were done in the 1980s and 90s to determine the levels of caffeine in the serum and breast milk of lactating mothers. In one study, 18 healthy breast-feeding women ingested around 145 mg of caffeine in coffee. In this study, caffeine concentrations in breast milk were measured 2 and 4 hours after the intake of coffee. The study had shown that the average breast-milk concentration was 0.82 mg/L and the serum caffeine concentrations measured in the babies ranged from less than 0.05 to 0.75 mg/L. The authors calculated the daily caffeine intake of the infants to range from 0.027 to 0.203 mg/kg/day (Hildebrandt and Gundert-Remy 1983). Berlin et al examined the breast milk levels of caffeine in 15 lactating women after ingesting a known amount of caffeinated beverage (36 to 335 mg). The peak levels of caffeine were achieved

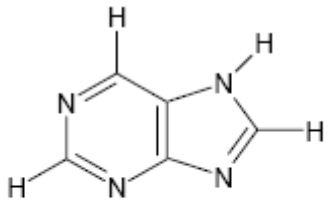
within 1 hour of ingestion and ranged from 2.09 to 7.17 µg/mL. The study had also shown that the amount of caffeine available for infant absorption ranged from 0.01 to 1.64 mg or 0.06% to 1.5% of the maternal dose (Berlin, Denson et al. 1984). Ryu JE studied 9 lactating women and their infants for 9 days. The women ingested 750 mg of caffeine daily for the first 5 days, and for the next 4 days they ingested no caffeine. For 7 mothers who were 11-22 days after delivery on the 1st day of study, the concentration of caffeine in samples of milk obtained by pooling aliquots from each feeding on day 5 averaged 4.3 µg/mL. Values ranged from less than 0.25 µg/mL to 15.7 µg/mL. The mean concentration of caffeine in sera of the infants on day 5 was 1.4 µg/mL and ranged from non-detectable to 2.8 µg/mL (Ryu 1985). It has been reported that breast milk/serum levels of caffeine ranged between 0.7 and 0.9 (McNamara, Burgio et al. 1992; Oo, Burgio et al. 1995), and the elimination half lives of caffeine in human milk ranged from 1.5 to 14.5 hours with average of around 6 hours (Berlin, Denson et al. 1984).

These studies indicate that in the usual amounts of caffeinated beverages taken by lactating mothers, the amount of caffeine ingested by the infants is small compared to the dose of caffeine used for treatment of AOP.

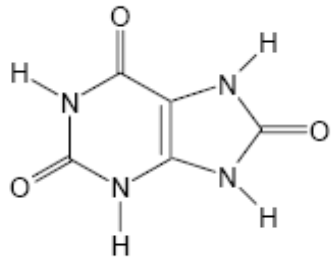
2.11. Conclusion:

Caffeine is commonly used in neonatal intensive care units. It is the preferred drug for treatment of AOP. It blocks adenosine receptors in the respiratory centers. Adenosine is an important neuro-modulator that is involved in many neurotransmitters' functions in the CNS. Adenosine receptors are widely

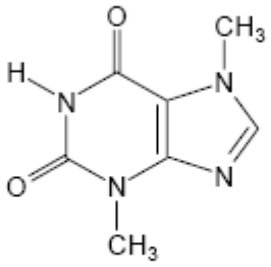
distributed in the brain. Caffeine, a non-selective adenosine receptor blocker, can affect most of these functions. For that, there was always a concern of the short and long term effects of caffeine on the developing brain of the preterm infant. Many human and animal studies were done to explore this concern. Despite recent studies by Schmidt et al. (2006;2007), indicating a benefit of caffeine for the preterm infant regarding bronchopulmonary dysplasia, cerebral palsy and cognition delay, in the short term, these studies were done in human newborns with complicated medical backgrounds. In this regard, the effects of caffeine *per se* on the brain of the preterm infant have not yet been elucidated, and more over the long term subtle effects on development remain unknown.



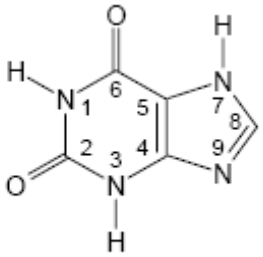
Purine



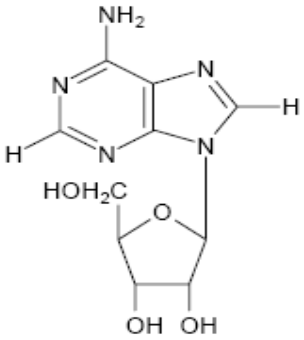
Uric acid



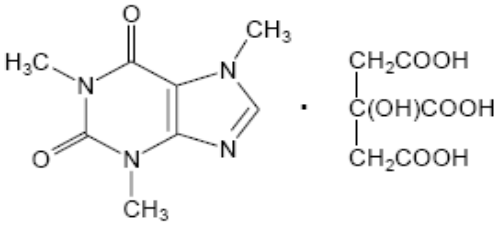
Theobromine



Xanthine



Adenosine



Caffeine citrate

Figure 2-1: The structures of caffeine and related molcules (Comer, Perry et al. 2001).

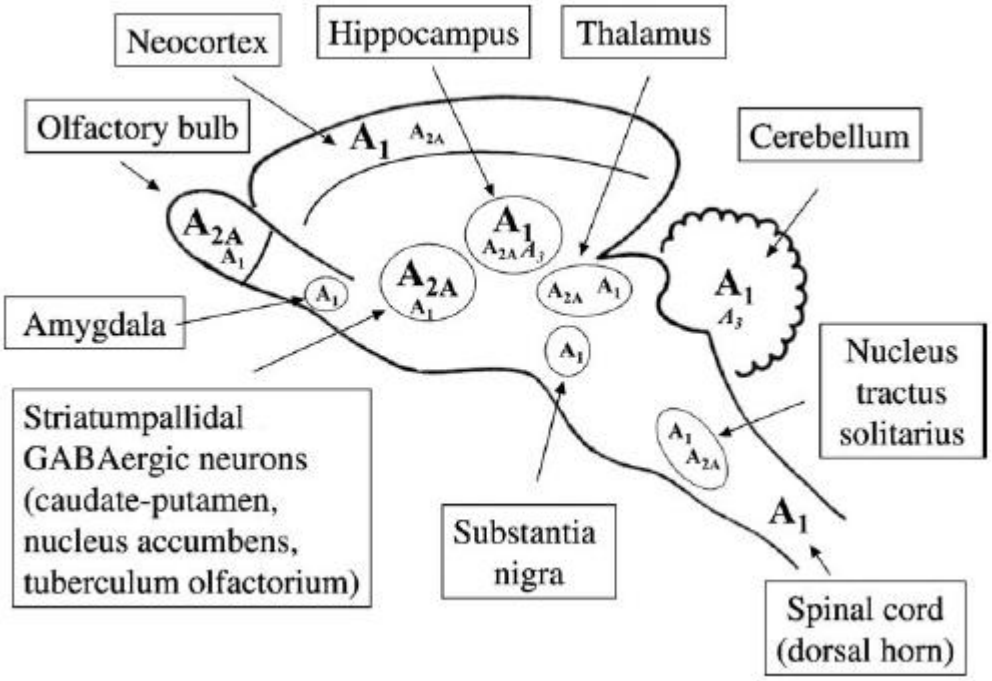


Figure 2-2: Distribution of adenosine receptors (A_1, A_{2A} and A_3) in the main regions of the central nervous system. High levels of expression are indicated by bigger alphabets (Ribeiro, Sebastiao et al. 2002).

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Chapter 3: Methodology and Experimental Protocol

3.1. Hypothesis:

Despite the common usage of caffeine around the world, in the newborn premature infant, and clinical trials which have shown benefit for both BPD and cerebral palsy, the direct effect of caffeine, in isolation, has not been fully elucidated. Previous animal studies from our laboratory (Black, Pandya et al. 2008), and others have shown that neonatal caffeine intake results in changes in some of the neurobehavioral functions (Fisher and Guillet 1997; Pan and Chen 2007; Li, Dai et al. 2008), expression of adenosine receptors (Guillet and Kellogg 1991; Gaytan, Saadani-Makki et al. 2006) and neuronal cell death (Kang, Lee et al. 2002; Black, Pandya et al. 2008). We, therefore, **hypothesize** in this research, that the use of caffeine, *per se*, in immature brains will have effects on the neurobehavioral and /or the neuropathological outcome of the developing immature animal (rat).

3.2. Rat Model:

The rat pup is commonly used as an animal model to study brain injury in the newborn, and the effect of caffeine on the brain. There are several advantages of using rat pups: 1) the large number of animals that can be handled in each experiment especially for neurobehavioral tests and neuropathological studies, 2) the large litter size can allow for randomization procedure which may reduce the confounding effect of possible differences between litters, and 3) the newborn rat is a non-precocial animal (Chapados and Cheung 2008). At birth the rat pup is

comparable to 22-26 weeks gestational age (GA) in humans (Fisher and Guillet 1997; Craig, Ling Luo et al. 2003; Clancy, Finlay et al. 2007).

3.3. Study Design:

In this study, rat neonates were injected with either saline or caffeine in doses and during a period of development that are comparable to the preterm human's infants who are treated with caffeine in neonatal intensive care units for apnea of prematurity. Several neurobehavioural and neuropathological tests were performed on these animals starting from the neonatal period until adulthood. All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care and Use Committee: Health Sciences, University of Alberta.

Long-Evans male and female adult rats were purchased from Charles Rivers Laboratories (Montreal, PQ), and housed in the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. The animals were locally bred by our research associate in the laboratory. Rat pups were allowed to deliver vaginally and date of birth was considered to be postnatal day 1 (P1). Litters were routinely culled to 10 pups within 48 hours of birth to ensure that all the pups had equal nutrient intake and growth. The pups were reared with their dams in conventional housing, weaned at P21 and then separated by sex and grown at 2 rats per cage until testing. All animals were maintained on a 12-hour light/dark schedule and received food and water *ad libitum* throughout the study.

On day 3 of life (P3), the rat pups were sexed and weighed then

randomized into one of the experimental groups: The treatment (caffeine) group and the control (normal saline) group. Between P3 and P7, the rat pups were subcutaneously (s.c) injected, between the scapulae, with either caffeine citrate at a dose of 20 mg/kg at P3, then 15mg/kg daily from P4 to P7, or sterile normal saline alone. All the injections were made in a volume of 0.05 mL. The dose of caffeine citrate was calculated daily according to the average weight of the litter on each day. Caffeine was prepared from purified caffeine citrate powder and dissolved in sterile normal saline for injections in each day. The daily injections were given at the same time, each day around 10:00 am.

Beginning at P3, rat pups were weighed daily until P14 and then at P21, P42 and finally at P160. See figure 3-1 for time line.

3.3.1. The Period of Caffeine Treatment:

The first caffeine injection was started on P3, which is comparable to 26-28 weeks gestational age (GA) in humans, and the last injection was on P7, which is comparable to 36-38 weeks GA in humans (Fisher and Guillet 1997; Craig, Ling Luo et al. 2003; Clancy, Finlay et al. 2007; Pan and Chen 2007). This is comparable to the GA range over which caffeine is administered to preterm infants for treatment of AOP: the median starting age is 28 weeks GA and the usual stopping age is 36-38 weeks corrected GA in extreme preterm infants (Comer, Perry et al. 2001; Bhatt-Mehta and Schumacher 2003; Baird 2004; Martin, Abu-Shaweesh et al. 2004; Martin and Abu-Shaweesh 2005; Schmidt, Roberts et al. 2006).

3.3.2. The Dosage of Caffeine Treatment:

Caffeine was given to the rat pups in the form of caffeine citrate (obtained from University of Alberta Hospital' pharmacy) at 20 mg/kg on P3, followed by 15 mg/kg/day from P4 to P7, as a single daily subcutaneous injection. This regimen was shown to give serum caffeine level of 5-15 mg/L in previous rat models (Guillet 1990; Fisher and Guillet 1997; Montandon, Kinkead et al. 2007; Pan and Chen 2007). This is comparable to the usual loading dose of 20 mg/kg/day of caffeine citrate, followed by maintenance of 5 mg /kg/day of caffeine citrate oral or intravenous in human preterm infants (Comer, Perry et al. 2001; Schmidt 2005; Schmidt, Roberts et al. 2006; Leon, Michienzi et al. 2007; Natarajan, Botica et al. 2007). This regimen in preterm infants produced serum caffeine levels between 5 and 20 mg/L in the majority of the infants (Comer, Perry et al. 2001; Schmidt 2005; Schmidt, Roberts et al. 2006; Leon, Michienzi et al. 2007; Natarajan, Botica et al. 2007).

Following each injection, pups were separated into their respective treatment groups in non-restrictive containers and maintained at 34.5^{o}C in a thermoregulated incubator for half hour before returning to their dam.

3.4. Animal Neurobehavioral Tests:

Early behavioral testing began on P4 and continued through to P21. Object recognition test was done in early adulthood at P63 and the Morris water maze test was done in mature adults at P160. During early behavioral testing, the rat pups were removed from their home cage for testing every morning between 9 to

10 am. Behavioral tests were done before caffeine injection to avoid the effect of caffeine dose on behaviour. Newborn rats have an immature thermoregulatory system and are highly susceptible to hypothermia (Kreider and Blumberg 2005). To avoid temperature related behavioral changes, newborn rats were tested in an incubator maintained at 34.5ºC where possible, or under a warming lamp (31ºC) for tests that could not be performed inside the incubator. The postnatal day of attaining each individual reflex for each pup was recorded, except for the righting reflex where the duration of time to accomplish the task was recorded. The following reflex tests were adapted from the original work of Fox, Lubics et al. and Tchekalarova et al. (Fox 1965; Lubics, Reglodi et al. 2005; Tchekalarova, Kubova et al. 2005).

3.4.1. Early Behavioral Testing: Neonatal (P4-P9), Post-Neonatal, Infantile and Pre-Juvenile (P10-P21):

1. Body Righting (P4-P7):

The animal was placed gently on its back and the time it took to turn over and rest in the normal (prone) position with all four feet on the ground (principally labyrinthine and body righting mechanisms) was video recorded. The scoring system was the time required for the pup to turn over, with maximum 15 seconds for the trial. The day in which pups could turn over within 5 seconds was recorded.

2. Hind-Limb Grasp Reflex (P4-P7):

When the hind foot is stroked with a blunt instrument, the foot is flexed to grasp the instrument. Five trials were given for each limb. Scoring was 0 for no

movement, 1 for flexion movement of one limb, and 2 for flexion movement of the two hind limbs. When all the pups in each litter had a score of 2 for two consecutive days, the test was stopped. The day that the pup had a score of 2 was taken as the day of appearance for the reflex.

3. Hind-Limb Placing (P4-P9):

Contact of the dorsum of the foot against the edge of an object will cause the foot to be raised and placed on the surface of that object when the animal is suspended and no other foot is contact with a solid surface. Five trials were given per day. A score of 0 was given for the negative test, 1 for one limb, and 2 for the two limbs. When all the pups in each litter had a score of 2 for two consecutive days, the test was stopped. The day that the pup had a score of 2 was taken as the day of appearance for the reflex.

4. Cliff-Drop Avoidance (P4-P9):

When the rat is placed on the edge of a cliff or table top with its forepaws and face over the edge, it will turn and crawl away from the cliff edge. This test was video recorded. Two trials were given each day, with a maximum time of 10 seconds for each trial. A score of 0 was given when the pup dropped without effort, 1 if dropped after an effort and 2 when the pup avoided the cliff. The day that the pup had a score of 2 was taken as the day of appearance for the reflex.

5. Gait (P5-P10):

The pup was placed in middle of a paper circle with a diameter of 15 cm. The test was video recorded. The score was the time needed for the 2 forelimbs to come out of the circle. A maximum time of 30 seconds was given for the trial.

The day that the pups had left the circle within 30 seconds was taken as the day of appearance for the behavior.

6. Bar Holding Ability (P12-P18):

This test primarily measures strength which is an important aspect of motor performance. The rats were held so that their forelimbs grabbed a horizontal wire bar, stretched horizontally 40 cm over a foam pad. The time that the pup was able to hold on to the bar was recorded using a stop watch. Each pup was given two trials per day and the best trial on that day was used for subsequent analysis.

