INVESTIGATING THE LINK BETWEEN PREECLAMPSIA AND THE ADVENT OF AUTISM IN SPRAGUE-DAWLEY RATS, A NEUROANATOMICAL LOOK AT THE SEROTONERGIC SYSTEM

BY

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A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

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Johannesburg, 2021

DECLARATION

I, Grace Chioma Austin-Ajah (student number: 2320081), declare that this thesis is my own work, supervised by Dr B. Maseko. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before, in full or in part, for any degree or examination at this or any other university. I furthermore declare that all the work conducted and reported in this thesis titled "*Investigating the link between preeclampsia and the advent of autism in Sprague-Dawley rats, a neuroanatomical look at the serotonergic system*" is my own from conceptualization of the project to the final thesis write up. Where I have used work from other investigators, it is explicitly indicated so as to respect and credit the thoughts and ideas of other researchers

…… ……....

Grace Chioma Austin-Ajah

Signed on 21st September, 2021 at Randburg, Johannesburg

DEDICATION

This research is most of all, dedicated to GOD Almighty.

Secondly, to Master Keanu Mdali Ndlovu

and

all the Autistic children all over the world

ABSTRACT

Background: Preeclampsia, a pregnancy complication that is characterised by hypertension, oedema and proteinuria, is a major cause of maternal and foetal mortality and morbidity. Preeclampsia has been associated with the advent of Autism from previous studies. Autism is a neurodevelopmental disorder that is marked by restricted repetitive patterns of behaviour, verbal and non-verbal communication deficiencies and impaired social interaction. To assess the appearance of autism in response to intrauterine exposure to preeclampsia, we employed an array of behavioural studies in the progeny from cadmium chloride-induced preeclampsic pregnancies and those of normal pregnancies, and in addition illuminated the serotonergic system of the rat pups to investigate the presence, or lack thereof, of any structural deviations from the norm in this crucial neuromodulatory system previously reported as altered in autistic brains.

Method: 10 Pregnant Sprague Dawley (SD) rats and 44 pups were used in this study. The experimental pregnant group received an intraperitoneal injection of 0.125mg/kg/day of cadmium chloride ($CdCl_2$) dissolved in 1ml sterile saline for every animal, while the control group received the same volume of sterile saline for a successive period of 6 days (gestational day 9-14). Systolic blood pressure and body weight of pregnant dams were measured in the morning of GD 0,9,13,16,19 and 20. BCA protein assay was conducted to assess protein level in urine. The resulting pups from the two groups were accessed for autistic behaviour such as repetitive, cognition, gross motor coordination, sensory and anxiety. We also investigated the serotonergic neurons in the rat pups using antibody for serotonin.

Results: The systolic blood pressure (SBP) of the treated group was significantly higher than the control group. There was a gradual increase in SBP from 132mmHg to 143mmHg in the treated group, whereas in the control group the SBP increased and later dropped toward gestational day (GD) 20 from 133.30mmHg to129.38mmHg. the proteinuria analysis revealed higher protein concentration in the cadmium exposed dams. The mean body weight of the treated group was 306.7g and that of the controls was 291.2g. We observed reduced exploratory, increased anxiety-like behaviour, and excessive repetitive behaviour evinced by greater digging in the preeclampsia exposed group as compared to the normotension-exposed group.

Conclusion: Preeclampsia induces autism-like behaviours in SD rat offspring, while the serotonergic neurons were clearly consistent with the shape, location and density of those in

typically developed brains, indicating that the cell bodies themselves are not altered by preeclampsia.

ACKNOWLEDGEMENTS

I would like to appreciate Dr. Busisiwe Constance Maseko for introducing me to this field of research in neuroanatomy, supervising me through my master's degree, contributing intellectually, guidance, morally and for always looking out for me.

I also would like to offer my special thanks to the new family I have adopted Professor and Dr(Mrs) Amadi Ihunwo for encouraging me to pursue a postgraduate study and also supporting me in their time and prayers. I am truly grateful.

To the staffs of Central Animal Services, God bless you richly for your unending supports to students and for the vast knowledge impacted to me during my time and study with the Animals. I wouldn't have done it without your team. Kudos.

To the team from school of Anatomy, staff; Prof P. Manger (allowing me access to the Neurolab), Mrs Hasiena Ali (the knowledge on laboratory procedures, time spent with me and words of encouragement, thank you ma), and all other members of staff, I appreciate you. To my fellow postgraduate students (Asmau, Bernice, Folusho, Arthur, Alice, Jacylyn, Thandi, Kemi) one love guys. To Dr. Pillani Nkomozepi (the HOD, School of Anatomy and Physiology, University of Johannesburg) thank you for trusting me in your laboratory and giving me the necessary support when it was most needed.

I would want to appreciate my Mum (Mrs Patience Ikechi Ndah-Igweh) your prayers are heard, and heaven also sends confirmation of them. To my families, siblings and friends, your supports and encouragements are all appreciated.

Lastly to my support system, best friend, my rock, bed mate, soulmate, my ATM machine and the husband of my youth Austin Azubuike Ajah, "*My love"*, my thanks will never be enough to appreciate all you do for us especially me. Your encouragement is second to none, your thought for me to go further in my academics is heavenly and heaven will bless you. My prayers are that Psalm 138:8 will find full expression in your life. Amen. I Love you my Husband Man.

TABLE OF CONTENTS

Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION AND AIM OF THE STUDY

1.1 Introduction

Autism in the past years was considered to be a rare childhood disorder, but in recent times has been more well-publicized and researched. It ranges widely from mild to severe cases and is therefore defined as a spectrum termed Autism Spectrum Disorder (ASD) (Lord *et al*., 2018). Autism Spectrum Disorder is a neurodevelopmental disorder characterised by deficits in social communication, social interaction, along with restricted, repetitive patterns of behaviour, interests or activities (Khan *et al*., 2012). Autism is a heterogeneous neurodevelopmental disorder with multiple causes with varied combinations and severity of symptoms (Bailey *et al.*, 1996; Amaral, 2011). ASD diagnostic mechanisms are based on behaviour, as it is assumed to result from early altered brain development and neural reorganisation (Bauman and Kemper, 2005; O'Reilly *et al*., 2017). However, in many cases, there are no reliable biomarkers to confirm or dispute these assumptions. Autistic individuals sometimes have comorbid neurological disorders such as epilepsy and intellectual disability (McCarthy *et al*., 2010). Although the basic causes of ASD are not fully understood, it is regarded as a multifactorial disorder. Findings from Gardener et al. (2009) support the view that exposure to perinatal risk factors such as maternal haemorrhage, umbilical-cord complications, foetal distress, gestational hypertension and preeclampsia increases the risk of ASD in offspring. It has been determined that the male to female ratio of ASD diagnosis is likely 3:1 (Loomes *et al*., 2017). The underlying mechanisms to this gender bias of autism are not well understood, but recent research involving human subjects suggests that genetic differences disproportionately predispose males to ASD, and that these phenotypes may be modulated by hormones such as testosterone (Werling and Geschwind, 2013). Different approaches such as neuroimaging, clinical assessment and neuropathology have been used to study the structural and morphological brain changes or abnormalities in ASD. One of the more consistent findings in ASD research is altered brain growth (Eric Courchesne, 2004).

Preeclampsia is a perinatal condition that affects pregnancies commonly in second half of gestation, and in very severe cases can lead to maternal and foetal morbidity or even mortality (Malik *et al*., 2019). The most prominent causal pattern for preeclampsia is of shallow placentation (Cha *et al*., 2012) characterised by hypo-perfusion which reduces concentrations of angiogenic growth factors and increases placental debris in the maternal circulation, culminating in a robust maternal immune response and damage to the maternal, placental and foetal circulatory systems (Redman, 2005). Classic features of preeclampsia

include progressive hypertension, oedema, and proteinuria. In severe variants, there is evidence of maternal brain, liver or kidney deterioration and/or placental insufficiency. This clinical syndrome is characterized by foetal growth restriction, reduced amniotic fluid, and suboptimal foetal oxygenation (Neerhof and Thaete, 2008). Although placental insufficiency may arise without maternal hypertension, failed placental vascular remodelling appears to be a unifying mechanism for both conditions (Triunfo *et al*., 2014). Women with preeclampsia are more likely to deliver early, either spontaneously or by elective intervention to prevent complications of maternal and/or foetal deterioration (Redman, 2005). Scholars have suggested that the effect of intrauterine exposure to preeclampsia is associated with a high risk of ASD (Mann *et al*., 2010) whereas others have found no significant association between the two disorders (Larsson *et al*., 2005). As a result, preeclampsia during pregnancy may disrupt certain cell groups in the developing brain.

Neuromodulatory systems are one such cell population of interest as they underlie crucial functions that ultimately enable normal brain processing and human behaviour. These are therefore widely studied around the globe in a bid to understand the functions and organization of these cell populations within the brain. Such studies have employed both human tissues and that of many other vertebrate species. Conditions such as gestational diabetes, high body mass index (BMI) and preeclampsia are known to alter the foetal brain's developmental environment and thereby increase the likelihood of neurodevelopmental disorders developing including autism(O'Brien *et al*., 2003; Kolevzon *et al*., 2007; Weissgerber and Mudd, 2015; Wang *et al*., 2016; Xu *et al*., 2017). Many brain systems have been found to show alteration after having developed in these suboptimal gestational environments, including cerebral grey matter areas, cerebellum, corpus callosum, basal ganglia and brainstem components (Courchesne *et al*., 2004). The present study examines some neurotransmitter components of the brainstem due to their significance in secreting a key neurotransmitter, namely the serotonergic system. The serotonergic system was previously reported to play a significant role in the development of autism (Hornung, 2003; Whitaker-Azmitia, 2005; Azmitia *et al*., 2011). We believe this system to be one of the keys to understanding autism and the more that is known about it, the closer we will get to understand some of the workings of autism; particularly because it projects extremely widely within the brain (Hornung, 2003; Williams *et al*., 2005). Structural deviations within the system itself are likely to result in many more downstream alterations to both cortical and subcortical circuitry which most likely will impact on the connectivity and therefore

functionality of those brain regions, which probably would lead to some of the more complex symptoms seen in autism. The previously reported alteration in cortical circuitry is very significant as it probably underlies many of the autism symptoms seen (Whitaker-Azmitia, 2005). Another motivating factor to investigate the serotonergic system further is its role in modulating not only its own pattern of development but that of other systems such as the GABAergic and glutamatergic systems as well, further emphasizing the importance of this system in normal brain functioning, and by extension neurodevelopmental disorders(Hornung, 2003; Whitaker-Azmitia, 2005; Williams *et al*., 2005). In a previous investigation of the serotonergic system in autistic brains, Whitaker-Azmitia (2005) reported that the neuronal morphology of serotonergic cell bodies was fundamentally different from neurotypical cell bodies and that the size of the cells was reduced, moreover, they reported that the nuclear density was reduced in autism. However, with human tissue being so scarce, this study and others like it, have been done on one or two brains, thereby limiting the conclusive nature of their findings. This work focuses on the serotonergic neuromodulatory system of preeclampsia exposed autistic rat models, with the aim of studying their neurodevelopmental reflexes/ behaviours, to ascertain autistic features, thereby establishing the advent of Autism Spectrum Disorder following exposure to cadmium chloride.

1.2 Aim

The aim of this work was to successfully induce preeclampsia in pregnant Sprague Dawley rats, study the behaviours of resulting rat pups to see if there is a correlation between preeclampsia and the advent of autism in rat pups; and also, whether the serotonergic system of developing brains is altered in any way during a preeclamptic pregnancy.

CHAPTER 2: PREECLAMPSIA

2.1 Introduction

Preeclampsia is a potentially dangerous pregnancy complication characterized by, among other features, high blood pressure. It is also a multisystem disorder unique to the latter half of pregnancy and could lead to severe maternal and foetal morbidity and even mortality. The condition is more common in first pregnancy or maternal age extremes (Ananth *et al*., 2013). The disorder is caused by pregnancy and cured by delivery, it has an overall incidence of 5-7% (Mammaro *et al*., 2009; Uzan *et al*., 2011). The risk factors appear to be changed considerably by underlying maternal, metabolic and cardiovascular health (Roberts and Hubel, 2009).

The maternal symptoms of preeclampsia result from suppression of the standard physiologic responses to pregnancy, and includes generalised vasoconstriction, increased response to vasoconstriction, increased Blood Pressure (BP), Increased capillary permeability, sudden severe oedema, increases in plasma volume, increased intravascular coagulation, reduction in organ perfusion, decreases in glomerular filtration rate (GFR), proteinuria, widespread vascular endothelial damage including glomerular endotheliosis and decreased intrauterine growth (IUGR) of the foetus (Uzan *et al*., 2011).

During placental development, the timeline shows a placental change over the course of pregnancy. A crucial change is when blood vessels in the lining of the uterus are remodelled, thereby increasing blood supply to the placenta, this process is called spiral artery remodelling. The remodelled artery functions to supply blood to the foetus during pregnancy, however, if there is no pregnancy, the spiral artery spans the tissue of the uterine wall, supplying blood to the lining. In a normal pregnancy, cytotrophoblast cells from placental villi invade the space around the spiral artery, replacing cells that normally line the vessel. This remodelling makes the vessel larger and optimizes the blood flow to the placenta to support its function. In an abnormal pregnancy, cytotrophoblasts invade the space in and around the spiral artery, but the artery is not completely remodelled and blood flow from the mother to the placenta is not optimized to support proper placental perfusion. (Brosens *et al*., 1972; De Wolf *et al*., 1980). In preeclamptic patients, the mechanism of cytotrophoblast invasion is incomplete (Meekins *et al*., 1994). Figure 1 is a depiction of this phenomenon.

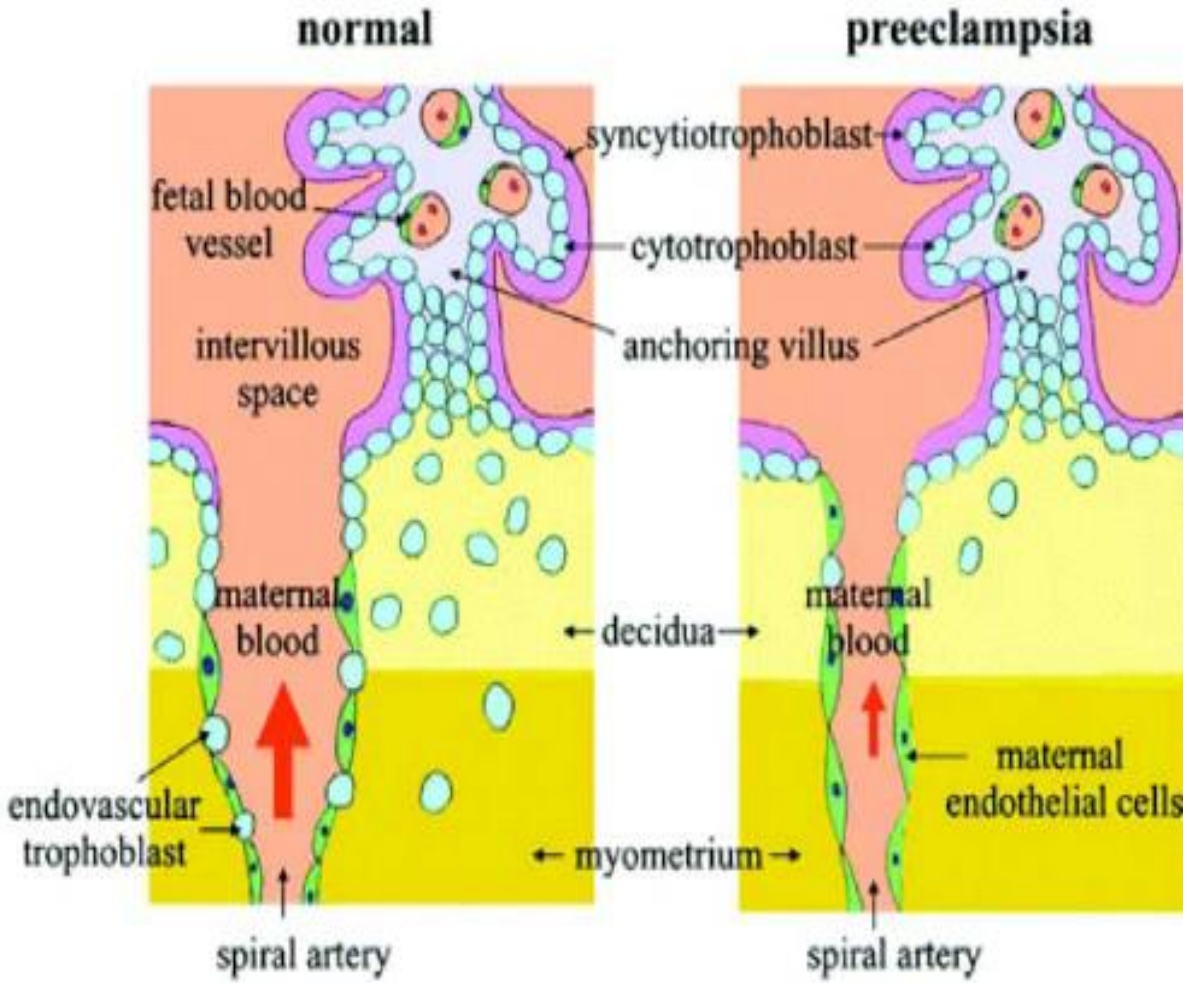


Figure 2. 1. Normal and abnormal placentation.

Decreased trophoblastic invasion seen in the case of Preeclampsia (Right), leading to reduced spiral artery diameters and therefore reduced perfusion ability in the preeclamptic pregnancy (McCrae KR; 2010)

Preeclampsia can be divided into mild and severe forms depending on the severity and type of symptoms presented. The mild form of preeclampsia is characterized by systolic blood pressure (SBP)≥140mmHg and diastolic blood pressure (DSP)≥90mmHg, and proteinuria>300mg/24hrs (Dhariwal and Lynde, 2017). The severe form of preeclampsia is characterised by severe hypertension (SBP)>160mmHg and (DSP)>110mmHg, and severe proteinuria (2g/24hrs) and/or symptoms of target organ damage as shown in Table 1 (Silva and Palmira 2014; Dhariwal and Lynde; 2016). Women with severe preeclampsia may present with headaches, visual disturbance, epigastric pain, nausea and vomiting, hepatic and renal insufficiency and pulmonary edema (AM J.Obstet Gynecol 2002).

2.1.1 Signs and Symptoms of Preeclampsia

Table 2. 1. Signs and symptoms of preeclampsia per organ.

(Silva and Palmira (2014), Dhariwal and Lynde (2016))

SYSTEMS	SIGNS/SYMPTOMS
Central Nervous System	Headaches, Visual disturbance Seizures
Renal system	Proteinuria, Oliguria, Hypertension Abnormal kidney tests
Vascular system	Severe hypertension
Cardiorespiratory system	Chest pain, Dyspnea, Pulmonary edema Low oxygen saturation
Hepatic system	Abnormal liver function, Nausea Epigastric pain
Hematologic system	Haemorrhage, Coagulation impairment Shock, Intravascular disseminated coagulation

A surprising finding was reported by Xiong *et al*. (2000) that smoking protects pregnant women from developing preeclampsia. They concluded that since smoking enhances the expression of the ligand vascular endothelial growth factor (VEGF) family which regulates the differentiation and survival of cytotrophoblasts, that this promotes normal uterine invasion (Zdravkovic *et al*., 2005). Nonetheless, it is still not recommended for pregnant women to smoke since smoking is a risk factor for several complications during pregnancy such as miscarriage, placental abruption, preterm delivery and reduced birth weight (Zdravkovic *et al*., 2005).

2.1.2. Risk Factors for Preeclampsia

The risk factors for preeclampsia can be classified into pregnancy-specific characteristics and pre-existing maternal features.

i. Pregnancy- Specific Features:

a. Parity

Nulliparity (female who has never given birth) is a strong risk factor, almost tripling the risk of preeclampsia (Duckitt and Harrington, 2005).

b. Placental factors

Excess placental volume as in hydatidiform moles, which is a non-cancerous tumour that develops in the uterus as a result of a non-viable pregnancy. There may or may not be an embryo or placental tissue present in this condition.

c. Multifetal gestation

Multifetal gestation is also associable with the development of preeclampsia in some cases(Sibai *et al*., 2000; Day *et al*., 2005).

ii. Pre-Existing Maternal Characteristics:

a. Age

Extremes of childbearing age such as above 35 years have been associated with preeclampsia (Wallis *et al*., 2008).

b. Race

Studies in the United States have reported that the association between the African-American race and preeclampsia has been confounded by a generally high prevalence of hypertension in this population; the hypertension is likely to be independent of pregnancy while other investigators report a higher risk of preeclampsia among African-American women.(Eskenazi *et al.*, 1991; Mittendorf *et al*., 1996; Knuist *et al*., 1998).

c. Pre-existing conditions

Many of the maternal risk factors for preeclampsia are similar to those of cardiovascular disease, e.g., hypertension, diabetics, obesity and vascular disorders are all associable with preeclampsia (Roberts and Hubel, 2009). Preeclampsia also occurs more often among pregnant women with autoimmune conditions such as systemic lupus erythematosus and antiphospholipid antibody syndrome (Duckitt and Harrington, 2005).

d. Family history

The risk of preeclampsia is almost tripled in a family with a past history of preeclampsia. (Duckitt and Harrington, 2005).

iii. Epidemiology of Preeclampsia

A review by the World Health Organization indicated that hypertensive disorders accounted for 16% of all maternal deaths in Africa, 9% in Asia while Latin America and the Caribbean were found as high as 26% (Khan *et al*., 2006). Data from the United States National Hospital Discharge survey from 1987 to 2004 reported that the rate of preeclampsia discovered during labor and delivery increased by 25% (Wallis *et al*., 2008). A study of a hospital managed by Health Care America Corporation shows that after obstetric

haemorrhage, preeclampsia was the second leading cause of pregnancy-related intensive care unit admissions (Porreco and Barkey, 2010).

iv. Causes of Preeclampsia

The exact cause of preeclampsia is not known since it involves several factors. Nevertheless, researchers are of the opinion that it begins in the placenta (the organ that nourishes the foetus throughout pregnancy) (Dekker and Sibai, 1998). In humans, sFlt-1 is important in the regulation of blood vessel formation in diverse tissues (Maynard *et al*., 2003). Early in pregnancy, this blood vessel develops and evolve to efficiently send blood to the placenta, but in women destined to develop preeclampsia, the blood vessels do not develop appropriately or function properly. They are narrower than normal blood vessels and respond differently to hormonal signalling, which limits further the amount of blood flow that can go through them (Egbor *et al*., 2006).

v. Causes of Abnormal Development of Preeclampsia

a. Damage to Blood Vessels

The plasma from women with preeclampsia impairs the ability of pre-constricted vessels to relax (Hayman *et al*., 2001). Further, the preeclamptic endothelial cell dysfunction due to an ischemic placenta is attributed to an alteration in the balance of circulating angiogenic and anti-angiogenic growth factors, which can cause hypertension and proteinuria. These observations led researchers to investigate the role of circulating factors like VEGF (Vascular Endothelial Growth Factor), lipid peroxidases and syncytiotrophoblast micro-fragments during pregnancy. The circulating levels of angiogenesis regulators like VEGF and PLGF (Placenta Growth Factor) are reduced during preeclampsia and may be responsible for many of the clinical symptoms of preeclampsia (Chau *et al*., 2017).

b. Immune System Problems

The interaction between decidual leukocytes and invading cytotrophoblast cells is essential for normal trophoblast invasion and development (Dekker and Sibai, 1998). Immune system Maladaptation may cause shallow invasion of spiral arteries by endovascular cytotrophoblast cells. The causes of impaired placentation in women who develop preeclampsia is not completely understood but may be in part due to faulty differentiation of extravillous

trophoblasts (EVT) with poor invasive properties, and in part due to changes in maternal decidual tissues, which regulate cytokines/ growth factors-mediated trophoblast behaviour (Malik *et al*., 2019).

c. Genetic Factors

A family history of hypertension and pre-existing hypertension before pregnancy increases the risk of preeclampsia (Trogstad *et al*., 2011). A single recessive gene or a dominant gene with incomplete penetrance could lead to the development of preeclampsia. The penetrance may be dependent on the foetal genotype (Dai *et al*., 2013).

d. Animal models of preeclampsia

There are many toxins previously used to study preeclampsia which did not a produce complete pre-eclamptic phenotype in vivo experimentally when administered to animals(Palmer *et al*., 2016) until the finding that over-expression of soluble fms-like tyrosine kinase-1 (sFLT-1) and cadmium chloride could produce a pre-eclamptic phenotype in pregnant rats (Maynard *et al*., 2003; Ronco *et al*., 2009). This breakthrough discovery initiated a decade of subsequent research, which has shown that sFLT-1 and cadmium chloride plays a major role in the pathogenesis of pre-eclampsia. The preeclampsia that develops from such models closely mimics the human condition with respect to the proteinurea, hypertension, oedema etc.(Ronco *et al*., 2009). The common clinical symptoms between human and preeclampsic Sprague Dawley rat includes hypertension. Proteinurea, renal dysfunction, decreased placenta perfusion and foetal growth restriction(Zhang *et al*., 2019). This is the motivation behind the choice of using Sprague Dawley rats in the current work, and the chosen mechanism of PE induction was Cadmium chloride as it is probably a major contributor to PE cases in the human population as well. This allowed us to simulate as closely as possible the human condition of PE.

2.1.3. Cadmium Chloride:

Cadmium is a transition metal (group II b) with eight stable isotopes that was discovered by the German chemist Strohmeier in 1817, because of the study of some of the impurities of zinc carbonate (Jacobo-Estrada *et al*., 2017). This metal is found in nature in many forms. The most common are elemental cadmium (Cd^0), and cadmium carbonate, chloride, oxide, sulphate and sulphide salts. Considering the natural sources, Cd is widely distributed in the

earth's crust. Normal rock wear and erosion results in the release of Cd, while volcanic activity on marine and terrestrial surfaces also contributes to its release (Jacobo-Estrada *et al*., 2017). Cadmium is a major environmental pollutant that causes multiple adverse medical effects in humans and animals (Kah *et al*., 2012; Thévenod and Lee, 2013). On a daily basis, the general populace is exposed to Cd through the contamination of food and drinking water and by the inhalation of cigarette smoke (Nawrot *et al*., 2010). The route of exposure impacts importantly on Cd absorption. For inhalation, which occurs mainly through tobacco smoke, Cd absorption is approximately 25% (range 5–50%); whereas the oral route, through contaminated water and food (offal and seafood), was estimated at 5% (range 1–10%) (Nawrot *et al*., 2010). Smoking one cigarette increases blood concentration per litre by approximately 0.1–0.2 μg of Cd, because each cigarette contains from 1 to 2 μg of Cd (Kosanovic *et al*., 2002). Nevertheless, Cd content can vary depending on the origin of the tobacco leaves: in studies on Mexican cigarettes, it was found that each cigarette contained from 2.5 to 2.8 μg of Cd (Saldivar. *et al*., 1991).

Cadmium can accumulate in various organs, mainly in the blood, liver, kidneys and reproductive system, due to its long biological life and low excretion rates. Nutritional deficiencies in protein and other essential minerals such as zinc, iron and calcium during pregnancy makes pregnant women more vulnerable to cadmium toxicity and thus leads to increased absorption and retention of cadmium (Nishijo *et al*., 2004). Given that preeclampsia is a multisystem disorder characterised by hypertension, proteinuria, oedema and often foetal growth restriction (Lyall *et al*., 2013; Minire *et al*., 2013); and given also that it is resolved after delivery, this suggests that the placenta is a major protagonist in the pathogenesis of preeclampsia (Raghupathy, 2013). Kippler *et al*. (2009) suggested that the placenta is the primary target for cadmium during pregnancy.

Exposure to cadmium during pregnancy increases the level of circulatory corticosterone (the main active Glucocorticoid (GC) in rodents) in mother and offspring, suggesting an involvement of the glucocorticoid system in some of the toxic effects induced by cadmium (Ronco *et al*., 2009). Effects of cadmium on cells could result in either a direct or indirect alteration in the brain development process. Cadmium interferes with cell cycle progression, proliferation and differentiation (Yang *et al*., 2004).

i. Sources of Cadmium

Considering anthropogenic sources, Cd is released from human-related activities. It is generally obtained as a by-product of zinc concentrates. The zinc:cadmium ratio in typical minerals ranges from 200:1 to 400:1. This metal may be produced in a secondary manner by the recycling of batteries, copper-cadmium alloys and powders from electric arc furnaces. It is estimated that the production of secondary Cd accounts for approximately 20% of the total production of metallic Cd. It is used in the manufacture of various products in common use, such as the nickel-cadmium batteries, the article that consumes most of the world's production of this element (90%). A percentage of Cd is also used in the manufacturing of pigments, coatings, stabilizers for plastics, non-ferrous alloys, electroplating, photovoltaic devices, etc. These activities related to the metal's extraction and manufacture, use and disposal of its products, as well as agriculture, have the potential to release Cd into the environment and to be sources of exposure for humans and other animals (Faroon *et al*., 2012; Thévenod and Lee, 2013). The human exposure to Cd is not without consequences as stated earlier, pregnant females particularly pose a great challenge as the metal cadmium influence on the placenta glucocorticoid synthesis and the immune system can cause the development of preeclampsia and possible decreased foetal weight.

