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The effects of maternal nicotine exposure during pregnancy or lactation on hypertension of the offspring

By

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DECLARATION

I, Abdalrauf Alforti, hereby declare that the dissertation "***The effects of maternal nicotine exposure during pregnancy or lactation on the hypertension of the offspring***" submitted by me for the Master's degree in Medical Biosciences at the University of the Western Cape, South Africa is my own original work and has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Abdalrauf M AB Alfourti.

Date: ……

Signed: ……

DEDICATION

I dedicate this master's degree to my mother and my wife for their encouragement, support and making my dreams a reality.

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First of all, I would like to acknowledge my Creator for giving me the faith, strength, good health, wisdom and giving me the strength and perseverance, without him I would not have finished this thesis.

This work would not have been done without the generous support and help received from many different persons. I would like to express my sincere gratitude for the valuable assistance I received through my master journey from the following people:

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ABSTRACT

Blood pressure and heart rate is known to increase during smoking. These effects are specifically associated with nicotine while the other components of tobacco smoke seem to be of minor importance. It is becoming increasingly clear that fetal nicotine exposure, through transfer of nicotine via the placenta, has numerous consequences that is detrimental to the health of the fetus and that these effects may last into adulthood or perhaps even manifest itself later in life. In this study, we investigated the effects of maternal nicotine exposure during pregnancy and lactation on the blood pressure of the male offspring. A preliminary study indicated that the female offspring do not become hypertensive. We were particularly interested to determine whether hypertension in the offspring could be prevented or attenuated by vitamin C (an antioxidant) therapy.

When confirmed to be pregnant, female Wistar rats were divided into four groups. The control group (group 1) received saline (1ml/day, s.c), the animals in group 2 received nicotine (1mg/kg/day in ml, s.c), group 3 animals received a combination of saline (1 ml/day s.c.) and vitamin C (1g/l in drinking water) and the animals in group 4 received a combination of nicotine (1mg/kg/day, s.c) and vitamin C (1g/l in the drinking water) during pregnancy and lactation. At weaning eight animals from group 2 received vitamin C in the drinking water (1g/l) until the end of the experiment. Blood pressure and body weight were measured every two weeks for 5 months. Blood samples were collected, and serum prepared at 3 weeks and at 5 months for biochemical analysis. Total antioxidant capacity (TAC), thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) assays were determined at 3 weeks; TAC was also measured at five months. After 5 months, the

animals were sacrificed and abdominal aorta was excised for histological (H and E staining) and IHC (AGE) evaluation.

From the data generated in this study, it was evident that maternal nicotine exposure during pregnancy and lactation increased blood pressure of male offspring but not female offspring. Exposure to nicotine during pregnancy and lactation significantly increased systolic blood pressure from 115±4.6 mm Hg in the first month to 147±6.1 mm Hg at five months ($P<0.05$), diastolic blood pressure was increased from 84±3.4 mm Hg in the first month to 110±7.2 mm Hg at 5 months ($P<0.05$) and MAP was increased from 94.8±3.8 mm Hg in the first month to 121±6.4 mm Hg at 5 months. Interestingly the MAP of the nicotine group was normal during the first two months. HR was similar in all groups at 5 weeks and 5 months. Maternal vitamin C supplementation in rats exposed to nicotine during pregnancy and lactation did not prevent development of hypertension of the offspring (MAP=121±6.4 mm Hg in the nicotine group vs 113.4±1.7 mmHg in the nicotine and vitamin C group, $P>0.05$). Supplementation with vitamin C in the drinking water after weaning significantly reduced blood pressure of the offspring (MAP=121±6.4 mm Hg vs 97.6±2.9 mm Hg respectively, $P<0.05$). No significant difference was found in any of the biochemical assays. Maternal nicotine exposure during pregnancy and lactation leads to alteration in aorta structure as evaluated by H and E staining. Structural alterations include protrusion of the intima and irregular arrangement of the vascular smooth muscle cells (VSMC) in the tunica media. Maternal vitamin C supplementation and vitamin C supplementation after weaning did not prevent the structural alteration of the aorta. Immunohistochemistry (IHC) indicated that the accumulation of AGE's in the nicotine group was stronger than in the control group. In conclusion, we show for the first time that hypertension induced by maternal nicotine exposure can be reversed after weaning by supplementation with vitamin C, an antioxidant.

Keywords

Antioxidant.

Blood pressure.

Fetal programming.

Hypertension.

Maternal exposure.

Nicotine.

Oxidative stress.

Vitamin C.

ABBRIEVIATION, NOTATION AND SYMBOLS

°C	Degree Celsius
µl	Microlitres
Ach	Acetylcholine
AGEs	Advanced Glycation End Products
ANG	Angiotensin
ANOVA	Analysis of Variance
ASVD	Arteriosclerotic Vascular Disease
BP	Blood Pressure
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CNS	Central Nervous System
CVD	Cardiovascular Disease
CYPs	Cytochromes P450
DAB	3, 3-Diaminobenzidine
DBP	Diastolic Blood Pressure
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EC	Endothelial Cells
ELISA	Enzyme Linked Immunosorbent Assay
FFA	Free Fatty Acid
HF	Heart Failure
HO_2	Hydroxyl Radical

HPA	Hypothalamic Pituitary Adrenal Axis
HPLC	High Performance Liquid Chromatograph
HR	Heart Rate
IgG	Immunoglobin G
IHC	Immunohistochemistry
IHD	Ischemic Heart Disease
LBW	Low Birth Weight
LDL	Low Density Lipoprotein
LPO	Lipid Peroxidation
MAP	Mean Arterial Pressure
MDA	Malondialdehyde
MI	Myocardial Infarction
nAChRs	Nicotine Acetylcholine Receptors
NAD	Nicotinamide Adenine Dinucleotide
NIDDM	Noninsulin Dependent Diabetes Mellitus
NO	Nitric Oxide
PKA	Protein Kinase A
PNS	Peripheral Nervous System
PVAT	Perivascular Adipose Tissue
RAGE	Receptors for Advanced Glycation End Products
RAS	Renin Angiotensin System
RF	Renal Failure
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

SADHS	South Africa Demographic and Health Survey
SBP	Systolic Blood Pressure
SCD	Sudden Cardiac Death
SHR	Spontaneous Hypertension Rate
SMC	Smooth Muscle Cells
SNS	Sympathetic Nervous System
SOD	Superoxide Dismutase
T2D	Type-2 Diabetes
TAC	Total Antioxidant Capacity
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substance
VPR	Volume Pressure Recording
VSMCs	Vascular Smooth Muscle Cells
WHO	Worldwide Health Organization
WKY	Wistar Kyoto Rats

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CHAPTER ONE

INTRODUCTION

The Fetal origin of adult disease theory was first introduced by Barker (1995). It proposes that alterations in the environment within which the fetus develops, such as nutritional status, oxygenation status and exposure to toxic substances, may change the structure, physiology and metabolism of the individual in such a manner that it predisposes the offspring to cardiovascular, metabolic and endocrine diseases in adult life (Godfrey & Barker, 2000). Changes in the environment within which the fetus and neonate develop might, therefore, have a profound effect on the future health of the individual.

Nicotine is the principal alkaloid present in the tobacco plant, which belongs to the nightshade family, and represents about 0.6-3.0% of the dry weight of tobacco (Tassew and Chandravanshi, 2015). Neuronal nicotinic acetylcholine receptors are pentamers with a unique conformation of alpha (α2- α10) and beta (β2-β4) subunits resulting in different receptor subtypes found throughout the brain, which is cause of nicotine addiction (Jiloha, 2010).In addition to being a stimulant drug, nicotine can also act as an antidepressant and is highly addictive (Hughes *et al*., 2014). Its neurochemical and metabolic effects are similar to those of other drugs such as cocaine, amphetamine and morphine, which have strong addictive properties (Pontieri *et al*., 1996).

Studies have also shown that exposure to nicotine is associated with the development of some chronic diseases such as, hypertension, obesity and type 2 diabetes (Gao *et al*., 2005; Gao *et al*., 2008). It has been reported that blood pressure increases during smoking, due to the presence of nicotine in tobacco smoke (Xiao *et al*., 2014). This because the nicotine affects the cardiovascular system in two ways: 1) it increases heart rate and 2) it causes narrowing of arteries which increases risk of developing peripheral artery disease (PAD), affecting the arteries that supply the kidneys, stomach, arms, legs and feet, and also leads to increases in blood pressure.

Epidemiological studies have demonstrated that exposure to maternal nicotine is associated with an increased risk of elevated blood pressure in postnatal life (Xiao *et al.,* 2008). Xiao *et al* (2008) reported that, in utero exposure to maternal nicotine (6 mg/kg/day) had no effect on baseline BP but significantly increased Ang II stimulated BP in male but not female offspring. On the other hand, Gao *et al* (2008)has showed that blood pressure was increased at 14 weeks of age in the nicotine (1 mg/kg/day) exposed male offspring.

Previous studies have reported that the harmful effects of nicotine is due to a disturbance in the capacities of endogenous antioxidant defences in which the activity of the antioxidant system is overwhelmed by reactive oxygen species generation (Halliwell and Whiteman, 2004). It has however also been suggested that the toxicity of nicotine induced oxidative stress can be modulated by antioxidants (McIntyre *et al*., 1999). Vitamin C is the major essential water-soluble antioxidant in human serum and is able to reduce the oxidative properties of toxic substances (Perai *et al*., 2014). As an antioxidant, vitamin C helps in

preventing oxidative stress by directly scavenging oxygen-derived free radicals, such as superoxide anions or hydroxyl radicals (Nagaraj and Paunipagar, 2014). An in vitro study has also shown advanced glycation end products (AGE's) to be part of the complicated interaction between oxidative stress and vascular damage (Wihler *et al*., 2005).

1.2. The objectives of this study:

In view of the above the objectives of this study were to determine whether:

1. Low dose (1 mg/kg/day) maternal nicotine exposure during pregnancy and lactation will induce hypertension in the offspring. We also wanted to determine whether both male and female offspring are affected.

2. Hypertension induced by maternal nicotine exposure is present at weaning or only develops later in life.

3. Hypertension induced by maternal nicotine exposure can be prevented or attenuated by vitamin C supplementation during the perinatal period or after weaning.

4. Low dose maternal nicotine exposure effects serum antioxidant capacity, aorta morphology and expression of AGE in the aorta.

5. The above can be prevented or reduced by vitamin C supplementation.

CHAPTER TWO

LITERATURE REVIEW

2.1. Nicotine

Nicotine is the principal alkaloid present in the tobacco plant, which belongs to the nightshade family, and represents about 0.6-3.0% of the dry weight of tobacco. It is a hygroscopic, colorless and an oily liquid that is readily soluble in alcohol (Tassew and Chandravanshi, 2015). Nicotine is a potent parasympathomimetic alkaloid and a stimulant drug (Zhou*et al*., 2014). The chemical structure of nicotine is shown in figure 2.1.

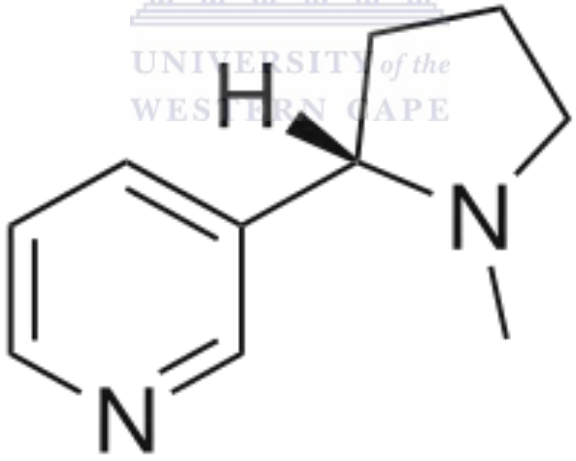


Figure 2.1: The structure of nicotine (Seeman *et al*., 1999).

When nicotine is exposed to ultraviolet light or other oxidizing agents, it transforms to nicotinic oxide, nicotine acid (Vitamin B3) and then to methylamine (figure 2.2), which may cause harm to the body.

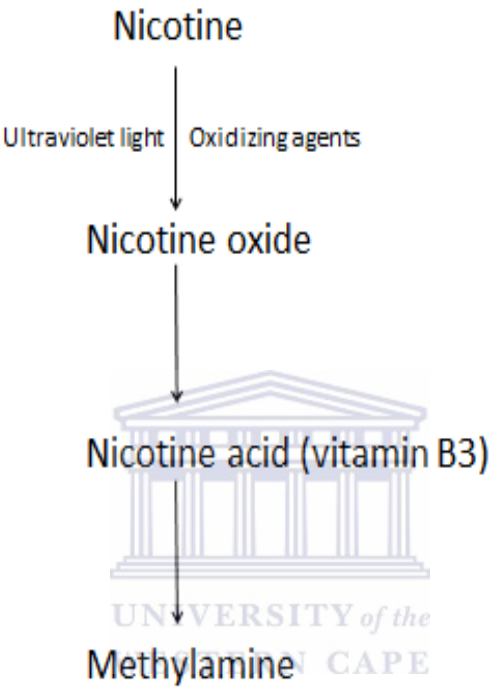


Figure 2.2: Illustration of how nicotine transforms to methylamine.

In addition to being a stimulant drug, nicotine can also act as an antidepressant and is highly addictive (Hughes *et al*., 2014). Its neurochemical and metabolic effects are similar to those of other drugs which have strong addictive properties such as cocaine, amphetamine and morphine (Pontieri *et al*., 1996). Studies have also shown that exposure to nicotine is associated with the development of hypertension, obesity and type 2 diabetes (Gao *et al*., 2005; Gao *et al*., 2008).

2.1.1-Biosynthesis, metabolism, excretion and therapeutic effects of nicotine

Nicotine biosynthesis from tobacco roots is promoted by cutting or removal of the apex of the tobacco plant (Xi*et al*., 2005). The pathway of nicotine biosynthesis in tobacco roots is still unclear, but some studies have suggested that the biosynthetic pathway of nicotine is through the interaction between the two cyclic structures that compose nicotine. The pyridine ring of nicotine is synthesized from niacin (nicotinic acid), while the pyrrolid one is synthesized from N-methyl-Δ^1-pyrrollidium a cation (Shoji and Hashimoto, 2013) through two independent synthetic pathways, the nicotinamide adenine dinucleotide (NAD) pathway for niacin and the tropane pathway for N-methyl-Δ^1-pyrrollidium cation.

The major organ responsible for the metabolism of nicotine is the liver, mainly by cytochrome P450 enzymes (CYPs) (Earla *et al.,* 2014). Nicotine metabolism is influenced by genetic factors, age and sex, use of estrogen-containing hormone preparations, pregnancy, kidney disease, medications, and smoking itself (Benowitz and Jacob, 1994). The lungs and kidneys have also been reported to play a part in the chemical breakdown of nicotine. In humans about 70–80% of nicotine is converted to cotinine through two stages. Firstly, nicotine is metabolized primarily by CYP2A6 to produce nicotine-$\Delta^{1'\ (5')}$-iminium, and secondly, it is the catalysed by a cytoplasmic aldehyde oxidase to produce cotinine (Wong, 2015). Cotinine is the most widely used biomarker of nicotine intake. It may be measured in blood, urine, saliva, hair, or nails. Only 10-15% of nicotine absorbed by a smoker appears as unchanged cotinine in the urine. The remainder is converted to metabolites, primarily *trans*–3′–hydroxycotinine (33–40%), cotinine glucuronide (12–17%), and *trans*–3′–hydroxycotinine glucuronide (7–9%)(Benowitz*et al.*, 1994; Hukkanen*et al*., 2005). Cotinine can also be

determined in the urine of infants whose mothers have been exposed to nicotine during pregnancy or lactation (Benowitz *et al.*, 2009). Figure 2.3 below shows the transformation of nicotine in the systemic circulation.