7. Normal Posture (P12-P18):

The rat pups normally crawl using mainly their forelimbs until about P10 when they start to use a quadruped stance. Starting from P12, rat pups were individually observed daily for stance and limb placement. The normal posture is scored as 1 and it was when the rat's limbs are beneath the body during rest and locomotion, while abnormal posture was scored 0 and it was when the rat's limbs were spread beside the body. The analysis was the average day in which all the pups had normal posture. The day that the pup had a normal posture was taken as the day of appearance for the behavior.

8. Accelerating Righting (P12-P17):

When the rat is suspended by the shoulders and the hips with the abdomen uppermost, and is suddenly released falling about 12 inches into a box lined with a thick piece of foam to cushion the fall. The successful rat will turn to land on all four limbs (labyrinthine response). The test began on P12 and was video recorded

to view the landing of the rats in slow motion. Two trials were given per day. A score of 2 was given for landing on the limbs, 1 for landing on the side of the body, and 0 for landing on the back. The day that the pup had a score of 2 was taken as the day of appearance for the reflex.

9. Auditory Startle (P12-P17):

A loud sharp noise causes an immediate startle response, seen as a sudden extension of the head and fore and hind limbs which are then withdrawn. The clap sound was used in this test. Scoring was positive or no reaction. The day that the pup had a positive reaction was taken as the day of appearance for the reflex.

10. Eye Opening (P14-P17):

Starting from P14, rat pups were individually observed daily for eye opening. In each day a score of 0 was given if both eyes were not open, 1 if one eye was open and 2 if both eyes were open. The day that the pup had a score of 2 was taken as the day of appearance for the reflex.

11. Visual Placing (P15-P18):

When the rat is suspended by the tail and lowered toward a solid object (e.g. a bar or table top) it will raise its head and extend the fore limbs in a placing response. Scoring was 1 for the above reaction and 0 for no reaction. The day that the pup had a score of 1 was taken as the day of appearance for the reflex.

12. Open Field (P21):

This test was adapted from the original work of Lubics et al and Kiss et al (Lubics, Reglodi et al. 2005; Kiss, Hauser et al. 2007).

A semi-sound proof room with a constant temperature was used for this test. The home cage containing the animals was transferred into the testing room at least 1 h before beginning the experiment for acclimatization. After the acclimatization to the testing environment, pups were placed in a Plexiglas box. The floor of the box was 45 cm X 45 cm and divided into 4 X 4 equal squares with sides of 30 cm high. Each rat was placed in the central area facing the same direction. A total of 5 min exposure was video-recorded and evaluated at later time in a blind manner. The testing apparatus was cleaned with disinfectant following each pup. Parameters measured include: motor activity (ambulation) which is expressed by the total number of squares crossed (with all four feet on one square) during the whole period of testing; exploratory behavior which was expressed by rearing (The pup will stand on the hind limbs and elevate the fore limbs to explore the environment) and head lifting (The pup will raise its head with all the limbs still on the floor to explore the environment); anxiety behavior which was expressed by the number of defecations and grooming.

3.4.2. Early Adulthood:

1. Open Field-The Second Round (P63):

See open field (P21) procedure for details. This test was done for 2 purposes: first to test ambulation, exploratory and anxiety behaviors at early adulthood age and secondly as the first part of the object recognition test to acclimatize the rat to the test box. The test was done 1 day prior to novel object recognition test.

2. Novel Object Recognition (P63):

Rats have a tendency to interact more with novel objects than familiar ones. This tendency has been used to study attention, memory and recognition. The test was adapted from Bevins and Besheer (2006). Animals are first placed in an apparatus and allowed to explore an object. After a prescribed interval, the animals are returned to the apparatus, which contains the familiar object and a novel object. Object recognition is distinguished by more time spent interacting with the novel object. The test was video recorded.

Procedure:

Two sample objects with one environment was adapted from (Bevins and Besheer 2006).

1. The 'identical' to-be-familiarized (sample) objects were put in the back left and right corners of the apparatus (Plexiglas box). The floor of the box was 60cm X 60cm and divided into 4 X 4 squares and the height was 50 cm. In our test 2 identical bottles filled with a red dye were used as the familiar objects (Fig. 2).
2. The animal was placed at the mid-point of the wall opposite the sample objects. When placed, its body was parallel to the side walls and its nose pointing away from the objects.
3. After the planned sample-object exposure time (10 min), the animal was removed from the apparatus for the training-to-testing interval (1h).
4. To test for object recognition after the training-to-testing interval, one of the familiar sample objects was placed in one back corner of the apparatus; the novel

object was placed in the other back corner. In our test a metal oxygen cylinder lid was used as a novel object.

5. The animal was placed as described in Step 2 and kept for 5 minutes, then removed.

The scoring was according to the time the rat spent in direct contact with the objects. Direct contacts include any contact with mouth, nose or fore paw, or when the nose was directed at the object within 2 cm distance from the object. The time with novel object versus familiar object, a difference score (novel object interaction – familiar object interaction), and a discrimination ratio (novel object interaction/total interaction with both objects) were calculated.

3.4.3. Mature Adulthood:

Morris Water Maze (P160):

The Morris water maze (MWM) is an open circular pool that is filled with water. The interior is made such that it is as close to being featureless as possible. It is a 'maze' because the animal must search in order to locate a relatively small goal (a hidden platform) that is submerged below the water surface and placed in different locations according to the trial and the day. It is a test of spatial learning for rodents that relies on distal cues to locate the submerged escape platform from start locations in an open swimming pool. Spatial learning is assessed across repeated trials (Vorhees and Williams 2006). The use of a distracter enhances the detection of spatial impairments. The presence of an extra cue (distracter cue) would have a disruptive effect on the rat and would prevent the rat from learning that a particular cue signaled the platform (Anisman and McIntyre 2002).

Equipment setup:

- The Maze: A plastic circular tank with a 210 cm diameter. The side of the tank was 51 cm in height with non-reflective interior surface.

- The platform: The goal (platform) was square (10 X 10 cm^2), constructed from plexiglas, and was submerged 2 cm below water surface.

- Water: The water temperature was kept at 20 ± 1°C.

- Room configuration: The maze was placed in a room with ample surrounding visual cues.

- Experimenter: An experienced experimenter ran all the tests and remained stationary in a constant location in each test.

- Lighting: Room lighting was indirect.

Procedure:

- One week prior to running the water maze, the rats were handled daily to get them used to being picked up, as well as reduce the amount of stressed the rats experienced.

- Spatial acquisition:

1. The animal was placed in the desired start position in the maze, facing the tank wall according to the day of test and trial number. The animal was released into the water at water level (not dropped). A timer was started the moment that the animal was released.

2. The timer was stopped when the animal reached the platform. A trial limit of 1 minute per trial was given. Animals not finding the platform within this time limit were placed on the platform.

3. The animal was left on the platform during the inter-trial interval. Inter-trial intervals of 15 seconds were given.

4. The animal was placed in the maze at a new start location and repeated the trial (Steps 1-3) until the animal had had four trials per day; it took more than 5 minutes per animal on the first day and progressively less time per animal each day thereafter.

5. On subsequent days, the trials were repeated. With four trials per day for a total of 4 days. The platform was put in different location in the maze for every trial, and was cued by a black rectangle box hung from the roof of the test room. The purpose of moving the platform is to see how rapidly the rats learn the process of finding the platform and learn to use spatial cues to find it.

6. Eighty animals were used for this test: Forty for the caffeine group and forty for the normal saline group. For half of the rats in each group, no other cue was present (no distracter). For the remaining rats a second cue hanging from the ceiling of the examination room was present and was irrelevant to the platform position (distracter cue). A different distracter cue was in a different location on each trial for each of the four daily trials. The four distracter cues were a black 8X8X8 cm^3 cube, a black plus sign, an 8-cm-diameter sphere, and an 8-cm-diameter black ring (Anisman and McIntyre 2002).

Scoring:

Latency, which is the time from start to goal (in seconds), and the path length from start to goal (in cm) on each day of testing were scored (Vorhees and

Williams 2006; Vorhees, Skelton et al. 2007). The results were averaged across four trials per day (mean ± SEM).

3.5. Animal Neuropathological Assessments:

For the purpose of neuropathology, animals were deeply anaesthetized with 5% isoflurane in 30% oxygen balance with nitrogen, followed by immediate decapitation. The brains were quickly removed and frozen in isopentane in dry ice. Brains were then stored at -70^{o}C until sectioning. Frozen brains were warmed to -20^{o}C before cryo-sectioning (Leica, cryocut 1800)(FOR HOW LONG?). Coronal brain sections (10 µm) were cut on a cryostat (-16 to -17^{o}C depending on the room's humidity and temperature). Sections were taken every 0.5 mm in all brains. Sections were then mounted on slides and stored at -20^{o}C to be stained for the planned neuropathological tests.

3.5.1. Caffeine Plasma Levels:

Caffeine plasma levels were determined at ½ hr, 4 hr and 24hr after the last dose on P7. The animals were decapitated and blood samples were quickly collected from the trunks of the animals into tubes and immerged into ice. Then the samples were centrifuged and the plasma samples were stored at -70^{o}C until the time of the analysis. Plasma caffeine levels were analyzed at another laboratory at the University of Alberta (Dr. Glen Baker's Lab) using high performance liquid chromatography (HPLC) technique which is well described in the literature (Di Iorio, Kleywegt et al. 2002).

3.5.2. Hematoxylin & Eosin Staining:

Hematoxylin and Eosin (H & E) staining, using a basic and an acidic stain (Hematoxylin and Eosin, respectively), takes advantage of the acidophilic and basophilic properties of different cell regions, allowing them to take up their preferred stain, and subsequently be visualized. It is a conventional popular staining method in histology and is the most widely used stain in medical diagnosis. This traditional histological staining method is technically simple and can be used to show cell degeneration based on changes such as neuronal size, vacuolation, and hyperchromatism (Schmued, Albertson et al. 1997). The procedure is well described elsewhere (provide reference). Briefly, sections were first fixed in 10% Neutral Buffered Formalin and then washed in tap water, followed by rinsing in distilled water. The sections were then dehydrated in a series of alcohol washes of increasing concentration, defatted in xylene, and subsequently rehydrated in a series of alcohol washes of decreasing concentration. At this point, the slides were immersed in Harris' Hematoxylin for two minutes, after which time they were washed in distilled water, dipped in acid alcohol, blued in tap water and rinsed in distilled water. Finally, the sections were briefly dipped in Eosin, rinsed in distilled water, dehydrated and cleared in 95 to 100% alcohol, immersed in xylene, and mounted on slides. H & E staining was done with P4, P8, P21 and P160 animals.

3.5.3. Fluoro-Jade B (FJB) stain:

Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. Fluoro-Jade stains the cell bodies, dendrites,

axons and axon terminals of degenerating neurons but does not stain healthy neurons, myelin, nor vascular elements. Cells of the meninges and the choroid plexus exhibit an affinity for Fluoro-Jade (Schmued, Stowers et al. 2005). Degenerating neurons stained with Fluoro-Jade appear bright green against a dark background (Schmued, Albertson et al. 1997). Fluoro-Jade stain was done at P4 and P8 in the following brain regions: caudate, globus pallidus, thalamus, hypothalamus, superior colliculus, nucleus accumbens, anterior cortex, post cortex, substantia nigra and hippocampus areas: CA1, CA3, CA4 and dentate.

The slides were first fixed in formalin then immersed in tap water followed by distilled water for 5 minutes each. Then slides were immersed in absolute alcohol for 3 min, followed by 70% ethanol for 1 min and distilled water for 1 min. The slides were then transferred to 0.06% potassium permanganate for 15 min and gently agitated on a rotating platform. After rinsing in distilled water for 1 min, the slides were incubated for 30 min in 0.001% FJB in the dark. A 0.01 % stock solution of the FJB dye was prepared by dissolving 10 mg of FJB (Chemicon, Temecula, CA) in 100 ml of distilled water. The 0.001 % working solution of Fluoro-Jade was prepared by adding 10 ml of the stock Fluoro-Jade solution to 90 ml of 0.1 % acetic acid in distilled water. Slides were then washed for 1 min in each of three changes of distilled water. Excess water was drained and the slides were rapidly dried on a dry plate of 37º C for 30 min. When dry, the slides were immersed in xylene and then cover slipped with D.P.X. (Aldrich Chemical Co, Milwaukee, WI), a nonaqueous and nonfluorescent plastic mounting medium. Sections were examined with an epifluorescence microscope

using a filter system suitable for visualizing fluorescein or fluorescein isothiocyanate (FITC) (eg, the Leica FITC filter cube B-2 has an excitation filter at 450-490 nm and a barrier filter at 520 nm). The resulting slides are very stable and require no special storage conditions or anti-fading agents (Schmued and Hopkins 2000).

Degenerating neurons were counted in FJB-stained sections at P4 and P8 in the following brain regions: caudate, globus pallidus, thalamus, hypothalamus, superior colliculus, nucleus accumbens, anterior cortex, posterior cortex, substantia nigra and hippocampus areas: CA1, CA3, CA4 and dentate. Regions were taken after consultation with a newborn rat brain atlas (Sherwood and Timiras 1970) and chosen based on previous research, to assess neural damage in areas most likely to be affected by caffeine based on their effect on brain receptors. Fluoro-jade B stained cells were counted under a fluorescent microscope at 400X magnification (area of 0.09 mm^2). Neuroanatomical landmarks were established to align the field of view of the microscope consistently across sections for each brain region counted (Kang, Lee et al. 2002; Black, Pandya et al. 2008).

3.5.4. NeuN (Neuronal Nuclei):

NeuN staining is a marker of nuclei of the neurons and is used to detect loss of neurons and abnormal neuronal morphology in rat brain (Lin, Fan et al. 2009). To evaluate the effects of caffeine on neuron loss, we detected NeuN immunopositive cells by immunohistochemistry at P21 and P160. Fewer neuronal

nuclei (NeuN) in any area of brain could be indicative of neuron loss and brain damage in that area.

NeuN Staining Procedure: Briefly, frozen sections were fixed in formalin, cleared in tap and distilled water, dehydrated in a graded series of ethanol washes, defatted in xylene, and subsequently rehydrated in a series of alcohol washes of decreasing concentration. Sections were then washed with 1% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with normal horse serum mixed with 0.1% Triton. Sections were then allowed to incubate with the primary antibody (monoclonal mouse anti-NeuN, Millipore) overnight. On the second day: After rinsing and 30 minutes of incubation with the secondary antibody (biotinylated horse anti-mouse IgG, Vector), the sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector Laboratories). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Vector).

Cell Counting Methodology:

Sections of rats' brains were cut and stained for NeuN as described above. Digital images of a 0.09 mm^2 area were captured using a Spot Flex digital camera attached to a Leica ATC2000 microscope at 400X magnification via Spot 4.5 software. Images from the nucleus accumbens, globus pallidus, CA1 region of hippocampus, thalamus, hypothalamus, substantia nigra and superior colliculus were taken after consultation with a newborn rat brain atlas (Sherwood and Timiras 1970). The saved images were then opened in Adobe Photoshop (v9.0)

and a grid was placed on the image to aid in counting. Cells that stained positively for NeuN were counted. For more detail, see section 3.5.3.

3.5.5. Neurofilament M:

Neurofilament M (NF-M) is an axonal marker as it is a key component of the heteropolymer intermediate filaments that provide structure to the axon. NF-M is abundant in the developing brain (Stone, Zhang et al. 2008).

As described before (Konakci, Streicher et al. 2005) the procedure is briefly as the following**:** First frozen sections were fixed in formalin, cleared in tap and distilled water, dehydrated in a graded series of ethanol washes, defatted in xylene, and subsequently rehydrated in a series of alcohol washes of decreasing concentration. Sections were then washed with 1% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with PBS containing 20% normal goat serum and 0.1% Triton for 1 hour at room temperature. Preparations were incubated in a mixture of primary antibodies against neurofilament (rabbit anti-neurofilament, catalogue no: AB1981; Chemicon, Temecula, CA) for 24 hours at 4°C. Then, preparations were incubated with the secondary antibody (1:400 goat anti-rabbit; Vector Laboratories Inc.) for 60 min at room temperature. The sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector Laboratories). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Vector). Between the incubation steps, specimens were extensively rinsed in PBS containing 0.1% Triton. After the staining procedure, specimens were rinsed in PBS, dehydrated, defatted again with xylene and mounted. To evaluate the effects of caffeine on axonal growth,

we assessed the staining intensity NF-M immuno-positive axons using densitometry at P8, P21 and P160 in the following areas of the rat brain: globus pallidus, thalamus, hypothalamus, superior colliculus, nucleus accumbens, substantia nigra and CA1 area of hippocampus.