2.1.4. Permits and Ethical issues

Permits and ethical clearance for the study were obtained from the University of the Witwatersrand Animal Research Ethics Committee (AREC) with the number 2018/09/041/B. The ethics certificate is included in appendix A.

2.2 MATERIALS AND METHODS

2.2.1. Husbandry

Ten 8- to 10-week-old (150 -200g) female Sprague-Dawley nulliparous females were used and mated overnight with a male; this was done by leaving each pair in a metabolic cage overnight. To confirm conception had occurred, vaginal plugs were located the next morning. The presence of the plug indicated that mating had occurred, which often leads to pregnancy and is considered as day 1 of pregnancy (GD 1).

The rats were obtained and housed at the Central Animal Services, University of the Witwatersrand. Once pregnant, each rat was caged individually and allowed *ad libitum* access to food and drink. Throughout the study, the cages were cleaned twice a week. The room temperature was maintained at a range between 24±2°C, and a 12-hour dark and light cycle (7:00 hours to 19:00 hours) was adhered to throughout the study.

2.2.2 Induction of Preeclampsia

To induce preeclampsia, we employed cadmium chloride. The pregnant dams were divided randomly into two groups of five animals each. Group 1 dams were the control group, while group 2 were the experimental group.

The experimental group received an intraperitoneal injection of 0.125mg/kg/day of cadmium chloride ($CdCl_2$) dissolved in 1ml sterile saline for a successive period of 6 days, starting on gestational day 9 and ending on 14 (GD 9-14), the protocol was derived from (Zhang *et al.*, 2019). The systolic blood pressure and the body weight of the pregnant rats were measured on the morning of Gestational Day 9, 13, 16,19 and on GD 20 between 0800 to 0900hrs. The control group also received 1ml of sterile saline (9gNaCl in 1000 distilled water) per intraperitoneal injection. The cadmium used herein was a reagent grade of 99.99% (D-91625, Sigma-Aldrich). All the parameters were noted in both groups. For all dams, around GD 21-GD23, the birth occurred. The pups were then allowed to suckle for 21 days from their mothers to grow and gain strength.

i. Measurement of Systolic Blood Pressure

Systolic blood pressures (SBP) were measured in conscious, restrained pregnant rats on Gestational Days 9,13,16,19, and 20. This was done in the mornings using a tail cuff monitor apparatus (NIBP250, BIOPAC Systems Inc., USA) (Fig 2). The NIBP250 incorporates a built-in pump that automatically inflates the blood pressure cuff to occlude the vessel in the tail (ventral caudal artery where blood pressure is monitored) of the rat. Once the pump reaches the inflation point it slowly deflates the cuff, providing a linear drop in pressure.

A touchscreen LCD monitors the pressure and pulse signals. The NIBP250 displays the marked values for the systolic and the diastolic blood pressures, as well as the calculated values for the mean blood pressure and the heart rate.

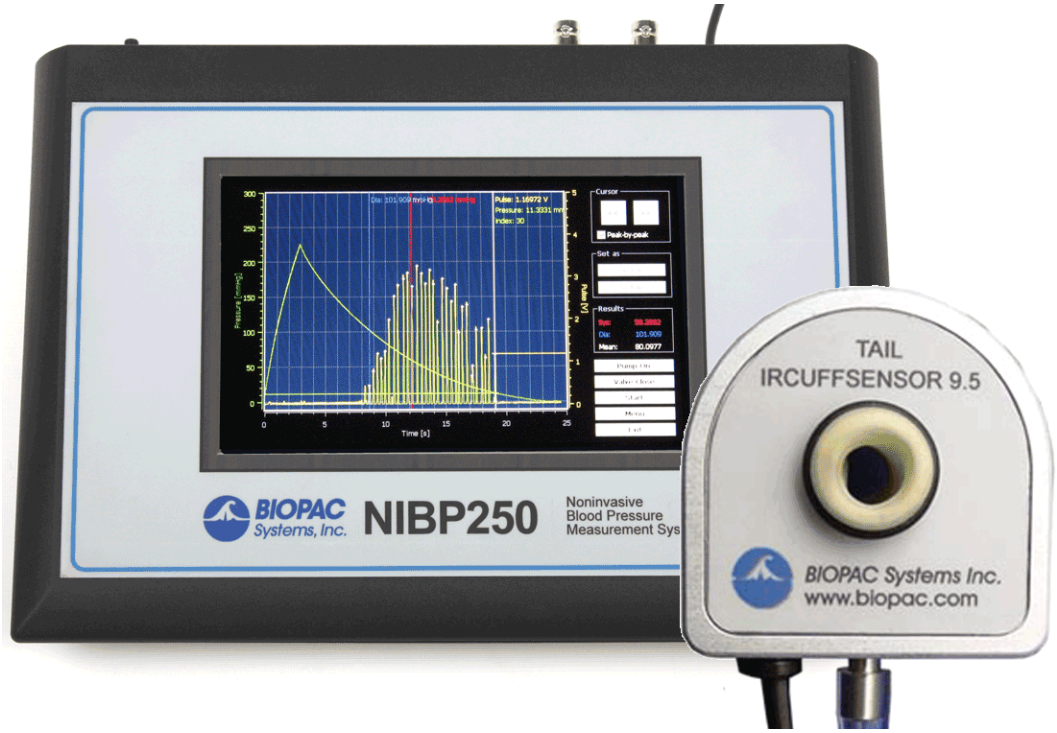


Figure 2. 2**.** NIBP250, A non-invasive Blood Pressure Measurement System that utilizes a tail cuff.

ii. Determination of urinary albumin excretion

On days 3 and 19 of pregnancy, the rats were placed in metabolic cages for a 24-hour urine collection. Rats were allowed free access to food and water. Urine protein concentrations were determined with a Bicinchoninic Acid (BCA) protein assay kit using bovine serum albumin as a standard. The detailed protocol is included in Appendix B.

2.3 RESULTS

Cadmium is a heavy metal known to induce preeclampsia (Zhang *et al*., 2019). Its primary target during pregnancy is the placenta (Kippler *et al*., 2009). We induced our subject pregnant Sprague Dawley rats with a low dosage of cadmium chloride for 6 consecutive days from gestational day 9-14. We then measured various indicators to confirm preeclampsia.

2.3.1 Measured Parameters

For us to ascertain the presence of preeclampsia, we measured the systolic blood pressure, the body weight of the pregnant rats and proteinuria (the amount of protein in the urine).

i. Blood pressure

There was a spike in the systolic blood pressure of the cadmium-injected experimental group, as seen in the below graph (Fig 2.3).

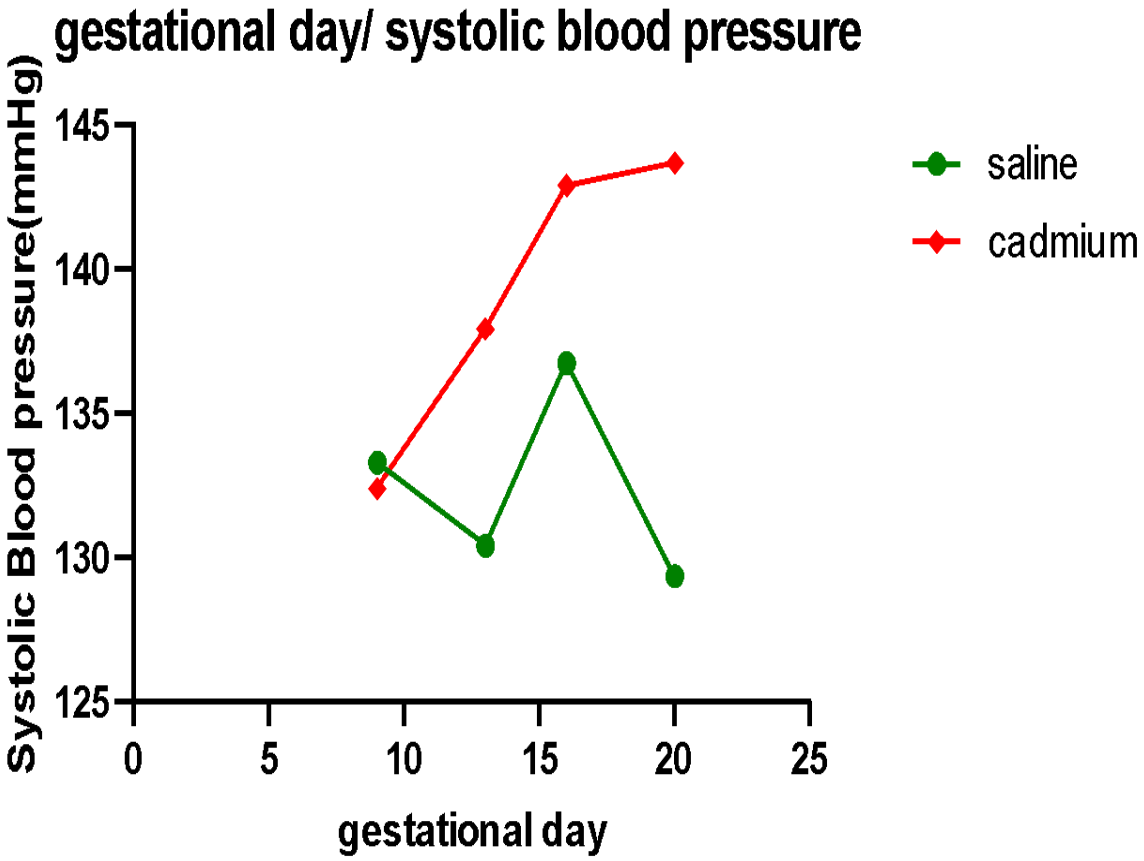


Figure 2. 3. Pregnant Sprague Dawley rats developed preeclampsia-like symptoms in response to cadmium chloride (CdCl2) injections.

Systolic blood pressure of the cadmium injected group spiked. Systolic level of Saline group (M = 132, SD = 3.01, n=5) and Cadmium exposed group (M = 139.3, SD = 4.52, n=5). The difference was significant, t (8) = 2.31, p = 0.02 (2 tail)

The observed systolic blood pressure steadily increased through the pregnancies and did so significantly when compared to the control group (P=0.02). In line with the research of (Zhang *et al*., 2019).

ii. Body weight

We also measured the maternal body weight and found an increase in the weight of the pregnant dams belonging to both groups. However, the difference between the increases in body weights were found to be statistically non-significant (p= 0.36; Fig 2.4).

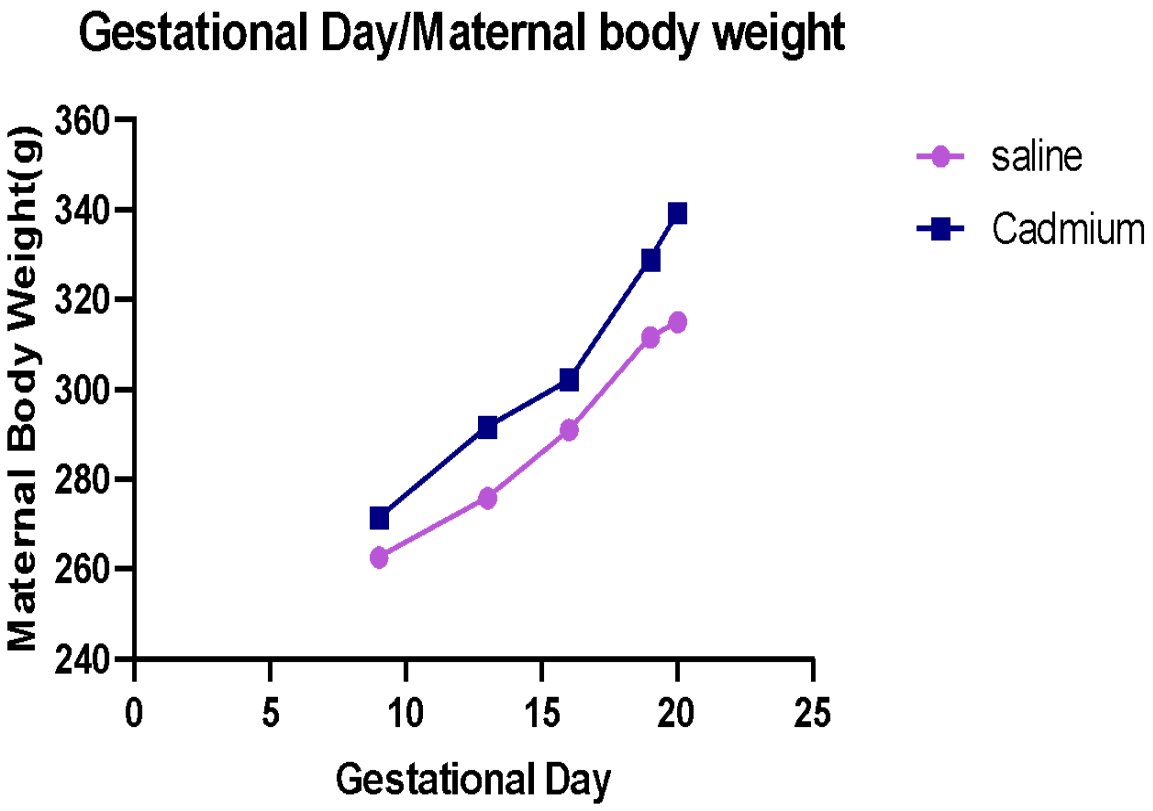


Figure 2. 4**.** Pregnant Sprague Dawley rats' body weight showed increases in both the cadmium chloride ($CdCl_2$).

(M = 306., SD = 27.6, n=5) and Saline group (M = 291.2, SD = 22.5, n=5). The difference was not significant, t (8) = 2.31, p = 0.36(2 tail).

iii. Proteinuria

A BCA protein assay was conducted to assess the level of protein in the urine of all dams. it was found that the cadmium injected rats had higher protein concentration in them on day 19 of pregnancy as compared to their saline injected counterpart. However, our statistical analysis showed no significant difference between the two. The protein concentration for the cadmium injected rats was 2589±60.10 and the saline rats was 1884±701.5 with a p - value = 0.4224

2.3.2 General Observational Findings

During the course of the research, some general observations were noted with respect to the adult pregnant Sprague Dawley rats, and later their progeny.

The Animals administered with cadmium were usually very quiet after administration of drugs as compared to the saline group, which were more excitable.

One of the cadmium dams had a miscarriage 3 days after completion of treatment(GD17) with cadmium chloride. We further observed that both the cadmium and saline dams consumed some of their pups, particularly in the second week (postnatal day 10). Offspring death also occurred in both groups, especially where litters exceeded 12 pups. In some of the saline litters, siblings showed visibly unequal weights and sizes (Fig 2.5). Given the significant rise in SBP of the cadmium exposed group, along with the detected proteinuria, the study's aim to induce preeclampsia was met successfully in the current study.

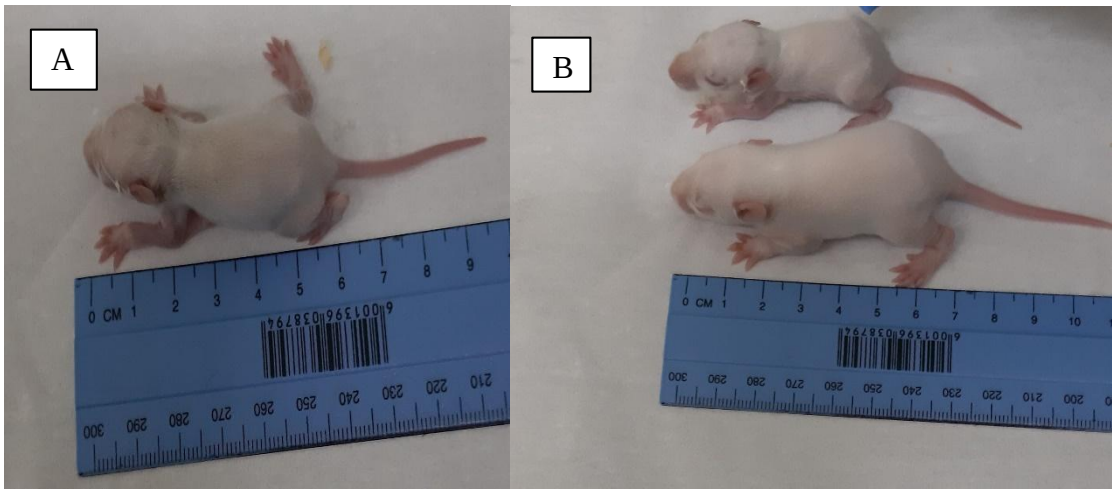


Figure 2. 5. Differing body sizes from sibling pups of a saline treated dam.

A indicates the length of the smaller pup, **B** indicates a larger and smaller sibling and their differences in size.

i. Litter size and body weights

Maternal exposure to Cadmium Chloride did not affect the litter size as all dams exposed to $CdCl_2$ had the normally expected (in Sprague Dawley rats) litter sizes of more than 10 pups (Hudson *et al*., 2019) per litter. The litter sizes for Cadmium exposed pups was 9.4±2.42 and the saline exposed group was 7.2±3.09 (n=10) (n=number of dams). No statistical significance was detected among the two group's litter sizes ($p = 0.59$).

According to Hudson *et al*. (2019), exposure of pregnant rats to Cadmium Chloride could lead to low birth weight in pups and increased heart size. Our findings were consistent with this pattern, in that the size of the cadmium exposed rat pups at birth were notably smaller than the control group's pups, however they did increase in weight and size as they grew. The average body weight of the cadmium exposed progeny was 92.71g±25.99 while the saline group had 91.3g±26.5 ($p = 0.97$).

2.4 DISCUSSION

Preeclampsia is a multisystem disorder characterized by hypertension, proteinuria, edema and often fetal growth restriction(FGR) (Minire *et al*., 2013). The symptoms resolve after delivery which suggests that the placenta is the principal contributor to the pathogenesis of preeclampsia (Raghupathy, 2013). Cadmium chloride has been shown previously to produce preeclampsia-like characteristics in Sprague Dawley rats (Zhang *et al*., 2019). The placenta is also a primary target for Cd during pregnancy (Kippler *et al*., 2009). Studies also by Gökalp *et al*. (2009) have shown further that intraperitoneal injection of cadmium produces hypertensive symptoms in rats.

For our purposes, a low dosage of cadmium chloride (0.125mg/kg) was given to the pregnant Sprague Dawley rats intraperitoneally for a period of six consecutive days (GD 9-14). We observed that there was a spike in systolic blood pressure of cadmium exposed pregnant rats, and detectable proteinuria levels that where higher than those of the control group, albeit we could not establish significant. It is probable that with greater sample sizes this significance will be established. We also observed that there was an increase in body weight of the pregnant dams, which is in line with earlier research (Wang *et al*., 2016; Zhang *et al*., 2019). The exposure of the pregnant rat to a low dose of cadmium chloride also led to miscarriage of

one of the dams, an observation also previously reported by Wang *et al.* (2016). The size of litters was not affected by the exposure of cadmium chloride; however, we did observe litters with low birth weight, which improved within days(Wang *et al*., 2016; Hudson *et al.*, 2019). Our findings demonstrated all these features of preeclampsia and are therefore consistent with previous findings. The body weights of dams in both groups increased as would be expected in pregnancy, with the cadmium exposed rats appearing to have greater weights. However, there was no statistically significant difference between the groups on their weight gains. Consistent with the work of Hudson *et al*. (2019) and Wang *et al*. (2016), we found pups within some litters that had low birth weights and sizes when compared to their own siblings. We suspect that the differences in body weight between siblings could be as a result of malnutrition and inability to feed optimally as a result of the number of pups in the same litter, this argument is consistent with that previously made by Chahoud and Paumgartten (2009). According to Agnish and Keller (1997), limited milk yield and impaired maternal behaviour have been identified as underlying causes of slower growth and development of pups reared in larger litters.

CHAPTER 3: AUTISM SPECTRUM DISORDER

3.1. INTRODUCTION

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders that are associated with restricted, repetitive patterns of behaviour, verbal & non-verbal communication, and social interaction (Bailey *et al*., 1996). Pathophysiologic mechanisms conclude that the physiological changes seen in the brain of children with ASD are likely to originate during foetal development (Stoner *et al*., 2014). Studies by Buchmayer *et al*. (2009) concluded that preterm birth, small-for-gestational age, low birth weight, antenatal haemorrhage, low Apgar scores, and medication use during pregnancy are some factors associated with ASD. Maternal pre-pregnancy obesity, diabetes mellitus and chronic hypertension are associated with developmental delay and specific impairment in motor skills, receptive and expressive language, adaptive communication and socialization (Krakowiak *et al*., 2012). ASD is regarded as a multifactor disorder with no single aetiological agent but rather with a range of genetic and environmental contributors (Brašić and Holland, 2006; Brašić and Holland, 2007); Engel and Daniels, 2011). Different approaches (such as neuroimaging, clinical assessment and neuropathology) have been used to study the structural and morphological brain changes or abnormalities in ASD. One of the consistent findings is altered brain growth (Courchesne, 2004). Two stages of brain growth abnormalities precede the clinical appearance of autism; a reduced head size at birth and then a sudden and excessive increase between 1-2month and 6-14months of age (Courchesne *et al*., 2004). Further neuroimaging reports have shown that an abnormal pattern of brain growth also occurs at the frontal lobe, cerebellum and limbic structure between 2-4years of age(Courchesne *et al*., 2004; Schumann *et al*., 2004; Courchesne and Pierce, 2005). These brain regions are involved in the development of social, communication and motor abilities that are often impaired in ASD.

The human brain is the command centre for the human nervous system. It receives signals from the body's sensory organs and outputs information to the muscles, autonomic nuclei and the endocrine system. The human brain has the same basic structure as other mammalian brains but is larger in relation to body size than any mammal.

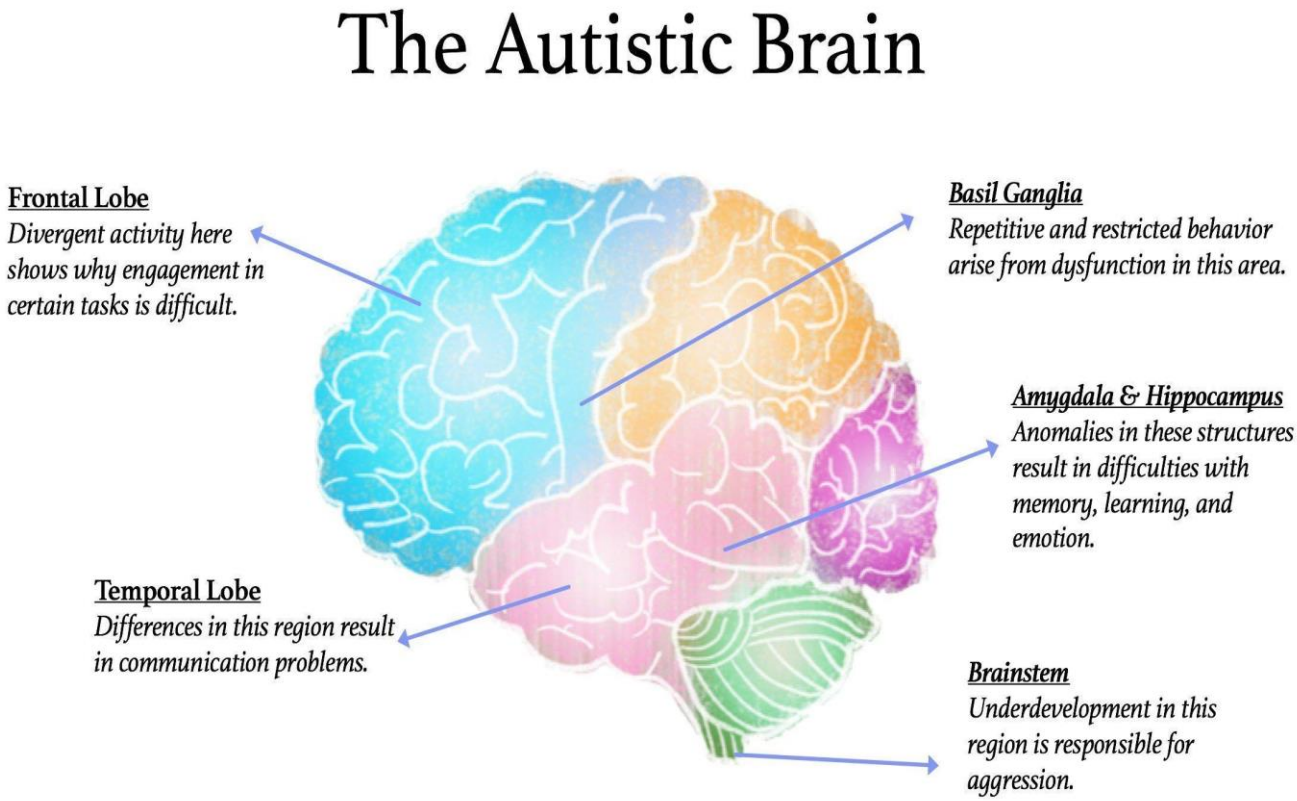


Figure 3. 1**.** The autistic brain briefly explained @ Q-Science.

Independent cortical areas in the temporal, frontal and occipital lobes are affected in Autism. Many major structures of the brain including the limbic system, cerebellum, corpus callosum, basal ganglia and brainstem are also affected. Studies have also shown structural abnormalities and inflammation in the brain associated with Autism. Gene expression patterns in the Autistic brain are also significantly different from typical brains.

3.1.1. Aetiology of Autism Spectrum Disorder:

There is no single cause of autism, it is generally accepted to be caused by abnormalities in the brain structure or functions. Examples include genetic factors, vitamin D deficiency, post-encephalic infections and auto-immune factors.

i. Genetics

Genetic factors play a major role in the development of autism, a finding that has become more and more commonly reported. From studies of identical twins with one child was autistic, the other was determined to have an 82% chance of also being autistic. That percentage was only 10% for fraternal twins. Genetic researchers now believe that as much as 90% of the behavioural phenotype of autism is related to inherited genes (Tick *et al*., 2016). Muhle *et al*. (2004) reviewed various genes such as Forkhead box P2 (FoxP2) which is involved with developmental language and speech deficits, while Reelin (RELN) is believed to be involved in memory formation, neurotransmission and synaptic plasticity in autism. The results showed that data from whole-genome screening suggest interactions of at least 10 genes in the aetiology of autism.

ii. Nutrition

Vitamin D etiological hypothesis: studies have linked the deficiency of Vitamin D during gestation, as a cause of autism. The deficiency of Vitamin D dysregulates the dozens of proteins involved in brain development which leads to increased brain size and enlarged ventricles, an abnormality previously reported in autistic children (Grant and Soles, 2009). Another example of nutritional risks that may lead to autism is oxygen supply depletion to the foetus, which may impair neurodevelopment and thus contribute to greater risk of autism spectrum disorder (Burstyn *et al*., 2011). A deficit in nutrients and oxygen can also lead to oxidative stress which encourages the release of protein into the maternal bloodstream in an effort to improve circulation (Walker *et al*., 2015). Because of the limited antioxidant capacity of the brain, the developing brain becomes vulnerable to oxidative stress (Chauhan and Chauhan, 2006). Oxidative stress resulting in the brain may lead to increased risk of ASD (Chauhan and Chauhan, 2006). Additionally, metabolic dysfunction (Krakowiak *et al*., 2012) and micronutrients (Lyall *et al*., 2014) are other potential biological mechanisms that may explain the increased risk of autism spectrum disorder. It is also important to note that seizures (Wu *et al*., 2008) are another known risk factor for Autism (Berg *et al*., 2011).

iii. Post-encephalic infections

A study carried out by Mankoski *et al*. (2006) states that cerebral malaria and infectious diseases are a common occurrence in African children under the age of 5 years; as a result, the population of African children with symptoms of ASD is higher.

iv. Other factors

Another association between preeclampsia and autism spectrum disorder may be the result of confounding factors such as parity, gestational diabetes, infections during pregnancy and pregnancy obesity. It is an established factor that gestational diabetes and body mass index (BMI) are associated with both the risk of preeclampsia (O'Brien *et al*., 2003; Weissgerber and Mudd, 2015) and neurodevelopmental disorders in children(Xu *et al*., 2014; Wang *et al*., 2016). This indicates that intrauterine exposure to preeclampsia increases the risk of Autism spectrum disorder.

Epidemiology of Autism is the study of the incidence and distribution of ASD. ASD continues to be over 4 times more common among boys (1 in 37) than among girls (1 in 151). In Africa, not much research has been done on autism, likely as a result of the belief that it is an illness of the more developed western world (Franz *et al*., 2017). To answer the question as to whether autism spectrum disorder occurs in Africa, children from Ghana, Kenya, Nigeria, Zambia, Zimbabwe and South Africa were screened for intellectual disability. 9 out of the 1312 screened met the eligibility criteria to be classified as having autism (Lotter, 1978). Another study was carried out in the Arab countries by Seif Eldin *et al*. (2008) on the prevalence of autism spectrum disorder among children with developmental disorders, the study however included Egypt and Tunisia. The prevalence of ASDs among children with developmental disorders in Egypt and Tunisia were 33.6% and 11.5% respectively. These findings highlight the state if under diagnosis of autism, as autistic individuals are often ruled as being "mentally impaired" or having "developmental delay or disorder" without the effort to specifically diagnose autism.