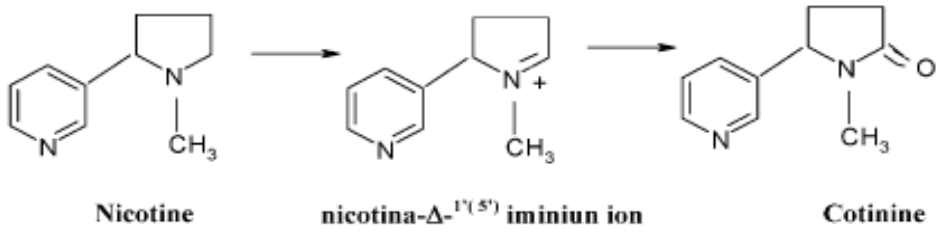


Figure 2.3: Transformation of nicotine into cotinine by cytochrome P 450 (Vellosa*et al.*, 2007).

Since the metabolism of cotinine is much slower than that of nicotine, the rate of metabolism of nicotine can be determined by measuring blood levels of cotinine after administration of a known dose of nicotine.

As mentioned earlier, excretion of nicotine could be via urine, feaces, bile, saliva, gastric juice, sweat and breast fluid (Mishra *et al.*, 2015; Nakajima *et al.*, 2002). When nicotine is given to animals (like rats), it has been shown that around 55% is excreted in urine. Animal studies have shown that the pH of urine has an effect on the rate of nicotine excretion. When the pH of the urine is alkaline, it leads to a decrease in nicotine excretion, and when the pH of the urine is acidic, it leads to an increase in nicotine excretion (Beckett *et al.*, 1965). Ascorbic acid or vitamin C may have an influence on urinary excretion and metabolism of nicotine as it

was reported by Evans *et al*. (1999) that ascorbic acid leads to an increase in the urinary excretion of cotinine and nicotine (Dawson *et al*., 1999).

Neuronal nicotinic acetylcholine receptors are pentamers with a unique conformation of alpha (α2- α10) and beta (β2-β4)subunits resulting in different receptor subtypes that are found throughout the brain, and the main cause of nicotine addiction are mediated via these receptors (Jiloha, 2010).The different areas in the brain contain different nicotine cholinergic receptor subtypes, which have different chemical conductance of sodium and calcium, and also different sensitivity to different nicotine agonists, resulting in correspondingly different pharmacological actions.

As a therapeutic agent, nicotine can be used as a drug in the treatment of some diseases (Raupach *et al*., 2012). It has rewarding psychopharmacologic effects such as tranquilization, weight loss, decreased irritability, reduction in craving for cigarettes and other tobacco products, increased alertness, and improved cognitive function (Cary, 2001). A few experimental systems have established that nicotine causes weight loss via reduced appetite and increased metabolic rate (Grebenstein *et al*., 2013). It can encourage the release of serotonin, which has an important role in reducing body weight. S-nicotine, a product of nicotine metabolism (Park *et al.*, 1993) has some beneficial effects on the treatment and protection against a number of diseases such as Parkinson's disease and Alzheimer's disease (Lopez-Arrieta and Sanz, 2001; Thiriez *et al*., 2011). Unfortunately, S-nicotine has side effects on the cardiovascular and digestive system, limiting its use as a therapeutic agent

(Girard *et al.*, 2000). (R)-nicotine, which is also found in cigarette smoke, has been studied for its anxiolytic effects (Salas *et al.*, 2003).

2.1.2. Harmful effects of nicotine

It has been reported that blood pressure increases during smoking, as a result of nicotine in tobacco smoke (Xiao *et al*., 2014). Nicotine affects the cardiovascular system in two ways: 1) it increases heart rate and 2) nicotine has effects on arteries by remodelling the artery and this leads to an increased risk of developing peripheral artery disease (PAD), affecting the arteries that supply the kidneys, stomach, arms, legs and feet and also leads to increase in blood pressure. The effect of nicotine on coronary heart disease is via accelerated atherogenesis, hyperlipidemia and dysfunctional endothelial cells (Benowitz, 1991). Nicotine can also play a role in stroke by altering the function of the blood-brain barrier and disruption of normal endothelial cell function (Hawkins *et al*., 2002). In addition, nicotine increases free fatty acid (FFA) levels, with some studies showing that people who suffer from heart disease always have high free fatty acid levels which facilitate the development of coronary heart disease (Papathanasiou *et al*., 2014).

In adults, it was shown that the cardio-ankle vascular index (CAVI) was increased by exposure to nicotine. The CAVI reflects the stiffness of some arteries in the body such as the aorta, femoral artery, and tibial artery (Noike *et al*., 2010). The cardio-ankle vascular index is used as a clinical marker for evaluation of atherosclerosis and arteriosclerosis in patient with essential hypertension (Okura *et al*., 2007). In hypertension patient's aortic stiffness is an

independent predictor of cardiovascular mortality, fatal and non-fatal coronary events, and fatal strokes (Laurent *et al*., 2001). Nicotine is believed to alter the structural and functional characteristics of vascular smooth muscle and endothelial cells by enhancing endothelial proliferation and increased atherosclerotic plaque formation. Neovascularization stimulated by nicotine has also been suggested to help the progression of atherosclerotic plaque. These effects lead to myointimal thickening, atherogenic and ischemic changes that increase the incidence of hypertension (Mishra *et al*., 2015). It has been reported that atherosclerosis and related diseases are due to vascular disorders including endothelial dysfunction (Rajendran *et al*., 2013). A study has indicated that vascular responses in adult offspring from adverse intrauterine environments were impaired in a gender-specific and age-dependent manner; with males more profoundly affected (Hemmings *et al*., 2005).

In adulthood, the relationship between nicotine and obesity is incompletely understood (Chiolero*et al*., 2007). In adults, studies show that nicotine could reduce appetite via increases energy expenditure (EE) (Hofstetter *et al.,* 1986). Other studies showed that light smokers have a lower body weight than do heavy smoker (Bamia *et al*., 2004; Chiolero *et al*., 2007).

In humans, exposure to nicotine during pregnancy and lactation leads to alteration in endocrine and glucose homeostasis in the adult offspring, a metabolic change which is consistent with the disturbed glucose metabolism that may lead to type 2 diabetes (Jiang *et al*., 2013).In developed countries, it has been suggested that maternal cigarette smoking during pregnancy is a significant environmental contributor to fetal growth restriction which may later in life increase the risk for adult disorders (Reeves and Bernstein, 2014; Wideroe *et al.*, 2003).Epidemiological studies have demonstrated that exposure to maternal nicotine is

associated with an increased risk of elevated blood pressure in postnatal life (Xiao *et al.*, 2008). Xiao *et al* (2008) reported that, in utero exposure to maternal nicotine had no effect on baseline BP but significantly increased Ang II stimulated BP in male but not female offspring. Gao *et al* (2008) also showed that blood pressure was increased at 14 weeks of age in the nicotine exposed offspring. They however did not measure BP in the young animals.

Exposure of the lungs to nicotine via cigarette smoking has been reported to cause cancer of the lungs (Health and Services, 2004; Warren and Singh, 2013). In adults, exposure to nicotine is associated with a higher rate of respiratory diseases such as bronchitis, bronchiolitis, pneumonia, and asthma (Jaakkola and Maritta, 2002; Maritz, 2013), while there is also an enhancement in the risk of respiratory infections in children who are already regularly exposed to nicotine (Gurkan *et al*., 2000). In addition to this, it has also been shown that in monkeys, nicotine exposure can alter lung development and is associated with enlargement of airspaces and reduction in alveolar surface area (Sekhon *et al*., 1999; Sekhon *et al*., 2001; Sekhon*et al.*, 2002).

The effects of nicotine in the pathogenesis of hypertension, obesity, type 2 diabetes, cardiovascular disease, and respiratory diseases as explained in the above paragraphs have been reported to be mediated by oxidative stress related pathways (Cai and Harrison, 2000). The mechanism by which nicotine induces such oxidative stress will be discussed later. The other pathophysiological process by which nicotine induces hypertension includes stimulating the release of catecholamines (Czernin and Waldherr, 2003; Paton, 2013). A study has shown

that alteration in central catecholamine metabolism is considered to be causally related to increased blood pressure (Dev and Philip, 1996).

2.1.3. Nicotine and Fetal programming

Fetal or developmental programming is defined as the concept that a maternal stimulus or insult at a critical period in fetal development has a long-term impact on the offspring (Barker *et al*., 1993; Rabadan-Diehl and Nathanielsz, 2013). In modern paediatrics and developmental psychobiology, fetal programming is a concept which occurs when important physiological parameters are reset during intra-uterine development by environmental events. This resetting can endure into adulthood and affect the following generation to produce a trans-generational non-genetic disorder (such as Willi syndrome and Angelman syndrome). An example of this is the impact of changes in local fetal cellular environments on gene expression during the development of tissues and organs, which can result in long-term consequences for the function of those tissues and organs during childhood and adulthood (Godfrey *et al*., 2007).

It is becoming increasingly clear that fetal exposure to nicotine has numerous consequences that is to the detriment of the health of the fetus, and that these effects may last well into adulthood. In pregnant women who smoke or use nicotine replacement therapy (NRT), nicotine crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation (Lambers and Clark, 1996; Mızrak *et al*., 2012). Exposure to nicotine during fetal development leads to diseases in adulthood, including obesity, hypertension, type-two diabetes, cardiovascular diseases (CVD) and some cancers.

Prenatal exposure to nicotine in rats is associated with increased postnatal body weight and higher levels of body fat in the fetus at gestational day 20 and during adulthood (Newman *et al*; 1999; Gao *et al*., 2005). Also, prenatal nicotine exposure has been reported to cause alterations in the central endocrine control of body weight homeostasis, which leads to increased body weight due to enhanced levels of the adipocyte hormone, leptin (Somm *et al*., 2008; Oliveira *et al*., 2009).On the other hand, a number of other studies have also shown that the main effect of nicotine during gestation is caused by vasoconstriction of uterine placental blood vessels, leading to a reduced blood flow to the placenta and a reduction in the delivery of oxygen and nutrients to the fetus, which leads to a reduction in weight of the fetus (Cnattingius, 2004).

In rats, exposure to nicotine during pregnancy and lactation has an effect on pancreatic development and postnatal beta cell survival and function (Bruin *et al*., 2007; Bruin *et al*., 2008; Bruin *et al*., 2008). Animal studies have demonstrated that fetal and neonatal exposure to nicotine causes a permanent loss of beta cell mass beginning at birth, and this resulted in reduced islet size and number, as well as a reduction in beta cell neogenesis.

It has been shown that there is an increased risk of hypertension in children born to women who smoke during pregnancy, and animal experiments have shown that this risk may be as a result of exposure to nicotine (Blake *et al*., 2000; Mund *et al*., 2013). Animal studies have also confirmed that exposure to nicotine during pregnancy causes elevated blood pressure in offspring after birth through dysfunction of endothelial cells and changes in the structure and function of the kidney (Pausová *et al*., 2003; Xiao *et al*., 2007; Mao *et al*., 2009). It has

suggested that nicotine has an effect on the regulation of vascular tone through alteration in per vascular adipose tissue (PVAT) composition and modulator function (Xiao *et al*., 2008), and this mechanism is associated with an increase in blood pressure (Gollasch and Dubrovska, 2004; Gao, 2008).

Some epidemiological studies have shown that exposure to maternal nicotine during pregnancy also leads to adverse pulmonary functional outcomes in the offspring (Leslie, 2013; England *et al*., 2015). Nicotine during pregnancy increases the risk of wheezing and asthma in the children up to 2 years of age (Lannerö *et al*., 2006; Rehan *et al*., 2012). Results from other studies suggest that there is also an impact on lung development and postnatal lung function as a consequence of maternal nicotine exposure (Hafström *et al*., 2005; Maritz, 2008; Campos *et al*., 2009). Accelerated aging of the lung, characterized by microscopic emphysema, enlarged alveolar volume, increased flattening of alveoli and decreased internal surface area for gas exchange with increasing age have also been reported by (Maritz and Windvogel, 2003).

Many studies have shown that fetal exposure to nicotine has an effect on the central nervous system (CNS) by alterations in cellular growth and activity of the central and peripheral nervous system (PNS), via cholinergic nicotine receptors (Palmer *et al*., 1992; Son and Winzer-Serhan, 2008; Dwyer *et al*., 2009). Prenatal nicotine exposure has an effect on neurobehavioral outcomes (such as attention-deficit hyperactivity disorder (ADHD) and decreased general cognitive functioning) in the offspring of women who were exposed to nicotine during pregnancy (Santiago and Huffman, 2014).

Animal models and human epidemiological studies, have reported that some diseases like dyslipidaemia and dysglycemia associated with disorders of metabolism of fatty liver disease, have relation to exposure to nicotine alone during fetal and neonatal development (Newman *et al*., 1999; Gao *et al*., 2005; Holloway *et al*., 2005). Table 2.1 summarized the harmful effects of nicotine on the body.

System/organ name	*Side effects*	*Reference*
Cardiovascular system		
- Hear rate	Increased heart rate and effect on arteries cause vasoconstriction.	Maryann and Gronisch, 2009
- Blood pressure	Increase risk of elevated blood pressure (Hypertension).	Xiao *et al.*, 2008
- Vascular smooth muscle	Alter the structural and functional characteristics of VSMC by enhancing endothelial proliferation and increased atherosclerotic plaque formation.	Noike *et al*., 2010
- Stroke	Altering the function of blood-brain barrier and disruption of normal endothelial cell function, causing stroke.	Hawkins *et al*., 2002
Respiratory system	Alter the structure and development of respiratory organs like the lungs.	Sekhon *et al*., 2002
Central nervous system	Alteration in cellular growth and activity of the central and peripheral nervous system via cholinergic nicotine receptors.	Dwyer *et al*., 2009
Body weight	Reduce appetite via increase energy expenditure and vasoconstriction of uterine placental blood vessels (Low body weight).	Hofstetter *et al.*, 1986
Type 2 diabetes	Alteration in endocrine and glucose homeostasis which lead to disturbed glucose metabolism.	Holloway *et al*., 2005
Pancreas	Reduced islet size and number as well as a reduction in beta cell neogenesis.	Bruin*et al*., 2008)
Cancers	Induces a dose-dependent increase in proliferation of cancer cells (lung cancer and breast cancer).	Dasgupta *et al*., 2009

Table 2.1: Summary of the harmful effects of nicotine on body tissue of adult**.**

2.2 Oxidative stress

An oxidation reaction can produce free radicals, which can cause damage to the cells (Rahal *etal*., 2014). A free radical can be defined as an oxygen containing molecule that has one or more unpaired electrons, thus making it highly reactive with other molecules. Under normal physiologic conditions, free radicals are constantly produced as byproducts of mitochondria electron transport in the cellular respiratory chain, and also are inevitable byproducts of many cellular and extracellular redox reactions (Droge, 2002; Laloi *et al*., 2004).