Densitometry (optical density):

Ten micron sections were stained for Neurofilament as previously described. The sections' images were taken with a Spot Flex Camera (Diagnostic Instruments, Sterling Heights MI) attached to a Leica GZ6E stereoscope (Leica Microsystems, Richmond Hill ON, Canada) using Spot 4.5 software (Diagnostic Instruments, Sterling Heights MI). Densitometry was measured using Image J ver 1.41 calibrated with a Kodak No.3 Calibrated Step Tablet scanned with an Epson Expression 1680 Professional scanner (O'Neill, Mitchell et al. 1989). A 0.035 mm^2 area was used to measure the Anti- NF M in the rat brain regions detailed in the above section. A region of the brain in each section that did not stain for Neurofilament M was chosen to be the background area for that section. The background was then subtracted from the densitometry readings to reduce variability due to staining done in different batches.

3.6. Statistical Analysis:

Unpaired student's *t*-test was used for two-group comparisons for newborn reflexes and neuropathology results. Two-way ANOVA was used for analysis of differences between more than 2 groups. The Morris water maze data was analyzed with a 2x2x2x4 (sex by treatment by distracter by day) ANOVA and the

novel object recognition and P63 open field data was analyzed by a 2x2 (sex by treatment) ANOVA. Data was expressed as mean ± standard error of the mean (SEM). A p-value of < 0.05 was considered to be statistically significant.

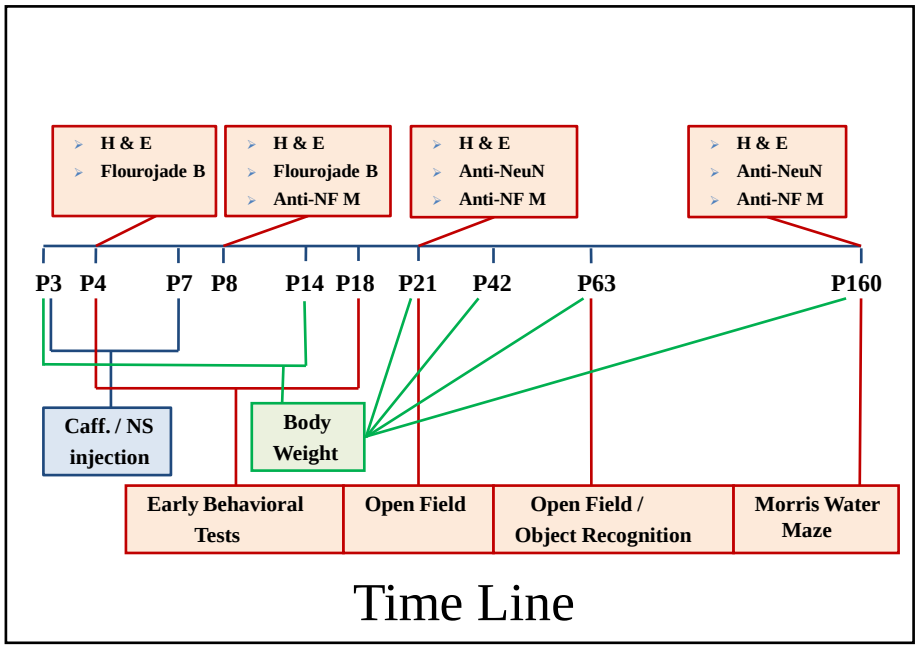

Figure 3-1: Study time line.

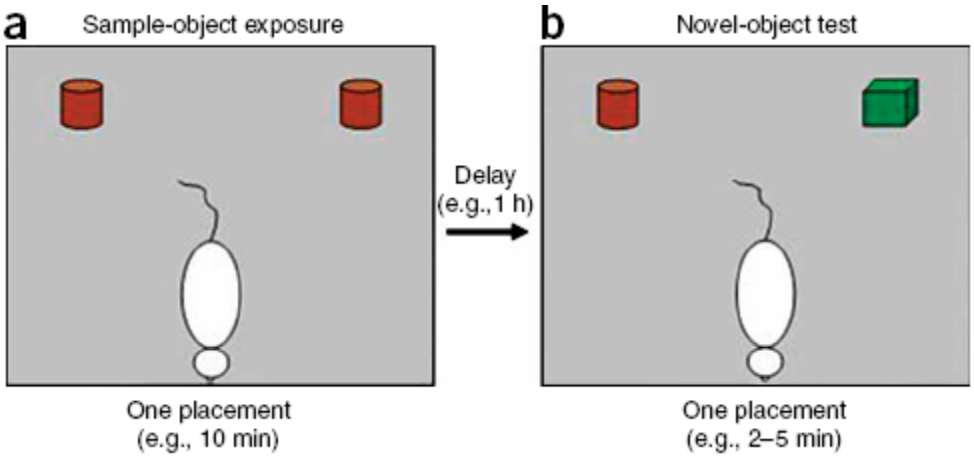

Figure 3-2: An image of the sample-object exposure phase (a) and novel-object test phase (b) (Bevins and Besheer 2006).

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Chapter 4: Results

We used a total of 209 rat pups in our experiments. The numbers of experimental animals are detailed with each test. There was no mortality during the course of the study.

4.1. Caffeine Levels:

A total of 30 animals were used for this test. Caffeine levels were measured at 0.5 h, 4 h and 24 h after the last dose of the caffeine course at P7 in both the plasma and the brain tissue. See Figure 4-1.

4.1.1. Caffeine Levels in the Plasma:

Caffeine Group:

- 0.5 h: 6 animals were used. The Mean ±SEM of caffeine levels was 12.62±0.68 µg/mL.

- 4 h: 6 animals were used. The Mean ±SEM of caffeine levels was 9.98±0.40 µg/mL.

- 24 h: 6 animals were used. The Mean ±SEM of caffeine levels was 1.22 ±0.29 µg/mL.

Normal Saline (NS) Group:

- A total of 7 animals were used to test the caffeine plasma level for the normal saline group as control and the levels were done at 24 Hr of the last caffeine dose and our results showed that caffeine was not detected in the plasma of the normal saline group.

4.1.2. Caffeine Levels in the Brain Tissue:

Caffeine Group:

- 0.5 h: 6 animals were. The Mean ±SEM of caffeine levels was 13.38±0.87 µg/gm.

- 4 h: 6 animals were used. The Mean ±SEM of caffeine levels was 10.77±0.87 µg/gm.

- 24 hr: 9 animals were used. The Mean ±SEM of caffeine levels was 1.37±0.28 µg/gm.

Normal Saline Group:

A total of 9 animals were used to test the caffeine levels in the brain tissue for the normal saline group as control and the levels were done at 24 h from the last caffeine dose and our results showed that caffeine was not detected in the brain tissue of the normal saline group.

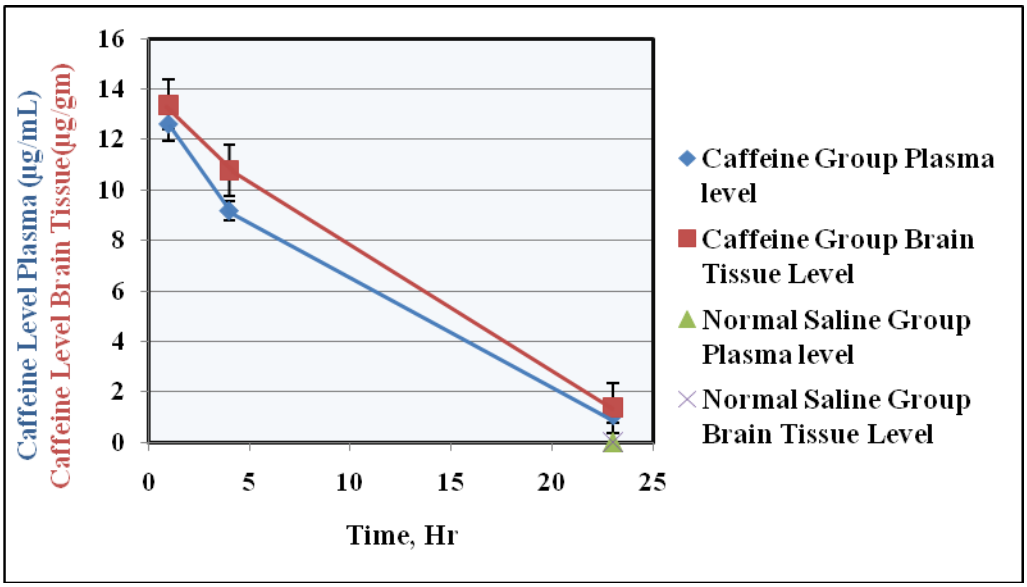

Figure 4-1: Caffeine levels in the plasma and brain tissue, taken at 0.5, 4, and 24 hours following the last dose of caffeine given on P7.

The levels of caffeine in plasma and brain are correlated using linear regression r^2=0.968, p<0.001.

4.2. Body Weight:

Animals were weighed daily from P3 to P14, then at P21**,** P42**,** P63 and finally P160. The total numbers of animals used for the body weight analysis were 55 at P3 to P21, 30 at P42, 36 at P63 and 80 at P160 (Table 4-1). The statistical analysis was done by 2 way ANOVA. Two way ANOVA test showed no effect of caffeine or sex on body weight between P3 to P21 and no interaction between sex and treatment. At P42, P63 and P160 we found no effect of caffeine on body weight and no interaction between sex and treatment. There was significant effect of sex on body weight; at P42: F (1, 29) =108.4, p < 0.001, at P63: F(1,35)=762.3, p < 0.001, and at P160: F(1,79)=1191.1, p< 0.001. The results are summarized in Figure 4-2.

Postnatal days	**Caffeine group**	**Normal Saline Group**
P3 to P21	27 (11 males and 16 females)	28 (12 males and 16 females)
P42 (Female)	7	7
P42 (Male)	8	8
P63 (Female)	10	10
P63 (Male)	8	8
P160 (Female)	20	20
P160 (Male)	21	19

Table 4-1: The numbers of animals for body weight.

Postnatal Age	Caffeine Group (Mean ± SEM)	NS Group (Mean ± SEM)
P3	7.50±0.16	7.51±0.16
P8	15.91±0.40	16.08±0.37
P14	27.68±0.51	28.22±0.47
P21	44.96±0.94	45.72±1.02
P42 (Female)	171.90±3.70	171.20±1.90
P42 (Male)	213.7±5.8	220.9±4.5
P63 (Female)	234.0±2.9	238.0±3.7
P63 (Male)	387.0±5.8	388.0±9.1
P160 (Female)	332.4±4.3	337.0±6.5
P160 (Male)	653.5±10.0	650.4±13.7

Table 4-2: The Mean±SEM for body weight in grams.

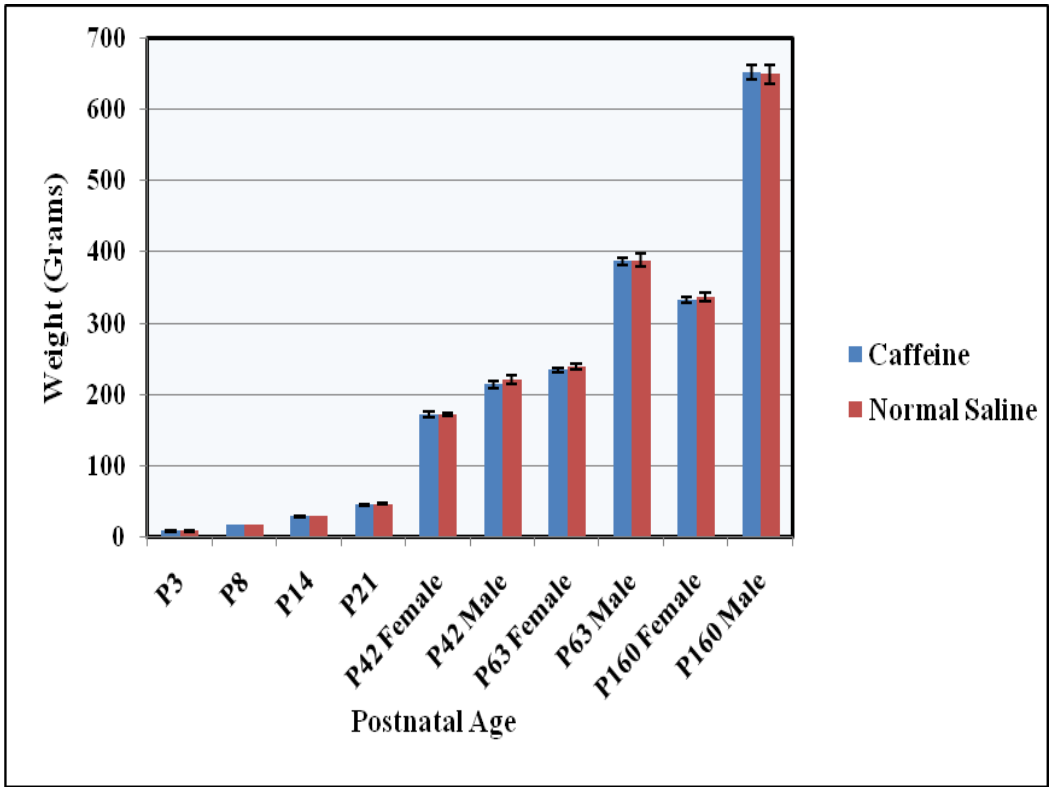

Figure 4-2: Body weight in grams.

4.3. Neurobehavioral Evaluation:

4.3.1. Early Behavioral Tests (P4-P18):

Since there were no significant differences between male and female rats less than 21 days of age the unpaired t test was used for statistical analysis. We didn't find any statistical difference between caffeine and normal saline groups for any of the early behavioral tests that were examined. See Table 4-3 and Figures 4-3 and 4-4. The numbers of animals were 27 in the caffeine group (11 males and 16 females) and 28 in the normal saline group (12 males and 16 females).

Behavioral Test (Postnatal date of appearance)	**Caffeine Group (Mean ± SEM)**	**Normal Saline Group (Mean ± SEM)**	**p Value**
Hind Limb Grasp	4.48±0.13	4.66±0.16	0.40
Hind limb placing	4.31±0.10	4.21±0.08	0.42
Cliff Avoidance	7.82±0.17	7.43±0.19	0.13
Gait	8.74±0.41	9.36±0.29	0.27
Auditory Startle	12.69±0.12	12.83±0.12	0.43
Accelerating Righting	15.63±0.33	16.04±0.26	0.34
Eye Opening	15.86±0.15	15.97±0.15	0.64
Visual Placing	16.42±0.10	16.41±0.08	0.42
Normal Posture	15.07±0.23	15.21±0.20	0.65

Table 4-3: Early behavioral tests (Postnatal date of appearance of appearance).