A 2012 review of autism prevalence rate around the world (excluding Sub-Saharan Africa), a median prevalence of 17/10000, approximately 1 in 588 was recorded. (Elsabbagh *et al*., 2012). The Centre for Disease Control (CDC) figures on ASD published in 2015 (which are believed to be more accurate) say that 1 in 45 children in the USA have ASD; the findings

were based on a parent survey which was designed to track the prevalence of developmental disorders in children aged 3 to 17 years (Zablotsky *et al*., 2015).

In Canada, the National Epidemiological Database for the Study of Autism in Canada (NEDSAC) ranks ASD as one of the most common developmental disabilities, affecting 1 in 94 children (Ouellette-Kuntz *et al*., 2012); while a pilot study that was conducted in Brazil reported a prevalence rate of 27/10,000 (Paul *et al*., 2019). The prevalence of ASD in regular school children in Quito, Ecuador was found to be 11/10,000 persons (Dekkers *et al*., 2015). The Leon survey in Mexico reported an overall prevalence of 87/10, 000 people (Fombonne *et al*., 2016).

Table 3. 1. Summary of Autism Spectrum Disorder prevalence by country

Country	Age range	Number of children	ASD prevalence	Author	Year of publication
Africa(Ghana, Nigeria, South Africa, Zambia, Kenya, Zimbabwe)	Under 18	1312	9	Lotter	1978
Canada			1/94	Ouellette-Kuntz *et al*.	2012
USA	3-17	43283	2.4	Zablotsky *et al*.	2015
Mexico	8	10000	87	Fombonne *et al*.	2016

Table 2 above is evidence that little has been done in this regard on the African continent. The knowledge and awareness about Autism Spectrum Disorder in Africa is still poor, thereby compromising opportunities for early recognition of the disorder and the introduction of appropriate interventions to alleviate severity. As a result of this, the need to engage in education of health care workers and the general populace in Africa on ASD cannot be ignored, because it would enhance early recognition and intervention which will improve prognosis in individuals with ASD, especially at early stages of life.

3.1.2. Diagnosis of Autism Spectrum Disorders

Diagnosis of ASD is on adherence to criteria described within the current diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (figure 3.2). Manifestation of deficits in social communication and restrictive, repetitive patterns of behaviour must be visible in early childhood before eight years of age (American Psychiatric Association, 2013; Vahia, 2013). However, variability of presentation is observed within directly associated core symptoms and those beyond explicit mention within the DSM-5, such as the commonly reported deficit in motor function (Lai *et al*., 2014).

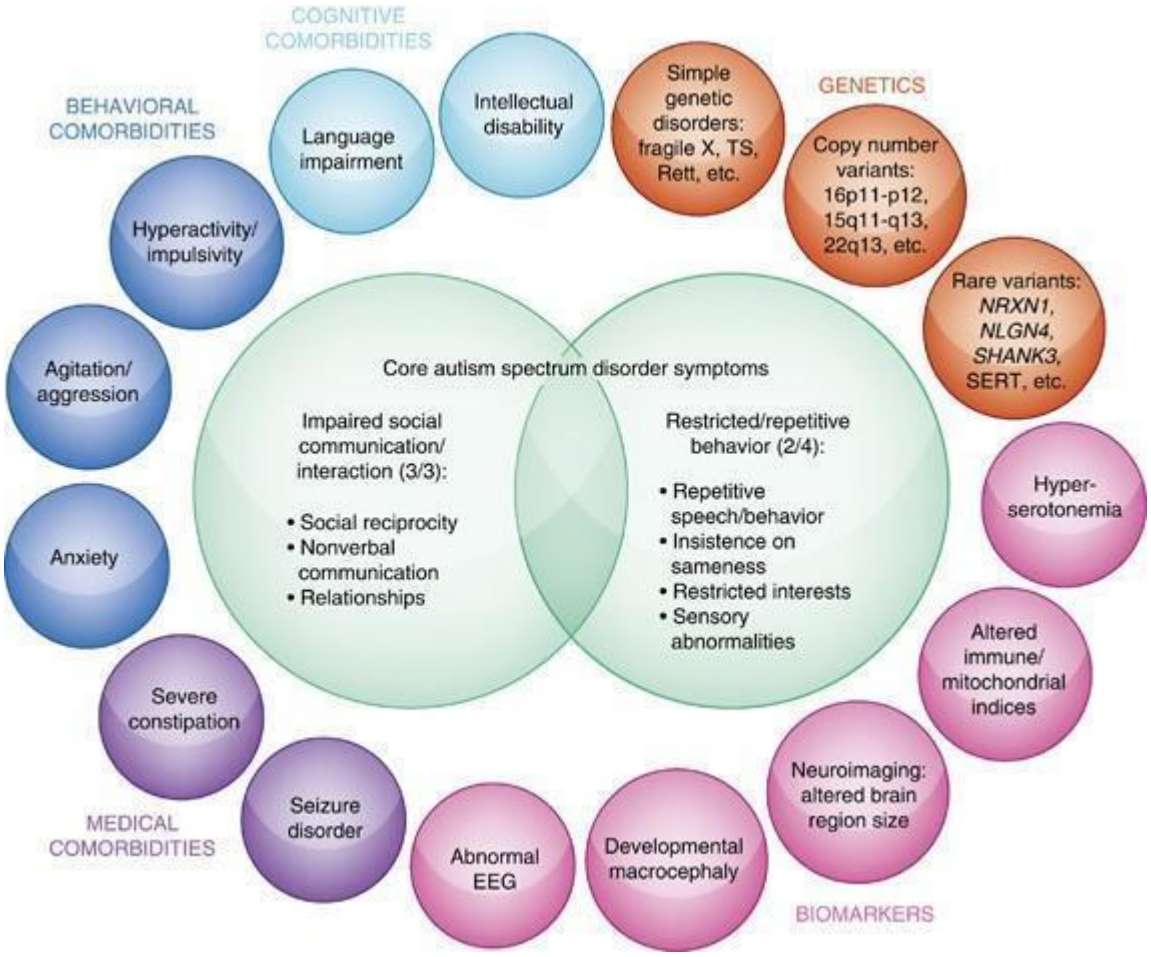


Figure 3. 2**.** Diagnostic criteria of Autism Spectrum Disorder with Associated Comorbidities.

Diagrammatic illustration of core diagnostic criteria of ASD as per the current DSM-5 (American Psychiatric Association, 2013)

i. Cognitive ability in Autism Spectrum Disorder

Cognitive ability is a set of brain-based skills that we need to carry out tasks ranging from the simplest to the most complex. They have to do mainly with the mechanism of how we learn, remember, solve problems and pay attention (Bunge *et al*., 2002).

Cognitive abilities as identified are as follows:

a. Visual-Spatial Processing: is the ability to understand where images and objects are in space. Examples include completing puzzles or reading a map.

b. Verbal Comprehension is the ability to draw on one's general knowledge and experience, and to reason with verbal information. Examples include recalling facts, vocabulary, communicating & reasoning.

c. Executive functions are terms that refer to mental skills that are necessary for regulating behaviour and emotions and goal achievement. Executive functions help in starting and completing projects/tasks, solve problems during conflict, organize our belongings, follow directions, and adapt to new situations.

d. Fluid Reasoning is the ability to solve new problems. An example includes completing a task for the first time.

e. Working Memory is the ability to remember information while processing it. Examples include remembering a phone number or multiple instructions (Luciana et al., 2005).

f. Processing Speed is the ability to process information quickly. An example includes the time it takes an individual to complete a test.

Cognitive ability in autistic individuals varies because they have specific cognitive strengths and weaknesses and these abilities are not static but change, and in most cases improve over time (Pellicano, 2010).

Developmental delay (DD) is a diagnosis applied to young children who have low cognitive function in addition to significant limitations in at least 2 other developmental domains (Shevell, 2008). Etiologic paradigms for DD are as diverse as the component conditions, and although genetic and congenital causes are implicated in up to 50% of affected children, environmental exposures (including antenatal toxin exposure, central nervous system

infections, hypoxic ischemic encephalopathy, cerebral dysgenesis, and early severe psychosocial deprivation) likely enhance risk during critical foetal and postnatal periods. (Srour and Shevell, 2014). Maternal prepregnancy obesity, diabetes mellitus and chronic hypertension during pregnancy have been associated with DD and specific impairments in visual reception, motor skills, receptive and expressive language, adaptive communication and socialization (Krakowiak *et al*., 2012). Prematurity and foetal growth restriction, both commonly associated with severe preeclampsia, are significantly and independently related to DD severity (Thomaidis *et al*., 2014).

ii. Intellectual Development in Autism Spectrum Disorder

Intellectual disability (ID) is a condition characterized by below average intellectual functioning (IQ<70) in conjunction with significant limitations in adaptive functioning. Intellectual disability may occur as an isolated phenomenon or accompanied with malformations, neurological signs, impairment of the special senses, seizures and behavioural disturbances (Schalock *et al*., 2010). According to Lense *et al*. (2011); approximately two-thirds of individuals with ASD have co-occurring intellectual disability (ID). However, according to Vivanti *et al*. (2013), ASD symptoms rather puts a child at risk of developing ID. And the risk increases with severity of symptoms, meaning that infants with ASD that do not have access to the appropriate input that supports the efficient organization and specialization of the brain, might ultimately develop an ID. A deduction from their model is that the more severe the ASD symptoms, the more the child would be "at risk" for developing an ID. Therefore, according to this view, ID is not a comorbid condition (i.e., an unrelated clinical entity), but rather a developmental outcome of the virtual "social deprivation" caused by the ASD symptoms.

iii. Social Ability in Autism Spectrum Disorder

The study of the evolution of ASD in children has informed our knowledge of early social behavioural development. Osterling and Dawson (1994) studied the birthday tapes of one-year-old children, the findings showed that children eventually diagnosed with ASD demonstrated significantly fewer pointing and showing behaviours and less looking at people and orienting to their name as compared with Typically Developing(TD) peers. Also, Osterling *et al*. (2002) found that children diagnosed with ASD or intellectual disability made

use of fewer gestures and showed more repetitive motor movements than typically developing peers. Other studies have used retrospective questionnaires to investigate specific deficits in children that develop ASD as compared to typically developing peers or those who proceed to have developmental delays but not ASD (Vostanis *et al*., 1994; Mitchell *et al*., 2011).

One of the most consistent social deficits in children with ASD is lack of non-verbal social gestures such as pointing, showing, and giving. At 8 months of age, pointing starts to develop and should make up the majority of gestures by 12 months of age (Rohlfing *et al*., 2017). Two types of pointing develop during childhood the Protoimperative and the Protodeclarative. Protoimperative pointing is a gesture that indicates what a child wants, is usually absent in young children with ASD although it sometimes develops in older children with ASD. Protodeclarative pointing is a joint attention gesture that is used to share experiences. Other important protodeclarative gestures that develop in early childhood include 'showing' and 'giving.' In 'showing' gestures, a child brings an object of interest to someone and extends their arms out holding the object toward the person's face to share their interest while in 'giving' gestures, a child places an object of interest in someone's hand to share the object of interest with the person. These protodeclarative gestures are characteristically absent in a child with ASD.

Quantitative measures of early behaviour have concentrated on visual attention. Reports from Klin and Jones (2008), say that children who are later diagnosed with ASD demonstrated different patterns of visual attention to point-light animation that simulated human movements at 15 months of age as compared to typically developing children. Eye movement studies demonstrated that children that developed ASD looked at the mouth rather than the eyes at 15 months of age when looking at faces (Sasson and Touchstone, 2014). The social skills deficits in individuals with high-functioning ASD (HFASD) have been attributed to deficits in several cognitive components, including the theory of mind and pragmatic competence (Berenguer *et al*., 2018), cognitive processing speed(Reinvall *et al*., 2017; Haigh *et al*., 2018) and metacognitive processes such as initiation and planning (Miranda *et al*., 2017). Deficits identified in autism provide some insight into the subtleties of the cognitive deficits in social function (Faridi *et al*., 2017). For example, although language is not seemingly abnormal in Autism, particular components of language competence may be deficient. Individuals with autism have adequate vocabulary and grammar skill but poor

inference and comprehension of narrative. Although those with Autism are thought to have a poor sense of humour, they actually have a deficit in gelotophilia (i.e., making others laugh at them) but show intact katagelasticism (i.e., laughing at others). Despite research defining the basic cognitive deficits that underlie ASD, it is important to remember that the key neurophysiological deficit is probably at a cellular level, suggesting a systems-level dysfunction of the nervous system rather than a specific neural pathway or region of the brain that is affected. Thus, the systems-level dysfunction in neural systems may express itself in a slightly different manner depending on other intrinsic and extrinsic factors, leading to the heterogeneity that we find in the ASD population.

iv. Motor Development and Delays in Autism Spectrum Disorders

Impairment in motor functions are most often the initial signs of abnormal development in people with Autism; and early identification of motor delays could be an indication of someone with high-risk genetic disorders (Bishop *et al*., 2017). Motor system development is important for an individual to be able to interact with the environment. The impairment in this system could be the first sign of atypical development (Fournier *et al*., 2010).

The development of motor function is linked to a child's capacity for language, cognitive and social development; so early emergence of motor skills and control has an impact on social and communicative development such as a child's ability to gesture and interact with other children in play (Iverson, 2010).

Motor control is a process of cognitively activating various brain regions and coordinating muscle movement to complete a particular task (Schmidt *et al*., 2018). It can be subdivided into gross motor control and fine motor control. Gross motor control is the modulation of large movement involving the upper and lower limbs in which compromised function could alter postural stability, gait and overall coordination. Deficits in gross motor control will result in reduced motor coordination, altered gait, postural instability and abnormal arm movement which are commonly observed in people with ASD (Bhat *et al*., 2011; Bedford *et al.*, 2016).

Due to the prevalent nature of gross motor coordination difficulty and its reliable detection as early as 1-2 years of age, it has been proposed to be an early indicator of ASD (Bhat *et al*., 2011; Leonard *et al*., 2014). Deficits in gross motor control, manifested as postural instability, may result in delayed onset of crawling, exacerbating avoidance of social

interactions through decreased opportunity (Iverson, 2010; Leonard *et al*., 2014). Additionally, postural instability severity has been correlated with severity of stereotyped, repetitive behaviours. Those with more severe stereotyped, repetitive behaviours display less postural symmetry and more postural wavering when performing balance related tasks, like standing on one foot with one's eyes closed (Travers *et al*., 2018).

On the other hand, fine motor control has to do with small, specific movements associated with dexterity, mouth and eye adjustment and facial expression. This motor control is often affected in individuals with ASD (Sipes *et al*., 2011; Leonard *et al*., 2014; Focaroli *et al*., 2016; St. John *et al*., 2016). Observation of the fine motor control in the ASD populace is seen as compromised "reach to grasp" development, planning movement when reaching out to grasp small objects, decreased dexterity and coordination of various digits (Mari *et al*., 2003). Deficits of fine motor control may be more sensitive to detection at an earlier age. There are reports of compromised fine motor function in infants assessed using the Mullen Scales of Early Learning (MSEL) standardized motor test, primarily observing grasping behaviour as fine motor assessment, as early as 6-7 months of age in at-risk populations (Bhat *et al*., 2011). Other researchers, however, have reported that detection of compromised fine motor control is not reliable prior to one year of age (Landa *et al*., 2012; Libertus *et al*., 2014).

Alterations in motor control development appear to have implications regarding development of communicative abilities. There is a relationship between motor control performance and socially relevant tasks such as locomotion to seek out social interactions, acquisition of verbal skills, and development of nonverbal expression (Iverson, 2010; Sipes *et al*., 2011; Nickel *et al*., 2013; Travers *et al*., 2018).

v. Brain Circuits Specifically Involved in Social Interactions

Social behaviour involves a wide range of cognitive processes, including perception, attention, memory, motivation, and emotion (Fernández *et al*., 2018). The medial prefrontal cortex, temporoparietal junction, and posterior temporal sulcus are developmentally involved in the theory of mind skills, while limbic structures including the amygdala, insula, and ventral striatum are intimately involved in emotional perception, expression, and regulation (Soto-Icaza *et al*., 2015). Structural and functional connectivity studies have found

differences between typically developing individuals and individuals with ASD in these areas involved in social cognition (Ha *et al*., 2015).

3.1.3. Immune Abnormalities

Autoantibodies to neural tissue, including neuron-axon filament proteins, cerebellar neurofilaments, myelin basic protein, caudate, and serotonin receptors are associated with Autism Spectrum Disorders (Ashwood *et al*., 2006). A study by Braunschweig *et al*. (2013) states that maternal antibodies are believed to bind to and disrupt the development of the foetal brain prenatally. Elevated cytokine levels in the cerebrospinal fluid and blood have been identified, particularly in cytokines associated with the innate immune system (Pardo *et al*., 2005). ASD has been associated with prenatal and postnatal infections, familial autoimmunity, and gastrointestinal inflammation, further suggesting a role for the immune system (Ashwood *et al*., 2006).

i. General pathophysiology of autism

Neuropathological Abnormalities have been reported in almost every brain region in individuals with ASD, from the lower brainstem to the cortex. The first reported abnormalities were in the cerebellum and brainstem, including changes in cerebellar volume (Courchesne *et al*., 2001; Courchesne, 2003), vermis agenesis (Bobylova *et al*., 2007), and loss of Purkinje and granule cells and changes in the inferior olive (Bauman and Kemper, 2005). Reductions in neuronal size and neuronal density were found in the limbic system (Bauman and Kemper, 2005), particularly in Cornu Ammonis CA1 and CA4, and hippocampal dendritic branching was reduced (Raymond *et al*., 1995). The trajectory of amygdala growth is unique in ASD with atypical age-dependent changes in dendritic spine density leading to amygdala dysfunction (Weir *et al*., 2018). Brain growth in some individuals with ASD appears accelerated during the first years of life (Courchesne, 2003) followed by being prematurely diminished by early childhood (Courchesne and Pierce, 2005a; Redcay and Courchesne, 2005). The early acceleration in head circumference is linked to increased brain volume (Courchesne and Pierce, 2005a; Herbert, 2005), non-neural tissue (Tate *et al*., 2007), extra-axial fluid (Shen *et al*., 2017). and gray matter (Courchesne *et al*., 2001; Courchesne, 2003) volume has been reported in ASD. Increased white matter volume has been attributed to more short association fibres in the frontal and temporal lobes

(Casanova, 2004). This is proposed to cause an imbalance between local and distant cortical communication and that it changes the whole brain network architecture (Li *et al*., 2014), leading to a deficit in large-scale cortical integration that is required for language, behavioural regulation, and social interactions. Other gray matter cortical abnormalities such as smaller, more compact, and numerous cortical minicolumns, particularly in the frontotemporal areas (Casanova *et al*., 2002; Casanova *et al*., 2006) are reported in ASD. This is associated with a reduction in peripheral neuropil space (Buxhoeveden and Casanova, 2002), a space that contains gamma aminobutyric acid (GABA) inhibitory interneurons (Favorov and Kelly, 1994; Mountcastle, 1997; Buxhoeveden and Casanova, 2002; Casanova *et al*., 2006; DeFelipe, 2011). The association of tics and repetitive movement and response to anti-psychotic medications implicates basal ganglia circuits in ASD. The basal ganglia are also essential in eye movement, coordination, sensory modulation, and inhibition control, all neurological functions that are often impaired in ASD (Subramanian *et al*., 2017). Interestingly, the cerebellum, another structure implicated in ASD, and basal ganglia are connected through a short di-synaptic pathway, highlighting their functional co-dependency (Subramanian *et al*., 2017). The cerebellum is recognized for its role in cognition and affect, given the rich connection with the cerebral cortex (Stoodley and Schmahmann, 2010).

3.2. BEHAVIOURAL ESSAYS

3.2.1. INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder whose major characteristics include deficits in social communication and social interaction, restricted and repetitive patterns of behaviour, interest or activities (Khan *et al*., 2012) among others. Rodents are among the most commonly used animal models in behavioural neuroscience because they show clinical symptoms that approximate closely the human neurodevelopmental disorders (Ellenbroek and Young, 2016). Rodents show enhanced cognitive abilities and a rich social repertoire, but with ASD deficits, the rodents may not be able to show or display these characteristics. Behavioural characteristics seen in neurodevelopmentally impaired rodents are defects in social interaction and communication, enhanced anxiety, seizures, abnormal pain sensitivity, eye blink conditioning and repetitive behaviour (Patterson, 2011; Ku *et al*., 2016). The core symptoms of autism include social

interaction, which is tested in a three chambered apparatus that offers the rodents a choice to spend time in a chamber with a reward or novel object. Another symptom is communication, which is evaluated by measuring responses to olfactory cues; and another is repetitive behaviour which is measured on insistence on sameness (Crawley, 2007). Based on the description by Crawley (2007) on the core symptoms of ASD, we carried out ten behavioural tests including the balance beam test, novel object test, elevated plus maze, T-maze, Y-maze, open field test, light-dark test, olfactory habituation test, hole-board test and marble burying test.

There were no biological markers identified for autism yet, thus the current diagnosis of autism is solely based on behavioural criteria (Silverman *et al*., 2010). Our aim of this study was to confirm if the core features of autism (Impaired social interaction, communication deficits, and abnormal repetitive behaviours) were present in our experimental animals, having developed in a preeclamptic environment.

3.2.2. Materials and Methods

This study was granted ethics approval by The Animal Research Ethics Committee (AREC) to Dr B.C. Maseko (2018/09/41/B). Funding was granted by the Thuthuka Foundation (BM_TTK190827471815). All animals were housed according to the standards set out by the Wits Animal Ethics Committee and housed under a regulated environment with a 12-12h light-dark cycle.

A total of 44 pups (21saline and 23cadmium-exposed) was used to observe behavioural phenotypic characteristics for autism while keeping them viable throughout tests over a series of weeks. In course of the study, any rat pup that falls of the testing apparatus will no longer be a part of that particular study. Behavioural testing began with training on postnatal day (PND) 23 and commenced from PND 24 to PND 56. Before and in between each trial, apparatus and chambers were thoroughly cleaned and disinfected with a 70 % ethanol cleaning solution. All tests were recorded using a USB Camera positioned directly above each apparatus. We used AnyMaze Software (Stoelting, IL, USA) for all quantification and analysis of our data. All behavioural testing and manual scoring were performed by researchers' blind to treatment groups.

i. Testing Room

All behavioural tests were conducted in behavioural testing rooms between 08:00 and

17:00. To habituate the rats to behavioural testing room conditions, rats were moved to behavioural room and allowed to move freely within their respective cages for about one to two hours before each test started daily. Behavioural tests were performed in rats from PND 24 to PND 56. Each behavioural test was performed on all animals within the same 24-hour window to ensure that all animals were the same age while encountering the test. After testing was concluded on each animal, the equipment was cleaned with 70% ethanol and hypochlorous water to eliminate olfactory cues. The behavioural testing rooms were illuminated at 100-lux intensity.

ii. Data analyses

Statistical analyses were performed using the SPSS software (IBM, Armonk, NY, USA). Data were analysed using the two-tailed student t-test, one-way ANOVA, or two-way repeated-measures ANOVA. Differences with a p-value <0.05 were regarded as statistically significant. Data are presented as box plots or means ± standard errors.

The following were the test studies conducted:

iii. Balance Beam test

This test is used to assess motor coordination and balance in rats and mice (Carter *et al*., 2001). The balance beam motor task has been reported to be more sensitive to slight alterations in motor coordination when compared to other motor tests, such as rotarod testing, when assessing drug induced impairment (Stanley *et al*., 2005). Autistic individuals have been shown to have deficient motor and coordination abilities (Pusponegoro *et al*., 2016). The goal of the test is to observe the rat's ability to stay upright and walk across an elevated narrow beam to a safe place. The protocol used is modified from Carter *et al*, (2001).

a. Materials and Methods

Sprague Dawley rat motor training began by preparing the balance beam apparatus (Figure 7), which consisted of a series of elevated, narrow beams suspended 50 cm from the ground with an enclosed escape platform of (22cm x22 cm), a middle traverse (Rod) distance of 80

cm and diameter of 1.5cm. The escape platform contained a box filled with bedding from the animals' cages for familiarity and there was a cushioned area directly below the elevated beams in the event of an animal falling. Motor skill testing was performed on all groups from postnatal day 23 through postnatal day 24. This testing consisted of using the same balance beam apparatus and was done for 60 secs per animal. The beam was cleaned with 70% alcohol after each test run. Animals were timed traversing the 80 cm beams Using Any-Maze Software (Stoelting IL, USA).

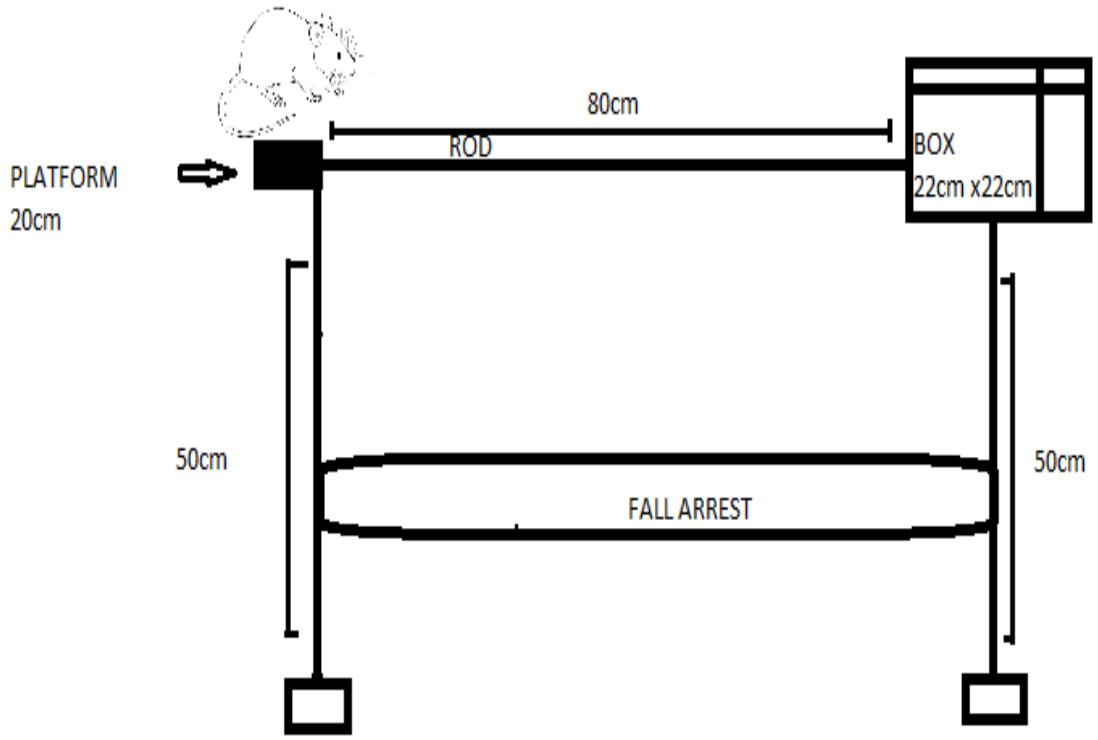


Figure 3. 3. Balance beam test for motor functions in rats

b. Results

The average time on the rod for the cadmium-exposed progeny during the balance beam test was 4.7 secs ±0.58 (n = 23), while the average time for the saline-exposed progeny was 9.27secs±1.52 (n = 21). There was a statistical significance between the observed groups, with a p-value of p =0.0058.

The average time for the cadmium-exposed pups on the platform during the test was 15.76 secs ±6.73(n = 23) while the average time for saline-exposed pups was 4.3secs±1.60(n = 21). There was no statistical significance p =0.1107.

The average speed for cadmium-exposed pups during the test was 0.08 m/s ±0.01, while the average speed for the saline group was 0.05m/s±0.01. There was a statistical significance in average speed between the two groups of pups (p =0.045).

The absolute turn angle (measures each vector movement of the animal that is from one position of the animal centre point to the next.) for cadmium-exposed pups during the test was 85.22deg ±16.21 while that of saline-exposed pups was 175.57deg±40.62. There was a statistical significance between the groups with regards to absolute turning angle (p =0.048).