Antioxidants are molecules that prevent the oxidation of other molecules and are hence very important in the human body (figure 2.5). The antioxidant system can be divided into two main groups: antioxidant enzymes and non-enzymatic antioxidants. The latter consist of both endogenous molecules, such as glutathione and nicotinamide adenine dinucleotide phosphate (NADPH), and exogenous antioxidants, such as ascorbic acid (vitamin C) and tocopheol (vitamin E) (Schreibelt *et al*., 2007). Should the concentration of free radicals within the blood increase greatly because of excessive exposure to harmful factors such as smoking, then the endogenous antioxidant defence system may be inadequate and hence cannot neutralize or prevent the accumulation of free radicals which may cause dangerous mutations that destroy cells and cause chronic diseases.

The antioxidants are distributed throughout the cell and body with only a small proportion accumulating in mitochondria, the cellular organelles where the generation of ROS may be high and the principle site of damage in the cells by free radicals. Mitochondria-target

antioxidants are able to reduce cell death arising from endogenous oxidative stress (Jauslin *et al*., 2003; Oyinloye *et al*., 2015). Research has shown that mitochondria-targeted antioxidants may be more effective as therapies than untargeted antioxidants. Vitamin C is an example of an antioxidant which targets the mitochondria (Augustin *et al*., 1997; Gruber *et al*., 2013).

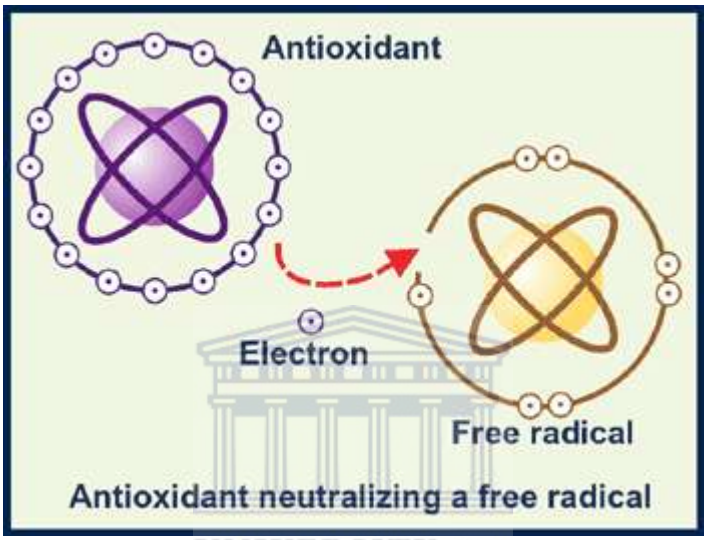


Figure 2.4: Antioxidant help by donating free electron and preventing cell damage (http://ins-jerusalem 2014.com/images/Antioxidants).

The term "oxidative stress" refers to the total intracellular and extracellular conditions that lead to the accumulation of free radicals (e.g. reactive oxygen species (ROS)) in the body, hence causing damage of cell membranes by peroxidation of membrane lipids, oxidation of protein, inactivation of enzymes, and damaging to the genetic material in the cell nucleus via impairment of ribonucleic acid (RNA) synthesis and degradation of deoxyribonucleic acids (DNA). Certain diseases are the end-results of some pathological process initiated by ROS such as superoxide, singlet oxygen, hydroxyl radicals, hydrogen and organic peroxides (Lee

et al., 2012; Chen *et al*., 2013; Yang *et al*., 2013). These highly reactive oxygen species are known to be cytotoxic and often cause tissue injury in diseases such as cardiovascular diseases (CVD), neurological diseases, malignancies, renal diseases, diabetes, cancer, rheumatoid arthritis, inflammatory problems, skin diseases, aging, respiratory diseases, liver diseases and different types of viral infections (Irshad and Chaudhuri, 2002) (see figure 2.5).

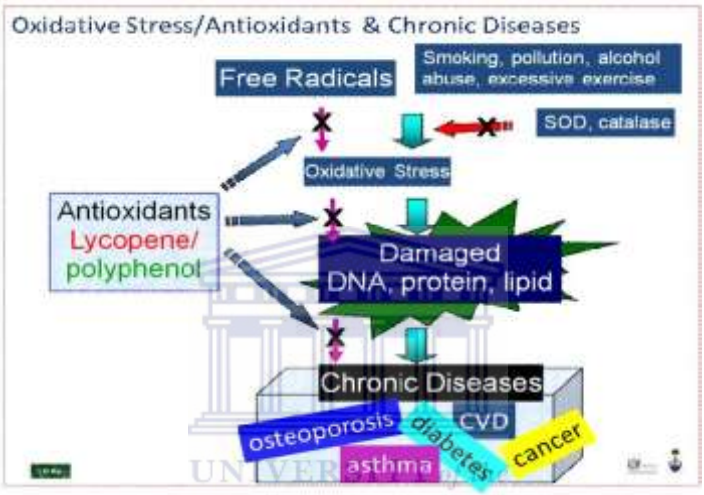


Figure 2.5: Oxidative Stress/Antioxidants & Chronic Disease (Rao and Rao, 2013).

Under conditions of oxidative stress, unsaturated fatty acids (UFA) can undergo lipid peroxidation, one of the biomarkers of oxidative stress, to indicate whether oxidative stress has a role in damage of cells and tissues (Hong *et al*., 2014). Other studies determined the development or presence of lipid peroxidation(LPO) in the serum, some of them based on production of malondialdehyde by enzymatic processes(Ayala *et al*., 2014). The thiobarbituric acid reactive substance (TBARS) is a well-established method for lipid peroxidation determination in serum and other biological fluids (Yagi, 1979). Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion

to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defence mechanism (Fukai and Ushio-Fukai, 2011). It has an important role to protect the cells and tissues of the body against ROS and particularly superoxide anion radicals (Van Raamsdonk and Hekimi, 2012). Total tissue antioxidant capacity (TAC) is an assay used to measure the ability of endogenous systems to resist oxidative damage and current research has shown the TAC assay to be a reliable biomarker for many different pathophysiological conditions like heart and vascular diseases, diabetes mellitus, neurological and psychiatric disorders, renal disorders and lung diseases (Kusano and Ferrari, 2008).

2.2.1. Oxidative stress and nicotine

Previous studies have reported that the harmful effects of nicotine is due to a disturbance in the capacities of endogenous antioxidant defences in which the activity of the antioxidant system is overwhelmed by reactive oxygen species generation (Halliwell and Whiteman, 2004). In nicotine administered rats, an increase in the generation of superoxide anion and hydrogen peroxide has been shown to lead to a decrease in the activities of the free radical scavenging enzymes such as SOD, catalase and glutathione peroxidase(Ashakumary and Vijayammal, 1996). Oxidative stress due to nicotine exposure has been reported in various tissues, including lung, vasculature and pancreas.

A recent study Dhouib *et al* (2015) has reported that lung damage in rats that were chronically exposed to nicotine for 18 weeks was due to oxidative stress. It reported that chronic nicotine administration caused a significant increase in malondialdehyde (MDA)

level, SOD activity and catalase (CAT) activity in lung tissue suggesting oxidative damage (Dhouib *et al*., 2015). Nicotine induced oxidative stress in pancreatic islet cells has also recently been reported by Bhattacharjee *et al* (2016). Oxidative stress in pancreatic tissue is associated with lipid peroxidation (Chowdhury and Walker, 2008). Research into the oxidative damage to organs by nicotine has also been reported in a study conducted by Akkoyun and Karadeniz (2016). The authors reported that neonates exposed to nicotine intrauterine showed decreased total glutathione (GSH), glutathione peroxidase (GSH-Px) and SOD antioxidant activities. This may explain why nicotine decreases the capacity of antioxidant in the tissue and increased MDA levels which is a measure of the oxidation of lipids by ROS; it suggests that lipid peroxidation participate in the development of damage due to nicotine (Akkoyun and Karadeniz, 2016).

2.2.2. Oxidative stress and hypertension

Oxidative stress had been suggested to be involved in the pathogenesis of hypertension and atherosclerosis (Alexander, 1995; Montezano *et al*., 2015). Laboratory animal studies have established that angiotensin II (ANG II)-induced hypertension in the rat is accompanied by oxidative stress in blood vessels (Ishizaka *et al.*, 1997; Ocaranza *et al*., 2014). Xiao *et al* (2011) reported antenatal nicotine exposure to increase the risk of hypertension in adult offspring as a result of programmed heightened oxidative stress and vascular reactivity via a Nox2-dependent mechanism. It has been suggested that the toxicity of nicotine induced oxidative stress can be modulated by antioxidants (McIntyre *et al*., 1999).

In the vasculature, the sources of ROS are from an assortment of different cell types including vascular smooth muscle cells (VSMCs), endothelial cells (EC), macrophages and fibroblasts. ROS has been shown to be an important factor in the regulation of many biological responses (Kunsch and Medford, 1999; Higashi *et al.*, 2009), including stretch of the vessel wall in a pathological process involved in the development of hypertension.

The increase of oxidative stress related to blood pressure is accompanied by a reduction in the most important antioxidant mechanisms hence antioxidant therapy (such as vitamin C) may relieve hypertension and invert the compensative up regulation of nitric oxide species isotypes in spontaneously hypertensive rats (SHR) (Vaziri *et al*., 2000).

2.3. Vitamin C as an antioxidant

Vitamin C (Vit C), also called ascorbic acid or ascorbate, is a water-soluble vitamin that is necessary for normal growth and development of the body. Vitamin C is the major essential water-soluble antioxidant in human serum and is able to reduce the oxidative properties of toxic substances (Perai *et al*., 2014). It is not synthesised in human due to a mutation in the gene coding for L-gulonolactone oxidase, which is necessary for the biosynthesis of vitamin C via glucuronic acid. It thus has to be supported in the human body via the intake of fruits, vegetables and vitamin C tablets (Carr and Frei, 1999; Naidu, 2003).

Some studies have shown that vitamin C acts in a non-enzymatic reaction as an electron donor to fulfil its role as an antioxidant in the prevention of many diseases. Vitamin C prevents other compounds from being oxidized by donating two electrons from the double bonds between the second and third carbon of its 6-carbon structure. As an antioxidant, vitamin C helps in preventing oxidative stress by directly scavenging oxygen-derived free radicals, such as superoxide anions or hydroxyl radicals (Nagaraj and Paunipagar, 2014). After the loss of an electron, the species formed is a free radical called ascorbyl free radical. Ascorbyl radical is relatively stable and fairly unreactive with a half-life of 10^{-5}seconds, which is why ascorbate is preferred as an antioxidant (Nagaraj and Paunipagar, 2014). Figure 2.6 below describes the chemical structure of the oxidation products of vitamin C.

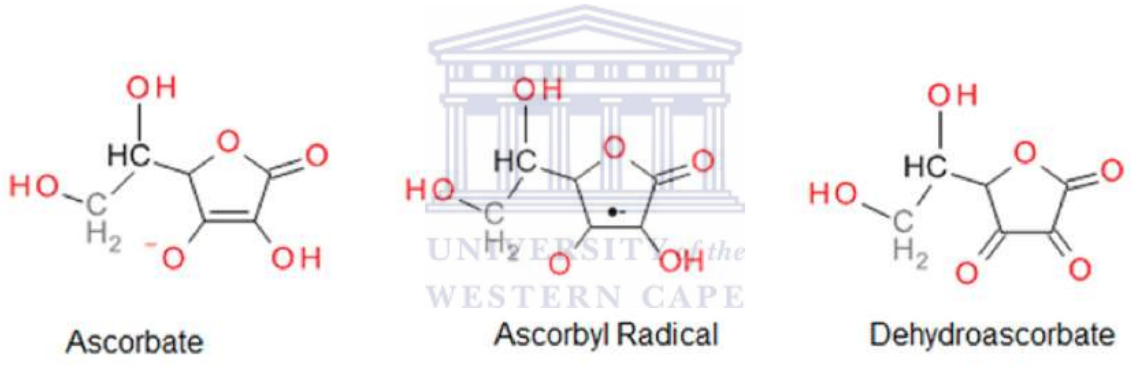


Figure 2.6: Vitamin C (ascorbate) and its oxidation products (Goszcz *et al*., 2015).

It has been demonstrated that vitamin C protects nitric oxide (NO) from oxidation and increases its synthesis, and by this protection, it increases endothelial NO and ameliorates endothelial dysfunction (Taddei *et al*., 1998). Evidence also indicates that vitamin C is beneficial to healthy subjects and those with cardiovascular disease (CVD) (Brown and Hu, 2001). A decrease in the concentration of vitamin C in plasma has been associated with hypertension and impaired endothelial function (Moran *et al*., 1993). Ascorbic acid has

beneficial effects on endothelium-dependent vasodilatation, and these beneficial effects may be related to the scavenging of oxygen free radicals (Akpaffiong and Taylor, 1998; Taddei*et al*., 1998). Ascorbate stimulates vasodilatation in the brachial and coronary arteries and by this dilation, vitamin C protects the body from many arterial diseases(Levine *et al*., 1996; Kugiyama*et al*., 1997).

2.4. Advanced glycation end products

Advanced glycation end products (AGEs) are proteins and lipid molecules that become non-enzymatically glycated when exposed to aldose sugars. AGEs are a complex and heterogeneous group of compounds that have been implicated in the pathophysiological process of some diseases, such as diabetes, renal failure, atherosclerosis and Alzheimer's disease (Singh *et al*., 2002;Goldin *et al*., 2006; Merhi, 2013), hence, the study of advanced glycation end products has become one of the most important areas of research today. While studies on AGEs have only commenced in the last 20 years, the initial chemistry behind their mode of action has been described since the early 1900s, when it was noted that amino acids heated in the presence of reducing sugars developed a characteristic yellow-brown color (Wihler *et al*., 2005). Endogenous sources of AGEs are usually found in areas of the body where proteins and lipid glycation occurs, leading to the generation of AGEs. AGEs can also be derived from exogenous sources such as tobacco and foods (Cerami *et al*., 1997).

In the body there are many variant receptors for AGEs, called receptors of advanced glycation end products (RAGEs). RAGE is a member of the immune-globulin superfamily of receptors

(Neeper *et al*., 1992; Sugaya *et al*., 1994)which become up-regulated when AGEs ligands accumulate in the body in a process of positive feedback activation (Mahajan and Dhawan, 2013). Such up-regulation takes placein endothelial and smooth muscle cells (SMC) (Schmidt*et al*., 2001).

Oxidative stress may induce formation of AGEs and AGEs may causes further oxidative stress (Jakus and Rietbrock, 2004). According to Yamagishi and Matsui (2010), there is evidence that AGEs and their signal-transducing receptor interaction evokes oxidative stress (Yamagishi and Matsui, 2010). An in vitro study has also shown AGE to be part of the complicated interaction between oxidative stress and vascular damage (Wihler *et al*., 2005). It has been demonstrated that the connection between AGEs and RAGE of endothelial cells leads to reduction in the defence mechanisms of the cellular antioxidants (e.g. glutathione and vitamin C) via generation of ROS, and this leads to further development of cellular oxidative stress (Basta *et al*., 2004). Current research has suggested a potential link between AGEs-induced vascular calcification and oxidative stress (Wei *et al*., 2013).The study used nicotine to induce the calcification of the aorta resulting in an increase in the expression levels of aorta AGEs (Wei *et al*., 2013). It has also been suggested that RAGEs may have a role in stiffening of the vasculature by forming cross links through the collagen molecule, or by interaction with their cellular transduction receptors (Geroldi *et al.*, 2005). Katz *et al* (2005) also hypothesized that nornicotine, a minor metabolite of nicotine, up regulate the expression of RAGE in the gingival of smokers and trigger the secretion of cytokines and reactive oxygen species which directly cause destruction of the tissues.