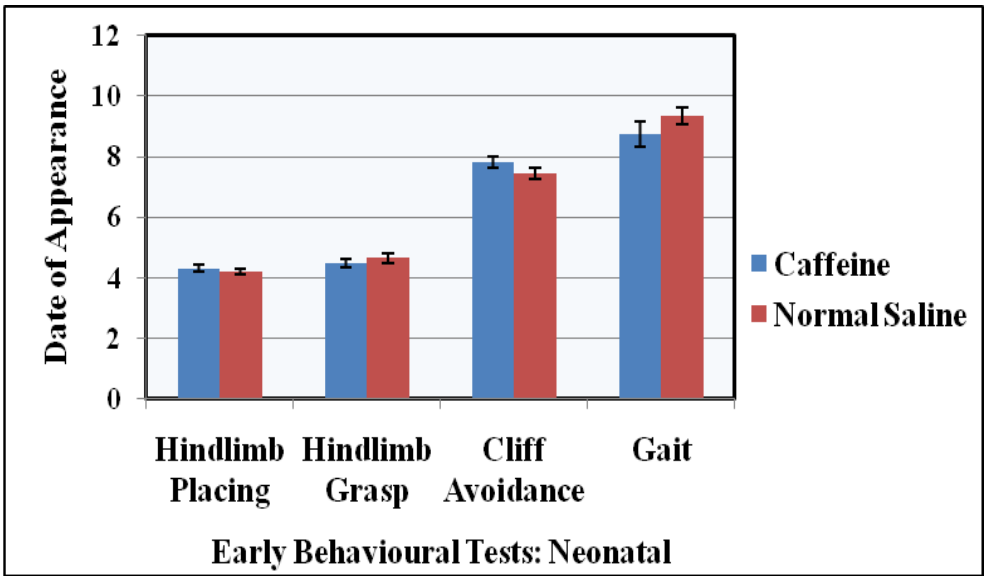

Figure 4-3: Early behavioral tests: Neonatal.

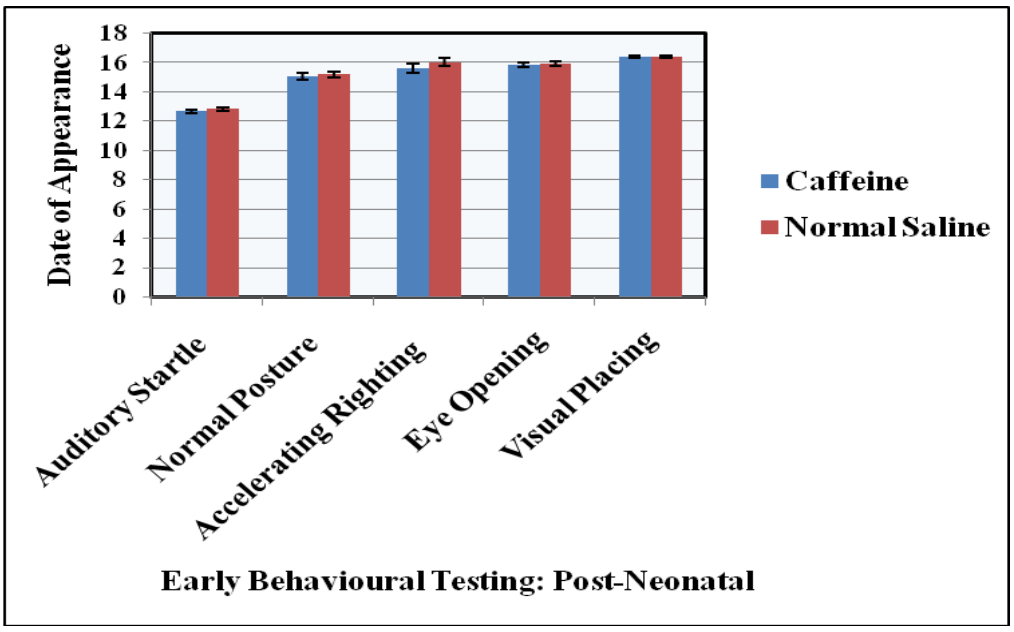

Figure 4-4: Early behavioral tests: Post-Neonatal.

The righting test was done daily from P4 until P7. The time required for the rat pup to achieve the test was recorded and summarized in Table 4-4 and Figure 4-5.

Postnatal age	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
P4	7.44±0.12	9.00±1.20	0.35
P5	5.98±1.06	6.2±1.07	0.89
P6	3.96±0.93	3.52±0.71	0.71
P7	4.76±0.95	4.99±0.97	0.87

Table 4-4: Body righting test (Time required in seconds).

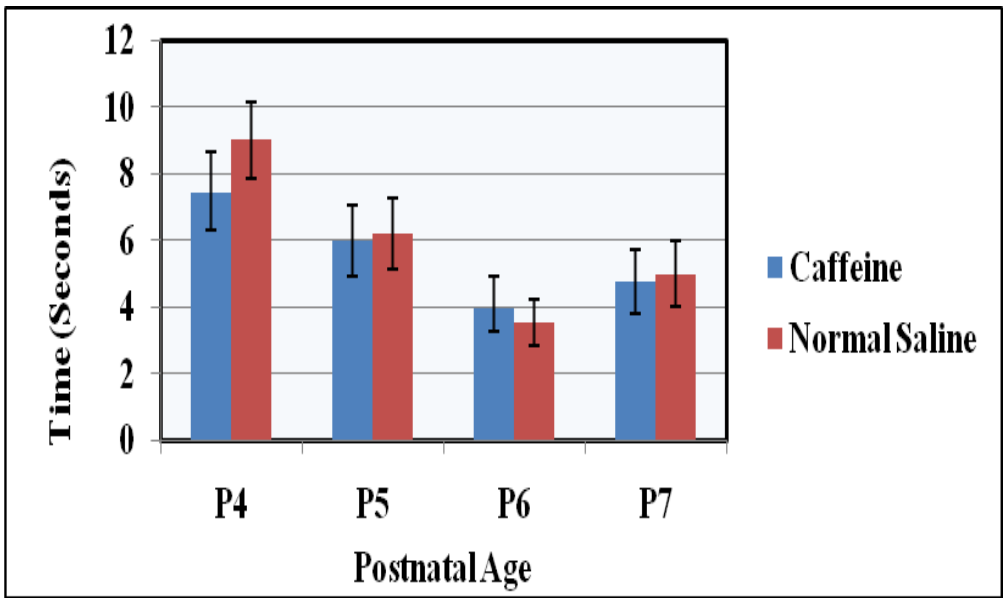

Figure 4-5: Body righting test (Time required in seconds).

For bar holding test, the best trial for each day was recorded and the results are shown in Table 4-5 and Figure 4-6.

Postnatal age	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
P12	7.5±1.4	8.1±1.5	0.77
P13	9.4±1.1	11.9±1.9	0.28
P14	19.2±2.4	22.3±3.0	0.43
P15	32.2±3.0	33.1±3.2	0.82
P16	33.9±3.4	34.5±3.3	0.89
P17	61.2±7.5	54.2±7.8	0.52
P18	54.7±8.2	40.4±8.1	0.22

Table 4-5: Bar holding test / Best trial score in seconds.

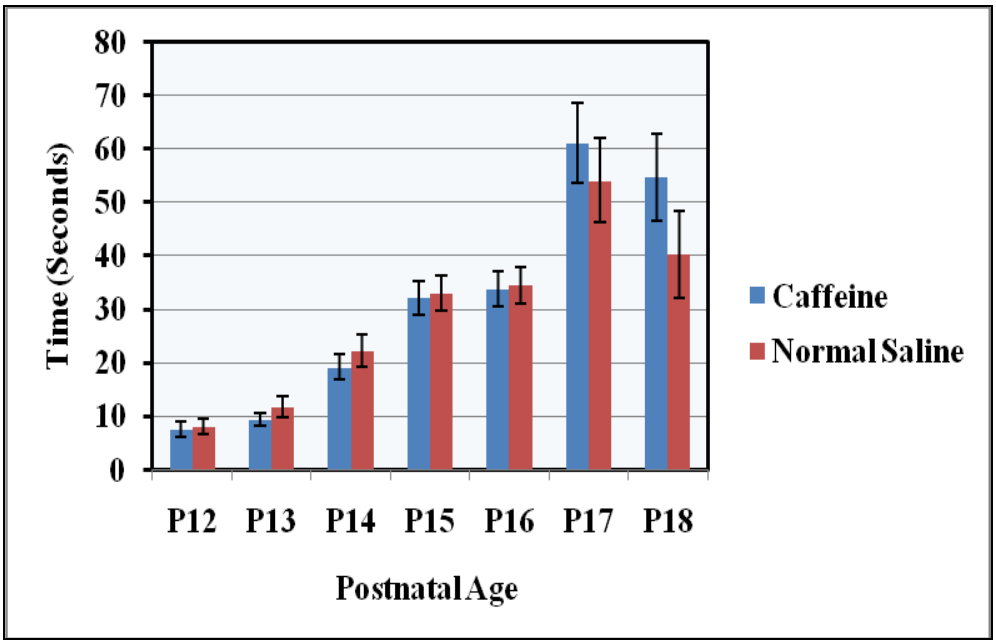

Figure 4-6: Bar holding test / Best trial score in seconds.

4.3.2. Open Field Test (P21):

Unpaired t test was used for statistical analysis. We didn't find any statistical difference between caffeine and normal saline groups for any of the behavioral tests that were examined. The numbers of animals were 27 in the caffeine group (11 males and 16 females) and 28 in the normal saline group (12 males and 16 females). The results are summarized in Table 4-6 and Figure 4-7.

Behavioral test	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	P Value
Defecation	2.67±0.23	2.50±0.27	0.65
Grooming	1.04±0.15	1.18±0.14	0.48
Head Lift	13.59±1.48	14.61±1.06	0.58
Rearing	39.00±2.67	40.68±1.66	0.59
Squares	75.15±4.32	79.96±3.43	0.74

Table 4-6: Open field behavioral tests at P21 (Number of each behavior per 5 minute period).

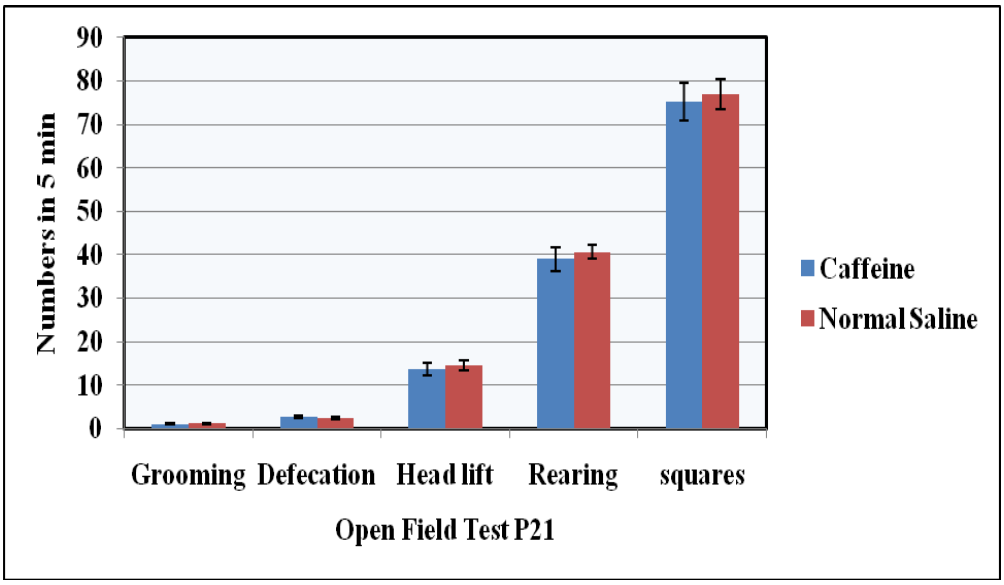

Figure 4-7: Open field behavioral tests at P21 (Number of each behavior per 5 minute period).

4.3.3. Open Field Test - The Second Round (P63):

Two way ANOVA test was used for analysis. It showed no effect of caffeine or sex on the open field behavioral tests at P63 and no interaction between sex and treatment. The numbers of animals were 18 in the caffeine group (8 males and 10 females) and 18 in the normal saline group (8 males and 10 females). The results are summarized in Tables 4-7 and 4-8 and Figures 4-8 and 4-9.

Behavioral test	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)
Defecation	1.00±0.47	1.67±0.18
Grooming	1.60±0.43	2.00±0.16
Head Lift	3.00±0.78	2.50±0.29
Rearing	48.80±4.70	42.80±5.00
Squares	91.70±6.70	75.50±6.30

Table 4-7: Open field behavioral tests at P63 in males (Number of each behavior per 5 minute period).

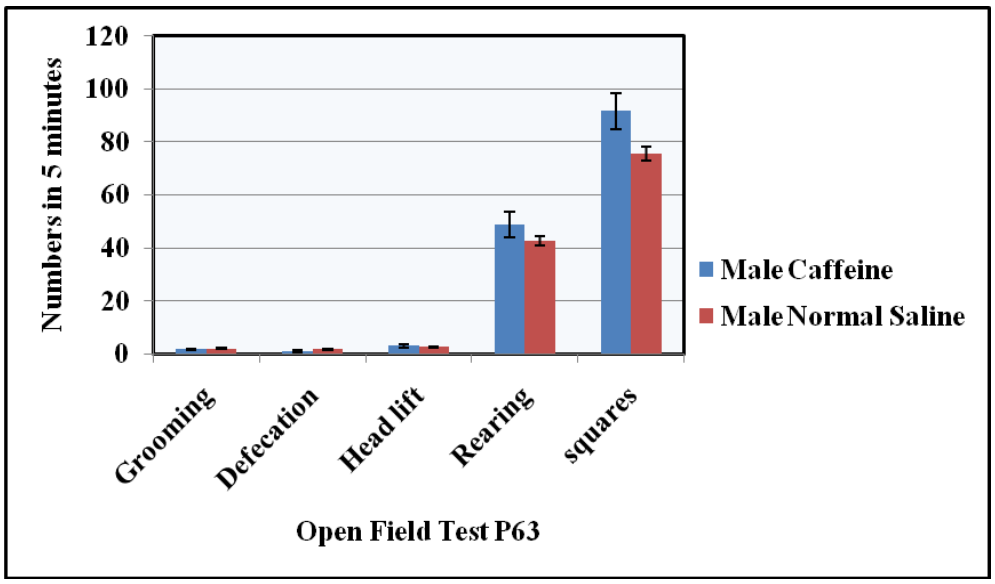

Figure 4-8: Open field behavioral tests at P63 in males (Number of each behavior per 5 minute period).

Behavioral test	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)
Defecation	2.00±0.84	1.08±0.54
Grooming	1.13±0.40	2.00±0.0.80
Head Lift	2.38±0.46	1.58±0.42
Rearing	48.80±4.70	50.50±3.50
Squares	80.30±3.50	87.60±5.70

Table 4-8: Open field behavioral tests at P63 in females (Number of each behavior per 5 minutes period).

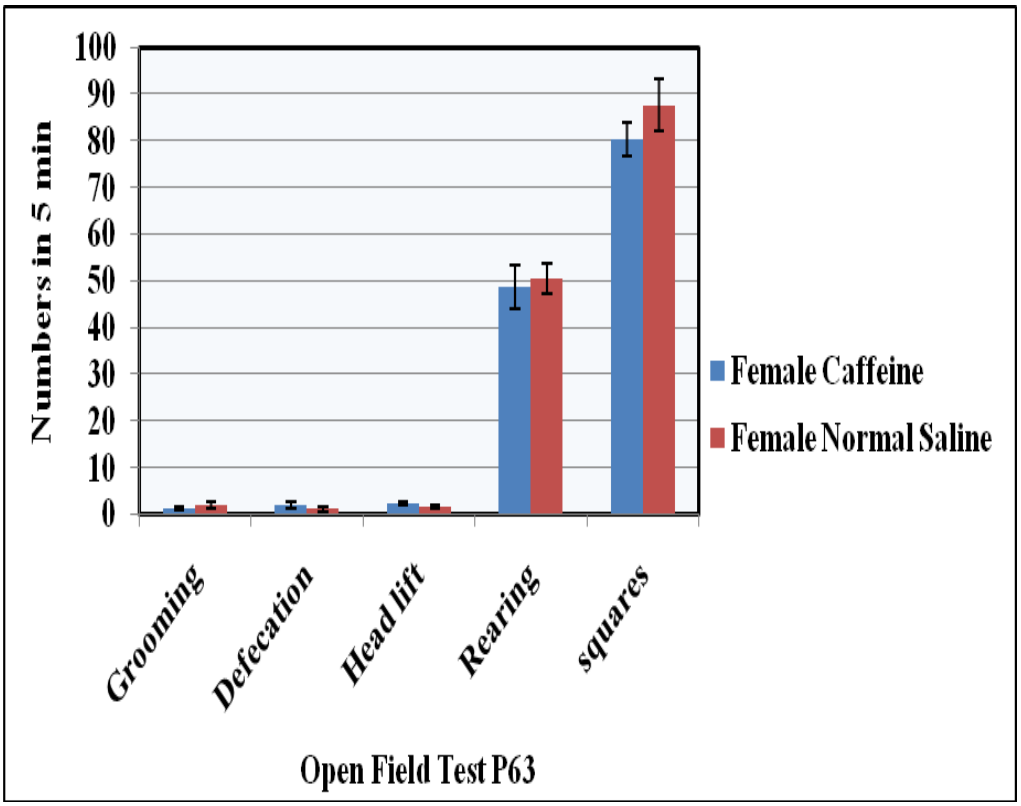

Figure 4-9: Open field behavioral tests at P63 in females (Number of each behavior per 5 minute period).