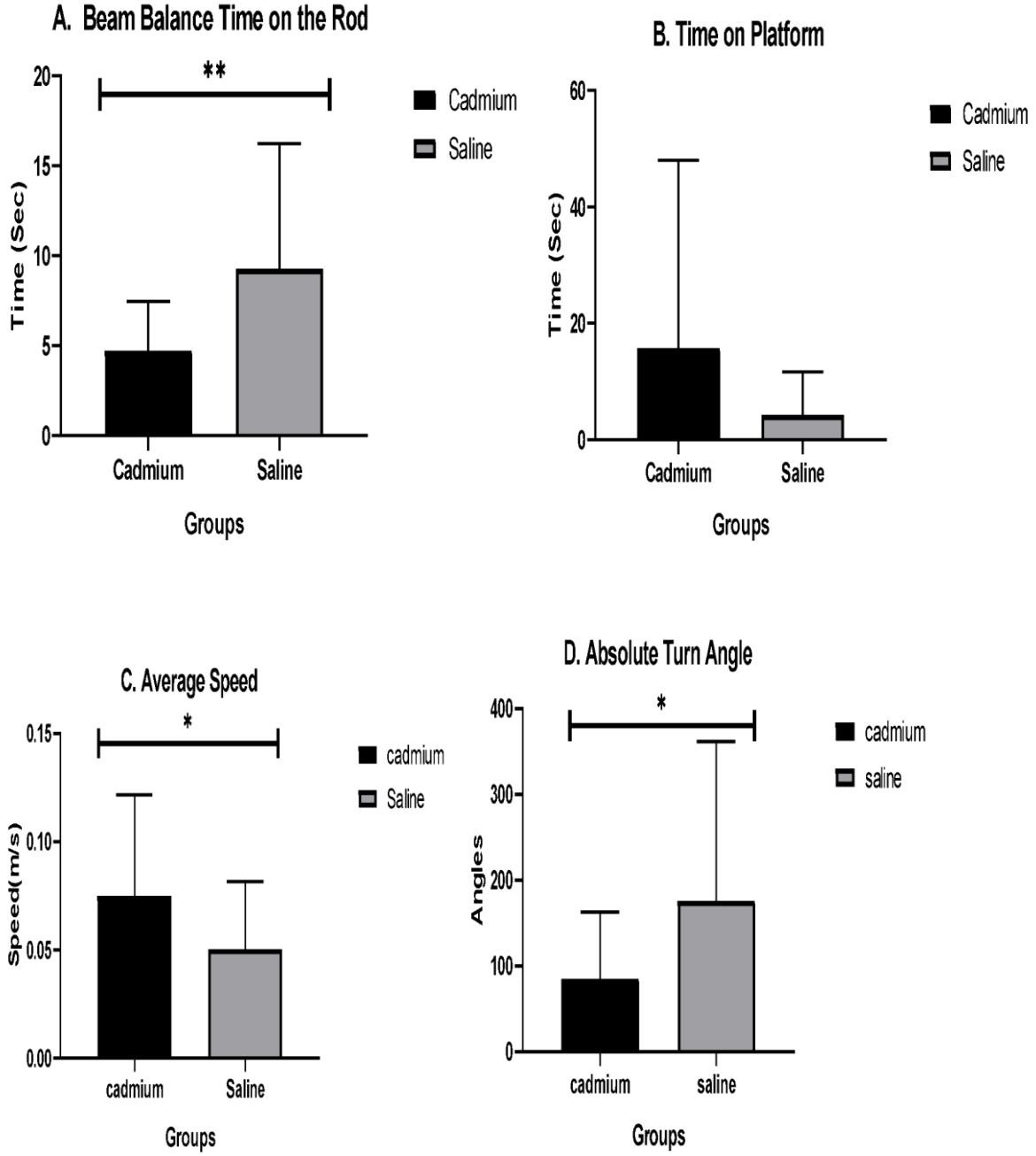


Figure 3. 4**.** Beam Balance motor functions test (**A**) time spent on the transverse rod.

(Cd-exposed v/s SAL, P-value = 0.0058, n=44), (B**) time spent on the platform, (Cd-exposed v/s SAL, p-value =0.1107, n=44), (**C**) Average speed on the rod*(Cd-exposed v/s SAL, P-value 0.045, n=44), (**D**) Absolute turn angle; *(Cd-exposed v/s SAL, P=0.048 n=44).

c. Discussion

The beam balance test was used to examine motor function between rat pups from preeclamptic and normotensive pregnancies. In the course of our studies, it was observed that the preeclampsia-exposed group had delays in coordination and in traversing the rod, which is also a typical feature in human autistic individuals, particularly in early childhood (Ketcheson *et al*., 2018), this feature would appear as difficulty in motor planning in humans. The study reveals that autism affects motor coordination, as evinced by the short time on the rod for the preeclampsia-exposed pups, which was a result of them having fallen off the rod rather than having traversed it quickly, and their increased time spent on the platform, although this aspect was found to not be statistically significant, it is still consistent with expectations given what is known of autistic motor difficulties. Conversely the normotensive pregnancy pups did not have the challenge traversing the rod and also explored the beam apparatus more than the other group, hence their increased time spent on the rod.

vii. Novel Object Recognition Test

Novel object recognition is a form of memory test that does not rely on spatial cues, but the animal is trained to recognize certain objects. It was originally described by Ennaceur and Delacour in 1988.

a. Materials and Methods

The test was done on postnatal day 25, in a rectangular box measuring 42cm x46cm x53cm, (L x W x H). During the testing phase, one of the familiar objects was replaced with a novel object. The animal normally should spend more time investigating the novel object relative to the familiar one. In addition to objects, animals can be trained to discriminate novel from familiar odours, tastes, and social partners (Crawley, 2008). After an initial 1min acclimatization phase, one sample object and one unfamiliar object were introduced, and object interaction (a measure of environmental familiarity) was recorded for 5min. Object interaction was defined as an event where the rat’s head was within 2 cm of the object and directed towards the object. Sitting or leaning on the object was not considered object

exploration (Lueptow, 2017). The object Interaction [%] for the sample object was calculated as (sample object interaction x 100) / (sample object interaction + novel object interaction), while object interaction [%] for the novel object was calculated as (novel object interaction x 100) / (sample object interaction + novel object interaction) (Antunes and Biala, 2012). Additionally, we measured the number of faecal bolus discharged and time spent in the field by the rats because they are measures for anxiety.

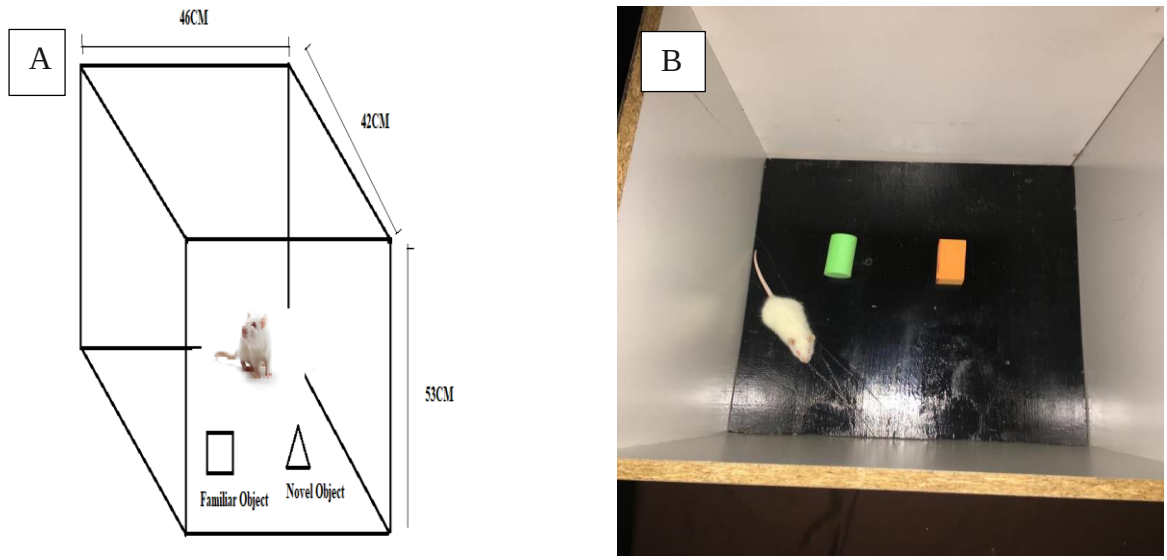


Figure 3. 5**.** Novel Object recognition apparatus.

A. shows a drawing of a rat within the apparatus, while **B.** shows a rat undergoing our test.

b. Results

During the behavioural assessment with the object recognition test, the distance covered by the two groups during the object recognition assessment were such that on Day 1, the cadmium-exposed progeny covered a distance of 5.20m±0.34 and the saline-exposed progeny covered 5.74m±0.28, however no significance could be found for these distances travelled between the two groups (p = 0.225). On Day 2, cadmium-exposed progeny travelled 3.90m±0.35 and the saline-exposed counterparts travelled 3.82m±0.35. Again, on day 2, no significance was found in the differences travelled between the two groups (p = 0.872).

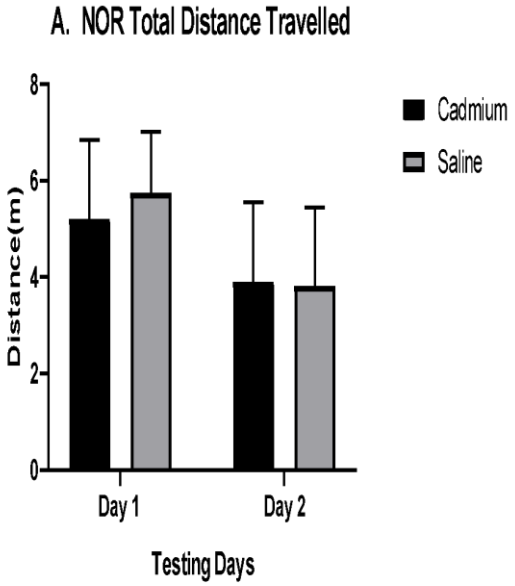
When observing time spent in the field, a statistical significance was recorded for both days. On Day 1, cadmium-exposed offspring remained for 76.46secs±1.94 and the saline group stayed 84.43secs±0.87 (p = 0.027). For Day 2, the cadmium-exposed group was 84.40secs±0.83 while the saline group was 88.10secs±0.50 (p = 0.00049).

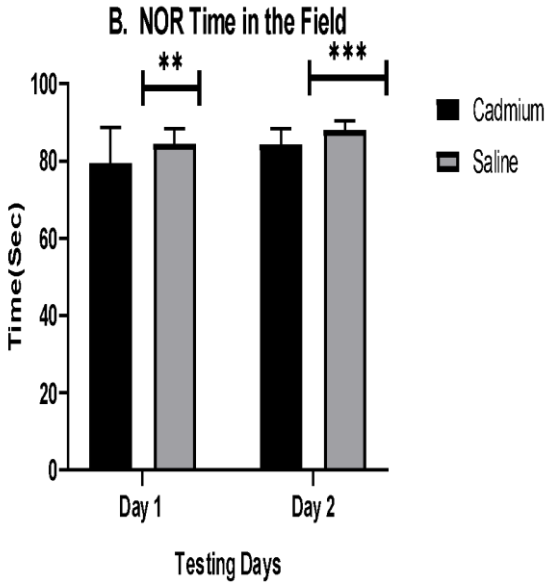
The number of faecal bolus discharged on both days are such that the cadmium pups

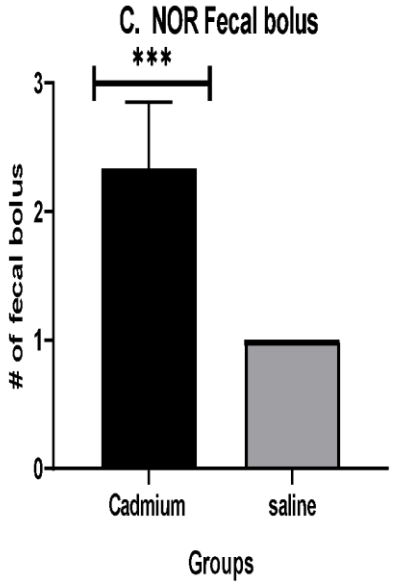
discharged 2.33±0.21 and the saline group was 1±0, we found a statistical significance between the faecal bolus discharges for each group (p = 0.0015).

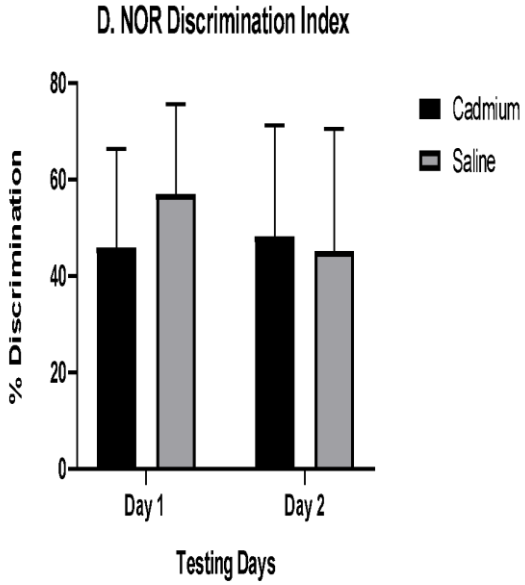
We calculated the percentage discrimination index and found that for Day 1, the cadmium-exposed progeny was 45.91%±4.27 while the saline group was 57%±4.07. No significance was recorded (p = 0.067). The measure for Day 2 was recorded as the cadmium-exposed group having 48.22%±4.81 while the saline group was 45.09%±5.55 with no statistical significance between the two groups (p = 0.673).

Time spent with objects (familiar and novel objects) was assessed and reported on both days. Day 1(A; Familiar) cadmium group was 7.78secs±0.86 and saline group was 6.33secs±0.94. p = 0.261, no significant. Day 1(B; Familiar) cadmium group was 7.12secs±0.81 and saline group 7.77secs±0.67. statistically not significant p = 0.539. Day 2(A; Familiar) cadmium group was 6.73secs±0.69 and saline group was 5.40secs±0.71. p = 0.188, no significant. Day 2(B; Novel) cadmium group was 7.24secs±0.87 and saline group 5.03secs±0.75. statistically not significant p = 0.062.


A. NOR Total Distance Travelled
Distance(m)
0
2
4
6
8
Day 1
Day 2
Testing Days
Cadmium
Saline


B. NOR Time in the Field
Time(Sec)
0
20
40
60
80
100
**
***
Day 1
Day 2
Testing Days
Cadmium
Saline


C. NOR Fecal bolus
# of fecal bolus
0
1
2
3
***
Cadmium
saline
Groups
Cadmium
saline


D. NOR Discrimination Index
% Discrimination
0
20
40
60
80
Day 1
Day 2
Testing Days
Cadmium
Saline

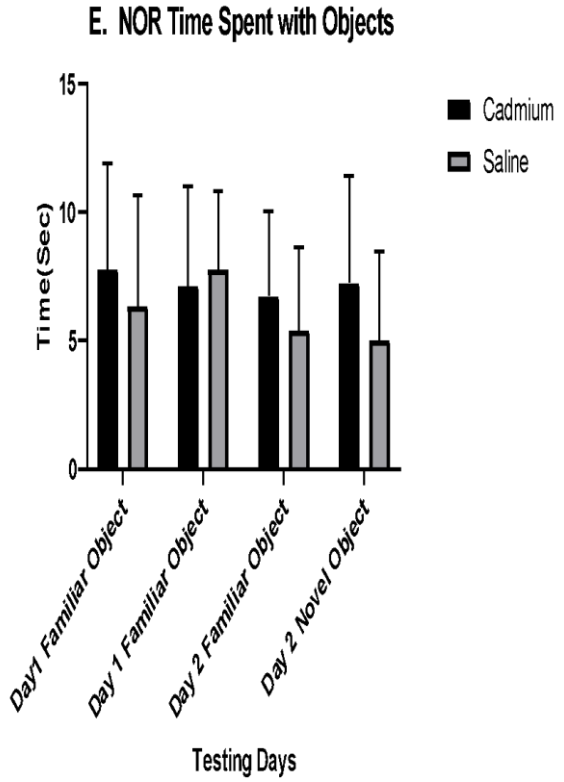


Figure 3. 6**.** Graphic representation of results from Novel Object Recognition (NOR).

(**A**)NOR total distance travelled; (Cd- exposed v/s SAL; Day 1 p value = 0.0.027, Day 2 p value = 0.872, n=44). (**B**). NOR Time in the field; *(Cd- exposed v/s SAL; **(Day 1 p value = 0.02); ***(Day 2 p value = 0.00049), n=44) (**C**). NOR faecal bolus; ***(Cd- exposed v/s SAL; p value = 0.0015, n=44), (**D**). NOR discrimination index; (Cd- exposed v/s SAL; Day 1 p value = 0.067, Day 2 p value = 0.673, n=44), (**E**)NOR time spent with objects; (Cd-exposed v/s SAL; Day 1; Familiar(A) p value = 0.261, Familiar(B) p- value=0.539, Day 2; Familiar (A) p value = 0.188, Novel(B) p value=0.062, n=44)

c. Discussion

The Novel object recognition test was used to check for memory and cognitive abilities of rat pups from preeclamptic and normotensive pregnancies. From the studies carried out, we observed that both the groups of animals on day 1of the studies showed the same exploration and also had the same affinity to both familiar objects presented to them. But on day two of the studies, the rat pups from the preeclamptic pregnancy showed higher preference towards the novel object (Murray, 2010), and as such spent more time exploring the novel object as compared to the normotensive pregnancy group. The greater faecal bolus discharged by the preeclampsia pups was indicative of higher anxiety levels as rats are known to discharge increased faecal matter when experiencing anxiety. This finding is consistent with various authors who have found that heightened anxiety levels very often accompany autism(Vasa and Mazurek, 2015) . Discriminative index is a measure of how well the animal was able to differentiate between the familiar and the novel object.

v. Elevated Plus-Maze

The elevated plus maze test is used for measuring anxiety-like behaviour in mice and rats, it measures fear of elevation/height (Walf and Frye, 2007)**.** The open and closed arms are considered to evoke the same exploratory drive; therefore, avoidance of the open arms is considered to be a result of the induction of higher levels of fear (Holmes *et al*., 2000). The animals were also observed expressing behaviours such as rearing (vertical movement against the side and/or end of the walls) and grooming (Holmes *et al*., 2000).

a. Materials and Methods

On postnatal day 26, rats were taken through the elevated plus maze task. The maze resembled a cross (+) sign, with two opposite closed and two opposite open arms, and elevated 70cm above the ground. Each arm was 10cm wide and measured 40cm from its end to the common square of the maze. The closed arms had Perspex walls 40cm high. The test duration was 5mins and rats were video recorded using Any-Maze software. Measures taken were time spent in each arm and number of entries into and exits from each arm. The total measure of time spent in the central square was obtained by calculation (total test time minus the time in open +closed arms). Total number of entries into the closed v/s open arms is a measure of anxiety, and the percentages of entries and time spent in each arm constitute a

measure for primary anxiety (Komada *et al*., 2008). In the elevated plus maze test, the maternal cadmium group had 22 subjects and the saline offspring had 20 pups.

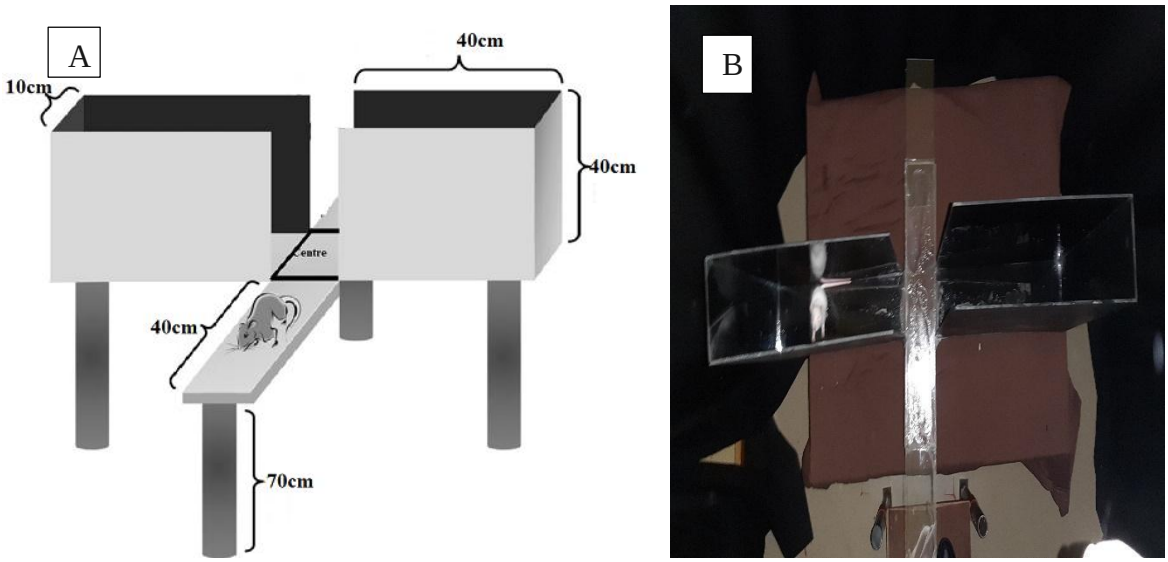


Figure 3. 7**.** Elevated plus maze.

A- showing a diagrammatic representation of the EPM, while **B** shows our rat actually undergoing the EPM test

b. Results

The total distance travelled during the test for the maternal cadmium group on the elevated plus maze was 6.9m ±0.50 (n = 22), while that of the maternal saline group was 8.36m±0.38 (n = 20). We found these differences in distance travelled on the elevated plus maize to be statistically significant, with the p value being (p = 0.027).

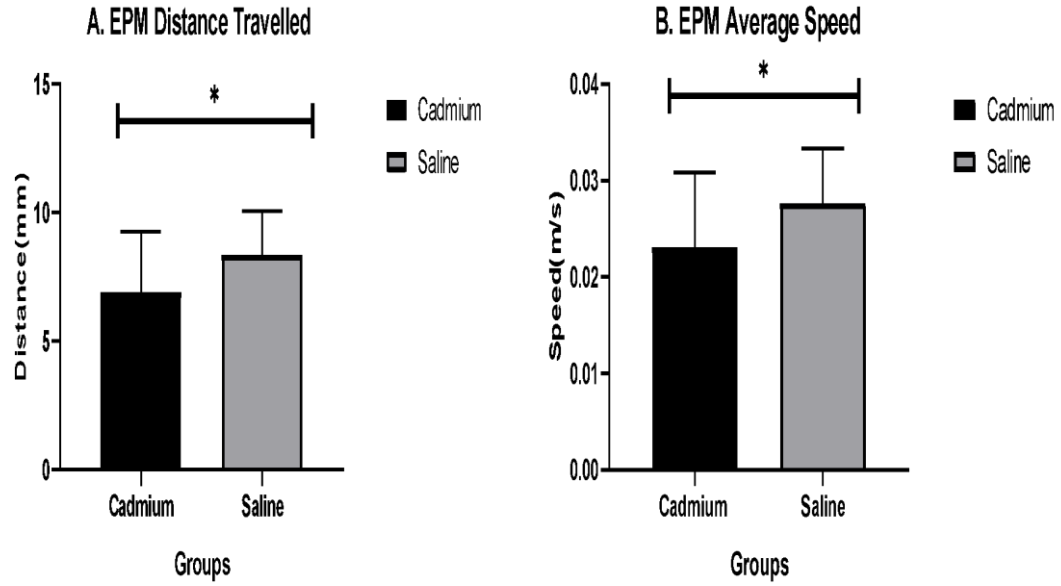
The average speed in the course of the elevated plus maze test for the maternal cadmium group was 0.0230m/s±0.0277, while the saline group was 0.0277m/s±0.0056. There was a statistical significance as we found the P-value to be p = 0.032.

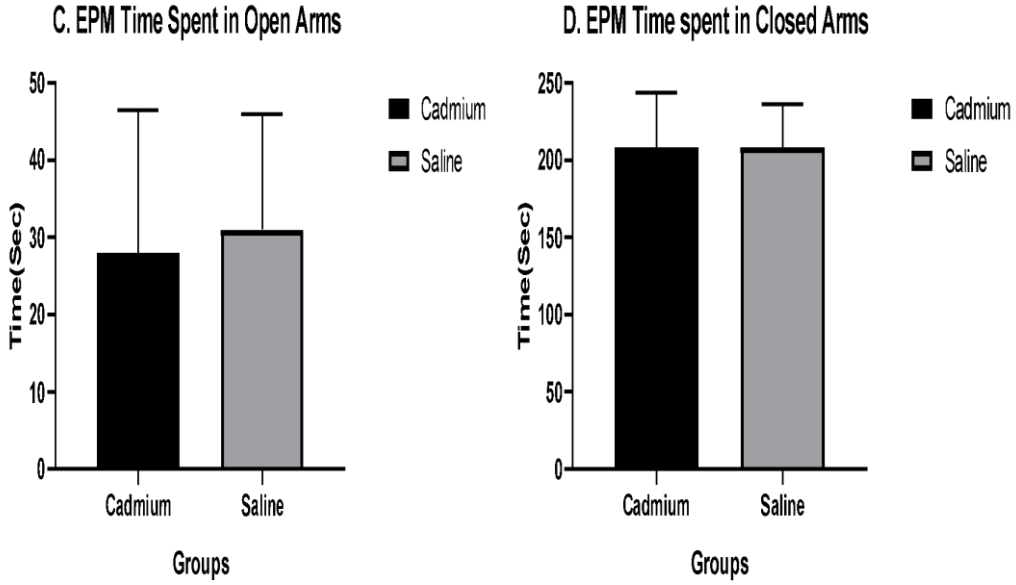
The time spent in the open arm of the elevated plus maze test for the cadmium-exposed progeny was 28.00sec ±3.94 while the saline group was 30.99sec±3.36. There was no statistical significance found between the groups (p =0.57). While in the closed arms, the maternal cadmium group spent 208.30sec±7.57, and the saline group was 208.40sec±6.26. We found no significance difference between the groups, since the P-value was p = 0.99.

The mean number of entries into the open arm of the elevated plus maze test for the maternal cadmium group was 4.23 ±0.63, while for the maternal saline group it was 4.60±0.47. There was no statistical significance between the differences in this case (p =0.160). While for the closed arms, the mean number of entries into the closed arms for the maternal cadmium group was 9.73±0.80, and for the maternal saline group it was 11.05±0.45. No statistical significance was found when comparing the two groups in this regard (p=0.64).

The time spent by animals at the centre of the elevated plus maze for maternal cadmium pups was 63.69sec ±4.82 while that of the maternal saline group was 60.61sec±4.03. There was no statistical significance in the difference between the groups (p =0.63).

The total freezing time both at the open and closed arms, found the cadmium-exposed progeny freezing for 74.96±10.84 and the saline group had freezing time of 48.18±5.26 with p = 0.0373, which is statistically significant.


A. EPM Distance Travelled
Distance(mm)
15
10
5
0
*
Cadmium
Saline
Cadmium
Saline
Groups
B. EPM Average Speed
Speed(m/s)
0.04
0.03
0.02
0.01
0.00
*
Cadmium
Saline
Cadmium
Saline
Groups


C. EPM Time Spent in Open Arms
Time(Sec)
50
40
30
20
10
0
Cadmium
Saline
Cadmium
Saline
Groups
D. EPM Time spent in Closed Arms
Time(Sec)
250
200
150
100
50
0
Cadmium
Saline
Cadmium
Saline
Groups

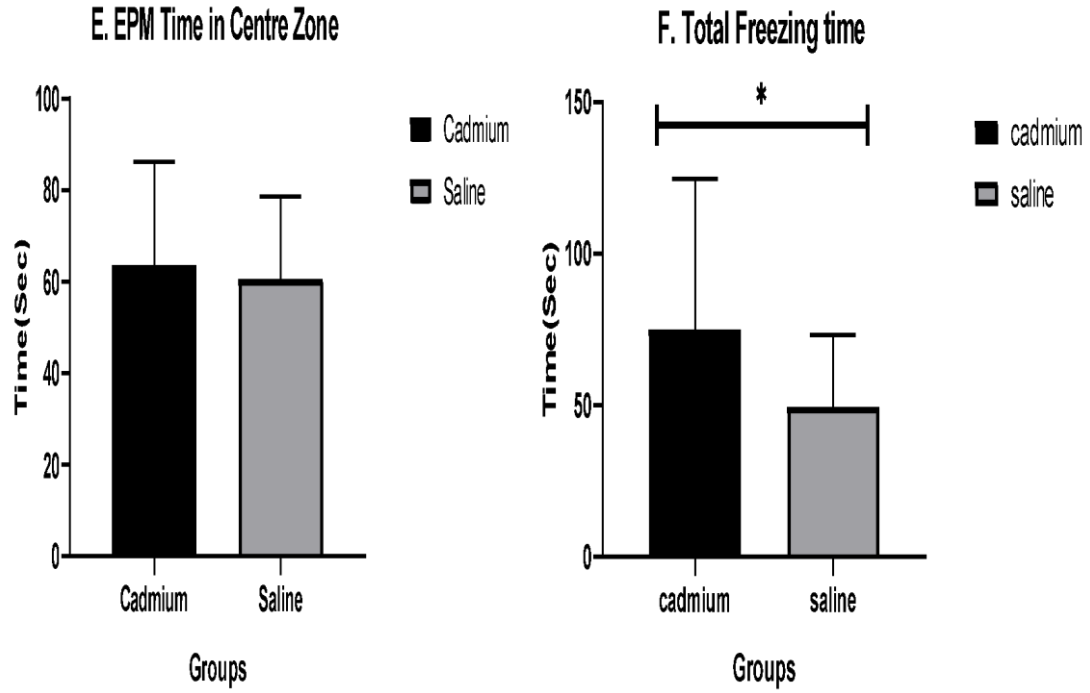
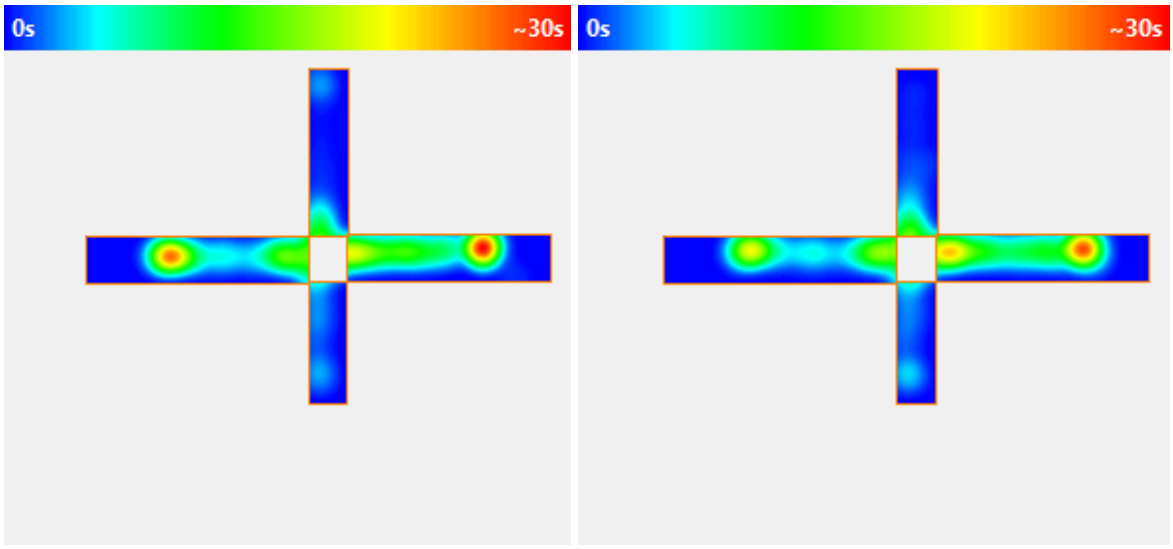


Figure 3. 8**.** Graphical representation of the results from the elevated plus maze, showing a comparison in each between the cadmium-exposed and the saline offspring.