CHAPTER THREE

MATERIALS AND METHODS

3.1.Ethical Clearance

Ethical clearance for the study was obtained from the Ethical Committee of the University of the Western Cape (project no: 10/2/17).

3.2.Materials

The materials that were used in this study are presented in Table 3.1 and 3.2 below.

Table 3.1: Biochemical assay kits and chemicals used in this study

Kits and Chemicals	SUPPLIER
Total antioxidant capacity (TAC) test kit.(Ca.No:MAK187)	Sigma-Aldrich (USA)
Thiobarbituric acid reactive substances (TBARS) test kit (Ca.No: 700870)	Cayman-chemical
Superoxide dismutases (SOD) test kit (Ca.No: 706002)	Cayman-chemical
Vitamin C (ascorbic acid)	Sigma-Aldrich (USA)
Pentobarbitone sodium	Norpham medical (SA)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (USA)
High performance liquid chromatography (HPLC) grade water	Kimix (SA)

Ethanol	Servochem (SA)
Formaldehyde	Merck (Germany
Di-Sodium hydrogen phosphate (anhydrous) (Na_2HPO_4)	Kimix (SA)
Sodium Di-hydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$)	Kimix (SA)
Xylene	Kimix (SA)
Parafine wax	Merck (Germany)
DPX	Kimix (SA)
Haematoxylin and eosin (H**&** E) stain	Merck (Germany)

Table 3.2: Equipment

Product	**Supplier**
Automatic tissue processor	Leica, TP 1020 Germany)
Autostainer machine	Leica XL (Germany)
Balance	ae-Adam, Keynes (USA)
Centrifuge	Kent Scientific (USA)
CODA blood pressures monitor	Kent Scientific (USA)
Embedding system	Bobo (China)

Heating pad	Doccol Corporation (USA)
Light microscope	Carl Zeiss (Germany)
Microtome	Leica, RM 2125RT (Germany)
POLARstar omega spectrophotometer	BMG Labtech (Germany)
Vortex	Kent Scientific (USA)
Water bath	Electrothermal (England)

Nicotine used for this study was a gift from Dr Windvogel of the Department of Physiology, Stellenbosch University.

3.3.Protocol for animal experimentation

The animals used in this study were virgin Wistar rats and were bred in the animal facility at the University of the Stellenbosch. Only animals free from visible signs of diseases and ill health were used in this study. Animals had free access to food (chow) and tap water. The rats were kept in a room with controlled temperature (23C°), controlled lighting (lights on at 7 a.m. and off at 7 p.m.) and controlled humidity.

First study

Female Wistar rats were given either saline (1 ml/day, s.c; control group), or nicotine (1 mg/kg/day in 1 ml s.c; the nicotine group), during pregnancy and lactation. The blood

pressure of both female and male offspring was measured at 6 months of age. The experimental design of this study is shown in next flow diagram (figure 3.1).

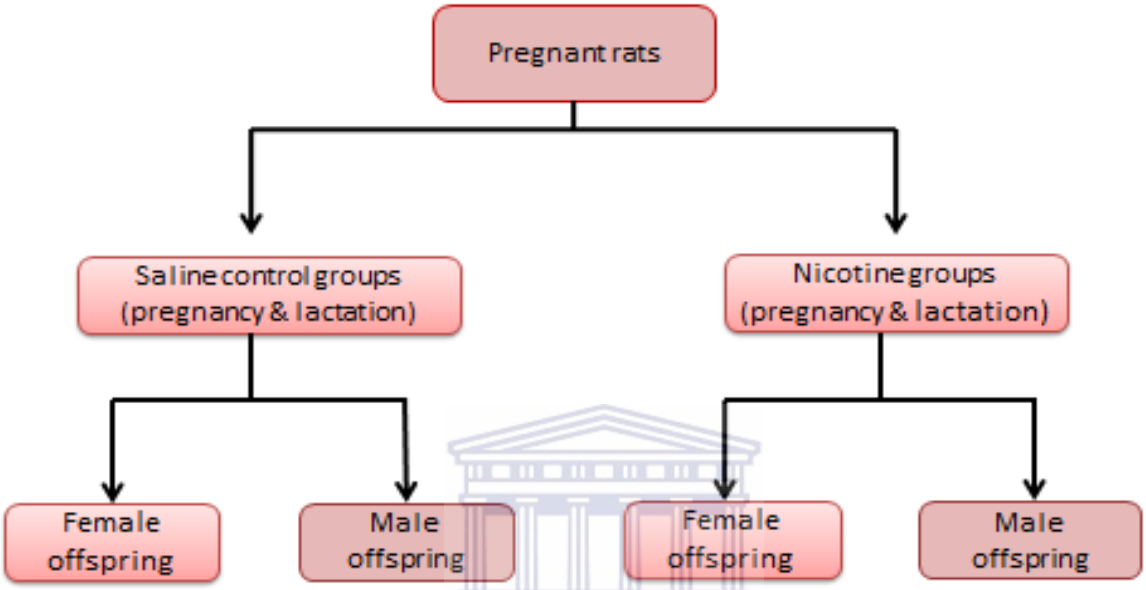


Figure 3.1: Flow diagram showing the experimental design for the first study**.**

Second study

In the second study, rats confirmed to be pregnant were randomly divided into four experimental groups. Group one (1) animals served as a control group while groups two to four (2-4) were treated as indicated below. Eight animals from group 2 received vitamin C after weaning (group 5).

Group 1: Saline (1 ml/day, s.c.).

Group 2: Nicotine (1mg/kg/day in 1 ml, s.c).

Group 3: Saline (1ml/day, s.c) and vitamin C (1g/l in the drinking water).

Group 4: Nicotine (1mg/kg/day in 1 ml, s.c) and vitamin C (1g/l in the drinking water).

Female rats were treated as indicated above from day 3 after mating up to weaning on postnatal day 21. It is important to note that the offspring in group 1-4 were not treated with nicotine, saline or vitamin C. The fetuses and neonates were thus exposed to nicotine via the placenta and mother's milk. Animals in group 5 received vitamin C (1g/l) in the drinking water from weaning up to 5 months of age. The experimental design is shown in next flow diagram (figure 3.2).

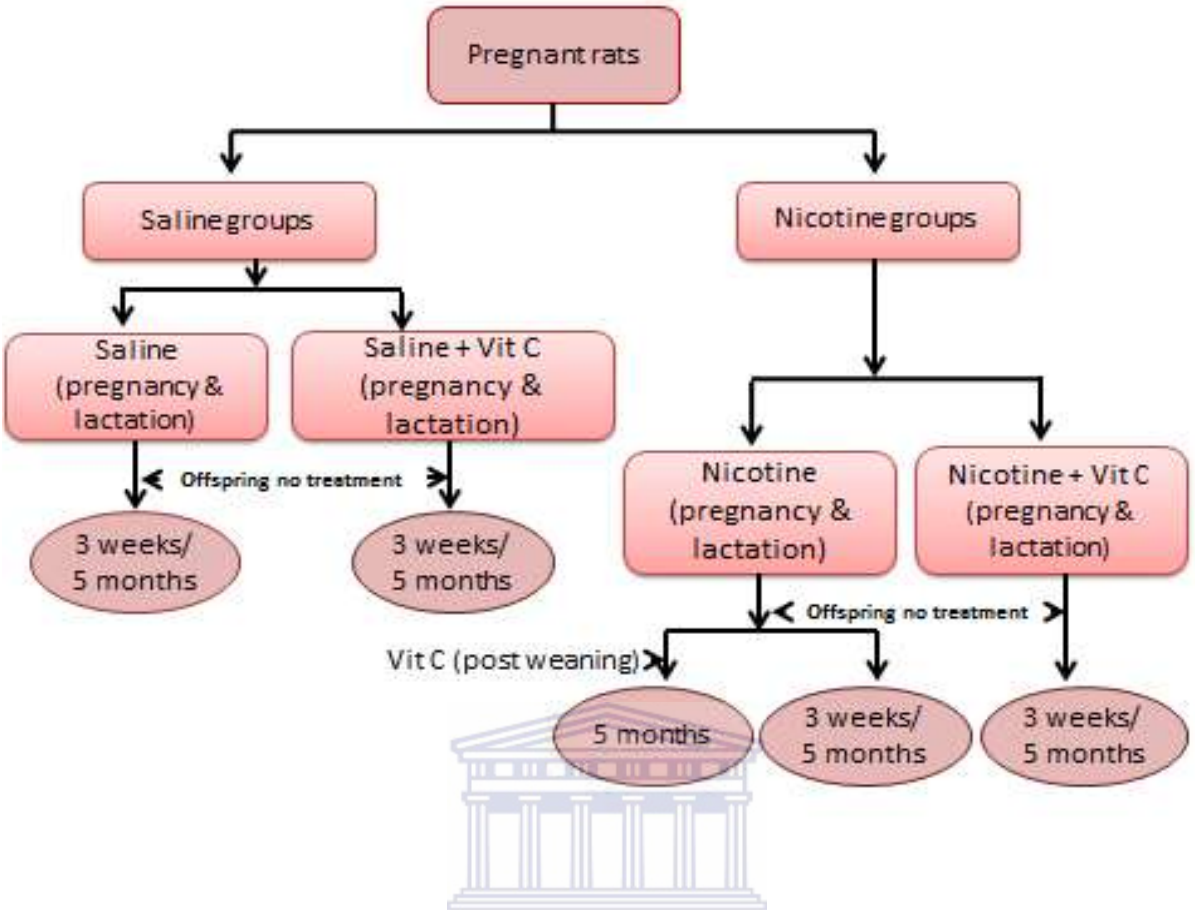


Figure 3.2: Flow diagram showing the experimental design for the second study**.**

Animals from group 1-4 (5 – 8 animals) were weighed (to determine the initial body weight) and sacrificed by exanguiation at 3 weeks of age, and blood samples were collected, and serum prepared as described below, for biochemical assays. Blood pressure of animals was measured every two weeks from 5 weeks of age. The weight of animals was also measured at two week intervals until 20 weeks (5 months) of age. At the end of the experimental period (5 months) animals were anesthetized with sodium pentabarbital (100 mg/kg i.p.) and killed by exanguiation (when withdrawal reflex was absent after performing a toe pinch). Blood samples were again collected, and serum prepared, for further analysis. To prepare serum blood samples were collected from the thorax by pipette and transferred to Eppendorf tubes

and left for 20 minutes to clot. The clear serum separated by centrifugation (3000 x g, 15 min), was transferred to new Eppendorf tubes and stored at -20^{o}C for further use.

After the rats were killed (at 5 months old), the abdominal aorta was removed from 20 rats (4 rats from each group) for histological and histochemical analysis. Aortic samples removed were fixed in 10% buffered formaldehyde solution (pH 7.2). A 10% buffered formaldehyde solution was prepared as follows: 4g of di-sodium hydrogen phosphate (anhydrous) (Na_2HOP_4) and 6g of sodium di-hydrogen phosphate di-hydrate ($NaH_2PO_4.2H_2O$) were dissolved in 900 ml distilled water. Once the chemicals were dissolved in the water, 100 ml formaldehyde solution was added to make up a 1000 ml buffered formalin solution.

3.3.1. Measurement of blood pressure

Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) were measured in rats by the tail-cuff method with a computerized blood pressure monitor (CODA blood pressure monitor, Kent Scientific, USA). This system uses a volume pressure method to determine blood pressure. The animals were put under light sedation to reduce handling stress, which could influence blood pressure measurement. To maintain blood flow to the tail, the animals were then placed on a heating pad while maintaining the ambient temperature at 30°C. The occlusion cuff was placed proximally on the tail of the animal and allowed to fit loosely for free movement of the tail. A volume pressure recording (VPR) cuff was placed distally behind the occlusion cuff. After that, about

three to six stable measurements of blood pressure were taken, and the averages of the readings were calculated.

3.3.2. Serum biochemical assays

Biochemical estimation of serum antioxidant systems was performed using a thiobarbituric acid reactive substance (TBARS) assay, superoxide dismutase (SOD) assay and total antioxidant capacity (TAC) assay.

3.3.2.1.Total antioxidant capacity (TAC) assay

The total antioxidant capacity assay is a spectrophotometric method that is used for measuring the total antioxidant capacity in human and animal serum. In the total antioxidant capacity assay kit, either the concentration of the combination of both small molecule and protein antioxidant, or the concentration of only small molecule antioxidants can be determined. The TAC assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 570 nm. The color intensity is proportional to the total antioxidant capacity. In this study the assay was done with serum collected at five weeks (initial collection of blood) and at five months (final collection of blood) of age.

In brief, the experimental protocol was as follows. The reagents and samples were allowed to warm to room temperature (about 30 minutes) prior to running the assay. All samples and

standards in this assay were run in duplicate on a 96-well plate. A standard curve was generated by adding different volumes (0, 8, 16, 24, 32, 40, 48 and 56 µl) of trolox standard to wells to get a different concentration. After that, water was added to each well of standard to bring the volume to 100 µl. 5 µl of serum was added to each of the sample wells followed by addition of 95 µl water to bring the final volume of 100 µl. For the assay reaction to develop, 100 µl of the Cu working solution was added to sample wells. The 96-well plate was protected from light using a thin foil during the incubation period. It was mixed using a horizontal shaker and incubated for 90 minutes at room temperature. Finally, the absorbance of standards and unknown samples was measured at 570 nm using a POLARstar omega spectrophotometer (BMG Labtech, Ortenberg, Germany). The standard curve generated was used to calculate sample TAC. The TAC activity of the samples was calculated using the equation obtained from the linear regression of the standard curve. Since the serum samples were diluted, values calculated were corrected by multiplying by the dilution factor (5x). The result was expressed as nmole of TAC per µl of sample volume.

3.3.2.2.Thiobarbituric acid reactive substances (TBARS) assay

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation (LPO) (Yagi, 1998). This method depends on the formation of the lipid peroxidation (LPO) end product, malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) to produce a thiobarbituric acid reactive substance (TBARS), a pink chromogen which can be measured spectophotometrically at 532 nm. Modifications of the thiobarbituric acid reactive substance (TBARS) assay by many researchers have been used to evaluate several types of samples including human and animal tissue and fluids, drugs, and foods (Richard *et al*., 1992).

The assay protocol of the experiment was as follows: 100 µl of MDA standards (malondialdehyde) with different concentration (0, 0.0625, 0.125, 0.25, 0.5, 1, 2.5 and 5 µM) and samples were added to a 5 ml tube, which was appropriately labeled. Thereafter, 100 µl of SDS solution and 4 ml of the color reagent were added to each tube and tubes were swirled to mix. The vials were capped and placed in a holder to keep the vials upright during boiling. The vials were boiled for an hour, after which they were immediately removed and placed in an ice bath for 10 minutes to stop the reaction. After 10 minutes, the vials were centrifuged for another 10 minutes at 1600 x g at 4 C°. 150 µl from each vial was loaded in duplicate to a clear plate (calorimetric version). The absorbance of the standards and unknown samples were read at 530 nm using a POLARstar omega spectrophotometer (BMG Labtech, Ortenberg, Germany). The standard curve generated was used to calculate TBARS of the samples. The result was expressed as nmol of MDA per ml of sample volume.

3.3.2.3. Superoxide dismutase (SODs) assay

Superoxide dismutases are metallo-enzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide thus forming a crucial part of the cellular antioxidant defence mechanism (Barik *et al*., 2005). The superoxide dismutase assay kit utilizes a tetrazolium salt for detection of superoxide radical generated by xanthine oxidase and hypoxanthine.