4.3.4. Object Recognition Test (P63):

Two way ANOVA test was used for analysis. We found no effect of caffeine or sex on the total time of interaction with the same objects (part 1) nor for the novel object test scores (difference score and discrimination ratio) and no interaction between sex and treatment. The numbers of animals were 18 in the caffeine group (8 males and 10 females) and 18 in the normal saline group (8 males and 10 females). The results are summarized in Tables 4-9 to 4-11 and Figures 4-10 to 4-12.

Sex	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)
Males	156.4±11.8	142.3±18.3
Females	136.4±10.2	133.6±10.9

Table 4-9: Object recognition test part 1: total time (seconds) of interaction with the same objects.

Figure 4-10: Object recognition test part 1: total time (seconds) of interaction with the same objects.

Sex	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)
Male	0.64±0.02	0.72±0.04
Female	0.65±0.03	0.68±0.04

Table 4-10: Object recognition test part 2 (novel object): discrimination ratio score = novel object interaction/total interaction with both objects.

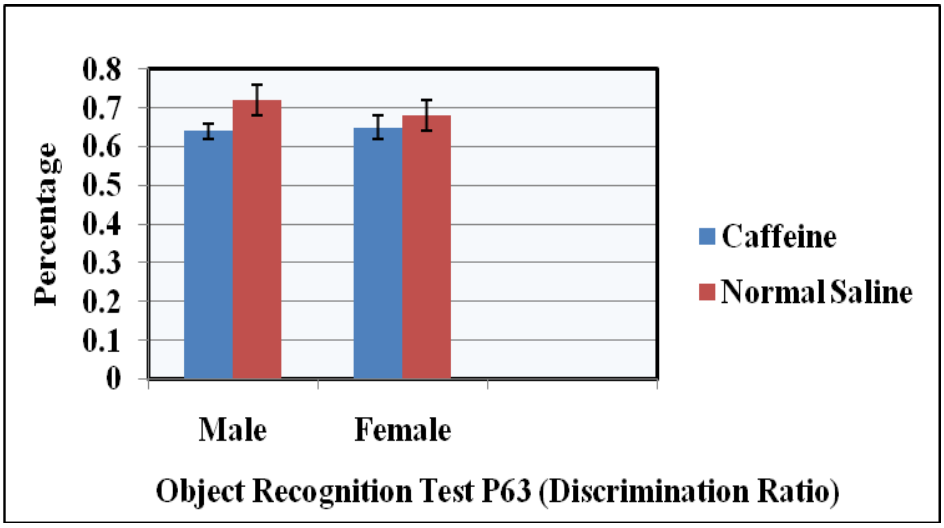

Figure 4-11: Object recognition test part 2 (novel object): discrimination ratio score = novel object interaction/total interaction with both objects.

	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)
Male	22.56±4.41	32.05±4.51
Female	19.42±3.77	21.35±4.50

Table 4-11: Object recognition test part 2 (novel object): difference score = novel object interaction - familiar object interaction.

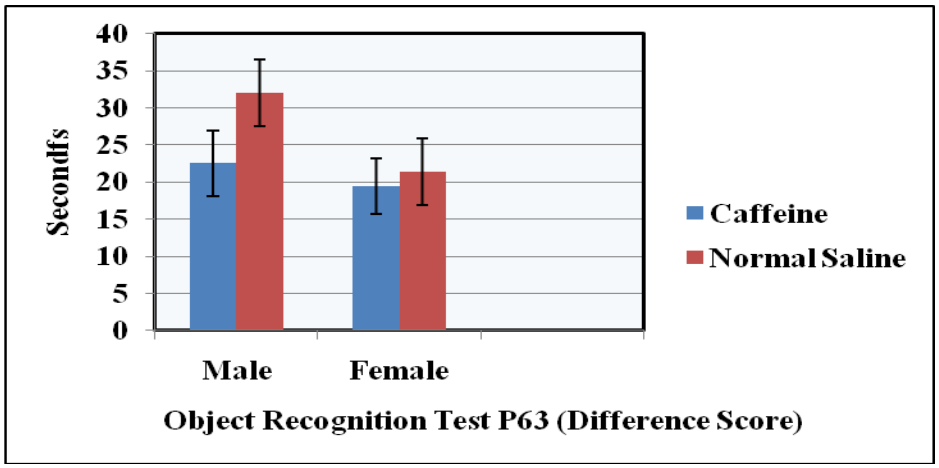

Figure 4-12: Object recognition test part 2 (novel object): difference score = novel object interaction - familiar object interaction.

4.3.5. Morris Water Maze Test:

Two way ANOVA was used for the statistical analysis of this test. The 2 scores analyzed were latency and path length and were analyzed from Day1 to Day4. Latency is the time from start to goal (platform) in seconds and the path length is the length from start to goal in cm. The results were averaged across four trials per day (mean ± SEM).

For latency we found no effect of caffeine treatment on the test all through the study from Day1 to Day4 and there were no interactions between sex and treatment, sex and cue, treatment and cue or sex and treatment and cue. There was an effect of the distracter cue on Day2 and Day4 with $F(1,79) = 4.47$, $P = 0.038$ at Day2 and $F(1,79) = 7.74$, $P = 0.007$ at Day4; the rats required significantly more time to find the platform in the presence of the cue (distracter). We found also an effect of sex on latency score at Day4 with $F(1, 79) = 8.86$, $P = 0.004$; male rats required significantly less time to find the platform.

For path length we found no effect of caffeine treatment all through the study from Day1 to Day4 and there were no interactions between sex and treatment, sex and cue, treatment and cue nor sex and treatment and cue. There was an effect of the cue (distracter) from Day2 to Day4 with F (1,79) = 6.90, P = 0.011 on Day2, F (1,79) = 4.93, P = 0.030 on Day3 and F (1,79) = 6.38, P = 0.014 on Day4; the rats required significantly more length to find the platform in the presence of the cue (distracter). We found also an effect of sex on path length score at Day3 and Day4 with F (1, 79) = 6.24, P = 0.015 at Day3 and F (1, 79) = 6.38, P = 0.005 at Day4; male rats required significantly less length to find the platform. The results are detailed in Tables 4-12 to 4-15 and Figures 4-13 to 4-16.

Treatment	Cue	Day 1 (Mean±SEM)	Day 2 (Mean±SEM)	Day 3 (Mean±SEM)	Day4 (Mean±SEM)
Caffeine	Distracter	37.65±3.90	18.70±3.23	11.85±1.86	9.35±0.88
Caffeine	No Distracter	31.86±4.18	14.97±3.79	10.27±2.77	7.07±0.86
Normal Saline	Distracter	40.51±1.32	23.83±1.20	11.13±0.88	10.41±0.27
Normal Saline	No Distracter	38.67±4.05	11.84±1.75	8.97±1.26	7.56±1.49

Table 4-12: Morris water maze test latency score (seconds) in males.

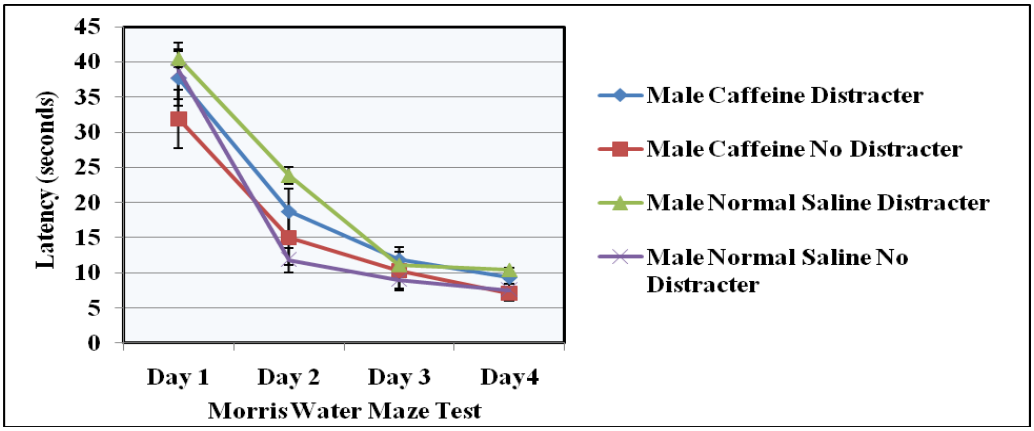

Figure 4-13: Morris water maze test latency score (seconds) in males.

Treatment	Cue	Day 1 (Mean ±SEM)	Day 2 (Mean±SEM)	Day 3 (Mean±SEM)	Day4 (Mean±SEM)
Caffeine	Distracter	2454±237	1265±265	691±126	473±57
Caffeine	No Distracter	2088±263	907±188	550±128	373±54
Normal Saline	Distracter	2618±293	1600±320	626±109	532±80
Normal Saline	No Distracter	2458±274	703±102	478±74	406±115

Table 4-13: Morris water maze test path length score (cm) in males.

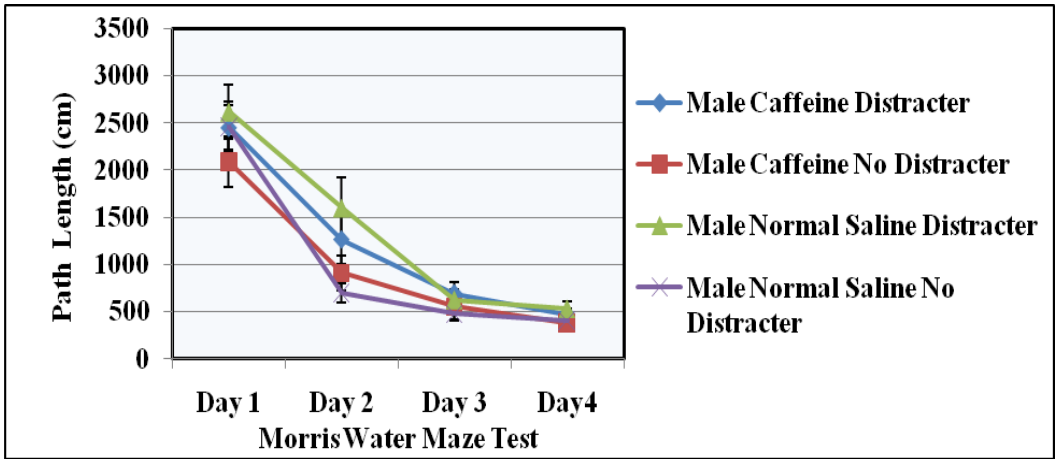

Figure 4-14: Morris water maze test path length score (cm) in males.

Treatment	Cue	Day 1 (Mean ±SEM)	Day 2 (Mean ±SEM)	Day 3 (Mean±SEM)	Day4 (Mean±SEM)
Caffeine	Distracter	34.64±3.08	17.35±2.73	16.08±2.86	14.43±2.60
Caffeine	No Distracter	35.21±4.24	15.44±1.99	13.58±1.64	8.78±1.18
Normal Saline	Distracter	34.21±3.27	19.77±3.16	14.82±2.60	12.36±1.49
Normal Saline	No Distracter	32.09±2.53	18.39±3.37	12.22±1.42	11.39±1.39

Table 4-14: Morris water maze test latency score (seconds) in females.

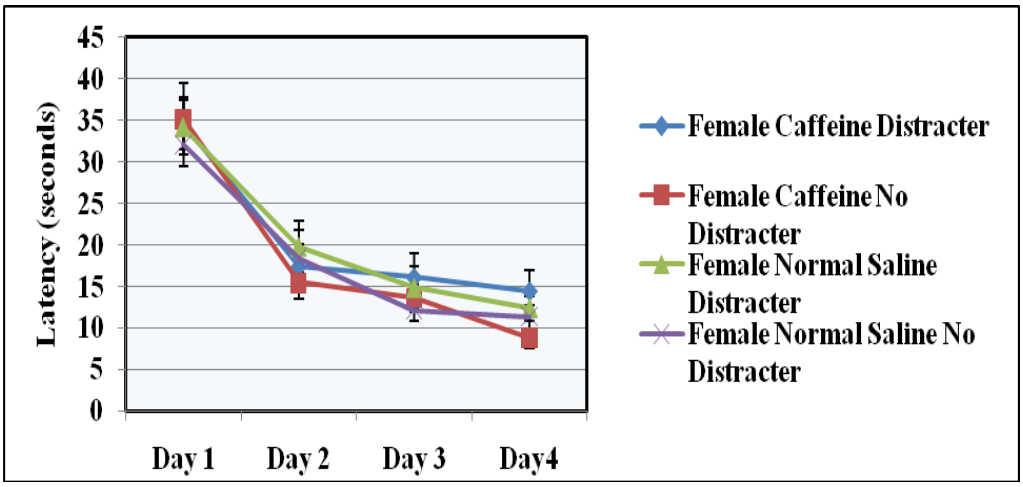

Figure 4-15: Morris water maze test latency score (seconds) in females.

Treatment	Cue	Day 1 (Mean ±SEM)	Day 2 (Mean ±SEM)	Day 3 (Mean±SEM)	Day4 (Mean±SEM)
Caffeine	Distracter	2206±211	1004±148	983±194	824±166
Caffeine	No Distracter	2216±336	869±116	679±79	403±56
Normal Saline	Distracter	2296±254	1284±226	927±183	713±111
Normal Saline	No Distracter	2103±171	1104±214	690±102	654±105

Table 4-15: Morris water maze test path length score (cm) in females.

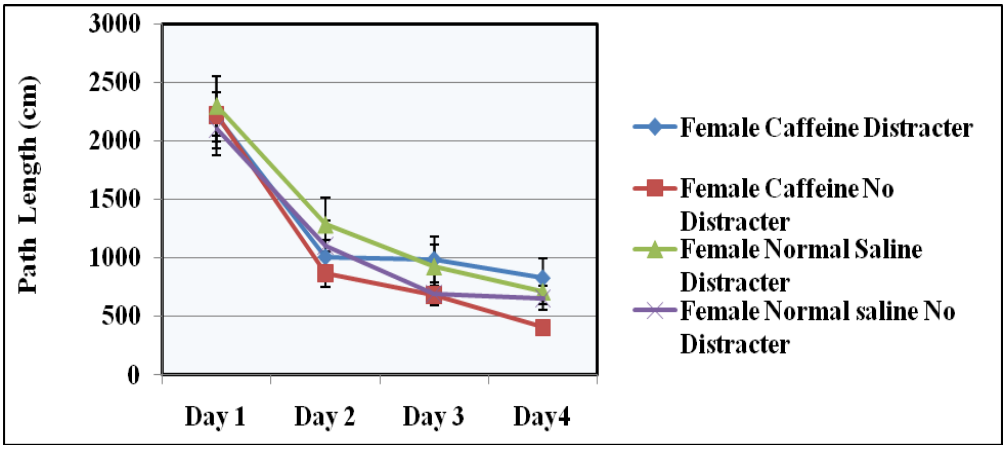

Figure 4-16: Morris water maze test path length score (cm) in females.

4.4. Neuropathologic Evaluation:

4.4.1. Fluoro-Jade B (FJB) Test:

Fluoro-Jade B stain was done at P4 and P8 in the following brain regions: caudate, globus pallidus, thalamus, hypothalamus, superior colliculus, nucleus accumbens, anterior cortex, posterior cortex, substantia nigra and hippocampus areas: CA1, CA3, CA4 and dentate. Six brains were used for analysis for each group (caffeine and normal saline).