A. Is the total distance travelled by each group on the maze *(SAL vs Cadmium exposed, P-value < 0.027, n = 42). **B** illustrates the average speed of travel compared between the groups *(Cadmium-exposed vs SAL pups, P-value < 0.032, n = 42. **C** and **D.** shows the amount of time spent on the open and closed arms of the maze (**C)**: cadmium-exposed vs SAL, p- value >0.57, n=42) (**D**) cadmium vs saline pup p >0.99, n=42). **E.** Time each the animals spent at the centre of the maze (Cadmium-exposed vs Sal, p- value > 0.63, n = 42). **F.** Is the total time it takes the animals to freeze on the maze *(Sal pups' vs cadmium exposed, p-value < 0.037, n = 42).



Cadmium-exposed group

Saline-exposed group

Figure 3. 9**.** Representative group Heat Map.

This shows how the time animals explored the elevated plus maze both the open and closed arms of the maze between the cadmium and saline group from the minimum time 0 secs to the maximum time 30 secs.

c. Discussion

The elevated plus maze was used for anxiety-like behaviour detection, from our studies, both the maternal preeclampsia group and the normative group spent almost equal amounts of time at the closed arm (figure 3.8D). Both the cadmium and the saline groups spent 10 times the amount of time in the closed arms than they did in the open arms, this indicates that both groups had a heightened level of anxiety. The control group explored the open arm more than the preeclamptic group. The experimental group had less vertical movement and grooming and was more static (freezing time), whereas the saline group had much of the vertical movement, rearing and less freezing time. There was reduced exploration by both groups (Walf and Frye, 2007). Given the number of entries into the closed v/s open arms for both groups was found not to be insignificant in their differences. We assessed that the anxiety levels in both groups were similar. In addition, the time spent by both groups in the open arms v/s closed arms was also not statistically different, suggesting that one group was not necessarily more anxious than the other.

vi. Light - Dark Box Test

The test is based on the natural aversion of rats to brightly illuminated areas and on their spontaneous exploratory behaviour in novel environments (Takao and Miyakawa, 2006).

a. Materials and Methods

A box with diameter (L-35cm× H-28 cm× W-25 cm) of a dual chamber apparatus, one compartment was brightly illuminated and the other was dark with an inter-leading opening as described by Umemura *et al*. (2017). The light - dark box test was done on postnatal day 28. Pups were placed in a dark compartment of the box and were allowed to freely explore both compartments for 5 min. The time that rats spent in the light compartment (35 cm×28 cm×25 cm) and the rate at which the animals entered the light compartment, the distance travelled and the latency to enter the light compartment are the measures used to check for anxiety-like behaviour (Ueno *et al*., 2020). Peeking out was assessed as the body remain in the dark chamber while the head and two paws were seen in the light chamber Results were analysed using ANY-MAZE software. The apparatus was cleaned with 70% alcohol between each test.

Figure 3. 10. Light-Dark Box test showing a rat occupying the dark chamber of the box

b. Results

The Light Dark box apparatus was employed to assess differences between the cadmium-exposed and saline-exposed pups. A few parameters were noted in the behaviour of rats as outlined below.

Mean total distance

The Mean total distance travelled during the test for maternal cadmium pups in the Light-Dark box was 1.49m ±0.13 while that of the saline group was 2.00m±0.13. There was a statistically significant difference when the two groups were compared ($p = 0.0085$).

Peeking out/ heads out

This is the number of peeks out from the dark into the light compartment in the Light-Dark box. Therefore, the maternal cadmium group having 7.96± 0.56 and saline group having 4.43±0.48. When comparing the differences between the two groups, we concluded a statistical significance ($p =< 0.0001$).

Time spent in the light compartment

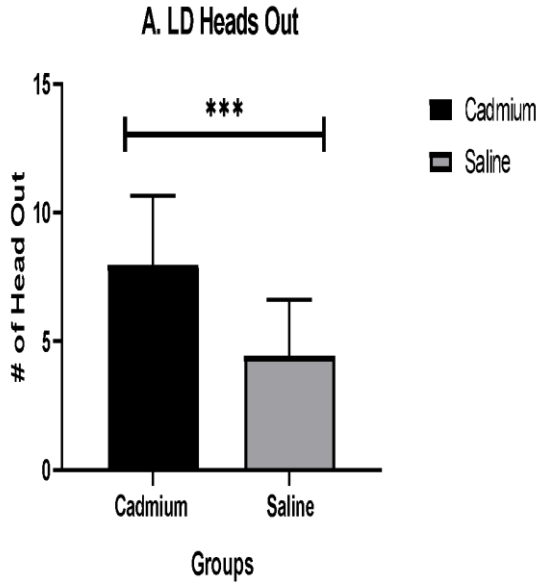
The time in the light compartment of the Light-Dark box for the maternal cadmium group was 97.77sec±9.347, and the saline group showed 99.87sec±5.97. There was no significant difference between the groups ($p = 0.854$).

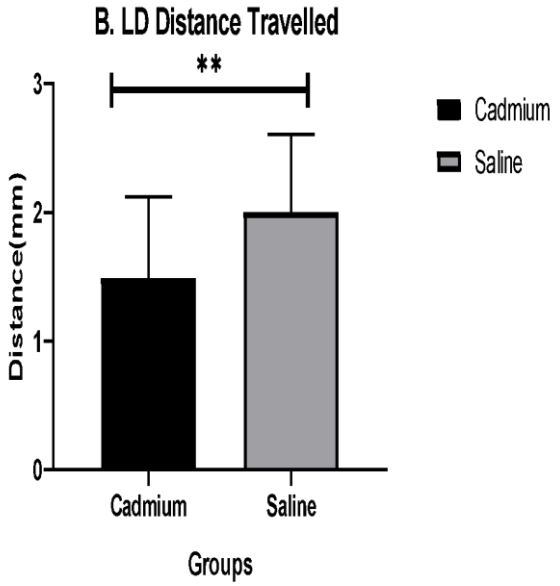
Rearing frequency

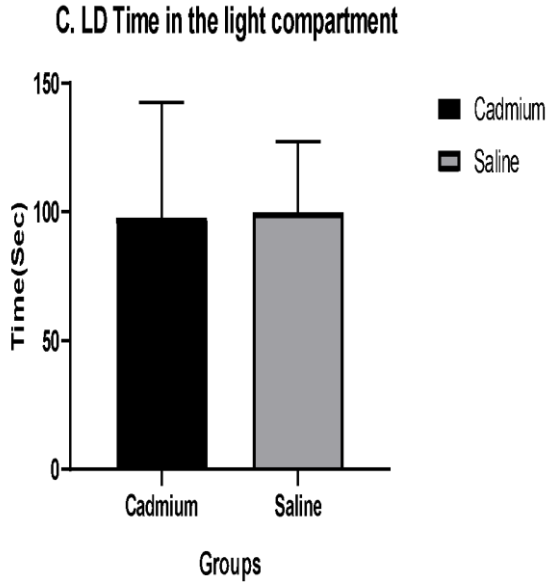
The rearing frequency in the Light-Dark Box of the maternal cadmium group was 0.041Hz±0.004, while that of the maternal saline group was 0.067Hz±0.023. There was no significant difference statistically ($p = 0.2807$).

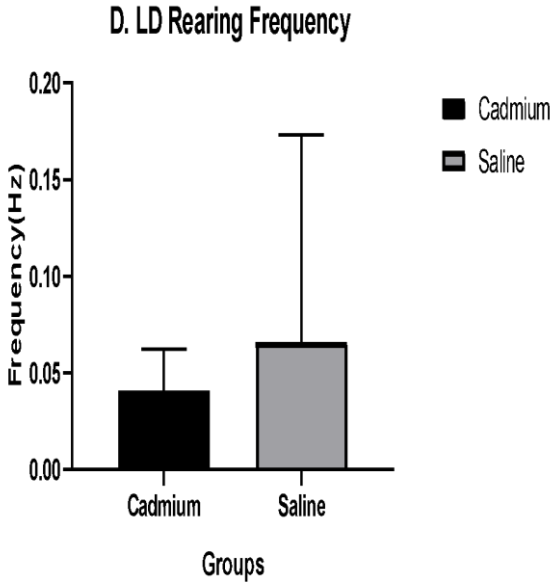
Speed in the light compartment

Speed in the light compartment of the Light-Dark Box for the maternal cadmium group was 0.019m/s±0.0008, while the saline group was 0.020m/s±0.0009. When comparing the two groups, we found that the difference between measures was statistically significant ($p = 0.0009$).


A. LD Heads Out
# of Head Out
15
10
5
0
***
Cadmium
Saline
Cadmium
Saline
Groups


B. LD Distance Travelled
Distance(mm)
3
2
1
0
**
Cadmium
Saline
Cadmium
Saline
Groups


C. LD Time in the light compartment
Time(Sec)
150
100
50
0
Cadmium
Saline
Cadmium
Saline
Groups


D. LD Rearing Frequency
Frequency(Hz)
0.20
0.15
0.10
0.05
0.00
Cadmium
Saline
Cadmium
Saline
Groups

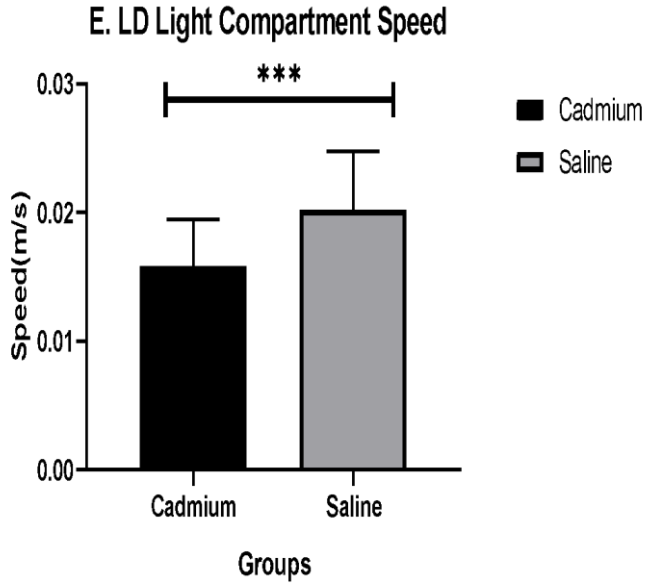


Figure 3. 11**.** Graphic representation of the light- dark experiment.

A. Illustrates the results from noting the peeking out between the two groups ***(Cd-exposed v/s SAL, P-value <0.0001, n=44), **B**. Illustrates the mean total distance travelled for both groups **(SAL v/s Cd- exposed, p value = 0.0085, n=44). **C.** The time the animals spent in the light chamber of the apparatus (Cd- exposed v/s SAL, p =0.854, n=44). **D**. Illustrate the rearing frequency in the light chamber (SAL v/s Cd- exposed, p = 0.28.7, n = 44). **E**. the speed of movement in the light compartment between the two groups ***(Cd- exposed v/s SAL. P –value =0.0009, n=44)

c. Discussion

During the light dark box test, the extent of exploration for both the preeclamptic rat pups and the normotensive rat pups was the same. Both groups spent an equal amount of time in the light compartment of the light-dark box, as seen in (figure 3.11C). The preeclamptic rat pups tended to peek with their heads into light compartment, attempting to explore and view the light compartment but still remaining in the dark (figure 3.11A). The control rat pups showed more vertical movement when they were out in the light compartment trying to explore the immediate environment.

v. Open Field Test

The Open Field test was developed by Hall and Ballachey (1932) and is used in a wide variety of tests involving exploratory behaviours.

a. Materials and Methods

The apparatus consisted of an open arena with white coated plywood with the square area measuring 44cm x 53cm (Length x Width) and contained by high walls (19cm Height) to prevent the animals from escaping the arena. On postnatal day 29, the subject animal was placed at the centre of the arena and allowed to move freely (Richter *et al*., 2016). Parameters used to measure the subjects' locomotion were distance travelled, time spent in the centre of the arena, horizontal activity and vertical activity (when the animal stand upright with its two limbs or standing on two paws and the other two resting on the wall of the apparatus) rearing or vertical activity is measured by counting the number of photobeam interruptions) (Shoji *et al*., 2016; Umemura *et al*., 2017). The test was run for 3 minutes for each run, after which apparatus were cleaned with 70% alcohol in preparation (removing of possible olfactory signals left behind by the previous subject) for the next animal's run. Analysis was done using ANY MAZE software.

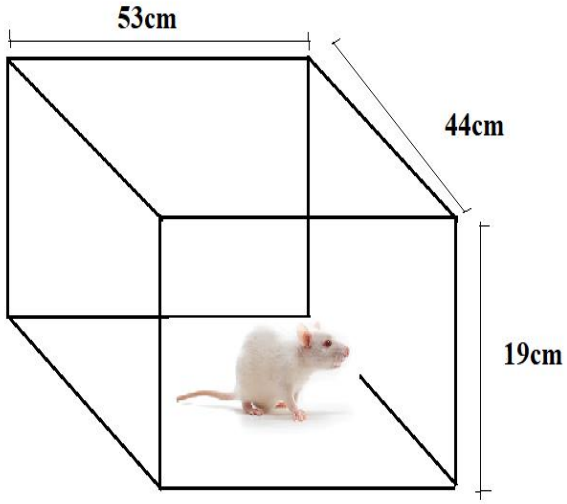


Figure 3. 12. An illustration of the Open Field Test

b. Results

Total distance travelled

During the open field behavioural assessment, the total distance travelled by the cadmium-exposed group was 10.54m/s±0.9, while the saline group was 11.09m/s±0.58. These differences were found to have no statistical significance ($p = 0.61$).

Time spent in the field centre

Animals were regarded as spending time in the field centre when the stay at the field centre for up to 200secs. The average time spent in the centre zone for the cadmium-exposed pups during the open field test was 16.45secs ±2, while the average centre zone time for the group was 13.53sec±1.29. when comparing the difference between the two groups, there was found no statistical significance ($p = 0.276$).

Number of entries into the field centre

Entry into the centre was defined by 65% of the animals' body including the head and centre entering into the field centre. The number of entries into the centre by the cadmium group during the open field assessment was 10.43±1.29, and the saline group was 9.71±0.88, no significant difference was detected in this parameter between the two groups ($p = 0.65$). This was an indication that exploratory activity did not differ from both groups.

Number of vertical activities

The number of vertical activities performed during the test for cadmium was 16.30±1.86 numbers, while for the saline offspring it was 16.52±1.25. Vertical activities are defined as when the animal stand upright with its two limbs or standing on two paws and the other two resting on the wall of the apparatus. The calculated P-value was 0.096, as such, we concluded that differences observed between the experiment and control groups had no significance statistically.

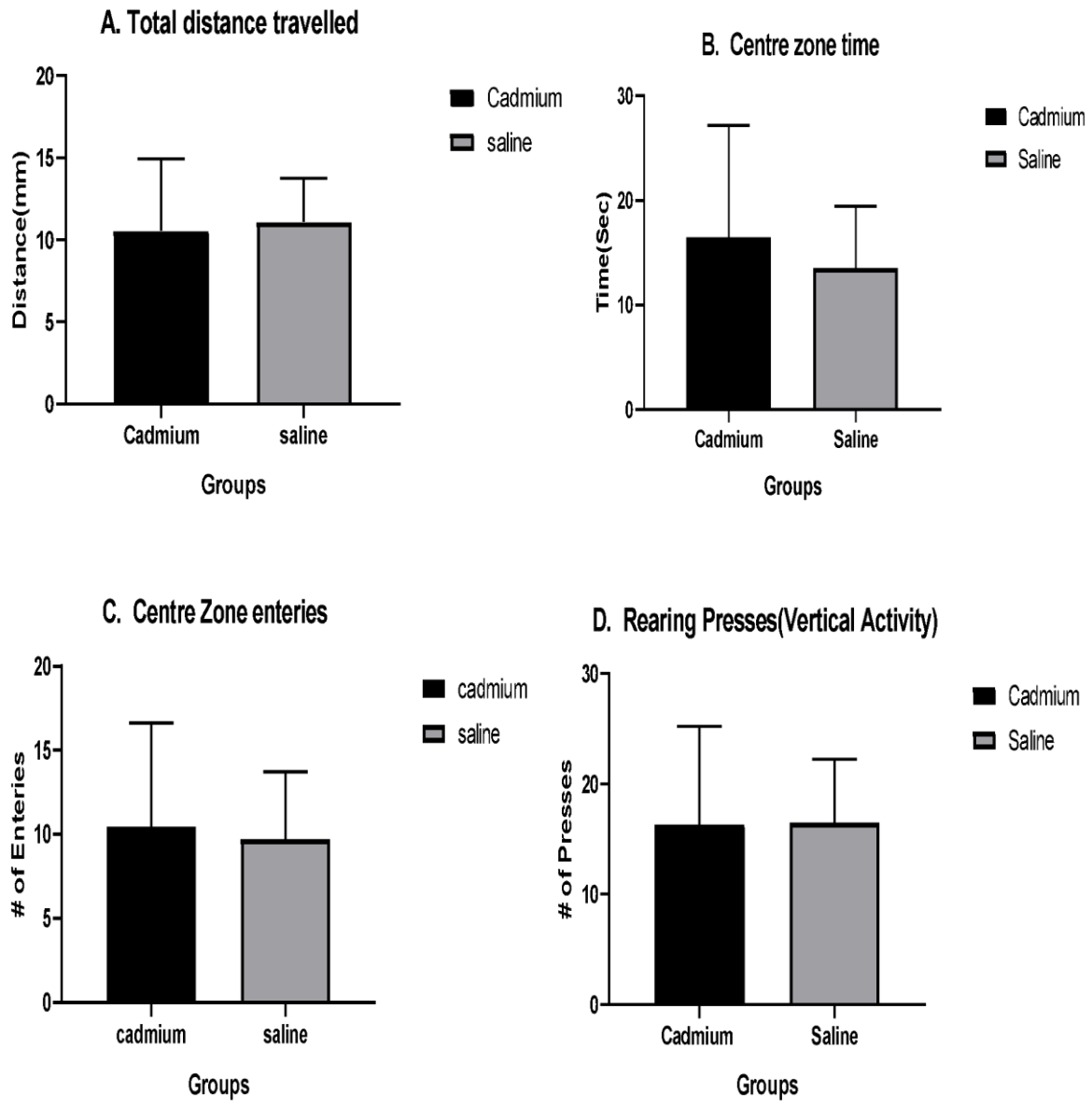
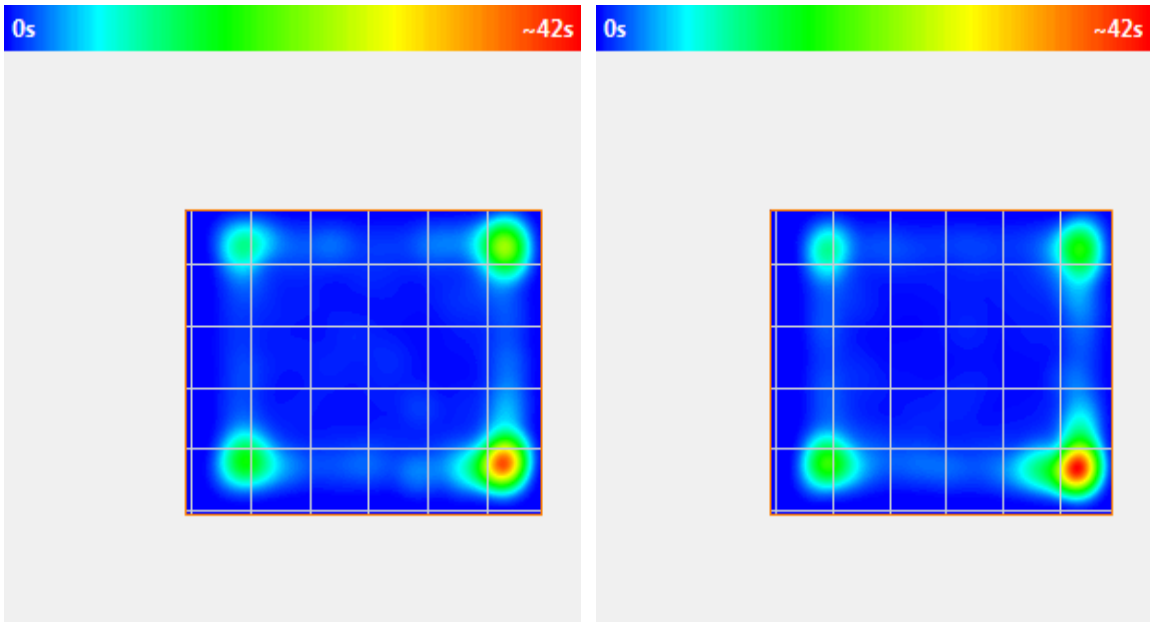


Figure 3. 13**.** Results of the Open Field Test, showing varying parameters that were examined and compared between the cadmium-exposed progeny and the saline-exposed progeny.

The parameters represented by each graph are as follows: A. total distance travelled within the field (SAL v/s Cd-exposed, p-value =0.61, n= 44) B. represents the time spent within the field's centre zone;(SAL v/s Cd-exposed, p value = 0.276, n =44) C. depicts the number of entries made into the centre zone by each group (SAL v/s Cd –exposed, the value of p = 0.65, n=44)and D. depicts the number of vertical behaviours that were observed between the two groups (SAL v/s Cd-exposed, p =0.096, n= 44).



Cadmium Group

Saline Group

Figure 3. 14**.** Heat map showing the activity of rat pups**.**

The red dot is an indication of the area the animals spent the most time(42seconds), in this case, at the bottom right corner of the field for both groups.

c. Discussion

Open field activity is used to measure anxiety levels and exploration rate of rats during behavioural activity. In the course of the study, both groups of animals avoided the centre region, however the preeclamptic offspring group attempted to explore a little more of the open space than the normotensive offspring. The groups had almost equal amounts of vertical activity, most of which was the supported rearing in which the animal leaned against the wall to assess its environment.

vi. Y-Maze Test

This test is exploiting the natural drive of rodents to explore novel environments, and tests the short-term spatial recognition memory (Grabrucker *et al*., 2016).

a. Materials and Methods

The Y-maze consisted of three identical arms arranged in the shape of a "Y." Each arm was 30cm long and 9cm wide with 15cm high walls, all made of wooden material which had been painted white. The test was done on postnatal day 32, where rats were placed in zone A of the Y-maze at the beginning of the test, which remained constant for all animals throughout the testing sessions. The rats were tested with no previous exposure or habituation to the maze. A spontaneous alternation was defined as an entry into three different arms on consecutive choices (Ueno *et al*., 2020). The percentage of alternation was calculated as the ratio of actual to maximum number of alternations. The total distance travelled (m), number of entries, and number of alternations were recorded and analysed using the ANY-MAZE software.

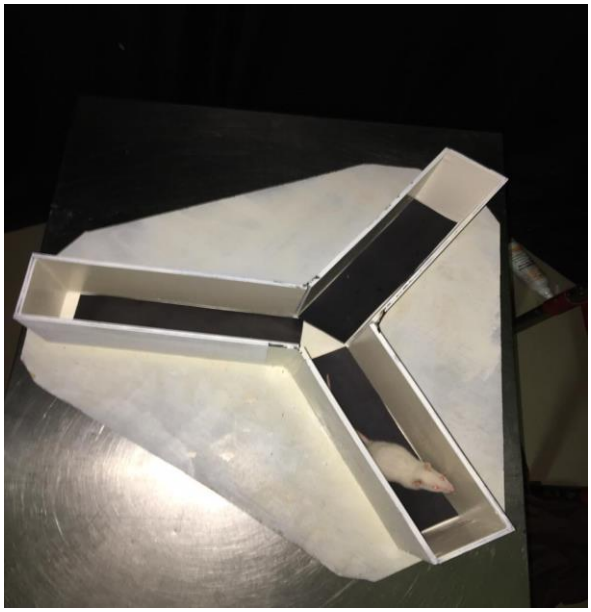


Figure 3. 15**.** A rat pup navigating the Y-Maze

b. Results

Total distance travelled

The Mean total distance travelled during the Y-Maze spatial working memory test for cadmium-exposed offspring was 4.24m ±0.32, while that of the saline-exposed offspring was 4.38m±0.19. There was no statistical significance when the differences were compared to between groups ($p = 0.746$).

Percentage (%) spontaneous alternation

The mean percentage spontaneous alternation movement during the Y-maze test for the cadmium group was 56.55%±3.53 while the saline group was 56.17%±4.26 with no significance statistically $P = 0.945$.

Speed of movement

The Average speed during the behavioural analysis on the Y-maze was calculated, finding that the maternal cadmium-exposed group had 0.023m/s±0.0018 while the saline group had 0.024m/s±0.0011. There was no statistical significance when comparing the differences, $p = 0.74$.

Number of entries into each arm

The number of entries into each arm of the Y-maze during the analysis was as follows:

Arm A: maternal cadmium group was 3.87±0.36 while the maternal saline group was 3.90±0.31. Our statistical analysis yielded $p = 0.94$, therefore no significance difference was found between the groups.

Arm B: maternal cadmium group was 4.09±0.41 while the maternal saline group 4.05±0.33. Our statistical analysis yielded $p = 0.94$, therefore no significance difference was found between the groups.

Arm C: maternal cadmium group entered arm C 5.43±0.45 times, while the maternal saline group entered the arm 4.90±0.25. With the P-value found as $p = 0.311$, we concluded no statistical significance when the differences were compared between groups.

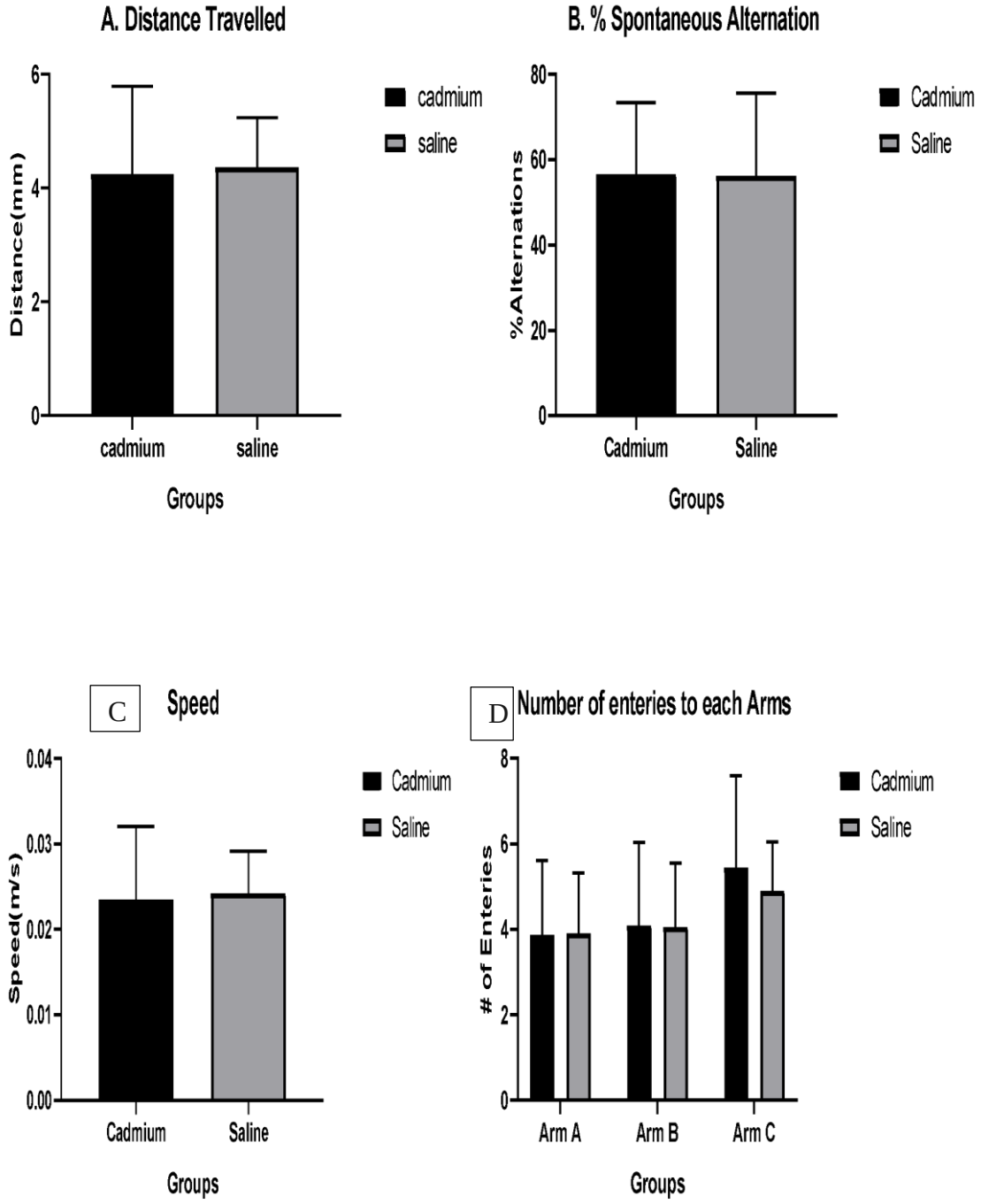


Figure 3. 16**.** Graphic representation of Data from the Y-maze experiment, illustrating each parameter.