In brief, the experimental protocol was as follows. All samples and standards in this assay were run in duplicate on a 96-well plate. For SOD standard wells, 200µl of the diluted radical detector and 10 µl of standards (SOD) with different concentration (1, 1.472, 1.752, 2, 2.89,

3.9971 and 4.75 U/ml) were added to each of the designated wells on the plate. For sample wells, 200 µl of the diluted radical detector and 10 µl of the sample were added to the sample wells. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all wells. The 96-well plate was covered, carefully shaken for a few seconds to mix, and then the samples were incubated for 30 minutes at room temperature on a shaker. After 30 minutes, the absorbance of standards and unknown sample was read at 450 nm using a POLARstar omega spectrophotometer (BMG Labtech, Ortenberg, Germany). The standard curve generated was used to calculate sample SOD activity. The SOD activity of the samples was calculated using the equation obtained from the linear regression of the standard curve. The result was expressed as U (units) of SOD per ml of sample.

3.3.3. Histology study

3.3.3.1.Tissue Processing

The aortic tissue was carefully removed from the 10 % buffered formaldehyde and placed on a clean glass tile and cleaned of connective tissue. The tissue was placed in a plastic tissue processing cassette that was properly labeled, and placed into a tissue processing rack of the automatic tissue processor (Leica TP 1020). The total duration of secondary fixation and wax impregnation was programmed for a 22-hour cycle (see table 3.3). Table 3.3 summarizes the tissue processing protocol.

3.3.3.2. Embedding

After the 22-hour tissue processing cycle was completed, tissues were manually removed from cassettes in order to begin the tissue embedding procedure. Tissues were transferred into a mould in a standing position. Then, slowly a very small volume of warm wax was run into the mould, to ensure that the tissue sample was fully covered with wax. The mould was then placed on the refrigerated plate of the embedding system just for a few minutes to permit the wax to start solidifying.

3.3.3.3. Sectioning

When the tissue had solidified at the end of the embedding phase, the cassettes were removed from the mould. The tissue blocks were trimmed and cut into 5 μm sections for haematoxylin and eosin (H & E) staining.

Table 3.3: Tissue processing procedure for light microscopy.

Step	Solution	Time (min)	Temperature (ºC)
1	70 % alcohol	1 hr	40
2	70 % alcohol	1 hr	40
3	80 % ethanol	2 hr	40
4	90 % ethanol	2 hr	40
5	100 % ethanol	2 hr	40
6	100 % ethanol	2 hr	40
7	100 % ethanol	2 hr	40
8	Xylene	2 hr	40
9	Xylene	2 hr	58
10	Parafine	2 hr	58
11	Parafine	2 hr	58

3.3.3.4.Mounting

The 5 μm tissue sections were obtained from the embedded tissue and transferred to labelled microscope slides. This was done by allowing the cut sections of wax ribbon to float in a warm (60-70˚C) water bath (Electothermal paraffin section mounting bath) allowing the wax ribbon with the tissue section to flatten for easy picking with the microscope slide. The slides were left overnight to allow the tissue to be fixed onto the slide. Sections that broke up easily when placed in the water bath were excluded from the study. The labelled microscope slide was placed on a slide rack and placed in an incubator (Heraeus) at 80˚C for ± 30 min, to further fix the tissue and melt the wax, after which they were then stored in microscope slide boxes until staining was executed.

3.3.3.5.Haematoxylin and Eosin Staining

Standard haematoxylin and eosin (H&E) staining was used to prepare tissues for evaluation of structure. Standard haematoxylin and eosin (H&E) staining was carried out by an autostainer machine (Leica Auto Stainer XL) at the University of Stellenbosch, Cape Town, South Africa. The staining protocol used in this study is explained in the table 3.4 below:

Table 3-4: Procedure of Haematoxylin and Eosin staining.

Step	Chemical solution	Time
Deparaffination and Rehydration		
1	Xylene 10 min	5 min
2	Ethanol (99%)	5 min
3	Ethanol (95%)	2 min
4	Ethanol (70%)	2 min
5	Distilled water	5 sec
Haematoxylin staining		
1	Haematoxylin	8 min
2	Running water	5 min
3	Ethanol (1% acid alcohol)	30 sec
4	Running water	5 min
5	Ammonia (0.2%)	45 sec
6	Running water	5 min
7	Ethanol (95%)	10 dips

Eosin Staining and Dehydration

1	Eosin	45 sec
2	Ethanol (95%)	5 min
3	Xylene	5 min
4	Mount for observation using DPX and slide cover slips	

3.3.3.6. Immunohistochemistry (IHC) staining for receptors of advanced glycation end products

Tissue sections were prepared and mounted as described earlier. All immunohistochemical (IHC) staining procedure was performed using an automatic Leica Bond Autostainer with the Bond Polymer.

Bond polymer detection was performed by incubating the specimen with hydrogen peroxide to quench endogenous peroxidase activity. The primary antibody was applied and Post Primary Immunoglobulin G (IgG) linker reagent was used as the postprimary antibody. Poly-HRP (Horseradish peroxidase) IgG antibody was used as the second antibody and the substrate chromogen, 3, 3'-diaminobenzidine tetrahydrochloride (DAB) was used to visualize the complex as a brown precipitate. Finally, hematoxylin (blue) counterstaining was performed to allow for visualization of the cell nuclei.

The method of immunohistochemistry (IHC) applied staining is illustrated in the table 3.5 below.

Table 3-5: IHC staining protocol (standard protocol of the autostainer).

Step	Type	Incubation Time	Temperature
1	Peroxide Block	5 min	Ambient
2	Bond Wash Solution	2 min	Ambient
3	Bond Wash Solution	2 min	Ambient
4	Bond Wash Solution	2 min	Ambient
5	Primary Antibody	15 min	Ambient
6	Bond Wash Solution	2 min	Ambient
7	Bond Wash Solution	2 min	Ambient
8	Bond Wash Solution	2 min	Ambient
9	Post Primary	8 min	Ambient
10	Bond Wash Solution	2 min	Ambient

11	Bond Wash Solution	2 min	Ambient
12	Bond Wash Solution	2 min	Ambient
13	Polymer	8 min	Ambient
14	Bond Wash Solution	2 min	Ambient
15	Bond Wash Solution	2 min	Ambient
16	Deionized Water	1 min	Ambient
17	Deionized Water	1 min	Ambient
18	Mixed DAB Refine	10 min	Ambient
19	Deionized Water	1 min	Ambient
20	Deionized Water	1 min	Ambient
21	Deionized Water	1 min	Ambient
22	Hematoxylin	5min	Ambient
23	Deionized Water	1 min	Ambient

24	Deionized Water	1 min	Ambient
25	Deionized Water	1 min	Ambient

After immunohistochemistry (IHC) staining, the tissues were rehydrated and cleared. The rehydration was done as described in the next protocol (see table 3.6).

Table 3-6: Rehydration protocol.

Step	Solution	Duration
1	70% alcohol	5 dips
2	96% alcohol	5 dips
3	96% alcohol	5 dips
4	99% alcohol	5 dips
5	99% alcohol	5 dips
6	Xylene	Dip for 1 min
7	Xylene	Dip for 1 min

Thereafter, the PDX mounting medium was used to cover the glass slide. The slide was left to dry at 25 °C. The protein expression in aorta tissue was evaluated by microscopy using a Zeiss Microscope with objectives at 200 X and 400 X magnification.

3.4. Statistical analysis

Data generated from this study was analyzed with GraphPad Prism 5.0. Results are shown as the mean ± SEM. Two groups were compared using a Student's t-test, whereas multiple groups were compared by ANOVA (in all cases variances between groups were found to be equal). $P < 0.05$ is considered significant.

CHAPTER FOUR

RESULTS

4.1. Preliminary study

4.1.1. Effect of nicotine exposure during pregnancy and lactation on the blood pressure of rat pups

In a preliminary experiment, the blood pressure of 6 months old offspring (female and male) from rat dams exposed to nicotine during gestation and lactation was determined. The blood pressure of male pups was significantly higher ($P<0.01$) when compared with the control group (MAP= 111 ± 11.55 mm Hg vs 129 ± 12.66 mm Hg in control and the nicotine group, respectively; figure 4.1B), whereas blood pressure of female offspring was not significant ($P>0.05$) between the control and nicotine group (figure 4.1A). This could mean that male rats exposed to nicotine during pregnancy and lactation might be more susceptible to, and possess a higher risk of hypertension. Because of this observation all subsequent experiments were performed on male offspring only.

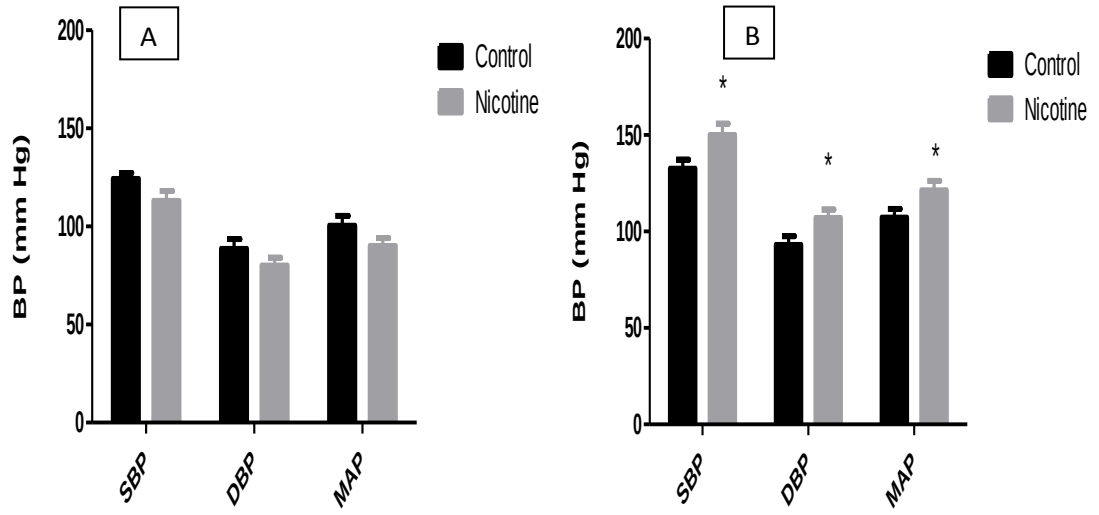


Figure 4.1: The effect of maternal nicotine exposure during gestation and lactation on the blood pressure of offspring at 6 months.

Female (A), Male (B), SBP= Systolic blood pressure, DBP= Diastolic blood pressure, MAP= Mean arterial pressure. Data is presented as the mean ± S.E.M, *P < 0.05.

4.2. Second study

4.2.1. Effect of maternal exposure to nicotine during pregnancy and lactation on the body weight of the male offspring

Body weight of male offspring was measured at 3 weeks after birth (weaning) and is considered as the initial body weight, and 5 months after birth (final body weight). The results show that the initial mean body weights of the four groups were approximately the same at week 3 (81.50 ± 2.9 g, 74 ± 2.50 g, 72.2 ± 2.40 g and 71.20 ± 4.30 g for saline, nicotine, saline + vitamin C and nicotine + vitamin C groups respectively; with P >0.05) as shown in table 4.1.

On the other hand, the final body weight of animals after 5 months shows that the mean body weight of the nicotine group (263 ± 6.6 g) and the group that received vitamin C after weaning (287.3 ± 6.3 g) was significantly reduced (P<0.001 and P<0.05, respectively) when compared with the control group (317 ± 5.4 g), see figure 4.2B. Animals exposed to nicotine and vitamin C during gestation and lactation (309.8 ± 5.3 g) and the group that received saline and vitamin C during pregnancy and lactation (303.5 ± 4.7 g) showed no change in body weight when compared with the control group (P>0.05; figure 4.2A).

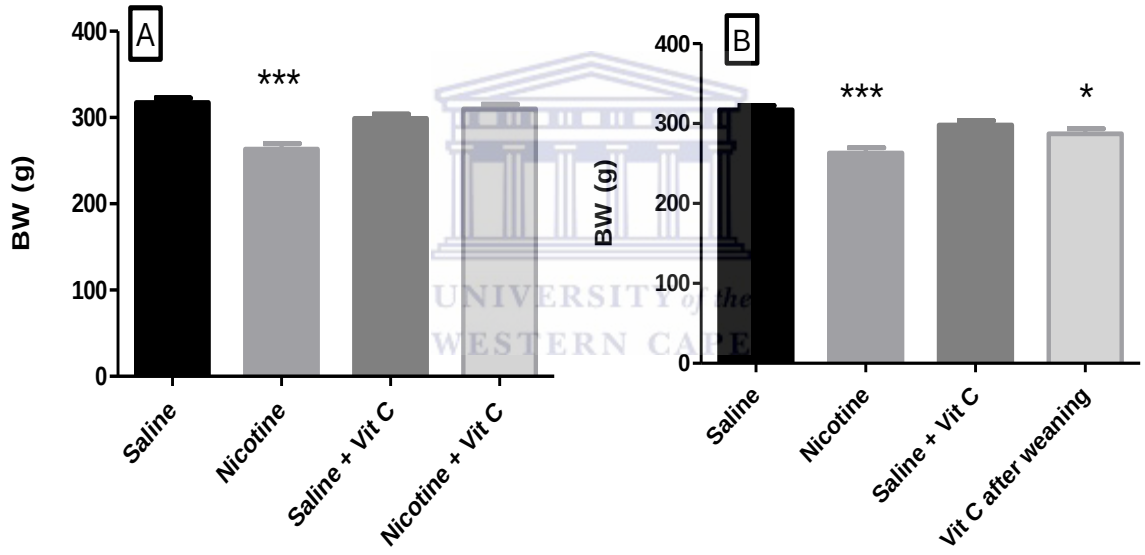


Figure 4.2: The effect of nicotine and vitamin C on initial (A) and final body weight (B) of male offspring.

Data is presented as mean ± SEM,*P < 0.05, ***P < 0.001 vs control.

Table: 4.1: Body weight of rats in the different groups at weaning (3 weeks) and at five months.

Variables	CN	N	S + C	N + C	CW
Initial BW (g)	81.5±2.9	74±2.5	72.2±2.4	71.2±4.3	
N	8	5	7	5	
P (comparison to control)		>0.05	>0.05	>0.05	
Final BW (g)	317±5.4	263±6.6	303.5±4.7	309.8±5.3	287±6.3
N	9	7	10	7	8
P (comparison to control)		<0.001	>0.05	>0.05	<0.01

CN= Control, N= Nicotine, S+C= Saline+vitamin C, N+S= Nicotine+vitamin C, CW= vitamin C after weaning, C= vitamin C. P compared with control. N= number of rats. Values are expressed as the mean ± SEM.

4.2.2. Effect of maternal exposure to nicotine during pregnancy and lactation on MAP and HR of the male offspring.

The blood pressure of the nicotine group (exposed to nicotine during pregnancy and lactation) and control group was measured over the 5-month period after birth. The results show that the blood pressure of both groups was similar for the first two months (postnatal day 65) after which a steady increase in MAP was noticed in the nicotine group up to five months of age. From 3 months onwards, the blood pressure of the nicotine group was significantly higher ($P < 0.05$) when compared with the control group (figure 4.3). In the nicotine group MAP increased from 94.8±3.8 mm Hg in the 1stmonth to 121 ± 6.4 mm Hg at the 5th month.