We found a significant increase in neuronal cell degeneration in the caffeine group in comparison to normal saline group at P4 in the following areas of the brain: globus pallidus, thalamus, hypothalamus, nucleus accumbens, substantia nigra and CA1 region of the hippocampus. At P8 there was no statistically significant difference between the 2 groups. The results are detailed in Tables 4-16 and 4-17 and Figures 4-17 to 4-20.

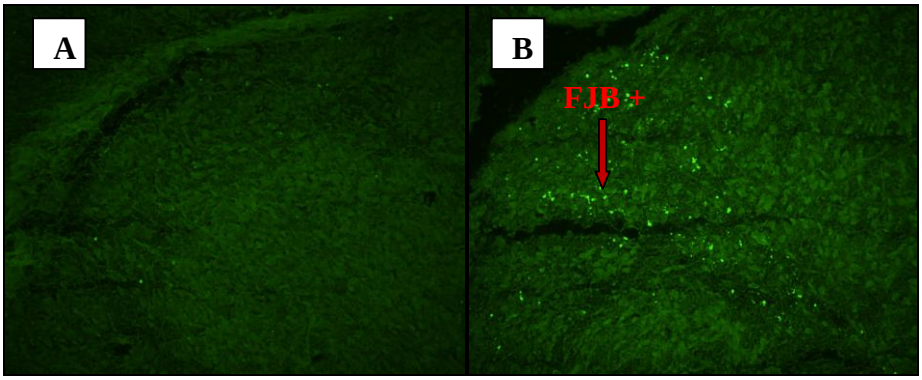

Figure 4-17: FJB-stained sections at P4 illustrating Thalamus in A (normal saline group) and B (caffeine group) at 40X magnification (FJB + cells appear with bright green dots).

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
Nucleus Accumbens	186.8±37.44	69.08±16.62	0.017
Globus Pallidus	185.6±21.56	97.03±15.95	0.011
Substantia Nigra	73.01±16.03	29.83±4.81	0.027
Thalamus	197.80±44.70	78.51±10.44	0.027
Hypothalamus	216.70±24.84	115.40±15.23	0.006
CA1	59.66±15.98	20.41±6.28	0.045
Superior Colliculus	166.40±31.45	107.60±12.31	0.112
Dentate	18.84±7.98	7.85±3.78	0.241
CA3	31.40±7.36	19.63±7.03	0.270
CA4	31.40±12.14	25.91±8.49	0.720
Caudate	18.84±3.20	13.35±4.11	0.317
Anterior Cortex	13.35±5.22	16.49±4.34	0.654
Posterior Cortex	32.19±9.05	18.06±5.76	0.217

Table 4-16. The number of degenerating neurons in different regions of the brain per mm^2 (numbers represent Mean ± SEM) at P4 manifested by FJB stained neurons. Statistically significant when p value < 0.05.

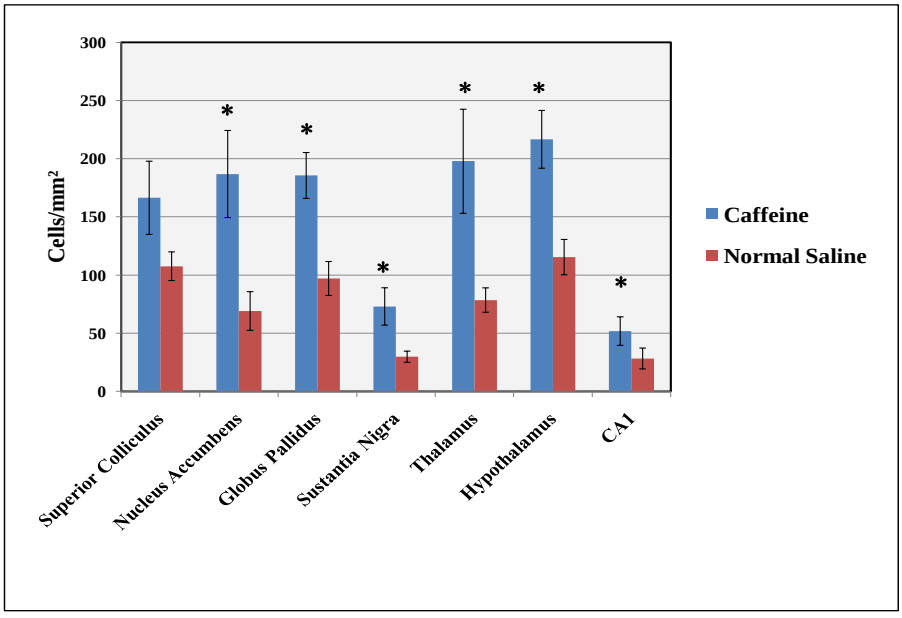

Figure 4-18: FJB-stain illustrating the degenerating neurons numbers per mm^2 in normal saline and caffeine groups at P4. P values less than 0.05 are marked by *.

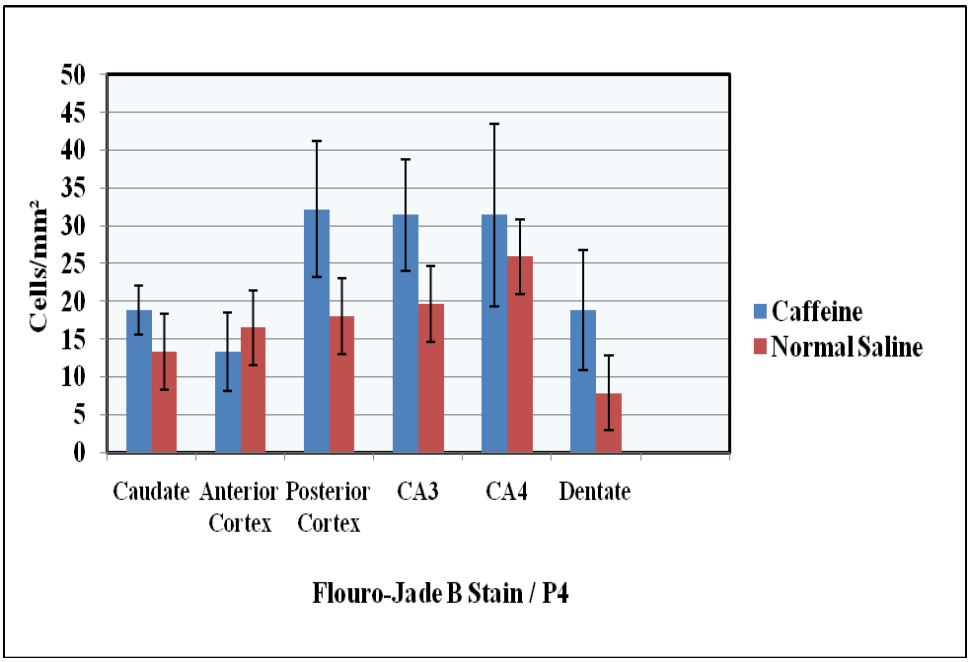

Figure 4-19: FJB-stain illustrating the numbers of degenerating neurons per mm^2 in normal saline and caffeine groups at P4.

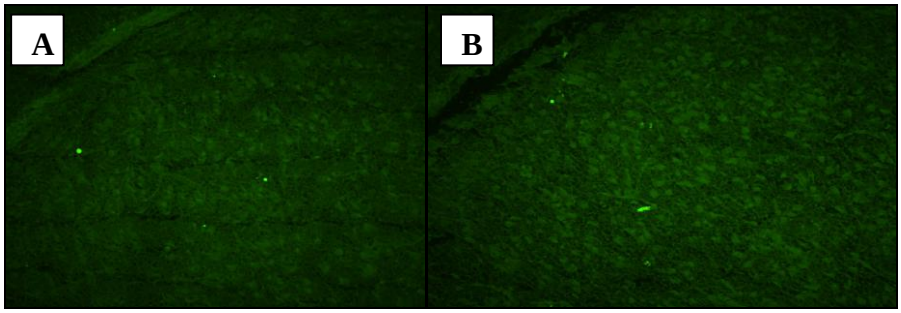

Figure 4-20: FJB-stained sections at P8 illustrating Thalamus in A (normal saline group) and B(caffeine group) at 40X magnification.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
Nucleus Accumbens	99.70±15.21	103.60±17.87	0.871
Globus Pallidus	41.61±9.38	35.33±15.03	0.730
Substantia Nigra	23.55±6.99	14.92±4.78	0.332
Thalamus	34.54±6.51	26.69±7.85	0.459
Hypothalamus	34.54±4.33	36.11±6.04	0.837
CA1	2.36±2.36	3.14±1.57	0.787
Superior Colliculus	32.19±8.01	26.69±8.83	0.655
CA3	3.14±1.57	7.07±2.02	0.156
Caudate	36.11±5.11	31.40±6.84	0.594
Anterior Cortex	14.13±5.44	8.64±3.53	0.417
Posterior Cortex	13.35±2.83	15.70±2.33	0.535

Table 4-17. The number of degenerating neurons per mm^2 in different regions of the brain (numbers represent Mean ± SEM) at P8 manifested by FJB stained neurons. Statistically significant when p value < 0.05.

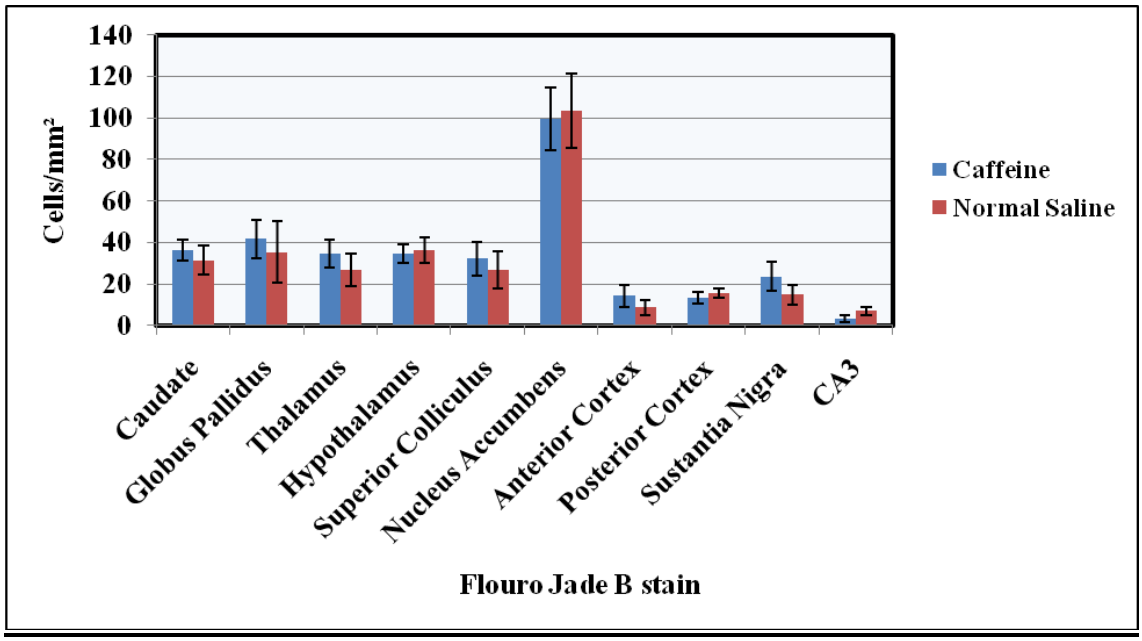

Figure 4-21: FJB-stain illustrating the numbers of degenerating neurons per mm^2 in normal saline and caffeine groups at P8.

4.4.2. Anti-NeuN Immunohistochemical Test:

Seven rat brains were stained with Neu-N from the caffeine group and compared with five brains in the normal saline group at P21. At P160, 6 rat brains (3 males and 3 females) were used for each group. The nuclei were counted in the following regions the brain: globus pallidus, thalamus, hypothalamus, nucleus accumbens, substantia nigra, superior colliculus and CA1 region of the hippocampus. Statistical analysis was done using unpaired t test and showed a statistically significant neuronal cell loss at P21 in CA1 region of the hippocampus ($p<0.01$) and the hypothalamus ($p<0.05$) in the caffeine group. At P160 there was no statistically significant difference between caffeine and normal saline groups.

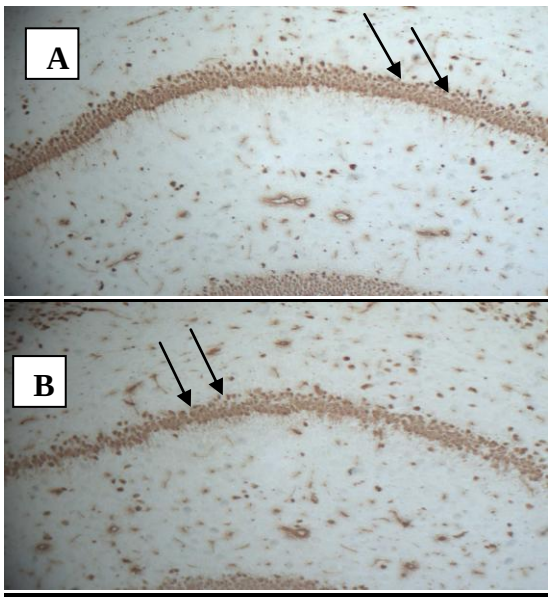

Figure 4-22: Anti-NeuN staining from CA1 of hippocampus at P21 in A (normal saline group) and B (caffeine group) at 40X magnification.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
Nucleus Accumbens	1594.35±100.28	1512.49±92.47	0.558
Globus Pallidus	268.11±38.94	235.42±20.89	0.499
Substantia Nigra	488.62±28.79	528.59±24.23	0.949
Thalamus	832.87±79.90	859.52±28.66	0.315
Hypothalamus	769.41±44.93	975.01±90.52	0.046
CA1	1054.97±47.81	1299.28±50.52	0.007
Superior Colliculus	675.82±67.77	670.74±19.11	0.778

Table 4-18. The number of neuronal nuclei in different regions of the brain per mm^2 (numbers represent Mean ± SEM) at P21 manifested by Anti-NeuN immunohistochemistry stain. Statistically significant when p value < 0.05.

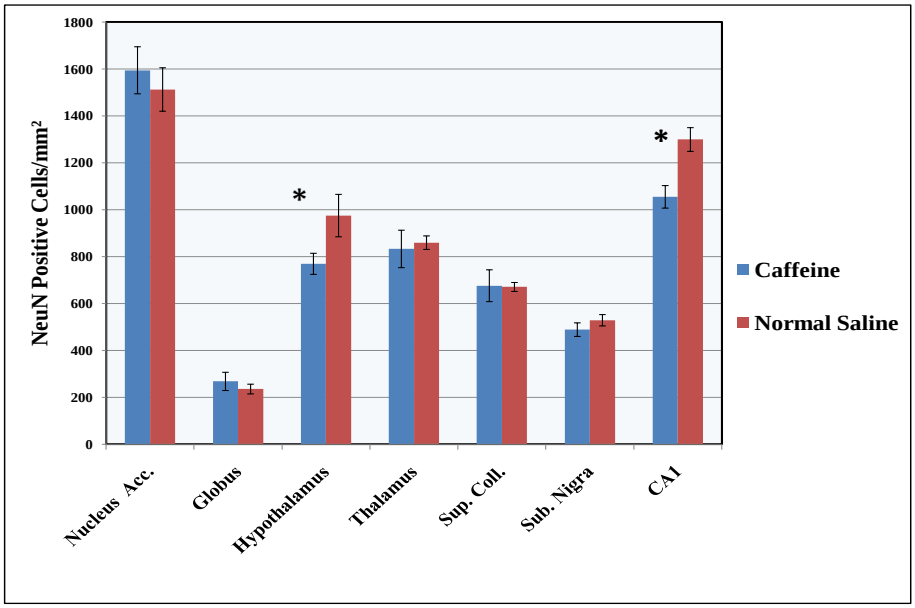

Figure 4-23: The number of neuronal nuclei in different regions of the brain per mm^2 (numbers represent Mean ± SEM) at P21 manifested by Anti-NeuN immunohistochemistry stain. P values less than 0.05 are marked by *.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	P Value
Nucleus Accumbens	1344.00±54.93	1314.00±88.92	0.783
Globus Pallidus	137.00±10.98	172.10±17.85	0.124
Substantia Nigra	401.60±63.95	447.90±33.89	0.537
Superior Colliculus	703.30±40.52	720.00±70.78	0.840
Thalamus	296.10±21.39	322.00±35.23	0.544
CA1	963.90±38.70	873.60±69.98	0.320
Hypothalamus	625.60±52.14	631.10±42.28	0.940

Table 4-19. The number of neuronal nuclei in different regions of the brain per mm^2 (numbers represent Mean ± SEM) at P160 manifested by Neu-N immunohistochemistry stain. Statistically significant when p value < 0.05.