A. illustrates the distance travelled within the Y-Maze for both groups (Cd –exposed v/s SAL p = 0.746, n= 44) **B**. illustrates the percentage spontaneous alternation for each group (Cd-exposed v/s SAL; p = 0.945, n = 44); **C**. illustrates the speed for each group (Cd-exposed c/s SAL, p = 0.74, n=44); **D**. illustrates the difference in entering each of the arms by both groups (Arms A: Cd- exposed v/s SAL, p = 0.94, n= 44. Arms B: Cd- exposed v/s SAL, p = 0.94, n= 44. Arms C: Cd- exposed v/s SAL, p = 0.311, n= 44)

c. Discussion

The Y-maze was used to test for the repetitive behaviour and spatial working memory of our rat pups. The two groups were exposed to the Y-maze apparatus, we did not find any significant difference between the cadmium exposed pups and the control group (fig 3.15), although we did notice more arm entries into arm C by the cadmium exposed group.

vii. T- Maze Test

Spatial learning, working memory and repetitive behaviour can be tested using the T-Maze, which requires an animal to use spatial cues to learn the location of a reward (Crawley, 2008). During training, food rewards were placed in each arm to encourage exploration.

a. Materials and Methods

The T-maze was made from a Perspex board, cut out to measure 15cm x51cm (W x L) for Arm A (start arm) and 15cmx10cm (W x L) for Arms B and C; and 25cm throughout in height with a centre platform of 10cm x 10cm (W x L). The test was done on postnatal day 35. On test day 1, the rats were taught to link only one arm with a reward. Once the animal entered the arm with the incentive, we baited it. This was done to allow access for exploration and working memory. On test day 2, a food reward was placed in one arm with a baiting in the maze's center, and the animal was placed in the start arm to locate the arm with the reward, and the system recorded the time it took the rat to consume all of the prizes, as well as the number of errors made (entries into an arm already visited). The amount of time it took to accomplish the work and the number of errors were the most important data points (Chang *et al*., 2017). After each test, 70% alcohol was used to disinfect the test apparatus to eradicate the smell of urine and faeces from previous test subjects.

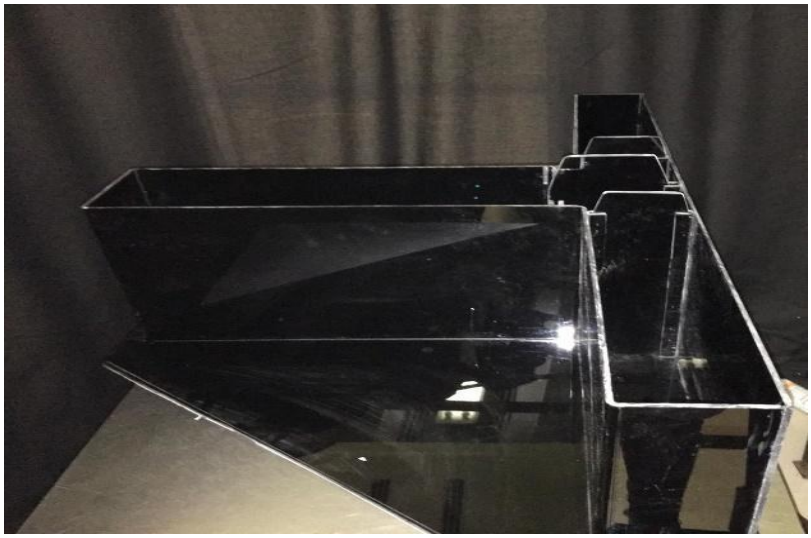


Figure 3. 17**. T-Maze** showing the characteristic three arms, made of black Perspex material.

b. Results

During the assessment on the T-maze apparatus, the number used for the maternal cadmium-exposed group was 23 and the maternal saline-exposed group was 21.

Time taken to complete task

The time to complete the task on the T-maze for **Day 1** in the cadmium-exposed group was 44secs±4.02 and the saline-exposed group was 25.99secs±3.36, with p = 0.0013 which is statistically significant; while on **Day 2** the cadmium-exposed group took 25.70sesc±3.9 and the saline-exposed group took 29.54secs±5.2, we found p = 0.556, which led us to conclude no significance in the differences.

Number of Errors while completing task

The number of Errors in trying to complete the task for each day was reported for **Day 1**, where the cadmium-exposed group made 0.74±0.14errors, and the saline-exposed group made 0.52±0.13 errors. No statistically significant difference was detected (p = 0.274); and reported also for **Day 2**, where the cadmium-exposed offspring made errors amounting to 0.74±0.18 and the saline-exposed group made errors amounting to 0.67±0.16. We found no significance in these differences from Day 2 as well (p = 0.764).

Distance travelled during the test

The distance covered by the groups during the T-maze assessment were found on Day 1 to be such that the cadmium-exposed group had travelled 2.21m±0.20 and the saline-exposed group, 1.98m±0.23, with no significance in the difference noted as p = 0.163. On Day 2, the cadmium-exposed group travelled 1.50m±0.16 and saline group travelled 1.33m±0.17. No significant was found statistically as p = 0.47.

Speed of task completion

The mean speed per group with which the task was completed was noted on Day 1 with the cadmium-exposed progeny moving at 0.056m/s±0.0038 while the saline-exposed group moved at 0.085m/s±0.015. We evaluated the differences and discovered p = 0.07, indicating that the different speeds have no statistical significance. Day 2 saw the cadmium-exposed group

moving 0.098m/s±0.023 and the saline-exposed group at 0.062m/s±0.0063. Statistically there was no significant difference between the two groups' speeds on Day 2 either ($p = 0.145$).

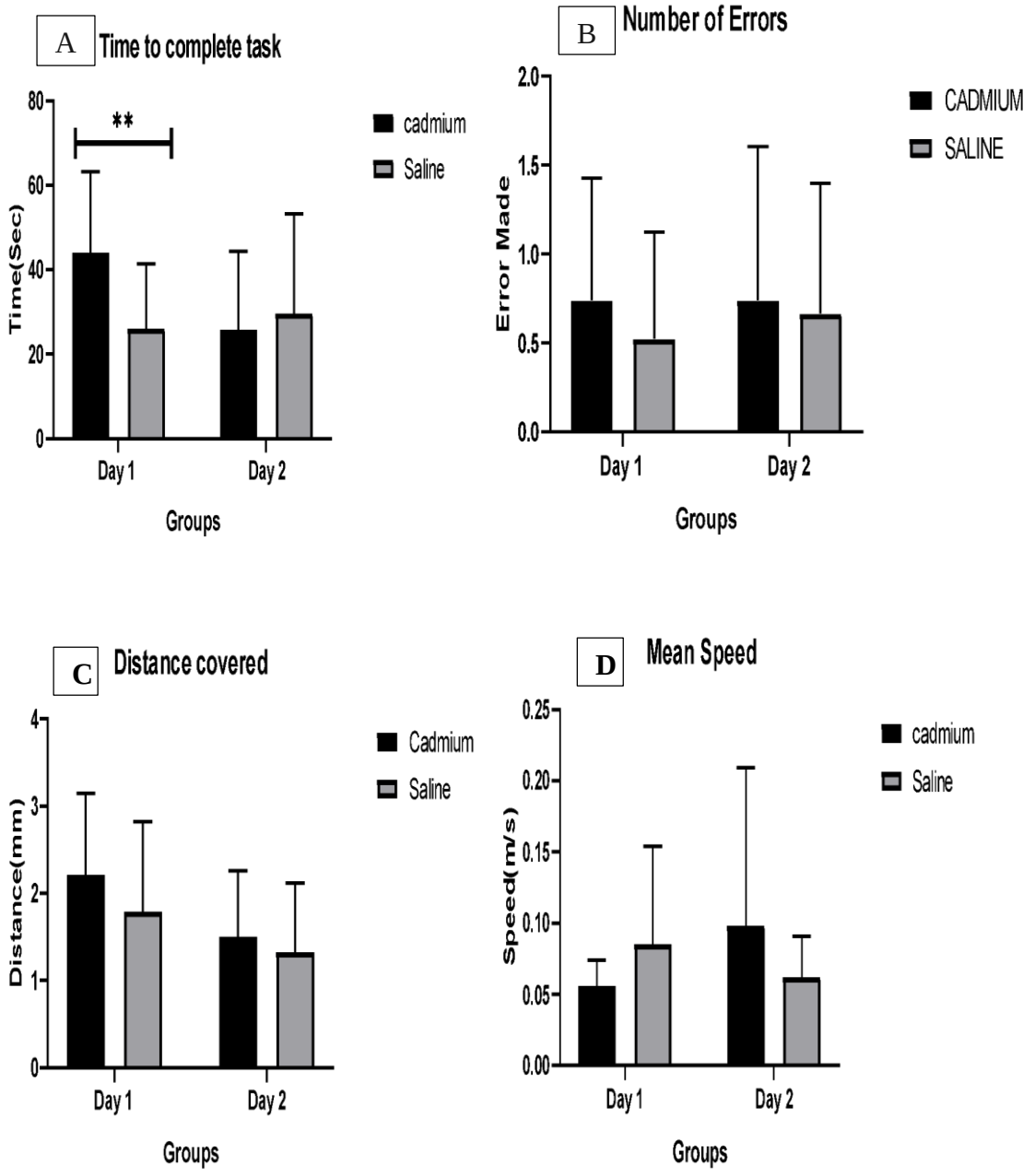


Figure 3. 18**.** Results from the T-maze test illustrating:

A. the time taken to complete task; (Cd- exposed v/s SAL; **(Day 1 p value = 0.0013), Day 2 p value = 0.556, n = 44). **B**. number of errors made during the task; (Cd- exposed v/s SAL; p -value for Day 1 p = 0.274, Day 2 p = value = 0.764, n = 44). **C**. the distance travelled within the task;(Cd- exposed v/s SAL; Day 1 p value = 0.163, Day 2 p value = 0.47, n = 44) and **D**. the mean speed at which each group moved during execution of the task; (Cd- exposed v/s SAL; Day 1 p value = 0.07, Day 2 p value = 0.145, n = 44).

c. Discussion

The T-maze was used for memory (spatial memory, which is defined as information that is only useful to a rat during the current experience with the task, and reference memory defined as information that is useful across all exposures to the task on the day of testing) and repetitive behaviour. During the T-maze task, the errors made, which indicate excess repetitive behaviour with the animal entering an arm already visited, the cadmium-exposed group exhibited more of the errors, an indication that they had more repetitive behaviour tendencies as compared to the saline-exposed group(Moy *et al*., 2008; Ueno *et al*., 2020).

viii. The Marble Burying Test

This test was carried out at 6 weeks of age on the rat pups to assess repetitive behaviours between the groups (Takeuchi *et al*., 2002; Andersen *et al*., 2010; Schwartzer *et al*., 2013).

a. Materials and Methods

A standard cage of 41x28.2x15.3cm (L x B x H) was filled with a 5 cm depth of wood chip bedding, and 10 marbles evenly spaced upon the bedding. Rats were placed individually for thirty minutes in the standard cage with marbles. After thirty minutes, the number of marbles buried was observed and recorded. In this process, a marble was considered buried if 2/3 of it were covered with bedding. The number of marbles buried was noted at the end of the experiment.

Figure 3. 19**.** Marble Burying setup, showing an individual undergoing the test.

b. Results

The number of marbles buried during the marble burying assessment were observed and found to be as follows: the cadmium-exposed group had buried 6±0.29 marbles; and saline-exposed group had buried 3.57±0.58. The differences between the groups were found to be statistically significant, $p = 0.0004$.

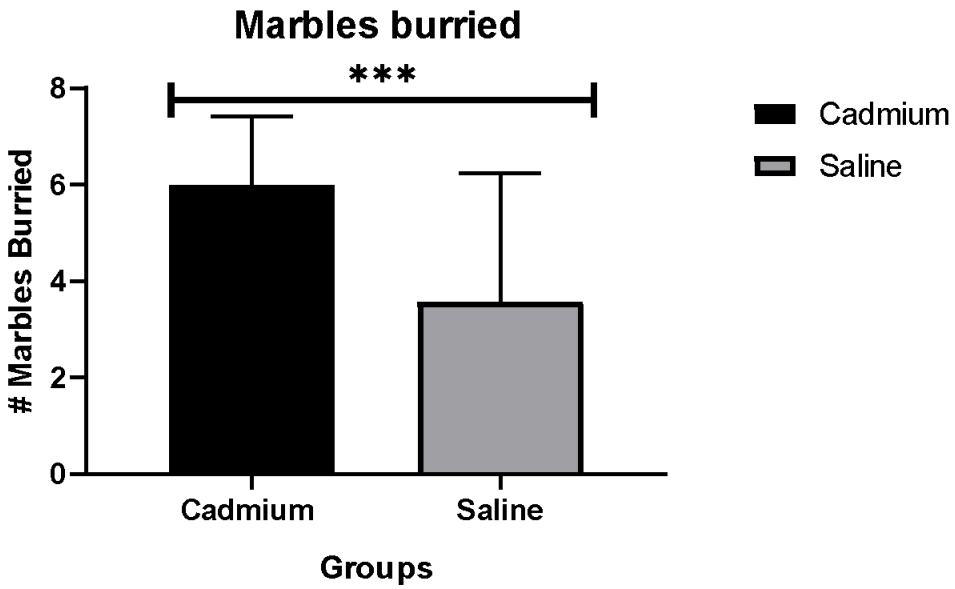


Figure 3. 20**.** Marble burying test results showing that the cadmium-exposed pups buried more marbles than the saline-exposed pups.

***($CaCl_2$ v/s SAL, p-value =0.0004, n=44)

c. Discussion

Repetitive or stereotyped kinds of behaviour are common with ASD patients. We assessed the extent of marble burying in both the cadmium-exposed group and the control group, we observed that the cadmium-exposed group buried significantly more marbles than the control group (Figure 3.20). The burying of marbles has been developed as a type of tool to assess repetitive behaviour(Thomas *et al*., 2009), therefore our findings suggest that as expected for autistic subjects, the $CaCl_2$ group progeny displayed a behaviour consistent with human autistic tendencies.

ix. The olfactory habituation test

This test was used to investigate social interactions. Olfactory cues have been found to play a critical role in social communication in many animal species, not the least of which are rodents. Compounds excreted in urine or by scent glands have been shown to modulate social and sexual behaviours (Beynon and Hurst, 2004; Arakawa *et al*., 2008). Olfactory habituation responses toward social odours and non-social odours have been used extensively to assess olfactory communication (Kas *et al*., 2014) in various autism rat models.

a. Materials and Methods

Rat pups were subjected to three repeated presentations of two odour samples: Banana extract, and urine from opposite sex. This process thus included presentation of both non-social odours (banana) and social odours (opposite sex urine). Each subject was placed in the testing room while in its own home cage with the lid close. The animal was allowed to acclimatize to the room for 30 mins. While in the home cage, each subject was then presented with two the odour samples, one at a time for 2mins each, this was repeated 3times for each odour. The number of sniffs and duration of sniffs were used to quantify olfactory response. This was noted as a subject pup placing its nose within 2 cm of an olfactory cue tip.

b. Results

During the Olfactory Habituation test, we counted the number of sniffs each rat gave to an odour, of which the odours consisted of both social and non-social odours. Two sets of odours were presented to each animal, with each set comprising of a pair of both a social and a non-social odour. Each of the three presentation of social/non-social odour pairs will be referred to as odour set 1,2 and 3.

Number of sniffs observed for each odour

For odour set 1 among the cadmium-exposed group and the saline-exposed group, the number of sniffs between the cadmium-exposed group with the social odour 1 and non-social odour 1was observed. The social odour 1 solicited 8.913±0.6409 sniffs and the non-social odour 1 received 5.304±0,7988 sniffs, a comparison between the social v/s non-social odour in cadmium-exposed pups was found to be significant (p =0.0010). For the saline-exposed group, social odour 1 received 6.905±0.5602 sniffs and the non-social odour 1 had received 5.810±0.6040 sniffs. This comparison did not yield a statistically significant difference between the odours (p = 0.1912).

For odour set 2, the cadmium-exposed group gave the second social odour 4.304±0.6012 sniffs, and the non-social odour 2 was given 4.000±0.6318 with no statistical significance p = 0.7288 between the odours. The saline-exposed group gave social odour 2 a number of 4.238±0.6283sniffs and the non-social odour 2 was given 3.762±0.5811. Upon comparison and analysis, we found that there was no statistical difference between the two odours' sniffing patterns from the saline -exposed rats (p = 0.5810).

Odour set 3 saw the cadmium-exposed group's social odour given 2.696±0.4847 sniffs and the non-social odour 3 had 2.130±0.6328 sniffs, with no statistical significance p = 0.3340 between the odours; while the saline-exposed group's social odour 3 had 3.333±0.4748 sniffs and the non-social odour 3 had 3.286±0.6139, with no statistical difference seen (p = 0.9514).

Relationship between the first and the third sniffs among cadmium and saline exposed groups.

a) the cadmium-exposed group at encounter with social odour 1 had 8.913secs±0.6409 sniffs and at social odour 3 had 2.696secs±0.4847 sniffs, with statistical significance between the two sets of social odours (1 and 3) (p = <0.0001). The saline-exposed group

at its encounter with social odour 1 had 6.905secs±0.5602 sniffs and at social odour 3 had 3.333secs±0.4748, the difference between the sniff numbers between the two social odours (from sets 1 and 3) was significant (p = <0.0001).

b) for the non-social odours, the cadmium-exposed group encountered non-social odour 1 and gave it 5.304secs±0.7988 sniffs and gave non-social odour 3 the lesser number of 2.130±0.6328 sniffs, this difference was found to be statistically significant (p = 0.0032). The saline-exposed group interacted with non-social odour 1 and gave it 5.810±0.6040 sniffs, and the non-social odour 3 was given 3.286±0.6139 sniffs. Differences between the two non-social odours (from sets 1 and 3) with respect to the control group was concluded to be significant (p = 0.0056).

Sniffs relationship between social and non-social odour cadmium and saline exposed group.

a) Social odour 1 showed the cadmium-exposed group had 8.913±0.6409 sniffs and social odour 1 for saline-exposed group had 6.905±0.5602 sniffs. We found that the cadmium v/s saline exposed groups' sniffs of social odour 1 had a statistical significance when compared (p = 0.024).

b) For the non-Social odour 1, the cadmium-exposed group sniffed 5.304±0.7988 times, and non-social odour 1 for the saline-exposed group was sniffed 5.810±0.6040 times, with no statistical significance when we compared the two (p = 0.6217).

The sniff durations in completing the task during the olfactory habituation test

a) The cadmium-exposed group interaction with social odour 1, took 3.357secs±0.2622 and the interaction time with non-social odour 1 was 2.102secs±0.3396 with p = 0.0054, indicating a statistically significant difference between the social and non-social interaction time in this group. While interacting with social odour 1, the saline group took 2.218secs±0.1256 and for non-social odour 1 the time elapsed was 1.850secs±0.2059, the difference of which was found to not carry statistical significance (p = 0.1347).

b) For social odour 2, the cadmium-exposed group spent 1.630secs±0.2217 on it, and the non-social odour 2 time elapsed was 1.543secs±0.2711 with no statistical significance (p = 0.8070) seen between the second set of social v/s non-social odours in the cadmium-exposed pups. The saline-exposed group while sniffing social odour 2, spent 1.357secs±0.2148 and with the non-social odour 2 the time spent was 1.304secs±0.2048. When comparing the two measures for saline offspring, it was found

that there was no significant difference between the second set of social v/s non-social odours *(p* = 0.8595).

c) For time elapsed during set 3 of the odours, the cadmium-exposed group's social odour 3 time spent was 1.630secs±0.2217 and that of the non-social odour 3 was 0.9609secs±0.2972, with no statistical significance (*p* = 0.4921) found. Considering the saline-exposed group, social odour 3 time spent was 1.090secs±0.1487, while the non-social odour 3 time spent was 1.173secs±0.2319, we found no statistical significance here *(p* = 0.7638)
d) Between the first and third sniff time, the cadmium-exposed group's social odour 1 time was 3.357secs±0.2622 and the social odour 3 time was 1.230secs±0.2492, which yielded a statistical significance when the two were compared (*p* = <0.0001). for the saline-exposed group, social odour 1 time spent was 2.218secs±0.1256 and social odour 3 time spent was 1.090secs±0.1487. When comparing these two measures we did find a statistically significant difference between the social odour 1and 3 in the saline-exposed group's time spent with the odours *(p* = <0.0001).
e) The cadmium-exposed group's Non-social odour 1 time was 2.102secs±0.3396 and the non-social odour 3 time was 0.9609secs±0.2972 which was statistically significant (*p* = 0.0151). The saline group's non-social odour 1 time was 1.850secs±0.2059 and the non-social odour 3 time was 1.173secs±0.2319 and *p* = 0.0349 which is significant statistically.

Time performance between Cadmium and Saline group are.

a) Social odour 1 time for the cadmium-exposed was 3.357secs±0.2622 and social odour 1 time for saline-exposed offspring was 2.218secs±0.1256, a difference which was found to be statistically significant (*p* = 0.00054).
b) Non-Social odour 1 time for the cadmium-exposed offspring was 2.10secs2±0.3396 and non-social odour 1 time for the saline-exposed offspring was 1.850secs±0.2059, and no statistical significance was found in these differences of non-social odour 1's between the two groups (*p* = 0.5390).

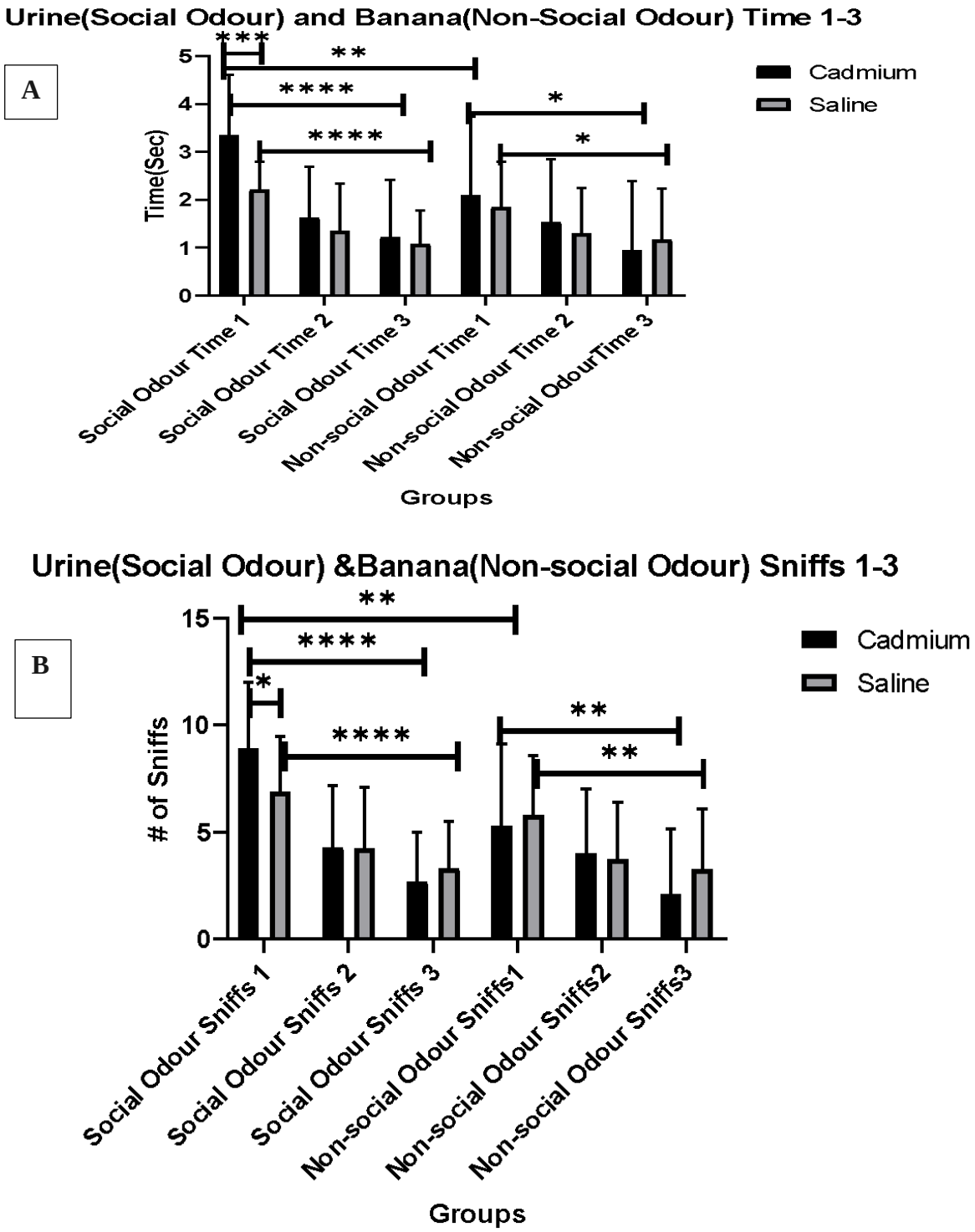


Figure 3. 21**.** Olfactory cues graphical illustration showing the performance between the Cd-exposed progeny and the Saline-exposed.

A. is an illustration of the duration(time) of sniffs between the Cd-exposed v/s SAL; **B**. illustrates the number of sniffs between the Cd-exposed v/s SAL.

c. Discussion

Olfactory habituation is used to assess sensory functions in rodents. We presented our rats (cadmium-exposed/preeclamptic and control offspring) with olfactory cues in banana extract and urine from the opposite sex. The preeclamptic and control offspring groups both spent a longer time with the social odours (urine from the opposite sex) when presented to them, we also observed that with each presentation of an odour, the time they spent with it was reduced. However, the initial presentation of the social odour to the cadmium-exposed progeny got them to be very active and excitable, a phenomenon not observed in control group. Conversely, upon the first presentation of the non-social odour, the control group became more active as compared to the cadmium-exposed group which had a delay of sniffing odours after the first presentation both for the social and non-social odours. This demonstrated a lack of interest in the cadmium group, where typically developing peers showed a clear interest. This tendency in cadmium-exposed pups is commonly observed in autistic individuals, and known as insisted of sameness or rigidity of interest.

x. Hole Board Test

This test was used to measure exploration in rodents, deduced from head dipping into the small holes at the bottom of the apparatus (Deacon *et al*., 2012).

a. Materials and Methods

This test was done on postnatal day 46, The box is made of a white wooden wall with 12 peripheral holes in the floorboard of 15-20mm diameter. The animal was placed in a corner of the box and allowed to explore it for 3mins. A head dip was recorded when the animal's head and ear entered the hole, but sniffing and small dips were excluded from the counts. We also noted the number of rearing presses done in the course of the test for each rat.

Figure 3. 22**.** Hole Board test showing the apparatus with holes in the periphery and centre of the board.

A rat has already been placed in the apparatus and is undergoing assessment.

b. Results

During the hole-board assessment, the number of head dips into each of the holes was summarized as follows:

Hole 1; the cadmium-exposed group had 2.13±0.2517 head dips, and the saline group had 1.17±0.27 head dips, no significance statistically with p=0.266, was found between the groups concerning Hole 1.

Hole 2; the cadmium-exposed group had 1.30±0.23 dips while the saline group had 1.57±0.24 head dips, we found no significance statistically with p=0.43.

Hole 3; the cadmium-exposed group had 1.39±0.17 head dips while the saline group had 1.42±0.28 dips with p=0.910, we concluded no statistical significance.

Hole 4; the cadmium-exposed group had 1.83±0.29 dips while the saline group had 1.57±0.23 with p=0.50 which is not statistically significant.

The number of Rearing presses during the hole-board test was noted, with the cadmium-exposed group having 11.43±0.92 presses, and the saline group having 7.71±0.73 presses. We compared the two and found that p=0.0030, and therefore concluded statistical significance.