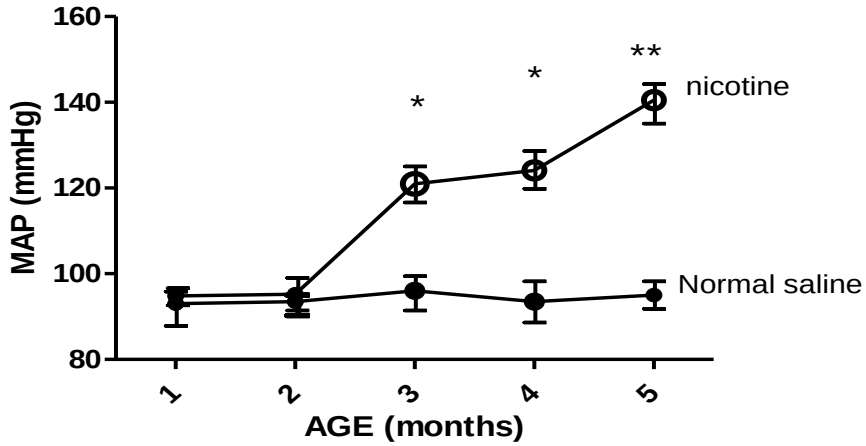


Figure 4.3: Blood pressure (MAP) in offspring over 5month period.

Data is presented as mean ± SEM, *P < 0.05, **P < 0.01.

Measurement of the baseline MAP at 5 weeks showed that the MAP of all groups was similar (P>0.05) and within the normal range (figure 4.4A and B).

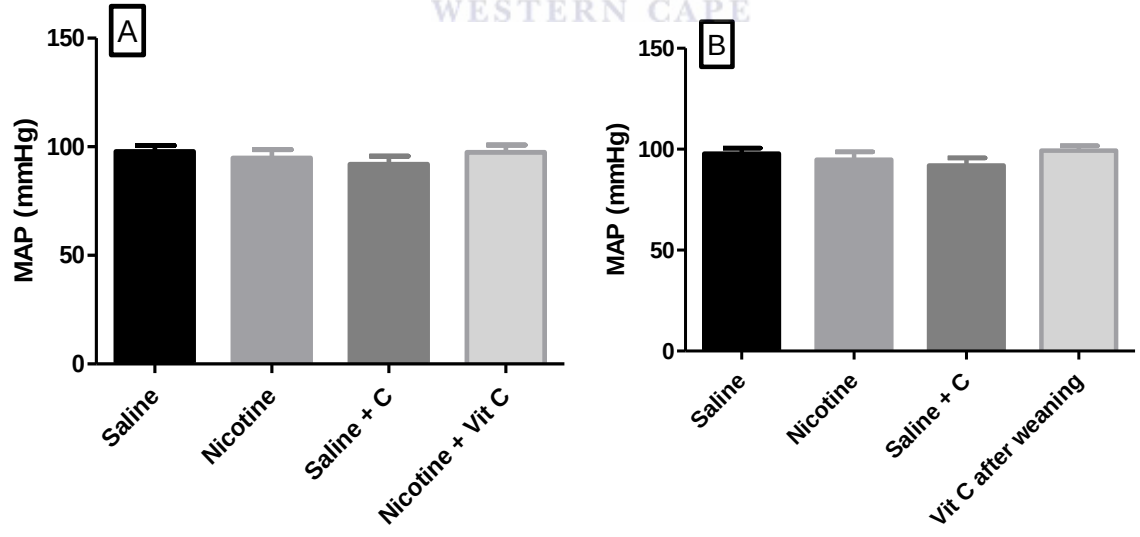


Figure 4.4: Mean arterial pressure in the different experimental groups at 5 weeks.

(A) Maternal vitamin C administration to the nicotine group during pregnancy and lactation. (B) Vitamin C administration to the nicotine group after weaning. Data is presented as mean ± SEM.

After the 5 month period, the MAP was significantly higher in the nicotine group (121±6.4 mm Hg; P<0.01) as well as the nicotine + vitamin C group (113.4±1.7 mm Hg; P<0.05) when compared with the control group (98.1±2.4 mm Hg; figure 4.5A). The MAP of the group that received vitamin C after weaning (97.6±2.9 mm Hg; P>0.05) was not significantly different from that of the control group (98.1±2.4 mm Hg), but it was significantly (P<0.01) less than that of the nicotine group (121±6.4; figure 4.5B). This shows that vitamin C administration after weaning reduces the nicotine induced increase in MAP.

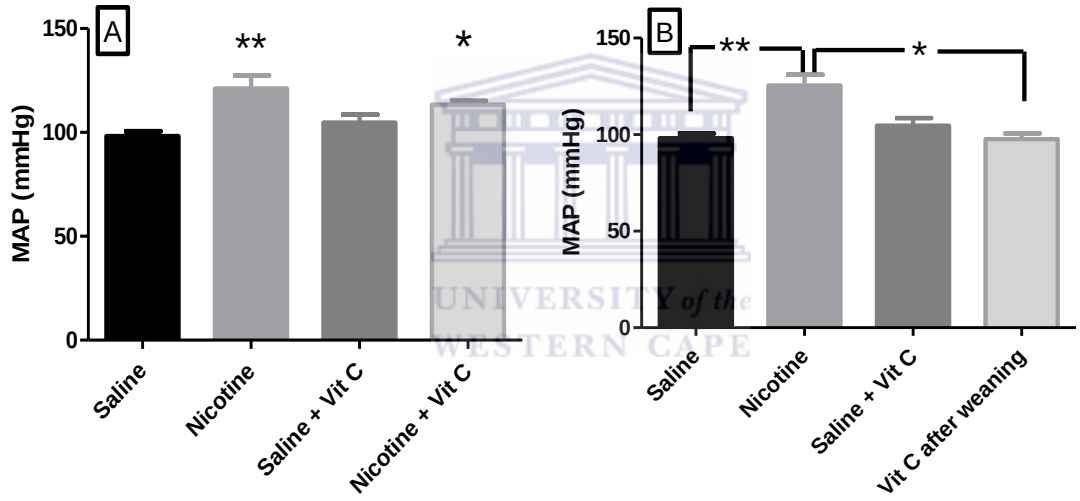


Figure 4.5: Mean arterial pressure in the different experimental groups at 5 months.

(A) Maternal vitamin administered to the nicotine group during pregnancy and lactation. (B) Vitamin C administration to the nicotine group after weaning. Data is presented as mean ± SEM,*P < 0.05, **P < 0.01 vs control.

After the 5 month period, both SBP and DBP were significantly higher in the nicotine group (SP = 147.1±6.1 mm Hg; $P<0.001$ and DP = 110.2±7.2 mm Hg; $P<0.01$, respectively) as well as the nicotine + vitamin C group (SP = 138.2±3.2 mm Hg; $P<0.05$ and DP = 102.5±3.4 mm Hg; $P<0.01$, respectively) when compared with the control group (SP = 121.1±1.7 mm Hg and DP = 83.9±3.0 mm Hg respectively) (table 4.2).

The SBP and DBP of the group that received vitamin C after weaning (SP = 116.5±3.5 mm Hg and DP = 88.2±2.7 mm Hg, respectively) was not significantly different ($P>0.05$) from that of the control group (SP = 121.1±1.7 mm Hg and DP = 83.9±3.0 mm Hg, respectively), but it was significantly ($P<0.05$) less than that of the nicotine group. This shows that vitamin C administration after weaning reduces the nicotine induced increase in SBP and DBP (table 4.2).

Table 4.2: MAP, SBP and DBP in the different groups at 5 weeks and 5 months of age.

Variables	CN	N	S + C	N + C	CW
Systolic blood pressure (SBP)					
Initial SBP (mm Hg)	118.5±2.8	115.1±4.6	113 ± 3.0	116.1±3.7	118.9±3.0
N	9	7	10	7	8
P (comparison to control)		>0.05	>0.05	>0.05	>0.05
Final SBP (mm Hg)	121.1±1.7	147.1±6.1	120.3±3.6	138.2±3.2	116.5±3.5
N	9	7	10	7	8
P (comparison to control)		<0.001	>0.05	<0.05	>0.05
Diastolic blood pressure (DBP)					
Initial DBP (mm Hg)	90.8±3.1	84.7±3.4	81.2±4.3	88.0±3.3	89.4±3.3
N	9	7	10	7	8
P (comparison to control)		>0.05	>0.05	>0.05	>0.05
Final DBP (mm Hg)	83.9±3.0	110.2±7.2	95.8±4.2	102.5±3.4	88.2±2.7
N	9	7	10	7	8
P (comparison to control)		<0.01	>0.05	<0.01	>0.05
Mean arterial pressure (MAP)					
Initial MAP (mm Hg)	97.8±2.7	94.8±3.8	91.9±3.7	97.4±3.4	99±3.0
N	9	7	10	7	8
P (comparison to control)		>0.05	>0.05	>0.05	>0.05
Final MAP (mm Hg)	98.1±2.4	121±6.4	104.1±4.6	113.4±1.7	97.6±2.9
N	9	7	10	7	8
P (comparison to control)		<0.001	>0.05	<0.01	>0.05

N = number of rats. CN= control, N= nicotine, SC= saline+vit C, NC= nicotine+vit C, CW= nicotine+vit C after weaning. Values are expressed as the mean ± SEM. P<0.05 is considered significant (compared with control).

The heart rate was not significantly different (P>0.05) between groups at 5 weeks (figure 4.6A and 4.7A) and 5 months (figure 4.6Band 4.7B).

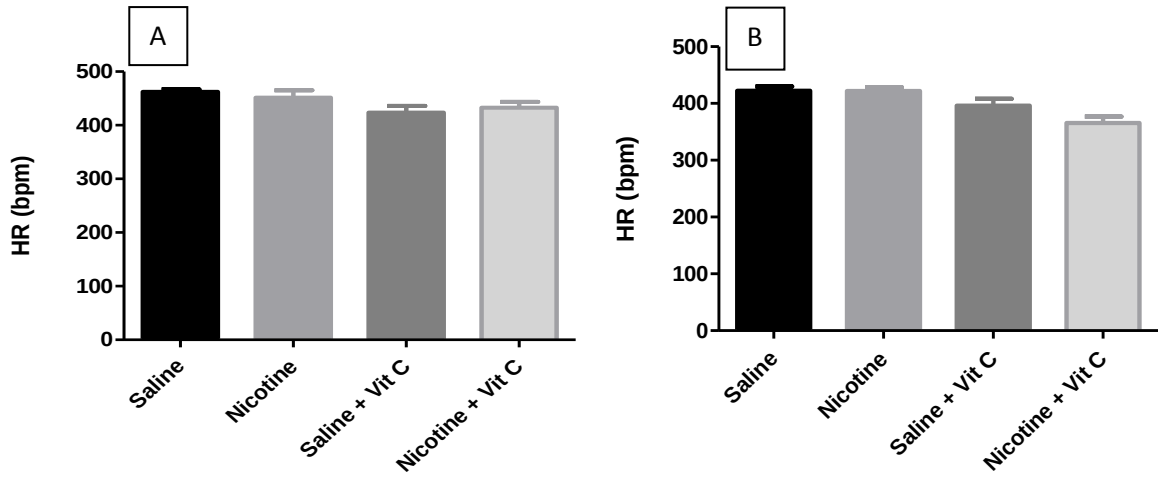


Figure 4.6: The effect of maternal nicotine exposure during gestation and lactation, and vitamin C co-administration on HR of the male offspring at five weeks (A) and at five months (B).

Data is presented as mean ± SEM.

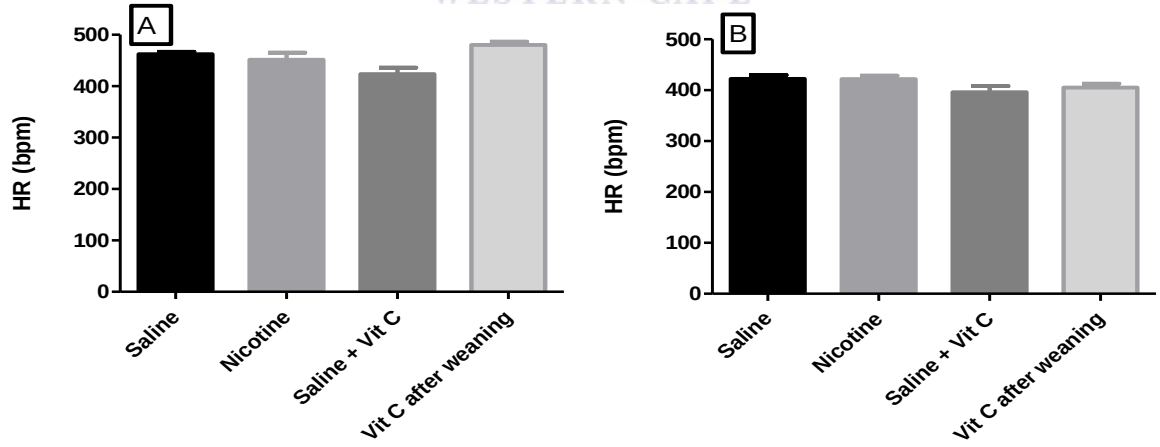


Figure 4.7: The effect of maternal nicotine exposure during gestation and lactation, and vitamin C administration after weaning on HR at five weeks (A) and at five months (B).

Data is presented as mean ± SEM.

Table 4.3: The effect of maternal nicotine and vitamin C exposure during gestation and lactation on HR of the different groups at 5 weeks and 5 months of age.

Variables	CN	N	S + C	N + C	CW
Heart rate (HR)					
Initial HR (bpm)	462±4.7	451.3±3.4	423.5±2.4	432.8±10.5	480±6.1
N	9	7	10	7	8
P (comparison to control)		>0.05	>0.05	>0.05	>0.05
Final HR (bpm)	422.4±7.3	421.9±6.1	396.2±11	365.4±11.4	405.1±7.7
N	9	7	10	7	8
P (comparison to control)		>0.05	>0.05	>0.05	>0.05

N= number of rats. CN= control, N= nicotine, SC= saline+vit C, NC= nicotine+vit C, CW= nicotine+vit C after weaning. Values are expressed as the mean ± SEM. P<0.05 is considered significant (compared with control).

4.2.3. Serum Biochemical Analysis

4.2.3.1. Lipid peroxidation (MDA)

The standard curve generated in the MDA assay shows a linear relation between absorbance and MDA concentration (figure 4.8). The MDA concentration of the samples was calculated using the equation (Y= 0.0329x + 0.0183) obtained from the linear regression of the standard curve.

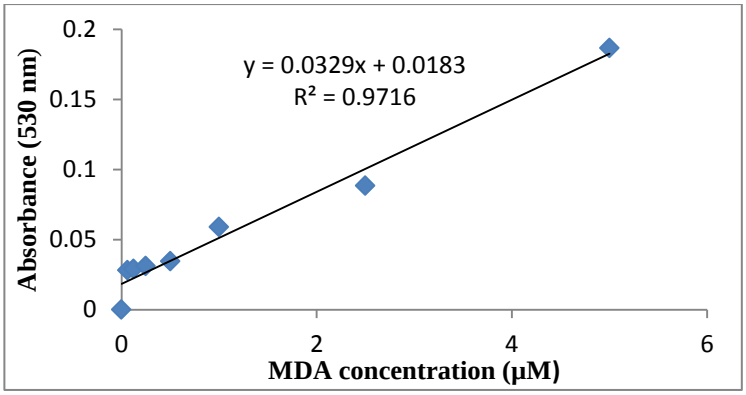


Figure 4.8: Standard Curve for the MDA assay.

Values are given as mean ± SEM

The results showed that at 5 weeks, the serum MDA content was not significantly different between the groups (P>0.05; figure 4.9). The values varied between 5.48 ± 0.6 µM (saline group) and 3.60 ± 0.6 µM (saline + vitamin C group).