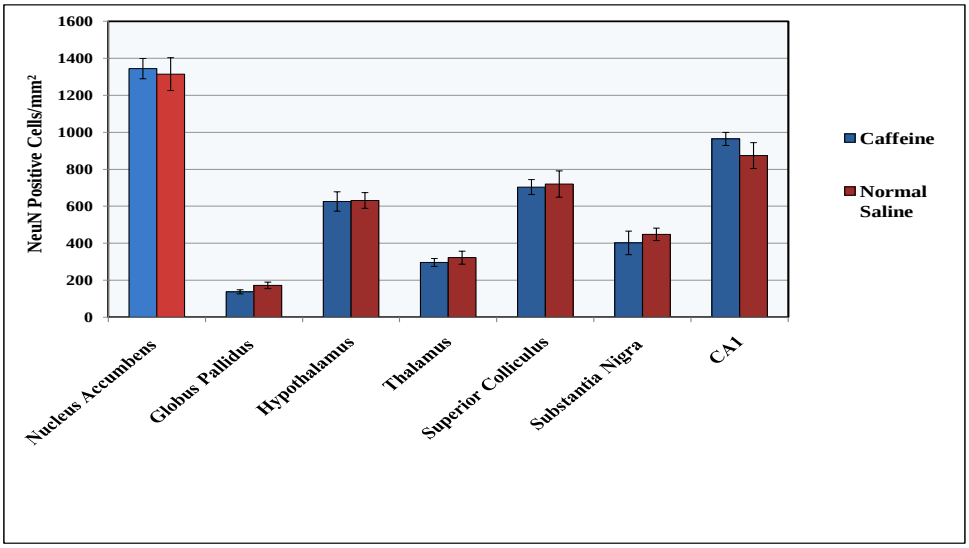

Figure 4-24: The number of neuronal nuclei in different regions of the brain per mm^2 (numbers represent Mean ± SEM) at P160 manifested by Anti-NeuN immunohistochemistry stain.

4.4.3. Anti-Neurofilament M Immunohistochemical Test:

Neurofilament M immunohistochemical test was done at P8, P21and P160. It was evaluated by using densitometry analysis. At P8, 6 rat brains were used from the caffeine group and 7 brains from the normal saline group. At P21, 7

rat brains were used from the caffeine group and 5 brains from the normal saline group and. At P160, 5 rat brains were used for each group. The test was done in the following regions of the brain: globus pallidus, thalamus, hypothalamus, nucleus accumbens, substantia nigra, superior colliculus and CA1 region of the hippocampus. Statistical analysis was done using unpaired t test and showed no statistically significant difference between caffeine and normal saline groups at any age group.

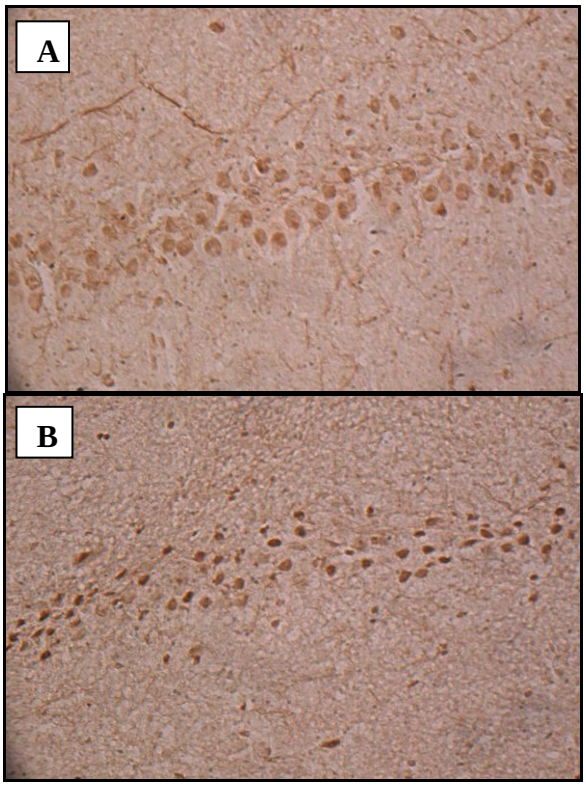

Figure 4-25: Anti-Neurofilament M immunohistochemical test from CA1 of hippocampus at P21 in A (normal saline group) and B (caffeine group) at 40X magnification.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	P Value
Nucleus Accumbens	0.090±0.007	0.090±0.013	0.985
Globus Pallidus	0.090±0.017	0.110±0.010	0.430
Substantia Nigra	0.110±0.006	0.090±0.009	0.112
Superior Colliculus	0.040±0.007	0.040±0.006	0.948
Thalamus	0.040±0.009	0.050±0.009	0.595
CA1	0.050±0.011	0.060±0.009	0.430
Hypothalamus	0.060±0.010	0.060±0.011	0.938

Table 4-20. Anti-Neurofilament M immunohistochemistry stain at P8 analyzed by optical density (numbers represent Mean ± SEM). Statistically significant when p value < 0.05.

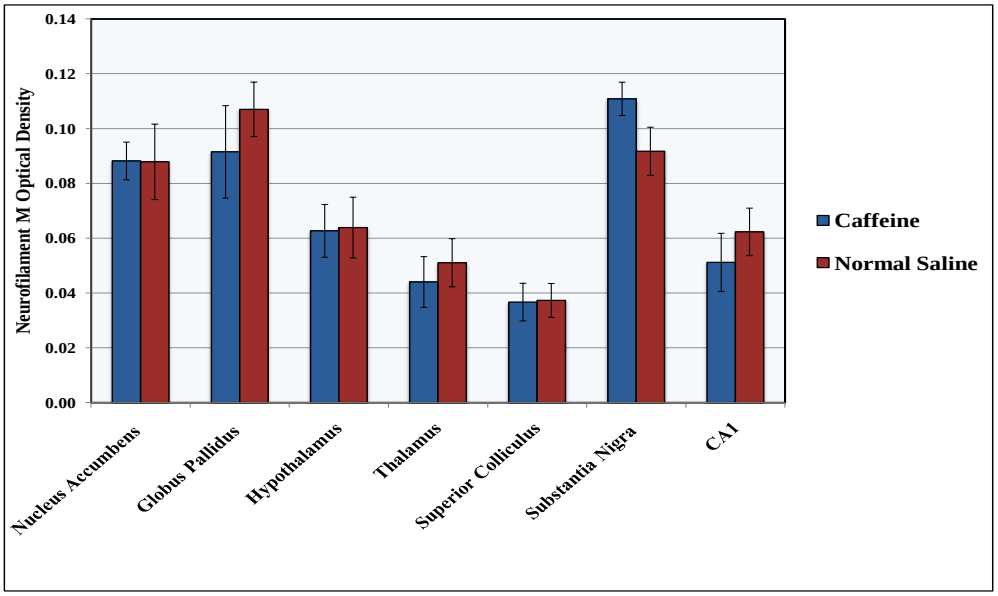

Figure 4-26: Anti-Neurofilament M immunohistochemistry stain at P8 analyzed by optical density.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	p Value
Nucleus Accumbens	0.050±0.008	0.040±0.007	0.34
Globus Pallidus	0.080±0.012	0.090±0.012	0.71
Substantia Nigra	0.140±0.014	0.150±0.024	0.77
Superior Colliculus	0.040±0.007	0.030±0.008	0.40
Thalamus	0.030±0.011	0.030±0.008	0.75
CA1	0.090±0.008	0.100±0.010	0.41
Hypothalamus	0.080±0.017	0.060±0.007	0.30

Tables 4-21. Anti-Neurofilament M immunohistochemistry stain at P21 analyzed by optical density (numbers represent Mean ± SEM). Statistically significant when p value < 0.05.

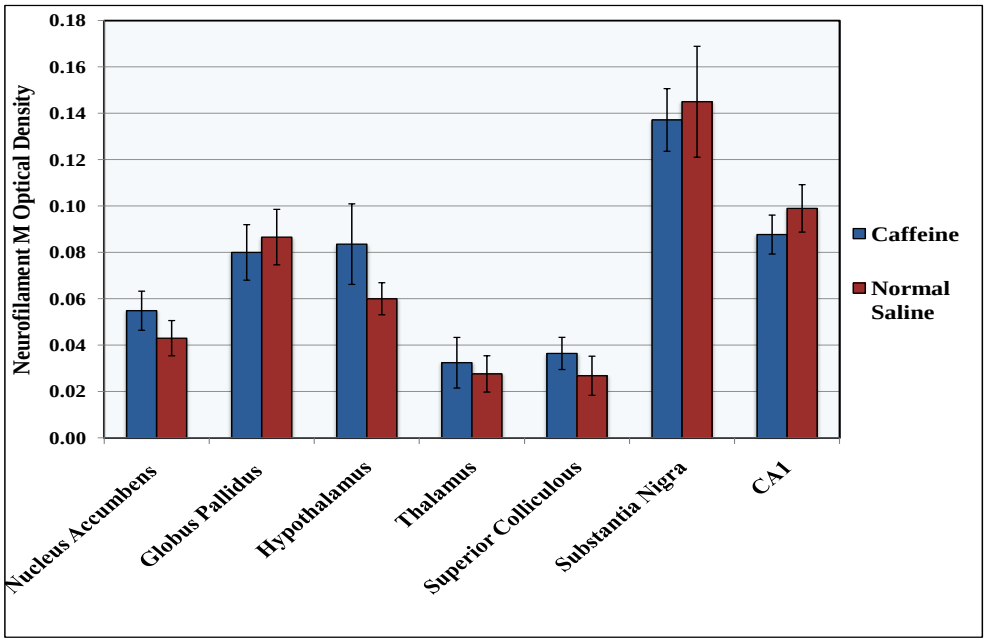

Figure 4-27: Anti-Neurofilament M immunohistochemistry stain at P21 analyzed by optical density.

Brain Area	Caffeine Group (Mean ± SEM)	Normal Saline Group (Mean ± SEM)	P Value
Nucleus Accumbens	0.100±0.014	0.110±0.009	0.83
Globus Pallidus	0.120±0.012	0.110±0.012	0.44
Substantia Nigra	0.150±0.016	0.160±0.014	0.49
Superior Colliculus	0.050±0.005	0.070±0.012	0.22
Thalamus	0.080±0.007	0.080±0.006	0.79
CA1	0.060±0.015	0.070±0.004	0.41
Hypothalamus	0.120±0.010	0.110±0.005	0.18

Table 4-22. Anti-Neurofilament M immunohistochemistry stain at P160 analyzed by optical density (numbers represent Mean ± SEM). Statistically significant when p value < 0.05.

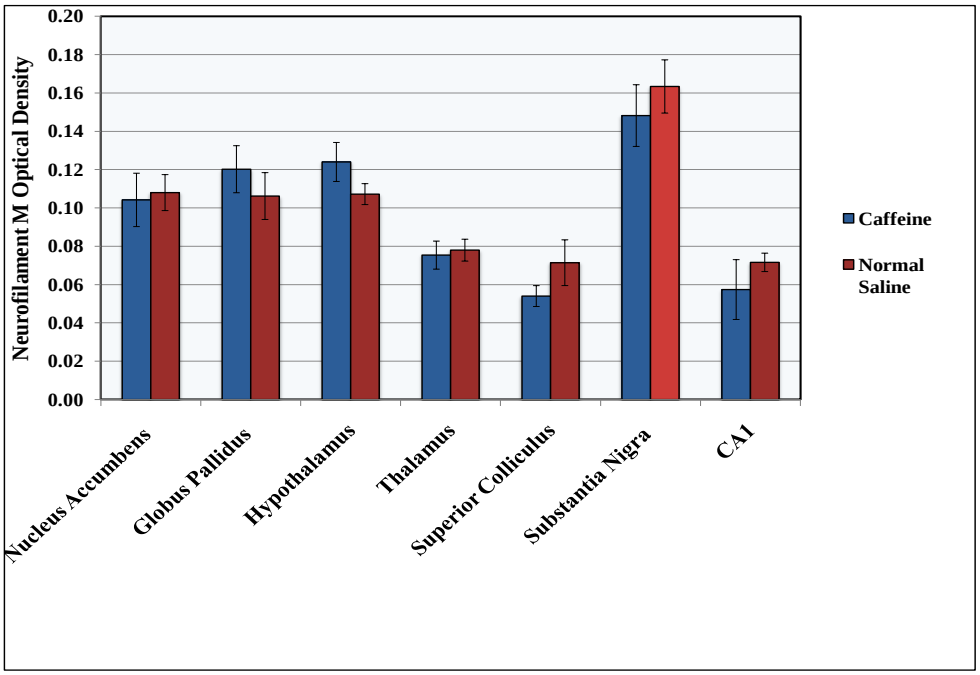

Figure 4-28: Anti-Neurofilament M immunohistochemistry stain at P160 analyzed by optical density.

Chapter 5: Discussion

The purpose of this study was to determine whether clinically relevant doses of caffeine, as utilized for apnea of prematurity, has long term effects on the developing brain, and whether these effects are manifested as behavioral and/or neuropathological abnormalities. To our knowledge, our study is the first study in which the rat pups were followed from the neonatal age (P4) until mature adults (P160) and tested for several neurobehavioral and neuropathological tests, making our study the longest and the most comprehensive one.

5.1. Caffeine Levels:

To our knowledge, our study is the first one in which caffeine levels were also measured in the brain tissue, in addition to the plasma. We found an excellent correlation between the caffeine levels in the plasma and those in the brain tissue. The levels of caffeine in plasma and brain are correlated using linear regression r^2=0.968, p<0.001. See Figure 4-1 and Table 5-1. These results are consistant with the volume of distribution of caffeine which is about 850 ml/kg, with a range of 365-1760 ml/kg (Charles, Townsend et al. 2008).

Time of taking caffeine level after the last dose at P7	**Plasma Caffeine levels (Mean ±SEM)**	**brain tissue Caffeine levels (Mean ±SEM)**
0.5 h	12.62±0.68 µg/mL	13.38±0.87 µg/gm
4 h	9.98±0.40 µg/mL	10.77±0.87 µg/gm
24 h	1.22 ±0.29 µg/mL	1.37±0.28 µg/gm

Table 5-1: The Caffeine levels in the plasma and brain tissue.

In clinical studies plasma caffeine levels were found to be between 5 to 20 mg/L in most preterm infants treated with caffeine for AOP (Leon, Michienzi et al. 2007; Natarajan, Botica et al. 2007; Charles, Townsend et al. 2008). In our animal study, caffeine levels dropped at 24 hours to 1.22 ±0.29 µg/mL in the plasma and to 1.37±0.28 µg/gm in the brain tissue. It can be argued that the 24 h levels of caffeine were low in our study and this could affect the results of neurobehavior and/or the neuropathology. We found that our results of plasma caffeine levels were consistent with Guillet et al's study which showed that the plasma levels of caffeine reached a peak between 5-15mg/L over the 24h period; however, at 24h the plasma caffeine levels dropped to around 2 mg/L (Guillet 1990). Beside that, in previous animal studies, caffeine was given in a dose and duration based on Guillet et al study and these studies showed that caffeine had an effect on the neurobehavior and/or the neuropathology of the brain of these animals (Guillet 1990; Guillet and Kellogg 1991; Guillet and Kellogg 1991; Etzel and Guillet 1994; Guillet 1995; Guillet and Dunham 1995; Fisher and Guillet 1997; Gaytan, Saadani-Makki et al. 2006; Pan and Chen 2007). In one study even low dose caffeine exposure, in the first 7 days of life, was enough to decrease hypoxic ischemic brain damage by 30%, although the mean ±SEM of plasma caffeine levels were only 0.22 ±0.054 mg/L (Bona, Aden et al. 1995). In this regard, it is possible that our study underestimated the effect of caffeine on neurobehavior and pathology. None the less, the concentrations achieved were similar to that seen in previous animal studies and early pathology displayed a significant increase in cell death, following loading.