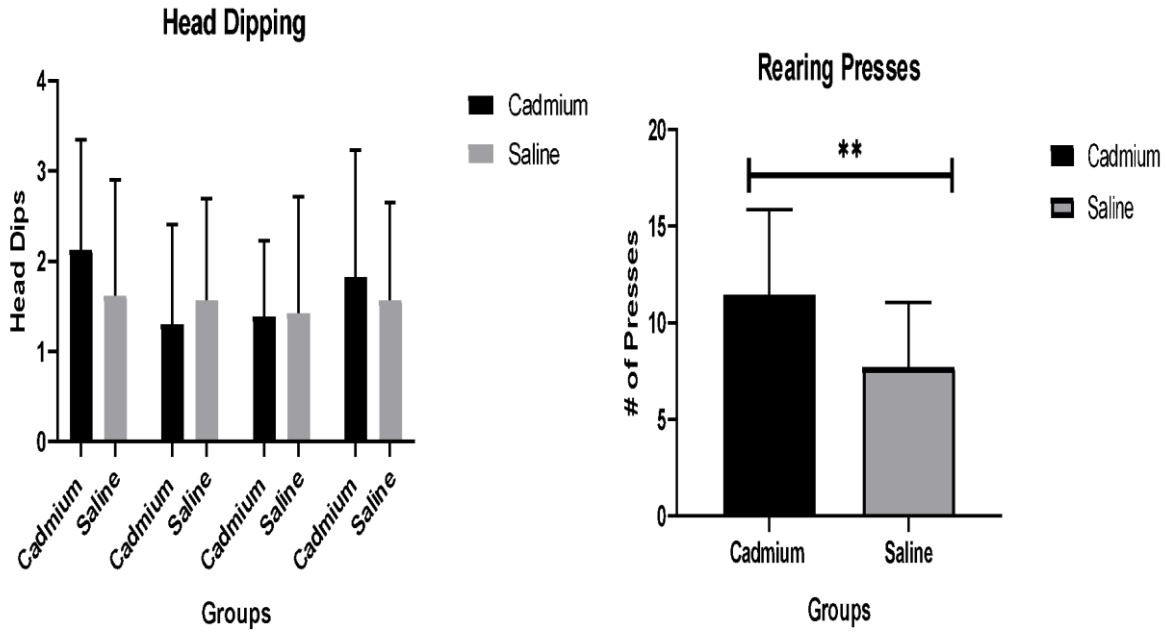


Figure 3. 23**.** Hole board graphical analysis between the cadmium-exposed and saline offspring.

A. shows amount of head dipping between the two groups (PE v/s SAL, hole 1 p =0.266; PE v/s SAL, hole 2 p =0.43; PE v/s SAL, hole 3 p =0.910; PE v/s SAL, hole 4 p =0.50;), **B**. shows number of rearing presses observed for each group **(PE v/s SAL, p = 0.0030).

c. Discussion

The hole-board test was used for exploration in rodents. During this test, it was observed that the rats had high preference for food reward at the periphery of the open field, and they avoided the centre space, an indication of increased anxiety. They explored the holes next to the walls, and we observed also that the cadmium-exposed had more of the supported vertical movement (rearing)trying to assess their environment for danger than the control group. Both the behaviours of rearing and remaining at the periphery of the field, are indicative of heightened levels of anxiety in the cadmium- exposed group.

3.3. DISCUSSION

One of the objectives of this research was the assessment of autistic features (cognitive, social and emotional behaviours) using standard behavioural phenotyping in pups from preeclamptic versus normotensive pregnancies. An array of behavioural tests was done on the Sprague Dawley rat pups beginning from Postnatal Day (PND) 24- 56. These included the Balance Beam Motor test, Open field for exploratory activities, elevated plus maze to name a few. The marble burying test shows a phenotype of repetitive behaviour in rodents and also their digging nature. Out of the ten tests conducted, the cadmium-exposed pups showed some positive effects, i.e., demonstrable autistic behaviour (e.g., anxiety, repetitive behaviour, restricted interest etc.). The T-maze and Y-maze used for memory assessment should not be a stand-alone test, as we learned from our study that the rat pups experienced difficulty in initiating or understanding the task before them. The results are discussed under the following sub-headings.

-Gross Motor Coordination and skills: Balance Beam, Open Field (line crossing, rearing)

-Anxiety: Open field, Light –Dark Box, Elevated Plus Maze

-Cognition, Spatial Working Memory: Novel Object, T-Maze, Y-Maze

-Repetitive Behaviour: Hole-Board Test, Marble Burying

-Sensory Function: Olfactory Habituation test

Gross Motor Coordination and Skills

Motor performance was examined through implementation of a balance beam motor task. The balance beam test revealed that intrauterine exposure to preeclampsia affects motor coordination, in that our offspring previously exposed to preeclampsia had compromised motor abilities when undergoing this test. This finding is consistent with observations in the study of Biscaldi et al. (2014), and that of Fournier et al. found in 2010; who both found this phenomenon in human autistic individuals The balance beam test allows greater observation of impaired subtle motor coordination and/or sensorimotor function (Carter *et al*., 2001). Postural instability or altered gait within the cadmium induced pregnancy offspring may be a reflection of poor motor coordination, both traits are commonly observed within population of humans with ASD (Bhat *et al*., 2011). This delay in motor coordination can be seen in a child's early life (Ketcheson *et al*., 2018), and can be associated with the defining core symptoms of autism identified by the diagnostic and statistical manual of mental disorders/DSM-5 (American Psychiatric Association, 2013). For instance, gross motor skills have been found to be linked with differences in social communication (Colombo-Dougovito and Reeve, 2017). Hence, it was anticipated that the intrauterine exposure of rat pups to cadmium chloride ($CdCl_2$) and subsequent maternal preeclampsia, would result in decreased coordination, which would be reflected by a greater traverse time on the rod. The cadmium exposed group spent more time on the platform before traversing the rod, an indication of a poorer ability for motor planning. This finding is reminiscent of those in human infant with autism, which show some level of developmental motor delays (Bhat *et al*., 2011; Ketcheson *et al*., 2018).

Anxiety

Autism is characterized by social withdrawal or awkwardness and a feeling of fear, worry and unease. Abnormal reactions such as "excessive fearfulness in response to harmless objects" are included as associated features of autistic disorder in the DSM-V (American Psychiatric Association, 2013; Sarkar, 2020)**.** Higher levels of anxiety have also been found to be associated with high levels of repetitive restricted behaviours (RRB) in children with ASD (Sukhodolsky *et al*., 2008). According to Joosten *et al*. (2009), children with the disorder may use their repetitive behaviours as a means of reducing anxiety; while Sofronoff *et al*. (2005) is of the opinion that RRB may be a consequence of anxiety. The maternal cadmium exposed group spent more time in the centre zone of the open field apparatus, suggesting that they were less anxious as compared to the control group; which is in line with

(Royce, 1977), that animals which spent most of the time in the field's central part must be considered as less-fearful or less-anxious rather than those which prefer perimeter area. Exploratory activity was reduced in all the experiments done to assess for anxiety, especially in the elevated plus maze, the animals spent equal time in the closed arms (Bailey and Crawley, 2009). The avoidance of the open arm zone in the elevated plus maze indicates the unconditioned fear of height and open space (Walf and Frye, 2007). Risk assessment in the Light-Dark Box (LDB) is also a means of accessing anxiety-like related behaviour in rats, this risk assessment includes a stretch-attend posture in which the head and fore-paws extend into the lighted area, but the rest of the body stays in the dark compartment (Karlsson *et al*., 2005), which was observed with the cadmium- exposed rats during the LDB test.

Cognition

Autistic individuals are thought to have a specific form of cognitive strengths and weaknesses which may include difficulties appreciating others' thoughts and feelings (a concept known as "theory of mind"), problems regulating and controlling their behaviour (executive function), and an enhanced ability to perceive details (Pellicano, 2010). Social communication and interaction difficulties are presumed to be underpinned by alterations in social cognition (SC) (Happé *et al*., 2017), referring to mental processes relevant for the understanding of agents and their interactions including the self. During typical development, an indirect processing of social information is present at an early age. Indirect SC is characterised as an unconscious, and automatic process without deliberate reflection. Later in life, with cognitive and linguistic development, an explicit form of SC, based on deliberate, verbal, rational and conscious consideration of mental states takes form (Heyes and Frith, 2014; Happé *et al*., 2017).

Theory of mind: Theory of mind (ToM) is defined as the cognitive ability to attribute mental states such as thoughts, beliefs, and one's intentions to other people. It implies an awareness that others have minds with mental states, information, and motivations that may differ from one's own, allowing an individual to explain, manipulate, and predict behaviour (Korkmaz, 2011; Miranda *et al*., 2017; Berenguer *et al*., 2018). ToM is a complex cognitive ability which functions by understanding the other person's perspective, differentiating between the mother's face and unfamiliar faces and recognizing facial emotions, all these are important in the development of perceptual components of ToM (Heyes and Frith, 2014).

Executive function: The "executive functions (EF)" encompass a broad spectrum of inter-related processes that are in charge of "guiding, directing, and controlling cognitive, emotional, and behavioural functions, especially during the active solution of new problems (Gioia *et al*., 2000). According to Johnston *et al*. (2019), executive function has been found to be impaired across a number of psychiatric and developmental disorders (response inhibition), and also impairment in performance of EF tasks may be a consequence of slowed processing speed. Studies have reported difficulties in timed tasks of executive function due to difficulties with initiation and psychomotor speed (Hill and Bird, 2006) and a subsequently slow and accurate response style in individuals with autism which was also observed during our analysis on T-Maze, the pups where slow with speed and decision on which arm to enter. In the Y-maze the animals had difficulty in alternating their movement as a result of difficulty in initiation (Johnston *et al*., 2019).

Repetitive behaviour

Repetitive behaviour is a core symptom of autism and comprises stereotyped responses, restricted interests, and resistance to change (Moy *et al*., 2008). We investigated the repetitive behaviour in Sprague Dawley pups using the hole board and marble burying tests. There are many forms of repetitive behaviours in rodents such as jumping, grooming, digging, backward flipping, or cage-top "twirling" and complex body movements (Angoa-Pérez *et al*., 2013). In humans, Turner (1999) grouped these repetitive behaviours into two classifications including lower-level repetitive behaviours, which are characterised by repetition of movement including stereotyped movements, self-injury, tardive dyskinesia, tics and repetitive manipulation of objects; and higher-level repetitive behaviours, which include circumscribed interests, obsessions, compulsions, rigid adherence to routines and rituals, insistence of sameness and abnormal attachments to objects. Other characteristics include perseveration, gesturing, spinning, finger flicking, rocking, posturing, and abnormal preoccupations (Bodfish *et al*., 2000; Angoa-Pérez *et al*., 2013). Turner is of the opinion that lower-level repetitive behaviours may not be exclusive to autism alone. Instead, they may be related to broader factors, such as level of cognitive ability or brain pathology, while the higher-level repetitive behaviours such as circumscribed interests may signify Pervasive Developmental Disorders (PDDs). However, some studies have indicated that the higher-level repetitive behaviours characteristic of autism may not become evident until a particular developmental level is achieved (Stone *et al*., 1999). According to Bodfish *et al*. (2000),

repetitive behaviour occurs in a wide range of neurodevelopmental disabilities such as mental retardation, psychiatric disorders such as schizophrenia, obsessive-compulsive disorder [OCD], and neurological conditions such as Parkinson disease, Sydenham chorea, or Tourette syndrome. Repetitive behaviour tends to co-occur in the autistic population both those with mental retardation as well as in those with high-functioning autistic behaviours or Asperger's Syndrome (Bodfish *et al*., 2000; Mooney *et al*., 2006). The marble burying test takes advantage of the tendency of rodents to dig in natural settings (*e.g.*, burrows, escape tunnels); while in the hole board test, the nose poking or head dipping tends to provide a direct measure of the exploratory behaviour (Moy *et al*., 2008). In the course of our study, both the cadmium-exposed and the saline-exposed rats had less head dipping during the hole-board test but had higher preference for the corner another sign of heightened anxiety. These findings were consistent with those reported by Moy *et al*. (2008). Our rats from both groups spent more time rearing (vertical activity) along the walls of the open field. In the marble burying activity, the cadmium-exposed rats expressed repetitive measures of digging consequently and the marbles were buried. However, Thomas *et al*. (2009) reported a view that marbles are buried once a rodent digs, which indicates that digging is necessarily part of burying for rodents anyway, and that it is therefore genetically influenced rather than atypical brain activity resulting in repetitive behaviour. These findings support the research of Kitaoka (1994) which states that digging in rodents has genetic background. Our findings suggest that the cadmium exposed progeny have a greater level of repetitive behaviour as they buried more marbles than their control counterparts, which is a trait common to autistic individuals. Even though a few authors have previously argued that digging is a natural trait for all rodents, we assert that all the rats in our study had this propensity to dig, therefore the presence of digging alone is not meaningful, however with our cadmium rats having dug significantly more than the controls, we are able to observe the digging as repetitive behaviours since our experimental group had a demonstrably higher level for this activity, i.e., was far more repetitive. The autism behaviour of repetitive tendencies can be anything that is done to a larger extent that typically developed counterparts, including the repetition of common human activities such as blinking and gesturing, we therefore assert that there is no reason that digging cannot be regarded as a repetitive behaviour.

Sensory function

Rodents are social creatures by nature, social transmission of food preference [STFP] in rodents has been patterned as part of the array for autistic behaviour in rodents (Crawley, 2007). Rodents depend majorly upon olfaction during typical social interactions. The olfactory cues enable them in looking for mate choice, distinguishing between individuals and determining health status (Arakawa *et al*., 2008). Anosmic rodents show abnormal aggressive, affiliative and social behaviour (Jakupovic *et al*., 2008). In rodents, there are two types of olfactory cues including the main olfactory system and the accessory olfactory system (Brennan and Kendrick, 2006). The accessory olfactory bulb processes non-volatile pheromones, whereas the main olfactory bulb primarily processes volatile odorants, and in rodents is responsive to social cues present in urine (Schaefer *et al*., 2001; Brennan and Kendrick, 2006). Both the accessory and main olfactory bulbs play a role in social behaviour (Schaefer *et al*., 2002; Jakupovic *et al*., 2008). Habituation is defined by a progressive decrease in olfactory investigation (sniffing) towards a repeated presentation of the same odour stimulus. Dishabituation is defined by a reinstatement of sniffing when a novel odour is presented (Woodley and Baum, 2003; Wersinger *et al*., 2007). In the course of this study, it was observed that the rats (both cadmium-exposed and saline –exposed rats) were more attracted and spent more time with the social odour that is the urine of the opposite sex than they did with the non-social odour (Banana scented nest) (Ryan *et al*., 2008). It was also noticed that the sniff duration reduced for each turn from the first to the third sniffs both for the social(Urine) and non-social(Banana) odours, a finding reminiscent of reports from Kepecs *et al*. (2006). The male and the female rats were driven more by the social odour, especially the males from the cadmium group which exhibited more of the vertical activity, i.e., grooming and some acrobatic display to show their satisfaction with the odours (Ryan *et al*., 2008). Based on the reaction we observed, and the time spent with each odour, we were confident that the animals were able to differentiate between odours (Wersinger *et al*., 2007).

CHAPTER 4: ACQUISITION OF BRAINS

4.1. INTRODUCTION

Forty-four, 6-week-old Sprague Dawley rat brains were used for this study. The animals were housed and handled according to the guidelines of the University of the Witwatersrand Animal Research Ethics Committee.

4.1.1. Permits and Ethical Issues

Permits and ethical clearance for the study were obtained from the University of the Witwatersrand Animal Research Ethics Committee (AREC) with number 2018/09/041/B**.** A copy of the ethics certificate is included as appendix A.

4.2 MATERIALS AND METHODS

4.2.1. Sacrifice and perfusion

Forty-four (44) animals aged 46 days old and weighing between 100- 150g were euthanized using 800 mg/kg of sodium pentobarbital injected intraperitoneally as described by (Zatroch *et al*., 2017). The rats underwent a trans-cardial perfusion with an ice cold 4% paraformaldehyde solution, preceded by cold phosphate buffered saline (PBS). A midline abdominal incision was made in each rat to expose the heart for the perfusion injections into it. Before the perfusion injections however, we collected additional tissues for future investigation related to the current work, such that blood was collected from each heart using 21 G needle mounted onto a 10mL syringe and transferred into heparinized blood tubes. The blood was centrifuged at 4000 x G for 15 minutes at 20^0C. Plasma was collected and placed into microtubes (Eppendorf, Hamburg Germany) and stored at -80^0C for later use.

4.2.2. Brain extraction

After successful perfusion, each rat was beheaded, and the soft tissue was dissected away from the skull. The skull bone was then opened using small ronjeurs. Bone was carefully removed in small pieces so as not to injure underlying brain tissue. Both bone and dura matter were dissected away to visualize the brain, which was carefully removed from the open skull.

4.2.3. Post-fixation treatment of the brain tissue

Following perfusion and extraction of brain tissues, the average brain weight for the saline group was 1.79g and that of the cadmium group was 1.75g. The brains were immersed in 4% paraformaldehyde overnight at 4^{o}C. Thereafter, the brains were stored in 30% sucrose for a few days or until they had sunk to the bottom of the container. They were then transferred to anti- freeze and stored at -80^{o}C.

4.3 DISCUSSION

The harvested brains were perfused and successfully fixed and stored appropriately following protocols that optimise the structural integrity for immunohistochemistry. The brain of the rat pups were collected and weighed after euthanizing. The average brain weight of the total (n = 44) brains collected was 1.77g.

CHAPTER 5: THE DELINEATION OF THE SEROTONERGIC NEURONAL SYSTEM

5.1 INTRODUCTION

5.1.1 Neuromodulatory System

Neuromodulation is the physiological process by which a specific neuron uses one or more chemicals to regulate diverse populations of neurons. These chemicals modulate and regulate the neuronal effect of other neurotransmitters, thereby causing direct excitatory or inhibitory effects on neurons (Aston-Jones and Cohen, 2005; Nadim and Bucher, 2014). Neuromodulators specifically bind to metabotropic, G-protein coupled receptors to initiate a second messenger signalling cascade that induces a broad, long-lasting signal. This modulation usually last from hundreds of milliseconds to several minutes (Nadim and Bucher, 2014). Effects of neuromodulators include altering intrinsic firing activity (DeRiemer *et al*., 1985), increase bursting activity, increase or decrease voltage dependent current, alter synaptic efficacy, (Harris-Warrick and Flamm, 1987) and synaptic connectivity reconfiguration (Klein and Kandel, 1980).

Neuromodulatory systems include; serotonergic, dopaminergic, noradrenergic, adrenergic, and cholinergic projections from the brainstem and basal forebrain regions to broad areas of the central nervous system (Briand *et al*., 2007). In the current investigation, we focused only on the serotonergic system.

5.1.2. The Serotonergic System in the Autistic Brain

Serotonin is synthesized from the essential amino-acid tryptophan. L-tryptophan is hydroxylated to 5-hydroxytryptophan(5-HTP) by the rate-limiting enzyme tryptophan hydroxylase which is subsequently decarboxylated by Aromatic-L-amino acid decarboxylase which is to be converted to serotonin eventually (Mulder, .2006). For millions of years,

serotonin (5-hydroxytryptamine, 5-HT) has existed as a signalling molecule across phylogeny (Hay-Schmidt, 2000).

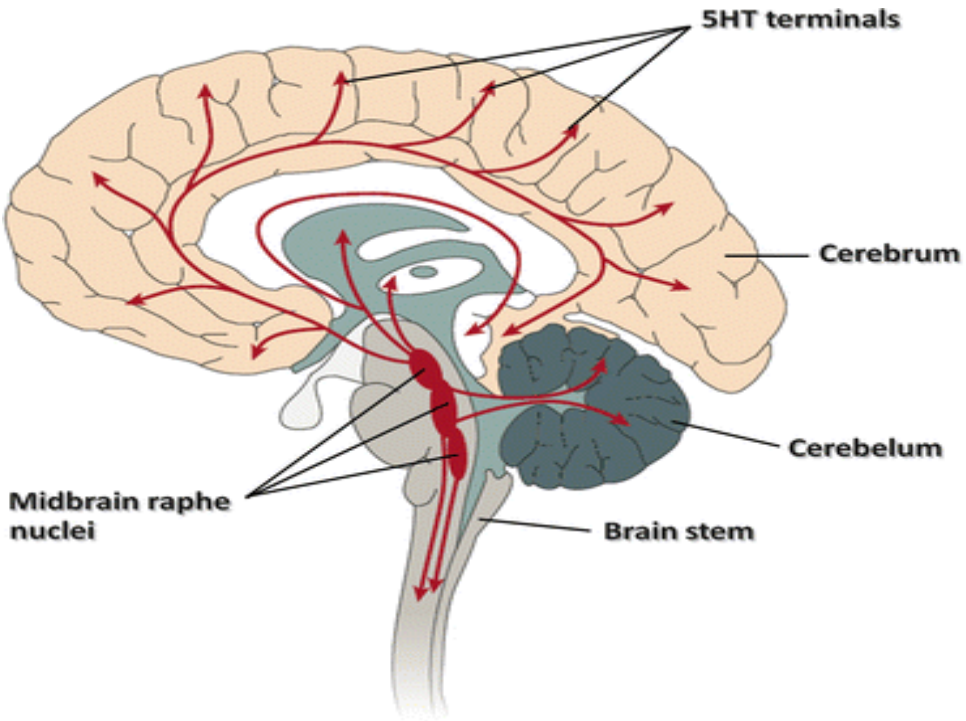


Figure 5. 1**.** Hyperserotonaemia in Autism: 5HT-Regulating proteins/springerlink

Tryptophan hydroxylase 1 (TPH1) and Tryptophan hydroxylase 2 (TPH2), are the two isoforms of tryptophan hydroxylase which are primarily responsible for 5-HT synthesis in the periphery and central nervous system (CNS) respectively (Lovenberg *et al*., 1967; Walther, 2003). Serotonin does not cross the blood brain barrier and, therefore, the brain's serotonin is exclusively synthesized in the central nervous system (CNS). It is catabolized there, by monoamine oxidase A and the product is secreted into blood (Mulder, 2006)

In the mammalian embryo, the serotonergic system is one of the most widely distributed, as well as the earliest to develop. The majority of neurons are located in the median and dorsal raphe nuclei (figure 5.1). They mainly provide fibres to the cortex first, and later to the hippocampus. The serotonergic system innervates almost all areas of the brain (figure 5.1), whereas serotonin presents in non-serotonergic cells, as well, where it acts as a developmental signal (Sodhi and Sanders-Bush, 2004). Serotonergic neurons can be detected in the human brain from the 5th gestational week (Sundström *et al*., 1993) and during the following months they grow and multiply rapidly.

The intense activity during the first stage of development as well as the early appearance of the serotonergic system, indicate its role in the developmental process. Serotonin is reported to influence neurogenesis and/or neuronal removal, neuronal differentiation, synaptogenesis etc (Whitaker-Azmitia, 2001). Serotonin holds an important role in dendritic development, including overall dendritic length, spine formation and branching in both the hippocampus and the cortex (Whitaker-Azmitia, 2001). In relationship to the cortex, the serotonergic system also regulates the development of the barrel fields, which constitute an area of the primary somatosensory cortex and demonstrate a transient expression of serotonin terminals (Whitaker-Azmitia, 2001). The early disturbance of the serotonergic system disrupts the developmental process and may contribute to several of the neuropathological changes seen in autism.

5.1.3 Neuroimaging Studies of Serotonin

There is vast a literature on Functional neuroimaging, into the synthesis as well as binding capacity of serotonin in individuals with autism. According to positron emission tomography (PET) studies, children aged 2 to 5 years normally undergo a period of increased serotonin synthesis capacity (200% of adult values), followed by a decline toward adult values between

the ages of 5 and 14 years. In contrast, patients with autism, aged 2 to 5 demonstrate a reduced capacity for synthesis, which, however, increases to reach and exceed adult's values by the age of 15 (Chugani *et al*., 1999). Therefore, the hyperserotonaemia, which probably appears in older patients, does not actually develop before an age when most diagnoses of autism are made (Gustafsson, 2004).

It has been indicated that the developmental pattern of serotonin synthesis in non-autistic children strongly resembles the profile of synaptic density in their frontal cortex, as demonstrated in PET and post-mortem studies respectively (Bethea and Sikich, 2007). The normal process of high brain serotonin synthesis and synaptogenesis during preschool years is highly disrupted in children with autism.

PET studies also revealed focal aberrations of serotonin synthesis with cortical asymmetry in serotonin uptake. Patients with reduced synthesis on the left side demonstrate high frequency of severe language impairment, whereas the decrease on the right side represents higher frequency of left- or mixed-handedness (Chandana *et al*., 2005).

5.1.4 Neuropathological studies of Serotonin

One of the major neuropathological findings in the autistic brain is the disruption of neocortical minicolumns (vertical columns through the cortical layers of the brain), which are the primary anatomical units with the lowest level of neocortical modularity. The disruption of minicolumns leads to aberrant cortical organization, clinically correlated with altered information processing and perhaps with the "hyperspecific" autistic brain. Minicolumns in autistic brains have been reported to be more numerous, but smaller and closely spaced, compared to controls. Furthermore, their constituent neurons are more dispersed, accounting for a normal cellular density, whereas the surrounding neuropil is significantly reduced (Casanova *et al*., 2002, 2003). The very detailed focusing, along with the failure to recognize broader contexts of information are probably some of the functional consequences of narrow minicolumns (Bethea and Sikich, 2007).

Another well replicated finding in autism is the alteration of the amygdala, affecting its volume, cell packing density and activation during processing (Haznedar *et al*., 2000; Stanfield *et al*., 2008). The central nucleus of the amygdala receives intense innervation from serotonergic dorsal raphe neurons. The amygdala is considered to play a significant role in

autism, mainly through its involvement in the developmental deficits in emotion processing and, generally, in social perception (Schultz, 2005).

5.2 MATERIALS AND METHODS

Immunohistochemistry

Brain sections were pre-treated at room temperature for 30mins under gentle shaking with an endogenous peroxidase inhibitor (containing 100% methanol, 0.1M PB, 30% H_2O_2) followed by three 10-minute rinses in 0.1M PB. The sections were then incubated for 2 hours in a blocking buffer (containing 0.25% Triton-X, 3% normal goat/ rabbit serum, 2% bovine serum albumin, in 0.1 M PB) under gentle shaking at room temperature. The sections then were transferred into a primary antibody solution containing the appropriately diluted antibody for 48 hours at 4°C under gentle shaking. To reveal serotonergic neurons, we used anti-serotonin (ab66047/Abcam raised in goat) at a dilution of 1:5000. After the 48-hour incubation period, sections were subjected to three 10-minute rinses in 0.1M PB before being incubated in a secondary antibody solution. This solution contained a 1:500 dilution of biotinylated anti-goat IgG (BA-1000, Vector Labs), in (3% normal rabbit serum, 2% bovine serum albumin, plus appropriate dilution of the secondary antibody, in 0.1 M PB) for 2 hours at room temperature under gentle shaking. Following that were three 10 min rinses in 0.1M PB, the sections were thereafter incubated for an hour in AB solution (Vector Labs). The sections were then transferred into three 10min 0.1M PB rinses before being placed in a solution containing 0.05% of 3.3 di-amino-benzidine (DAB) in 0.1M PB solution for 5 min. To each 1ml of solution, 3.3 µl of 30% H_2O_2 was added, and chromatic precipitation was visually monitored under a low power stereomicroscope until the desired background staining was reached. The precipitation process was stopped by placing the sections in 0.1PB and rinsed twice more in the same solution. Sections were mounted on glass slides coated with 0.5% gelatine and left to dry overnight, after which the slides were dehydrated in a graded series of alcohols (70%, 95%, 100%, 100%) and cleared twice in xylene before being cover slipped with Depex as the mounting solution. A low power stereomicroscope was used to examine the sections,

Nissl

The slides were placed first in a solution of equal quantities of chloroform and alcohol (DEFAT) overnight; next they were rehydrated by placing them into a series of alcohol baths of decreasing concentrations for a specified time in each. They were then submerged and agitated in Cresyl Violet for 1-2 minutes, followed by distilled water for a further 1-2 minutes to wash off any excess dye. To achieve the desire level of staining, the sections were monitored under a low power stereomicroscope until optimal staining was obtained. The dehydration process was done thereafter, submerging the slides in a series of alcohol baths of increasing the concentrations. They were then cleared twice in xylene and cover slipped using Depex, then left to dry.