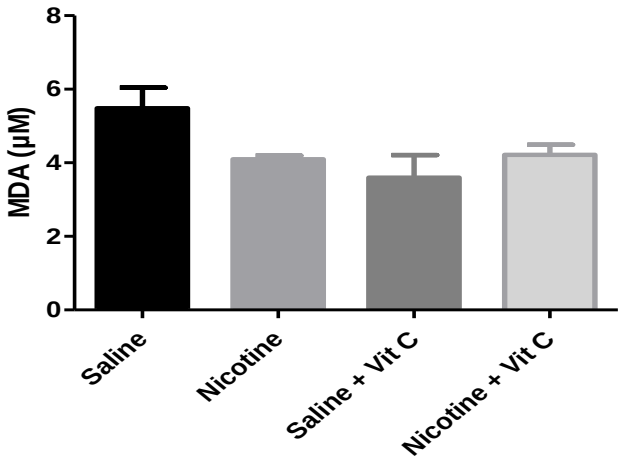


Figure 4.9: MDA content of different groups in rat serum at 5 weeks.

Values are given as mean ± SEM.

4.2.3.2 Superoxide dismutase activity (SOD)

The standard curve generated in the SOD assay shows a linear relation between absorbance and SOD concentration (figure 4.10). The SOD activity of the samples was calculated using the equation (Y= 0.6209x + 0.0641) obtained from the linear regression of the standard curve.

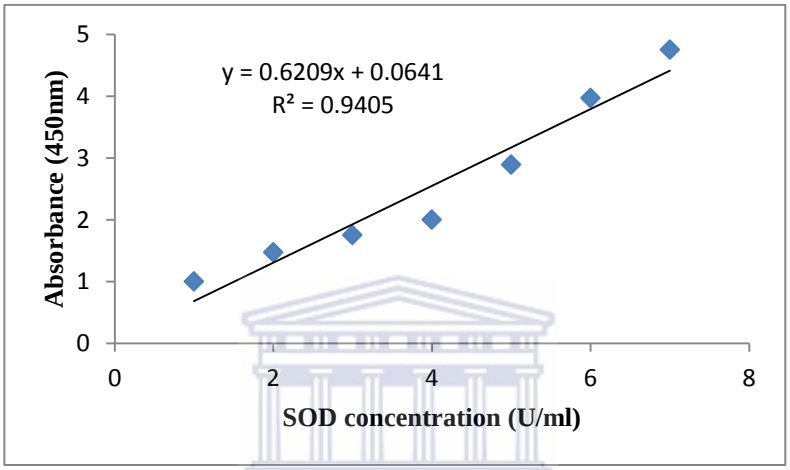


Figure 4.10: Standard Curve for the SOD assay.

Values are given as mean ± SEM.

The results showed that at 5 weeks, the serum SOD activity was not significantly different($P>0.05$) between the groups (figure 4.11). The values were varied between 4.03 ± 0.5 U/ml (saline group) and 2.93 ± 0.3 U/ml (nicotine + vitamin C group)

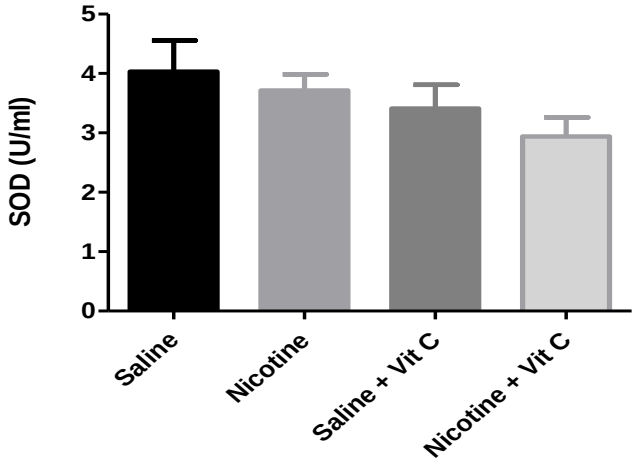


Figure 4.11: SOD activity of the different groups in male rat serum at 5 weeks.

Values are given as mean ± SEM.

4.2.3.3 Total antioxidant capacity (TAC)

The TAC of serum samples at 5 weeks and at 5 months was calculated using the equations (Y= 0.0196x + 0.011 and Y= 0.0245x + 0.1244; respectively) obtained from the linear regression curve generated during the assay (figure 4.12).

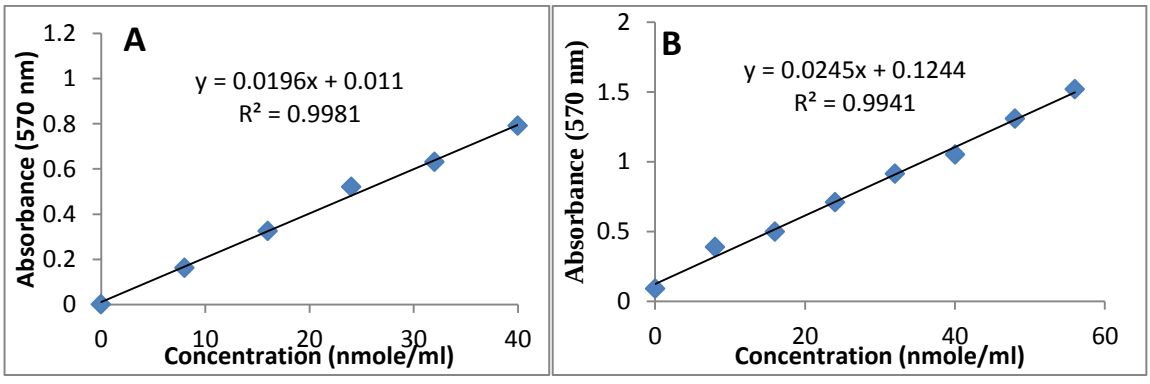


Figure 4.12: Standard Curve of the TAC assay at five weeks (A) and at five months (B).

Values are given as mean ± SEM.

Total antioxidant capacity levels in the serum of all groups either at five weeks or at five months showed no significant differences (P>0.05; figure 4.13 and table 4.4). At five weeks, the values varied between 19.2 ± 0.8 nmol/ml and 15.8 ± 2.6 nmol/ml for the nicotine group and nicotine + vitamin C group, respectively, whereas at five months the values varied between 18.3 ± 1.5 nmol/ml and 14.3 ± 1.4 nmol/ml for the saline group and nicotine + vitamin C after weaning group, respectively.

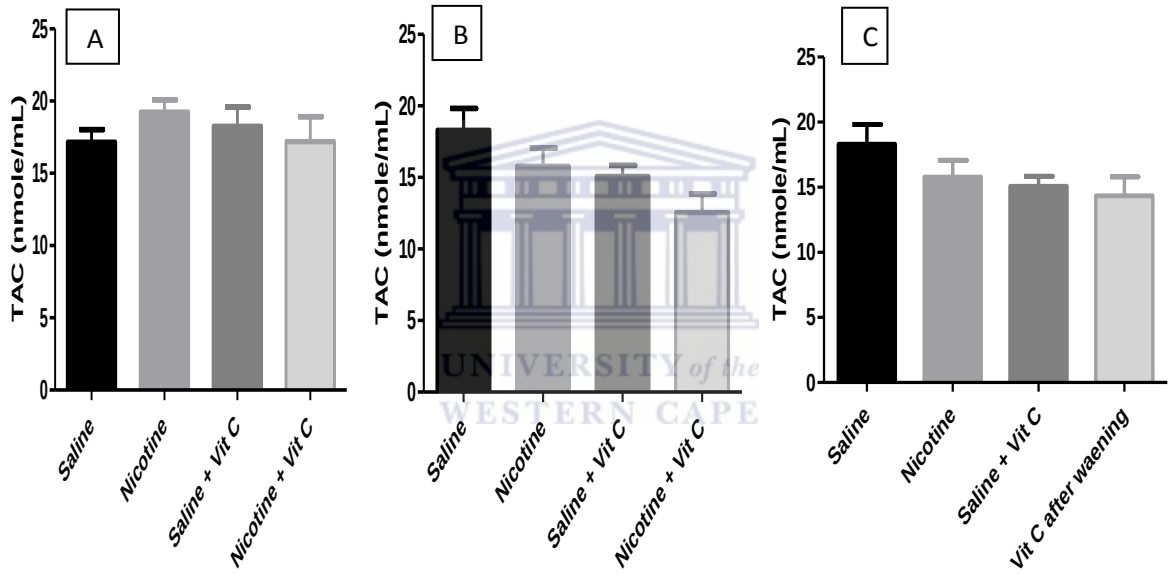


Figure 4.13: TAC level of the different groups in rat serum at 5 weeks (A) and at 5 months (B and C).

Values are given as the mean ± SEM.

Table 4.4: The summary result of biochemical assays done on the serum samples.

Variable	CN	N	SC	NC	CW
Lipid peroxidation (MDA)					
LP	5.4±0.6	4.9±0.9	3.6±0.6	4.2±0.2	
N	4	4	4	4	
P (comparison to control)		>0.05	>0.05	>0.05	
Superoxide dismutase (SOD)					
SOD	4.0±0.5	3.7±0.2	3.4±0.3	2.9±0.3	
N	5	5	5	5	
P (comparison to control)		>0.05	>0.05	>0.05	
Total antioxidant capacity (TAC)					
TAC (five weeks)	17.5±0.8	19.2±0.8	15.8±2.6	17.2±1.7	
N	5	5	5	5	
P (comparison to control)		>0.05	>0.05	>0.05	
TAC (five months)	18.3±1.5	15.7±1.2	15.0±0.7	14.6±2.3	14.3±1.4
N	7	7	7	7	7
P (comparison to control)		>0.05	>0.05	>0.05	>0.05

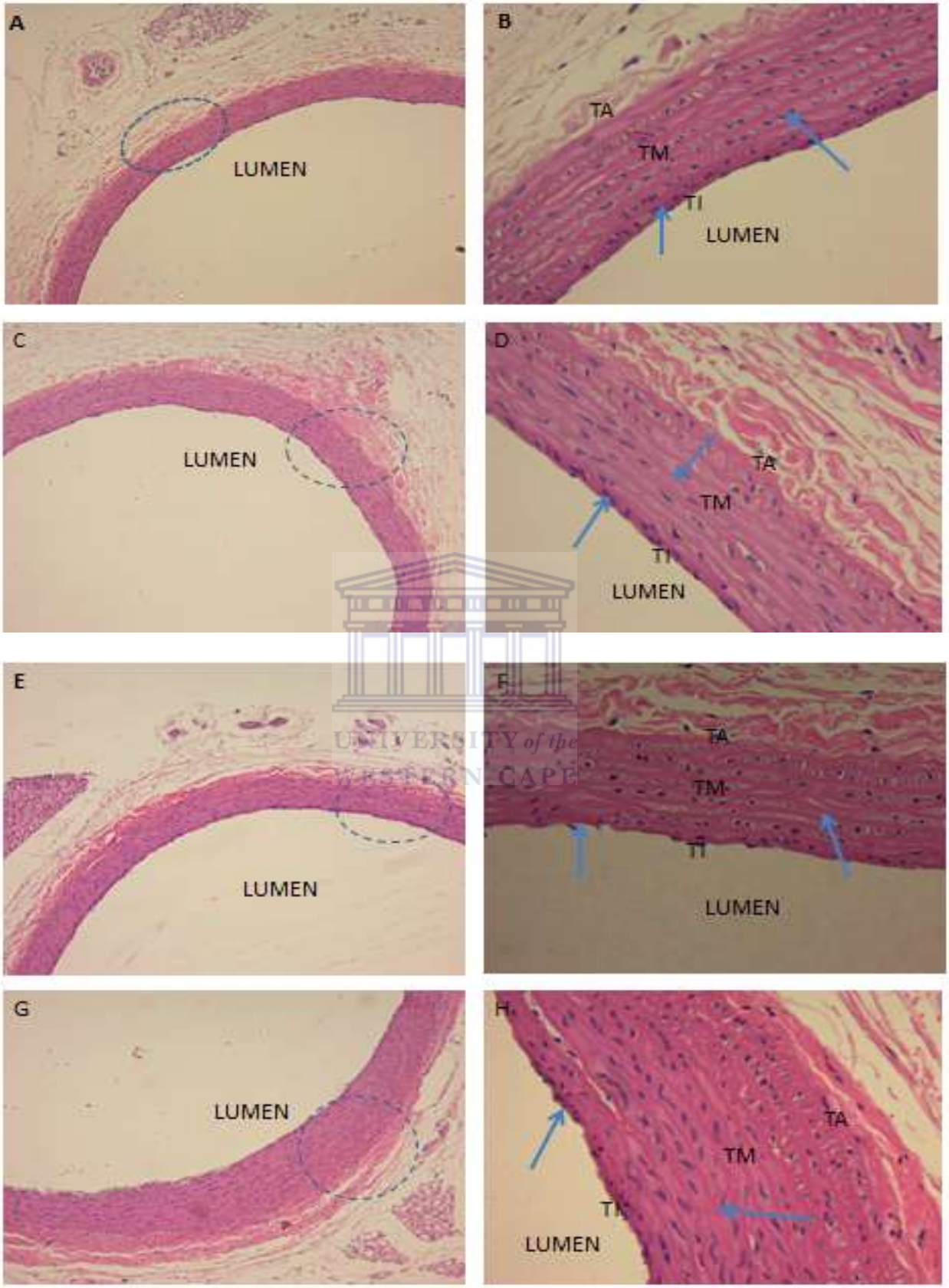
N = number of rats. CN= control, N= nicotine, SC= saline+vit C, NC= nicotine+vit C, CW= nicotine+vit C after weaning. Values are expressed as the mean ± S.E.M. P compared with control. TAC in nmole/ml; SOD in U/ml and MDA in µM

4.3. Histology Results

4.3.1 Haematoxylin and Eosin staining

Representative photomicrographs of transverse sections of the abdominal aorta from all the groups are shown in (figure 4.14). Sections of the control (saline) and saline + vitamin C group show regularity of the aorta wall and a normal arrangement of the three layers (tunica adventitia, tunica media and tunica intima), as well as the normal linear arrangement of smooth muscle cells (SMC) and endothelial cells (EC) (figure 4.14B and 4.14 D).

Photomicrographs show irregular and abnormal alignment of the aorta wall with an irregular arrangement of the vascular smooth muscle cells (VSMCs) at the tunica media layers in the nicotine, nicotine + vitamin C groups, as well as in the group that received vitamin C after weaning (figure 4.14F, H and K) respectively. In these groups the elastic fibres appear wavy and intima protrusions are also observed.



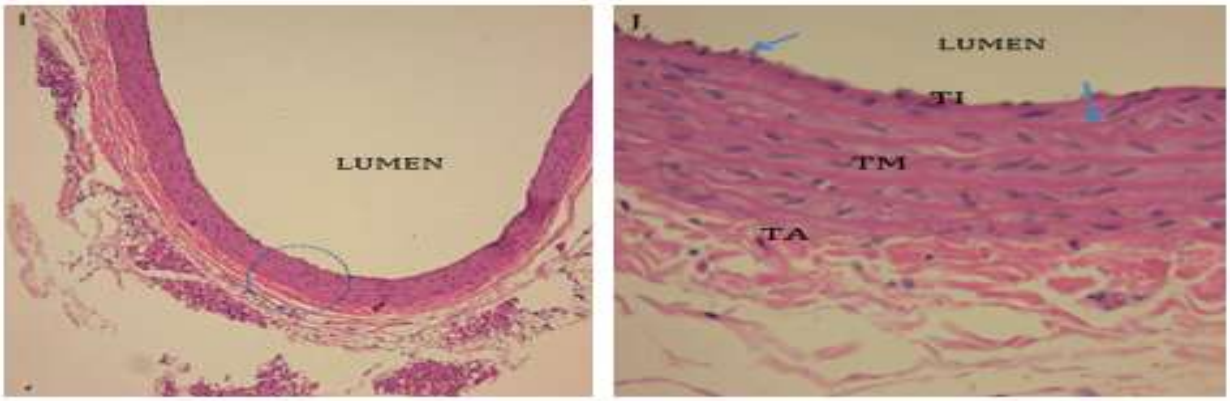


Figure 4.14: Transverse sections of the abdominal aorta stained with hematoxylin and eosin (H and E stain).