5.2. Mortality:

There was no mortality during the course of the study. This finding is consistent with the study by Black et al which showed no mortality in the animals that received caffeine treatment (Black, Pandya et al. 2008), and the caffeine for apnea of prematurity (CAP) trial in preterm human infants which showed that the rate of death did not differ significantly between caffeine and the control groups in the short term nor at follow up to 18-21 months (Schmidt, Roberts et al. 2006; Schmidt, Roberts et al. 2007).

5.3. Body Weight:

We found no effect of caffeine treatment on the body weight all through our study starting from P3 and lastly at P160. The results are summarized in table 4-2 and figure 4-2. Our results are consistent with Pan et al's study who found no effect of caffeine on the body weight in his rat model study (Pan and Chen 2007).

Although the CAP trial validated a reduction in the weight gain in the caffeine cohort, with the greatest difference noted after 2 weeks of starting treatment, this effect was transient and the difference between caffeine group and the control one resolved at 4 weeks of treatment (Schmidt, Roberts et al. 2006). Furthermore, the study's follow up to the corrected age of 18 to 21 months showed that the mean percentile for body weight did not differ significantly between the two groups (Schmidt, Roberts et al. 2007). The reason why in rat model studies there was no effect of caffeine on the body weight, while the CAP trial showed a transient effect of caffeine on the body weight, is that rats have

different metabolism than human preterm infants, they were normal pups and feeding was *ad libitum*, while human preterm infants were sick and their fluid and caloric intake were restricted.

These results indicate that the dose of caffeine used in the treatment of apnea of prematurity has no lasting effect on the body weight indicating its safety in this regard.

5.4. Neurobehavioral Tests:

Our study showed no statistical difference between caffeine and normal saline groups for any of the early neurobehavioral tests that were examined. These behavioral tests represent primitive reflexes (hind limb grasp, hind limb placing, cliff drop avoidance, and gait tests), labyrinthine and body righting mechanisms (body righting), strength and motor performance (bar holding ability), labyrinthine response (accelerating righting), and developmental milestones (auditory startle, eye opening, visual placing, and normal posture tests). These tests were done between P4 to P18, which represent the period from neonatal to infantile age.

Open Field tests were done at P21 (pre-juvenile age) and were repeated at P63 (early adulthood age). These tests represent locomotion and ambulation (number of squares crossed within the time of the test), exploratory (head lift and rearing) and stress and anxiety behaviors (defecation and grooming). Our study showed no statistical difference between caffeine and normal saline groups for all the above behaviors.

Novel object recognition test, which is used to study attention, memory and recognition, was done at P63. We found no statistical difference between caffeine and normal saline groups in this test.

Spatial learning, cognition and attention neurobehaviors were tested by the Morris water maze test. This test was done at P160 (mature adults). We found no statistically significant effect of caffeine on this test.

In summary, our findings in regard the effect of neonatal caffeine exposure on the developmental milestones are consistent with Pan et al's study which had shown that the developmental landmarks (milestones) including earflap opening, lower eruption incisor, and eyelid opening examined at P3, P10, and P12, respectively, did not differ significantly between the neonatally-treated caffeine group and the control one (Pan and Chen 2007).

Hong-Zhen Pan demonstrated also in his rat model that neonatal caffeine exposure results in thermal hyperalgesia in a hot plate test, low anxiety in the dark-light box test and the elevated plus-maze in comparison to controls. In the same study, it was also demonstrated that neonatal caffeine exposure produced memory impairment in step-through avoidance learning task in both males and females rats. These tests were done between P35 and P42 (Pan and Chen 2007). In a previous study, neonatally caffeine-treated rats required more trials to avoid the electrified grid than did control rats in both males and females at P28. The effect of caffeine seems not to be long lasting as at P70-90 there was no effect of caffeine treatment on learning (Fisher and Guillet 1997).

Why these studies showed that neonatal caffeine exposure caused changes in some of the neurobehaviors later in life, while our study didn't show any effect of caffeine on the short or the long term neurobehaviors, is not clear. However, the neurobehavioral tests that were done in our study were different from those in the above studies; to detect stress and anxiety we used open field behavioral tests (defecation and grooming), while in Pan et al' study, a dark-light box and the elevated plus-maze tests were used to assess these behaviors. It is possible that these tests are more sensitive to detect stress and anxiety behaviors than our tests. Nevertheless, putting all these tests together, they indicated that the effects of caffeine on stress and anxiety behaviors are subtle, at best.

For memory and learning, also other types of tests were done than ours, and they were done at an earlier age. In Pan et al's study, the step-through avoidance learning tasks were done between P35 and P42 (Pan and Chen 2007). In Fisher et al's study, the electrified grid showed a difference between the caffeine group and the control at an earlier age: P28, while there was no difference at the age of P70-90 (Fisher and Guillet 1997). For memory, cognition, learning and attention behaviors we used well known tests in the literature: the object recognition (Bevins and Besheer 2006) and the Morris water maze tests (Anisman and McIntyre 2002; Vorhees and Williams 2006; Vorhees, Skelton et al. 2007), and done at later ages: P63 and P160 respectively.

Given that there are no long-standing neuropathological abnormalities in our model, at the dose given, it is not surprising that behavioral abnormalities have not been detected. Irrespective of the fact that we used a range of tests across

the ages, we would anticipate abnormalities to be subtle. Either our findings will hold true in future studies, or more specific and sensitive tests for anxiety and learning are required.

5.5. Neuropathological Tests:

Our neuropathological tests showed significant increase in neuronal cell degeneration, manifested by Fluoro-Jade B stain, in the caffeine group in comparison to normal saline group at P4 (One day after the loading dose of caffeine citrate of 20 mg/kg) in the following areas of the brain: globus pallidus, nucleus accumbens, thalamus, hypothalamus, substantia nigra and CA1 region of the hippocampus. Kang et al showed that administration of three high caffeine doses of 50 mg/kg per dose at 5h interval on postnatal day 7, caused caspase-3-dependent neuronal cell death (apoptosis) in parietal cortex, temporal cortex, caudate putamen, dentate gyrus, thalamus, and hypothalamus (Kang, Lee et al. 2002). In another study Black et al showed, in a rat model, that caffeine citrate at a dose of 100 mg/kg given subcutaneously at P7 caused significant cell death measured by Fluoro-jade B and activated caspase-3, at 12 and 24 hour post-caffeine injection in the cortex, caudate, nucleus accumbens, hypothalamus, hippocampus and superior colliculus (Black, Pandya et al. 2008). Our findings were similar to these two studies in regard to the areas that are involved in neuronal cell death caused by caffeine and indicate that most of caffeine effect is evident in the grey matter areas of the neonatal brain, mainly thalamus, hypothalamus, nucleus accumbens and hippocampus.

Kang et al and Black et al studies suggested that the cell death caused by caffeine may be apoptotic in nature (Kang, Lee et al. 2002; Black, Pandya et al. 2008). Kang suggested that caffeine might trigger the caspase-dependent apoptosis by interfering with the intracellular Ca^{2+} homeostasis which leads to neuronal cell apoptosis (Kang, Lee et al. 2002). These explanations are consistent with one of the mechanisms of action of caffeine which is discussed in chapter 2. Caffeine, at high concentrations (≥250 μM), causes mobilization of calcium from intracellular storage sites and inhibition of voltage-sensitive calcium channels. This action may contribute more to the toxic effects of caffeine (Comer, Perry et al. 2001). Excessive elevation of intracellular calcium can lead to the activation of hydrolytic enzymes and trigger mitochondrial permeability transition which then causes apoptotic cell death (Blomgren, Leist et al. 2007). Although the loading dose of caffeine at P3 in our study is lower than the ones given in Kang and Black studies, it is possible that it may be enough to trigger the above mechanism of neuronal cell death. Beside this explanation, caffeine by blocking adenosine A_1 receptors can affect the release of the excitatory neurotransmitter (glutamate) pre-synaptically and can affect glutamate binding to NMDA receptors post-synaptically (Ribeiro, Sebastiao et al. 2002) .

Our study added to these findings that the effect of chronic low dose of caffeine is transient as we showed that at P8 (One day after the full course of caffeine citrate treatment) there was not a statistically significant difference between the caffeine and the normal saline groups in regard to neuronal cell death. Anti-NeuN immunohistochemical test confirmed this transient effect of

caffeine, as it showed a statistically significant neuronal cell loss at P21 in the hypothalamus and CA1 region of the hippocampus in the caffeine group, while at P160 there was no statistically significant difference between caffeine and normal saline groups. This may indicate that the cell death occurred after caffeine treatment may be caused by early than normal timing of programmed cell death (PCD) in these vulnerable developing brains of the neonatal rats, as apoptosis is one of the morphological manifestations of PCD (Blomgren, Leist et al. 2007); however, we don't know the exact explanation of this finding.

We didn't validate any effect of caffeine on the axonal formation as the Anti-Neurofilament M immunohistochemical test did not show any statistically significant difference between caffeine and normal saline groups at all ages: P8, P21 and P160.

The chronic use of caffeine could have a neuroprotective role in case of brain injury, as it was shown in several animal models. In these studies it was found that the neurological deficits, the cerebral edema and the inflammatory cell infiltration that occurred after the brain injury were all significantly attenuated with chronic caffeine intake. Myelination was enhanced and ventriculomegaly was reduced in hypoxia-exposed neonatal pups treated with caffeine. Furthermore, it was demonstrated that low dose caffeine exposure, in the first 7 days of life, decreased hypoxic ischemic brain damage by 30% in the 7-day-old rats. (Bona, Aden et al. 1995; Back, Craig et al. 2006; Li, Dai et al. 2008). The above results can be explained by the findings that chronic caffeine treatment attenuated glutamate release and inflammatory cytokine production, effects that

were correlated with an upregulation of brain A_1 receptor mRNA (Li, Dai et al. 2008).

These animal studies are consistent with the clinical study; the CAP trial which showed that, at 18 to 21 months follow up, treatment with caffeine as compared to placebo reduced the incidence of cerebral palsy and cognitive delay, while the rates of death, deafness and blindness did not differ significantly between the two groups (Schmidt, Roberts et al. 2007).

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Chapter 6: Conclusions, Limitations and Future Directions

6.1. Conclusions:

In conclusion we did not validate any effect of caffeine on the body weight nor the overall neurobehavioral outcomes. While analysis of the early neuropathology shows that caffeine caused neuronal cell death at P4 in the following areas of the brain: globus pallidus, nucleus accumbens, thalamus, hypothalamus, substantia nigra and CA1 region of the hippocampus, (manifested by FJB stain) and neuronal cell loss in CA1 and the hypothalamus regions at P21 (manifested by anti-NeuN immunohistochemical stain), the long term results have shown that there is no long-lasting effect on neuropathological outcome.

Caffeine is a commonly used medication in preterm infants for the treatment of apnea of prematurity. Our study provides new information which suggests that despite the early neurobehavioral and/or neuropathological alterations occurring in the brains of the developing rat brains, as shown in our study and previous ones, caffeine treatment did not cause overt permanent brain damage in the developing rat brain.

The clinical implications of our results are that the use of caffeine, in a manner similar to that utilized for the treatment of apnea of prematurity, has no significant effect on long-term functional or pathological outcome on the developing brains. These findings are consistent with current clinical studies, and

indicate that caffeine, in the doses used in treatment of apnea of prematurity, may be, in general, a safe medication for use in preterm infants.

However, our study also indicate that the use of caffeine, in a dose and duration that are comparable to human preterm infants, may have a transient effect on increasing apoptotic cell death. This effect seems to disappear quickly as no long term adverse alterations were seen. Perhaps the greatest point to be made is that the use of medications in the newborn developing brain needs to be approached with caution. Generally, there are two groups of preterm infants who are given caffeine for treatment of apnea of prematurity in the first few months of their lives:

1) The first group is the group of extreme preterm infants. This group of infants usually has a complex medical illness with severe respiratory distress syndrome, ventilation, intraventricular hemorrhage, chronic lung disease, the recurrent hypoxic/hyperoxic episodes and others. This is the group in which the CAP trial showed a benefit of caffeine treatment in regard of cerebral palsy and cognitive delay. In this group, caffeine may have neuroprotective functions, and for that the benefits of caffeine overweigh its risks, indicating the safety of using of caffeine in this group.

2) The second group is the preterm infants with the late onset prematurity; those who are born after 32 weeks gestational age. Apnea of prematurity in this group is usually not severe and they are most commonly clinically stable. In this group, the use of caffeine for treatment of apnea of prematurity should be in a wise way, taking in consideration the risks and the benefits of caffeine administration.

Though transient in nature, the early increase in cell death may have resulted in subtle changes of synaptogenesis that were not tested for in our study. Long term subtleties should be determined, and are the focus of future studies.

6.2. Limitations:

We used normal rat pups in our study, in order to validate the effect of caffeine *per se* on the developing brains. However, the preterm infants who are treated with caffeine in neonatal intensive care units have complex medical illnesses: hyaline membrane disease, AOP, bronchopulmonary dysplasia, infection, mechanical ventilation, etc. It will be very interesting and challenging to create an animal model that resembles the clinical status of the preterm human infants in neonatal intensive care units to study the effect of caffeine.

One limitation of this study is the difference in metabolism between the rat pups model and human preterm infants which is manifested in the caffeine plasma levels. In human studies, the clinical doses of caffeine used in treatment of AOP resulted in caffeine plasma level to be more than 5 mg/L in most of cases. In our study and previous rat model caffeine plasma level dropped to less than 5 mg/L at 24h.

Another limitation of this research is that, although we tried to do a comprehensive research, we couldn't do all the neurobehavioral or the neuropathological tests that were done in the literature to test the effect of caffeine. This is for many reasons; first, some behavioral tests required specific arrangements and special equipments in the examining room that are very

expensive and difficult to be available in every laboratory; second, these tests required a lot of training and time to perform them. The same above notes apply for neuropathology as well. So it will be extremely difficult to do every neurobehavioral and neuropathological test reported in the literature.

In regard of neuropathology the limitations are: 1) the densitometry method, used to analyze Neurofilament M immunohistochemical test, can't detect if there are abnormalities in the structure, the configuration or the connections of these axons. This can be assessed by using more specific neuropathological stains to give more specific details of the neuronal structures such as synaptogenesis, the length and branching of axons and dendrites configuration by using synaptophysin or Golgi staining (Stroemer, Kent et al. 1998; Juarez-Mendez, Carretero et al. 2006), 2) though we believe that the neuronal degeneration shown in our results are apoptotic in nature depending on previous studies; however, the golden method of detecting apoptosis is to look for the cell morphology using electron microscope. It will be interesting if these tests could be done.

6.3. Future Directions:

The results of my study and review of literature bring up few ideas for future directions in research:

1) It was shown in the literature that exposure to caffeine within the neonatal age in rat pups increased the subsequent expression of adenosine A_1 receptors in the thalamus and the cerebellum (Etzel and Guillet 1994) and also in cortex and hippocampus (Guillet and Kellogg 1991). To complete

our study it is planned to evaluate the expression of adenosine A_1 receptors in the rat brain. We will concentrate on the brain regions where we found a neuropathological effect of caffeine treatment on the FJ B stain and the NeuN immunohistochemical stain which are: globus pallidus, thalamus, hypothalamus, nucleus accumbens, substantia nigra and CA1 region of the hippocampus. It will be very interesting if we find a difference in the expression of adenosine receptors between caffeine and normal saline groups in these areas.

2) Doubling the dose of caffeine or administration of caffeine in 2 divided doses, to keep the 24h plasma caffeine levels above 5 mg/L, to be more comparable to the plasma caffeine levels in human preterm infants who are treated with caffeine for apnea of prematurity.
3) To use diseased animal models that mimic the complex status of preterm infants in NICU like:
    - Hypoxia/hyperoxia
    - Intrauterine growth retardation
4) As I found differences between the results of some of the neurobehavioral tests in my study and previous studies such as anxiety and stress, more specific tests are required to determine the effect of caffeine on these behaviors.

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