Myelin

The sections used for myelin staining were refrigerated for 2 weeks in a 5% formalin solution before being mounted on 1.5% gelatine coated slides (Gallyas, 1979; Limacher-Burrell *et al*., 2016). The myelin series of slides was stained with a modified silver stain following the protocols outlined in Gallyas (1979) and Limacher-Burrell *et al*. (2016)

5.2.1. Abbreviations

3N – oculomotor Nucleus

4N – Trochlear Nucleus

5HT - 5-hydroxytryptamine

7N – Facial Nucleus

ASD – Autism Spectrum Disorder

B9 - supralemniscal nucleus

BC – brachium conjuctivum

BMI – Body Mass index

$CdCl_2$ – Cadmium Chloride

CLi - Caudal Linear group

CVL - Caudal Ventrolateral nuclei

DD – Developmental Delay

DRD – Dorsal Raphe Nucleus

DRif - Dorsal Raphe Nucleus interfasciculus

DRI – Dorsal Raphe Nucleus Lateral Part

DRV – Dorsal Raphe Nucleus Ventral Part

DSM-5 - Diagnostic and Statistical manual of Mental disorders

EF – Executive Function

EPM- Elevated Plus Maze

ID – Intellectual Delay

IO – Inferior olives

LDB- Light-Dark Box

Mlf – Medial Longitudinal Fasiculus

MnR – Median Raphe Nucleus

PE – Preeclampsia

PET - Positron Emission Tomography

PMnR - Paramedian raphe nucleus

PND – Postnatal day

RMg – Raphe magnus nucleus

ROb - Raphe Obscurus nucleus

RPa – Raphe Pallidus nucleus

RtTg – Reticulotegmental Nucleus of the pons

RtTgP – Reticulotegmental Nucleus of the pons pericentral part

RVL – Rostral Ventrolateral nuclei

SAL – Saline

SC – Social Cognition

Scp – Superior cerebellar peduncle

SSRI - Selective Serotonin Reuptake Inhibitor

ToM – Theory of Mind

5.3 Results

We conducted immunohistochemical labelling in order to illuminate the serotonergic cell system within the brains of both our experiment and control groups. Serotonin containing neurons were found located throughout the brainstem. The serotonin positive cells were described using the atlas of Dahlström and Fuxe (1964) alphanumeric grouping B1-B9. The density has been divided into three categories(a); low density (b); medium density (c); high density. In the main, the nuclei were located at the midline, spanning the entire brainstem from the level of the superior cerebellar peduncles rostrally, to the spinomedullary junction caudally.

Our study revealed serotonin immunopositive (5-HT+) cell bodies located at the same area in both groups, i.e. in the midline or “raphe” region of the brainstem. We noted in addition that the intensity of the cell bodies appeared higher in the cadmium treated group than in the saline treated group. For ease of reference, we have divided the raphe nuclei into two previously described subgroups under two broad clusters, the rostral and the caudal clusters. There were no noticeable aspects (size, shape, density or fibre orientation) that were different between the two studied groups of offspring.

5.3.1: The rostral cluster

The rostral cluster is located in the upper portion of the brainstem, i.e., the midbrain and the pons, providing ascending serotonergic innervation to the forebrain and midbrain. This cluster comprises of the principal (and more caudal) subdivision of the dorsal raphe nucleus DR/B7 and CDR/B6, the central superior raphe nucleus which is made up of the caudal linear nucleus more rostrally, then the median raphe nucleus caudal to that (CLi + MnR/B8), the supralemniscal raphe nucleus (Sul/B9) and the pontine raphe nucleus(PnR/B5).

a. CLi (Caudal Linear group)

This group was identified most rostrally to all other serotonergic groups, ventrally along the midline, and contained a high density of neurons that were spherically shaped and multipolar. The nucleus was located along the raphe/midline region, and anterior the decussation of the superior cerebellar peduncles (brachium conjunctivum).

b. B9 (Supralemniscal nucleus)

This nucleus was identified anteriorly in the midbrain, with cell bodies spanning laterally from the CLi nucleus, forming a low density cluster. They were localized around the lemniscus medialis(ml) as shown in fig. 5.2E, with somata appearing round to fusiform, and fibres oriented in a mediolateral direction. The supralemniscal nucleus formed a lateral extension of the median raphe and part of the ventrolateral tegmentum of the pons and midbrain.

c. Median Raphe (MnR)

The density of serotonin immunopositive cells was high in this nucleus and each cell body was intensely stained. We observed two distinct columns on either side of the midline, therefore called “pararaphe columns” in the midbrain tegmentum. The columns were ventral to the Dorsal Raphe inter-fasciculus nucleus (DRif). The MnR neurons were found just rostral to the decussation of the superior cerebellar peduncle (brachium conjunctivum). The neurons were ovoid in shape and exhibited fibres that projected mediolaterally.

d. Dorsal Raphe (DR) nuclear complex

The dorsal raphe is the largest collection of serotonergic neurons in the brainstem and is divided into several nuclei which extend from the periventricular grey matter of the pons and extend rostrally into the periaqueductal grey matter to the level of the oculomotor nuclei in the midbrain. We found this nuclear complex to be the same in both our groups when compared to rat atlases. The complex was divisible as per previous guidelines into different nuclei including the *Dorsal Raphe dorsal (DRd), Dorsal Raphe ventral (DRv), Dorsal Raphe Nucleus interfasciculus (DRif), Dorsal Raphe Nucleus Lateral part (DRI), Dorsal Raphe Nucleus peripheral (DRI) and Dorsal Raphe Nucleus caudal(DRc)* with each containing serotonergic immunopositive cells.

e. Dorsal Raphe dorsal (DRd):

The DRd neurons were identified located just ventral to the inferior border of the cerebral aqueduct, dorsal to the oculomotor nerve nuclei as seen in fig. 5.2A, and the neurons were ovoid in shape and were multipolar, and the density was high and the fibres tended to be oriented in a superoinferior direction.

f. Dorsal Raphe ventral (DRv):

There was high density of 5-HT+ neurons located dorsal and medial to the oculomotor nuclei, within the periaqueductal grey matter that was identified as the DRv nucleus (ventral to the DRd). see fig 5.2 Aand D below. The neurons, like those of the DRd were multipolar and ovoid, with fibres travelling in general supero-inferior orientation.

g. Dorsal Raphe Nucleus interfasciculus (DRif):

This nucleus was observed extending ventrally from the DRv, located in between the bilateral medial longitudinal fasciculi and the oculomotor nuclei, at the most ventral portion of the periaqueductal grey matter midline. The shape was similar to that of the DRv and DRd, however the fibres appeared to all travel in a specifically supero-inferior direction.

h. Dorsal Raphe Nucleus Lateral part (DRI):

The DRl nucleus was seen having a low density of 5-HT+ neurons, appearing to extend laterally from the DRd, located lateral and dorsal to both the DRd and DRv, within the

periaqueductal grey matter of the midbrain (figure 5.2A below). The cells were large and ovoid in shape, and their fibers were mainly parallel to the walls of the periaqueductal grey. These nuclei are bilateral and continuous caudally to become a low density nucleus adjacent to the periventricular walls within the pons (other investigators have named the nuclei differently at the pontine region, as the Dorsal Raphe caudal nucleus).

5.3.2. The Caudal Cluster

The caudal cluster was identified and classified into the following group of nuclei: Supragenualnucleus (SGeR/B4), Nucleus Raphe Magnus (RMg/B3), Nucleus Raphe Obscurus (ROb/B2) and Nucleus Raphe Pallidus (RPa/B1). The parapyramidal serotonergic neurons can be added to this group (Rostral and Caudal Ventrolateral nuclei (RVL and CVL)).

a. Raphe Magnus Nuclues (RMg)

This was found as the rostral-most nucleus of this group centred at the pontomedullary junction, at the level of the facial nucleus. It was restricted to the pararaphe position, forming two columns of low density, large 5-HT+ neurons on either side of the midline. The neurons were moderately large and triangular. Some were bipolar and others multipolar in form, and located within the tegmentum.

b. Raphe Pallidus (RPa)

The 5-HT+ neurons of the RPa were found at the ventral midline of the medulla and extended to the anterior pole of the inferior olive (IO). These were located ventrally to the RMg nucleus at a similar level in the medulla, albeit slightly more caudal to it, bordering both the pyramids in a mediodorsal position between. The neurons were fusiform in shape and multipolar, with dendrites extending parallel to the dorsal aspects of the pyramids.

c. Rostral and Caudal Ventrolateral nuclei (RVL and CVL)

In the medulla region, starting rostrally at the level of the facial nerve nucleus and extending caudally to the spinomedullary junction, there was in the ventrallateralmost region of the

medulla, (lateral to the pyramids), identified two clusters of 5-HT+ neurons that did not co-occur. More rostrally we observed the RVL with a moderate density of cells that started to lessen at the level of the nucleus ambiguus, giving way to the less dense CVL nuclei which then extended caudally to the spinomedullary junction. Both nuclei had neurons that were intensely stained and bipolar and multipolar in form with ovoid cell bodies. The dendrites tended to extend in an orientation parallel to the medullary floor which was adjacent to them.

d. Raphe Obscurus (ROb)

The 5-HT+ neurons of the raphe obscurus were found dorsal to the RPa and in the pararaphe position of the caudal medulla that extends from the caudal pole of the facial nucleus to the pyramidal decussation near the spinomedullary junction. The neurons were fusiform and ovoid in shape, and bipolar and multipolar form

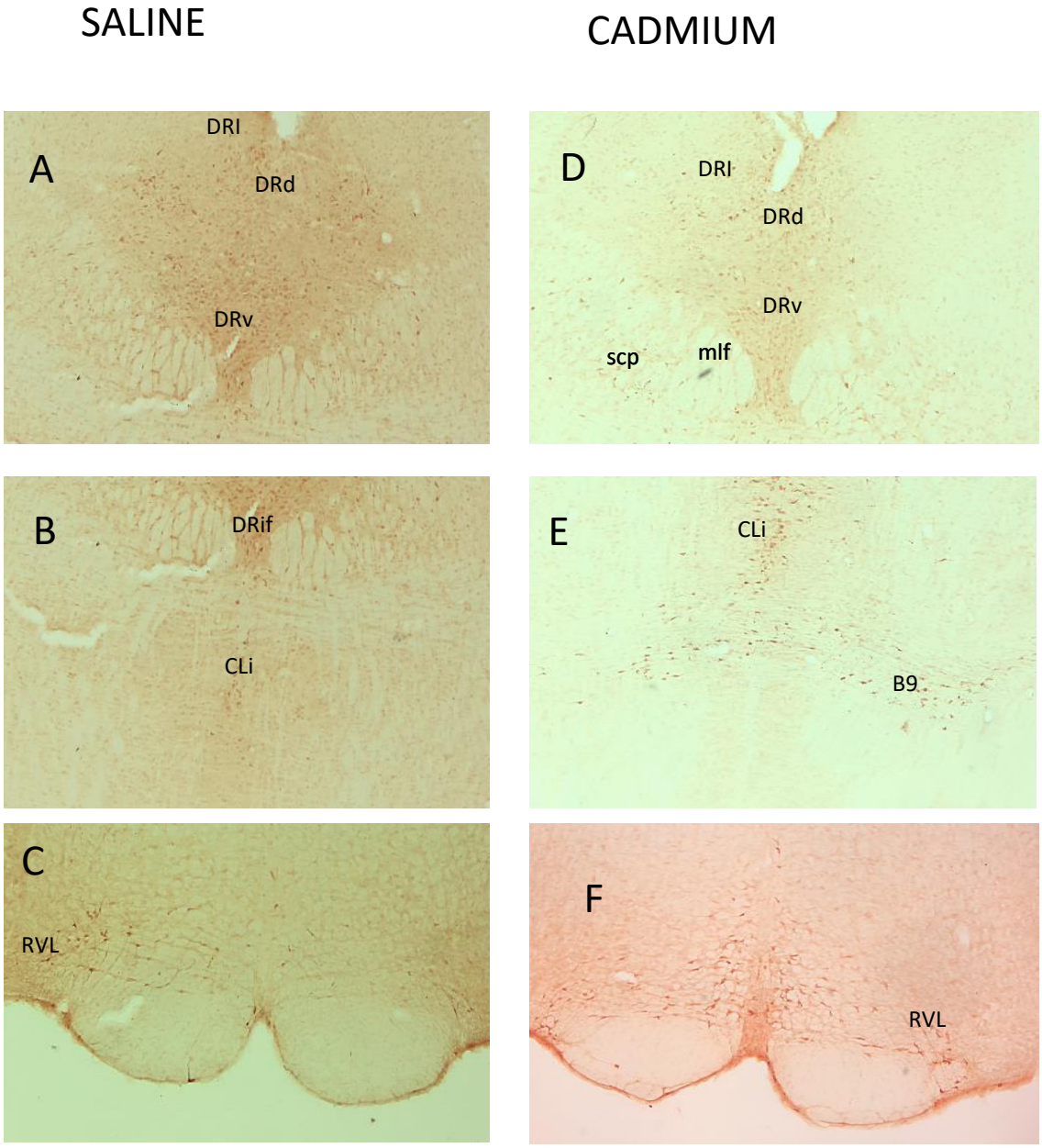


Figure 5.2. photomicrographs showing neuronal clusters

The neuronal clusters are immunohistochemically reactive for serotonin in the brains of Sprague Dawley rats. (**A-C)** show brains from typically developed rats, developed in saline injected pregnancies; **(D-F)** show autism-like rat brains, developed in cadmium chloride injected pregnancies; **(A, B, D)** DR (l, d, v and if), all subdivisions of the dorsal raphe including the lateral, dorsal, ventral and interfasciculus; **(B,E)** CLi, caudal linear nucleus located at the pontine region; **(E)** Sul/B9, the supralemniscal nucleus (B9); **(C,F)** RVL, rostral ventrolateral nucleus. In all photomicrographs, dorsal is up and ventral is down, and all images captured at 5x magnification.

5.4 Discussion

The current investigation was aimed at describing the nuclear parcellation of the serotonergic system in the Sprague Dawley rat, following brain development in an intrauterine environment insulted by preeclampsia, versus the same system in typical brain development following a normotensive pregnancy. Our study revealed similarities with those previously conducted in rodents (Steinbusch, 1981; Törk, 1990; Jakeman *et al*., 1994; Bacqué-Cazenave *et al*., 2020). The serotonergic neurons were observed in the brainstem at the midline (raphe position) and near the midline (pararaphe position). They were readily divisible into a rostral and a caudal cluster, a descriptive mechanism consistently followed when describing the system in laboratory rats (Dahlstro¨m and Fuxe, 1964; Fuxe *et al*., 1969), highveld gerbils (Moon *et al*., 2007), and most other mammals studied so far (Bjarkam *et al*., 1997; Maseko *et al*., 2007). To date, the only recorded exception in this nuclear organization of the system is the presence of serotonergic neurons in the hypothalamus of the monotremes (Takeuchi *et al*., 1982; Martin *et al*., 1985; Manger *et al*., 2002).

Our study revealed a nuclear parcellation of the serotonergic system that was identical to that previously described in rats (Jacobs and Azmitia, 1992), and this applied to both the preeclampsia exposed and saline exposed progeny. We found that all the nuclei previously described were present and in all the expected regions and levels of density, and we found also that there were no additional nuclei evident over and above those previously described in other rats. From the present studies, the serotonergic distribution in the brain of the SD rats appears to be the same with what has been previously recorded (Jacobs and Azmitia, 1992). The two groups we worked on showed similar distribution of serotonin which is in line with the classification of (Dahlström and Fuxe, 1964). We noticed that for both the cadmium and saline exposed groups of animals, no difference was seen in the pattern of distribution of the serotonergic neurons. The dorsal raphe (fig 5.2A) contains a moderate density of 5HT+ neurons while the B9 contains a low density.

CHAPTER 6: DISCUSSION

6.1 Overall Discussion

The goal of our study was to use a low dose intraperitoneal injection of cadmium chloride to induce preeclampsia in pregnant rats, exposing the developing brains to the condition intrauterine, with the overarching goal of determining whether preeclampsia is more likely to result in autistic outcomes in the offspring. In comparison to the saline-injected dams, we discovered identical hypertension symptoms in our cadmium-injected dams during the pregnancies, including weight gain, severe and prolonged rises in systolic blood pressure, and a larger amount of protein in the urine. The saline-injected dams (controls) had an increase in systolic blood pressure as well, but it decreased as the pregnancy progressed, similar to what happens in humans (Walker *et al*., 2015; Curran *et al*., 2018). When the pups were delivered, there were noticeable disparities in their body weights and sizes, with the preeclamptic offspring having significantly lower birth weights than the controls. Low birth weight has previously been linked to foetal congenital cardiac abnormalities in humans (Jin *et al*., 2016) and altered heart morphology and endothelial function in rats during adulthood (Jin *et al*., 2016).

Children with ASD are twice as likely to have been exposed to preeclampsia in utero, according to research. Preeclampsia raises the risk of ASD in afflicted offspring and has become increasingly common in recent years (Mann *et al*., 2010; Walker *et al*., 2015). In addition, non-autistic children born after preeclamptic pregnancies have recently been documented with brain structural changes that are comparable to those seen in ASD (Rätsep *et al*., 2016).

Exposure to a low dose of cadmium chloride caused resorption in one dam, a frequent symptom of preeclampsia (Wang *et al*., 2016). Although the preeclampsia children were little at birth, they grew and caught up in size to those from normal pregnancies, according to the research (Hudson *et al*., 2019), which was consistent with our own findings. Preeclampsia and ASD are worsened by gestational diabetes, parity, pregnancy obesity, and body mass index, according to Weissgerber and Mudd (2015). Preeclampsia and neurodevelopmental problems in children are known to be linked to body mass index (BMI) and gestational diabetes (Xu *et al*., 2014). This adds to the growing body of evidence that preeclampsia during pregnancy raises the risk of Autism Spectrum Disorder.

A battery of ten behavioural test were carried out to ascertain symptoms (cognitive, social and emotional behaviours) of ASD resulting from preeclamptic pregnancy. When selecting the behavioural assay test for autism we considered aspects that would reveal symptomology approximating the human condition as closely as possible. These include: those tests that would focus on different areas of learning and memory, such as the working memory and spatial memory (Antunes and Biala, 2012; Kirschmann *et al*., 2019); some core autistic features such as communication (Crawley, 2007; Moy *et al*., 2008; Ryan *et al*., 2010 and Teegarden, 2020). Out of the ten tests we conducted, we found in seven that the observed differences between the preeclampsia exposed and the saline exposed pups were indeed statistically significant. We observed significantly different behaviours between the groups when assessing the features such as anxiety, repetitive behaviours, insistence on sameness, all of which are hallmarks of human autistic ideation (Turner, 1999; Angoa-Pérez *et al*., 2013). We observed heightened anxiety with the hole-board test, open field and elevated plus maze in our pups that had developed under preeclamptic exposure. The animals 'preference for the peripheral regions in the hole-board test, and the avoidance of the centre space in the open field test, both showed some level of anxiety, which is typically seen in human autists (Sarkar, 2020). (Sukhodolsky *et al*., 2008) is of the opinion that a high level of anxiety is necessarily a cause of repetitive behaviours in children with ASD, while Joosten *et al*. (2009) linked the repetitive behaviour in autistic individuals as a means of reducing anxiety. Whichever scenario is most indicative of the truth, both views still fundamentally acknowledge the presence of anxiety when repetitive behaviour arises, this is in support of our conclusions in the current study, which is that our preeclampsia exposed progeny were more repetitive in their behaviour, and therefore more anxiety prone. Spending more time in the centre field of the open field is a suggestion that the animals were less anxious (Royce, 1977). According to Ramos, (2008), many behavioural test of anxiety have been linked to body activity and locomotion. From our findings on the activity displayed by our cadmium-exposed progeny in the elevated plus maze, open-field, light-dark box we are of the opinion that anxiety should also be a measure of the subject emotion. Given the number of faecal bolus left after the test (Holmes *et al.,* 2000*).*

Rearing behaviour, which consists of subjects standing vertically on both hind paws, has also been used as a measure of anxiety because it is considered as an exploratory behaviour. In the open field and elevated plus maze, the cadmium exposed progeny showed significant with the activity as sign also of high level anxiety. There does exist some contradiction in the field

however in this regard in that some researchers have reported that increased rearing corresponds with increased anxiety (Borta and Schwarting, 2005), while others say that decreased rearing is an indication of increased anxiety (Costall *et al*., 1989). Rodents are naturally social creatures and rely heavily on olfaction during a typical social interaction (Arakawa *et al.*, 2008). This was evinced by the sniffing behaviour during the olfactory habituation test. We observed reduced sniffs for each of the three turns or presentations of the same odour for both the social (opposite sex urine) and non-social (banana) odours. This was consistent with reports from Kepecs *et al*. (2006). The cadmium group showed more interest when presented with the social odour (opposite sex urine), and less interest in the non-social odour (banana); a trait seen in autistic individuals (Ryan *et al*., 2010). Cognition was measured using the T-maze and Y-maze, where we observed that the preeclampsia exposed progeny were slow in speed and decision on which arm to enter in the T-Maze; while in the Y-maze, the animals had difficulty in alternating their movement (changing between alternating arms), as a result of difficulty in motor planning and initiation, another reported feature of autism (Johnston *et al*., 2019).

We further examined the nuclear parcellation of the serotonergic system within the brains of both groups using immunohistochemistry targeted against serotonergic cells. We were particularly examining any discrepancies in somata sizes, location, orientation, form and density. We examined carefully each 5-HT+ cluster of neurons in the brainstem, and found that these were located, sized and organized identically between the preeclampsia exposed and the saline exposed progenies. The system in our preeclampsia exposed model was in no way different to that of the control group, and indeed that of older such descriptions of the serotonergic system in other rats (Dahlström and Fuxe, 1964). Both groups had the system in and near the raphe position of the brainstem starting at the mesencephalon rostrally and ending at the spinomedullary junction caudally. We observed all aspects to be the same including the orientation of the dendrites observed emanating from the somata.

The serotonergic system is a highly conserved system in the central nervous system, to the extent that even across mammals that vary greatly, the system still appears largely similar across (Dahlström and Fuxe, 1964; Moon *et al*., 2007). All mammals have similar serotonergic systems, with the exception of monotremes (Manger, 2002), which have a serotonergic nucleus in the hypothalamus. Our results showing a conserved serotonergic system are consistent with this nature of the system's conservation.

6.2 Concluding Remarks

The introduction of cadmium chloride to Sprague Dawley pregnancies gives rise to preeclampsia, a condition occurring in up to 8% of human pregnancies and increasingly being linked to the advent of autism spectrum disorders. The condition triggered a sustained hypertensive state, higher weight gains in affected dams, but low birth weights in offspring, another trait associable with autistic outcomes. When tested, the affected offspring displayed autistic ideations in seven out of ten behavioural tests chosen to investigate autism, including repetitive behaviours, heightened anxiety and cognitive inflexibility or insistence in sameness. The serotonergic system was found to resemble that of controls, which is consistent with a vast body of knowledge showing the system to be highly conserved.

6.3 Future Directions

Multiple tests need always be employed to conclude a diagnosis of ASD as some behaviours may appear contrary to what is expected, thereby requiring other tests to confirm the observations and conclusions. The serotonergic system was found conserved within the brainstem with regards to the cell body aspects, however the system may still show variations at other levels, e.g., where its projections travel and at the terminal boutons as they synapse onto distant cell bodies in the cortical and subcortical regions, and on other cells' projections.

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APPENDIX

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2018/09/41/B

APPLICANT: Dr B C Maseko

School: School of Anatomical Sciences; Department: N/A; Location: N/A

PROJECT TITLE: Can gestational hypertension lead to autism-like behaviors and abnormal serotonergic development in the Sprague Dawley offspring?

Category: B; Species and Numbers involved: *80X Sprague Dawley pups*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 28 Aug 2018. This approval remains valid until 9 Feb 2022.

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ Date: 11/02/2020

(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: _____ Date: 11/02/2020

(Registered Veterinarian)

CC: Student supervisor: N/A
Director Central Animals Service: Dr Kim Jardine

FOR RESEARCH USE ONLY!

BioVision
BioVision Incorporated

BCA Protein Assay Kit II

rev. 9/14

(Catalog # K813-2500; K813-5000, Store at Room Temperature)

I. Introduction:

Biovision's BCA Protein Assay kit provides a colorimetric detection and quantification of total protein content even in the presence of detergents. The Kit is based on the chelation of bicinchoninic acid (BCA) with the cuprous cation (Cu^{+1}), which is generated by reduction of cupric cation (Cu^{+2}) with the protein in an alkaline condition. The Cu^{+1}-BCA chelate is a water-soluble complex and exhibits a strong absorbance at 562 nm that is linear over a wide range of protein concentrations between 25-2000 µg/ml. In general, protein concentrations are estimated with reference to a commonly used protein standard. The Kit also includes Bovine Serum Albumin (BSA) as a protein standard for estimation of total protein content of samples.

II. Applications:

Measuring total protein concentration of pure proteins, extracts or lysates.

III. Kit Contents:

Components	K813-2500	K813-5000	Cap Code	Part Number
BCA Reagent A	500 ml	2 X 500 ml	NM	K813-xxxx-1
BCA Reagent B	25 ml	25 ml	NM	K813-xxxx-2
BSA Standard (2 mg/ml)	5 x 1 ml	10 x 1 ml	White	K813-xxxx-3

IV. User Supplied Reagents and Equipment:

- Sterile Eppendorf tubes, test tubes, spectrophotometer, microplate and microplate reader.

V. Storage and Handling:

Store all components of the kit at room temperature. Read the entire protocol before performing the experiment.

VI. Preparation:

- **Preparation of BSA Standards:**

  Prepare BSA Standards as suggested in the table below by diluting BSA Standard using de-ionized water or same diluent as that of the protein samples. Other similar dilutions can also be used within the assay range of 25-2000 µg/ml. One tube of BSA Standard is sufficient to make diluted solutions in triplicates. The diluted standard solutions can be used for up to one week when stored at 4 °C.

Vial	Volume of BSA (µl)	Volume of diluent (µl)	Final BSA Concentration (µg/ml)
1 (**Stock**)	300 of 2 mg/ml Stock	0	2000
2	300 of 2 mg/ml Stock	100	1500
3	300 of 2 mg/ml Stock	300	1000
4	300 of vial 3	300	500
5	300 of vial 4	300	250
6	300 of vial 5	300	125
7	100 of vial 6	400	25
8 (**Blank**)	0	400	0

- **Preparation of Protein Samples**: Prepare different concentrations of samples by diluting with water or an appropriate diluent to a concentration within the assay range (25-2000 µg/ml). It is recommended to use three different concentrations of samples & perform the assay in duplicates or triplicates.
- **Preparation of BCA working reagent**: To prepare BCA working reagent, mix BCA Reagent A with BCA Reagent B in the ratio of **50:1**. Upon mixing, green colored turbidity will be observed that should disappear upon further mixing to give a green colored solution. Each sample replicate requires 200 µl of BCA working reagent for microplate assay or 2 ml for test tube procedure. Prepare sufficient amount of BCA working reagent solution needed for all BSA Standards & Samples.

  Note: It is recommended that BCA working reagent should be prepared fresh. However, the prepared reagent is stable and can be stored at room temperature for several days in a closed container.

VII. Assay Protocol:

BCA Assay can be performed in a microtiter plate format or test tube format.

A. Microplate Procedure:

1. Add 25 µl of each BSA Standard and protein samples into microtiter plate wells.
2. Add 200 µl of BCA working reagent to the Standard & sample wells, mix thoroughly for 30 s.
3. Cover the plate and incubate at 37 °C for 30 min or room temperature for 2 h. After incubation, cool the plate to room temperature.
4. Set the absorption wavelength of a microplate reader to 562 nm and read all Standards and samples (OD_{562}).

B. Test Tube Procedure:

1. Add 100 µl of each BSA Standard and protein samples into a 4 ml test tube.
2. Add 2 ml of the BCA working reagent and mix well.
3. Cover the tubes and incubate under either one of following conditions:

- 37 °C for 30 min or at room temperature for 2 h (Assay range is 25-2000 µg/ml)
- 60 °C for 30 min (Assay range is 5-250 µg/ml)

4. After incubation, cool the tubes to room temperature.
5. Set the absorbance wavelength of a spectrophotometer to 562 nm. Blank the instrument by using water or the diluent only.
6. Read absorbance (OD_{562}) of all Standards and samples.

C. Calculations: Subtract OD_{562} of Blank (0 Standard, #8) from all readings. Plot the Standard curve, OD_{562} (on Y-axis) vs Standard BSA concentration (on X-axis). Obtain the equation from the plot **Y** = aX + b. Use the obtained value of slope (a) to calculate protein concentration in samples.

$$\textbf{Protein concentration in sample: } C = DX = \textit{Dilution Factor} \times \frac{(Y-b)}{a} = \mu g/ml$$

Where **Y** = OD_{562} of protein sample

X = concentration of protein sample

a = Slope of the BSA Standard curve

b = Y-intercept of the Standard Curve

D = Dilution factor of protein sample

Alternatively, get the sample concentration from the Standard curve. Then calculate protein concentration in sample:

C = DX

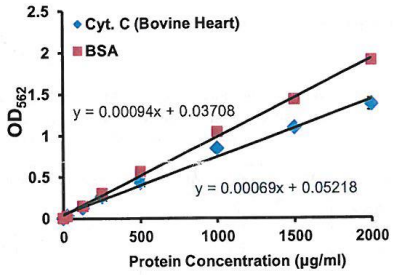

Figure: Typical absorbance plots obtained for BSA and Cytochrome C from Bovine Heart (Cat. # 2120) using a microplate procedure (37°C for 30 min).

VIII. RELATED PRODUCTS:

EZLys™ Bacterial Protein Extraction Reagent (8001)
EZLys™ Tissue Protein Extraction Reagent (8002)
EZLys™ lysozyme, human (8005)
Western Blot Substrate Kit (K820)
Protein Carbonyl Content Assay Kit (K830)
Protease inhibitor cocktails (K271, K272, K277, K278, K279)
EZLys™ Yeast Protein Extraction Reagent (8003)
EZLys™ Mammalian Protein Extraction Reagent (8004)
Protein Quantitation kit (K810)
BCA Protein Quantitation Kit (K812, K814)
Protease & Phosphatase inhibitor cocktails (K283, K284)

FOR RESEARCH USE ONLY! Not to be used on humans.