Plates A and C are photomicrograph of the transverse sections of saline (control) and saline+vit C groups. Aorta show regularity in the aortic wall and normal alignment of the three tunics (TA= Tunica adventitia, TM= Tunica media, TI= Tunica intima). Plates B and D are the higher magnification of plates A and C, and show the intima (TI), media (TM) and adventitia (TA). Plates E, G and I show photomicrographs of the transverse sections of the aorta from the nicotine, nicotine+vitamin C and nicotine+vitamin C groups after weaning. Irregular and abnormal alignment of the aortic wall of the three groups was observed. Plates F, H and K are the higher magnification plates E, G and I respectively. Blue arrows show the tunica intima (TI), abnormal arrangement of vascular smooth muscle cells (VSMC) in the media (TM) and adventitia (TA).

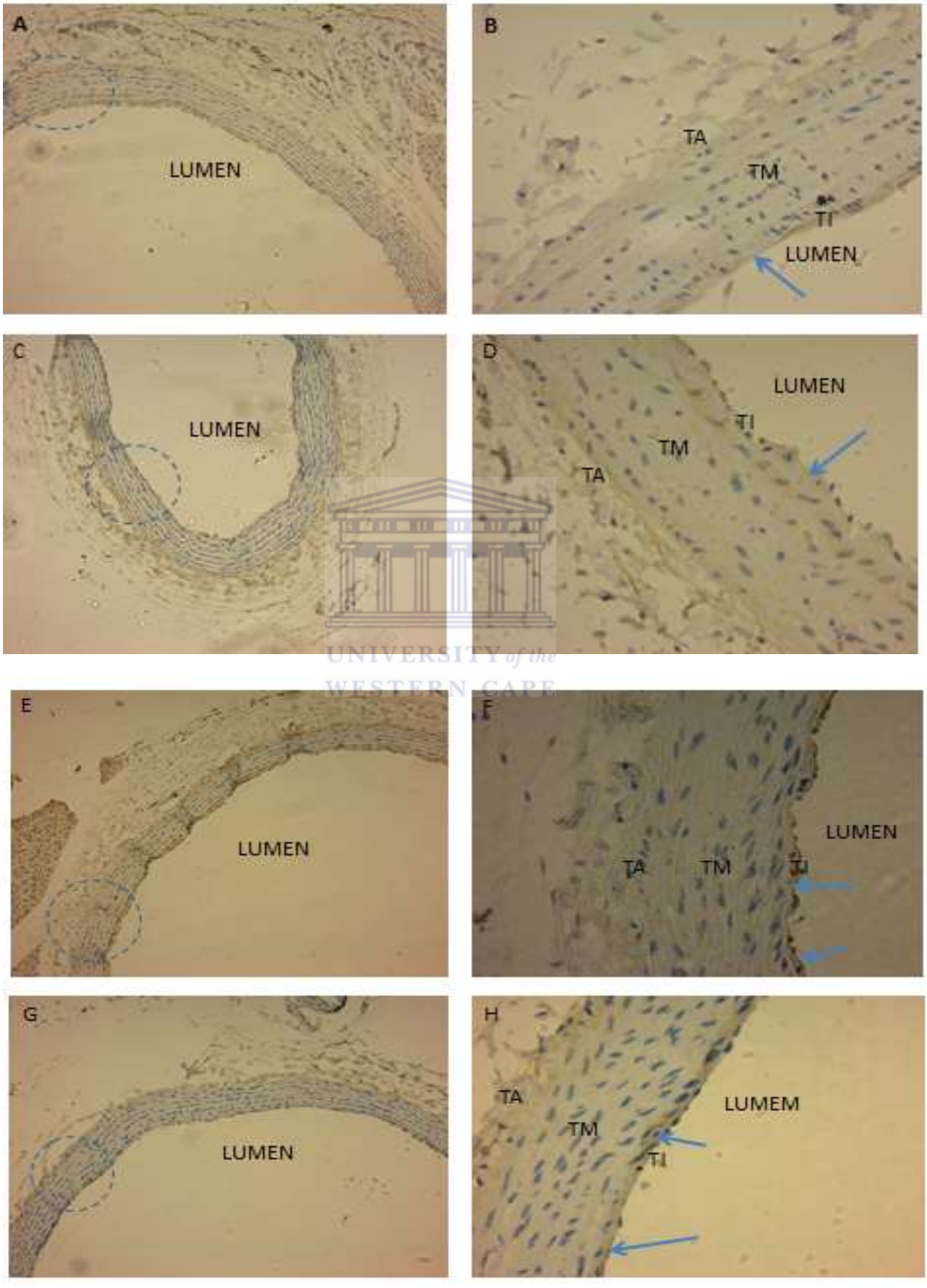
The images in plates A, C, E, G and I were taken at 200X magnification, whereas the images in figure B, D, F, H and K were taken at 400X magnification.

4.3.2 Immunohistochemistry (IHC)

To assess the expression levels of advanced glycation end products (AGEs) in aortic tissue, immunohistochemistry (IHC) was performed on sections from the abdominal aorta using an antibody to AGEs. Representative photographs of immunostaining of aortic tissue in all the groups are shown in images 4.15A - K. In this study, the brown stain represents the presence

of the AGEs protein. Expression levels of AGEs were observed in all the groups but the staining intensity differed amongst groups.

The saline and saline + vitamin C groups (figure 4.15 B and D, respectively) showed very little expression of AGEs, whereas the expression level of AGEs in the nicotine, nicotine + vitamin C and nicotine + vitamin C after weaning groups (figure 4.15 F, H and J, respectively) was much stronger.



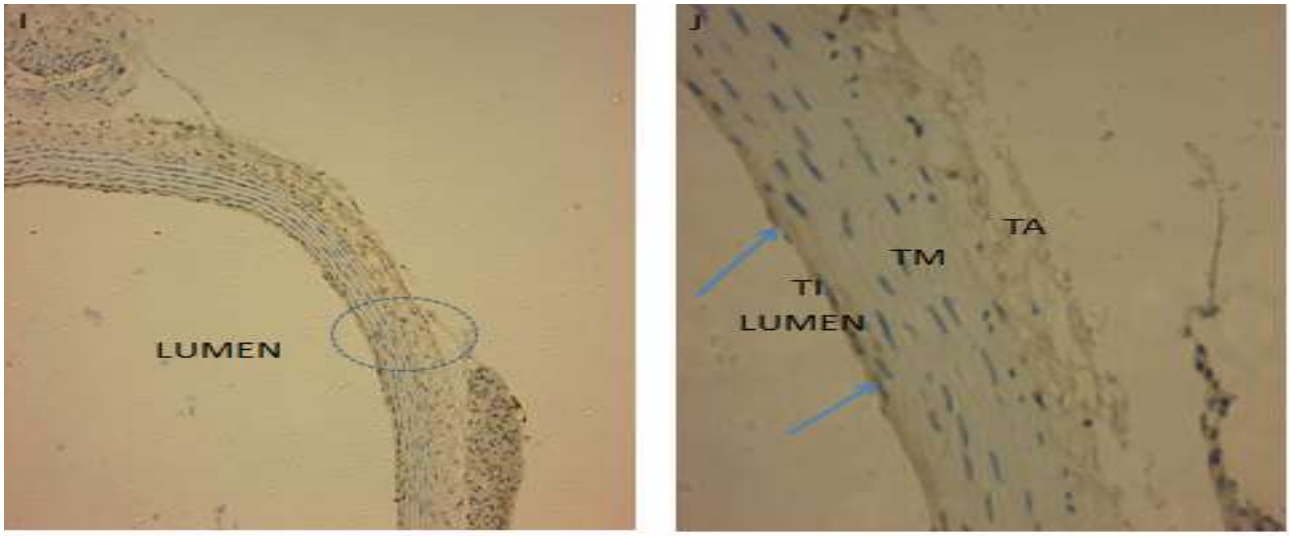


Figure 4.15: IHC of AGEs expression in abdominal aortic tissues from the five groups.

Control (plates A&B), normal saline + vitamin C (plates C&D), nicotine (plates E&F), nicotine + vitamin C (plates G&H) and nicotine + vitamin C after weaning (plates I&J). The expression of AGEs in the all of the groups was indicated with a blue arrow. (TA= Tunica adventitia, TM= Tunica media, TI= Tunica Intiman).

The images in plates A, C, E, G and I were taken at 200 x magnifications, whereas the images in plates B, D, F, H and K were taken at 400 x magnifications.

CHAPTERFIVE

DISCUSSION

5.1. Introduction

Events that occur during fetal development are linked to long-term health in the adult. So for example, intrauterine growth restriction (IUGR), which sometimes occurs as a result of inadequacy of nutrients or disruptions in placental structure or function, could lead to a decrease in the expression of genes that are responsible for nephrogenesis. Survival of the fetus under these conditions, most times, results in low birth weight (LBW) and a deficit in nephron number, which are associated with hypertension in adulthood (Jones *et al*., 2012). Hypertension is a strong independent risk factor for well-known cardiovascular and cerebrovascular morbidities that include myocardial infarction, stroke and atherosclerosis (Staessen *et al*., 2003).

A previous study has shown that sex hormones play a role in the progression of hypertension in adults subjected to intra uterine growth restriction(Black *et al*., 2015). It has been reported that while both male and female intra uterine growth restriction offspring are hypertensive early in life, only males remain hypertensive into adulthood (Ojeda *et al*., 2007).

5.2. Body weight

A strong relationship exists between the maternal nutrient intake during pregnancy and the body weight of infants (Parlee and MacDougald, 2014). In developing countries, the low birth

weight infants have been shown to be affected by intrauterine growth restriction during pregnancy (Ramakrishnan, 2004). In pregnant women the placenta is the organ that transports nutrients, respiratory gases and wastes between the mother and the fetus (Tarrade *et al.*, 2015). Placental blood flow is thus essential for the fetus to grow, as well as the removal of waste products during metabolism. Maternal smoking during gestation was reported to have an adverse effect on the placenta function as it results in placental vasoconstriction. This vasoconstriction, in turn leads to a reduction of more than 40% in utero-placental blood flow (Birnbaum *et al*., 1994; Anblagan *et al*., 2013), which will compromise nutrient supply to the developing fetus (Larsen *et al*., 2002).

According to Mothibeli (2013), the body weight of 14 and 21 day-old offspring was not affected by maternal nicotine exposure during pregnancy. The results of this study concur with these findings and show that maternal exposure to nicotine had no effect on the body weight of male offspring at 3 weeks of age. In contrast to our findings after 3 weeks, the body weight of animals in the nicotine group was significantly lower after 5 months (figure 4.2B). Previous work has indicated that body weight loss induced by nicotine, probably is due to its effect on certain neurotransmitters that act in the control and regulation of appetite and satiety in the hypothalamus (Chatkin and Chatkin, 2007). Mineur *et al* (2011) also established that nicotine reduces the food intake and body weight of young offspring by influencing the hypothalamic melanocortin system and synaptic mechanisms involved in nicotine-induced decreases in appetite.

5.3. Effects of maternal nicotine exposure during pregnancy and lactation on blood pressure of female and male offspring

Previous studies have indicated that the nicotine present in cigarette smoke has led to an increase in blood pressure and heart rate during smoking (Middlekauff *et al*., 2014; Omvik, 1996). Furthermore, it was also established that maternal exposure to nicotine induces hypertension in male offspring (Gao *et al.*, 2008; Xiao *et al*., 2014).

My preliminary study demonstrated that maternal nicotine exposure during gestation and lactation increased blood pressure of male offspring but not of female offspring (figure 4.1); hence all my subsequent experiments were performed only on male offspring. These results correspond to studies conducted by Tao *et al* (2013) and Xiao *et al* (2008), where that BP was higher in the adult male offspring than in the female offspring as a result of nicotine exposure during pregnancy and lactation. This suggests a protective function of female sex hormones, such as estrogen, on perinatal exposure to nicotine induced hypertension. Xiao *et al* (2013) suggested that estrogen has a role in the sex difference of perinatal nicotine-induced programming of vascular dysfunction. They hypothesized that estrogen may counteract heightened reactive oxygen species production, resulting in protection of females from developmental programming of the hypertensive phenotype in adulthood.

The result of this study also indicates that blood pressure in male offspring increased from the age of 12 weeks onwards for the male offspring of nicotine-exposed animals, this is supported by Ojeda *et al* (2007) who reported that while both male and female intra uterine growth

restriction offspring are hypertensive early in life, only males remain hypertensive into adulthood.

The systolic blood pressure increased from 115.1±4.6 mm Hg in the first month to 147.1±6.1 mm Hg in the fifth month, diastolic blood pressure increased from 84.7±3.4 mm Hg in the first month to 110.2±7.2 mm Hg in the fifth month, and mean arterial pressure increased from 94.8±3.8 mm Hg in the first month to 121±6.6 mm Hg in the fifth month. Our results at five months of age are similar to previous studies which also indicated that the blood pressure of offspring exposed to nicotine during pregnancy and lactation was increased at 14 weeks of age (Gao *et al*., 2008). Gao *et al* (2008) however, did not measure the blood pressure in the very young animals.

Nicotine may lead to an increase of blood pressure via nicotine receptors found on peripheral chemoreceptor cells in the central nervous system. The peripheral chemoreceptor cells are located near the carotid arteries and aorta and bear a significant stimulatory effect on ventilation and sympathetic activity (Gonzalez *et al*., 1994; Ciarka *et al*., 2005). Ciarka*et al* (2005) hypothesized that nicotine elevates peripheral sympathetic nerve activity to smooth muscle, causing vasoconstriction. In addition, heart rate is also increased due to sympathetic nervous system activation (Triposkiadis *et al*., 2009). Our results show that exposure to maternal nicotine did not raise HR although an increase in the blood pressure was observed, suggesting that the observed hypertension is probably not due to activation of the sympathetic nervous system. This will however require further investigation.

5.4. Effect of vitamin C supplementation on development of hypertension in animals exposed to nicotine during gestation and lactation

In human, a decrease in the concentration of Vit C in plasma has been associated with hypertension as well as impaired endothelial function (Juraschek *et al.*, 2012). Houston (2005) suggested a dose-dependent relationship between blood pressure and plasma ascorbate levels. It was also shown that ascorbic acid (2.4 mg/100ml) has beneficial effects on endothelium-dependent vasodilatation, and these beneficial effects could be related to the scavenging of oxygen free radicals (Akpaffiong and Taylor, 1998; Taddei *et al*., 1998). Administration of 1g of vitamin C per day reduced SBP by 7 mm Hg and DBP by 4 mm Hg (Bates *et al.*, 1998).

It is possible that the intake of nicotine during gestation and lactation via tobacco smoking or via nicotine replacement therapy could reduce the blood and tissue vitamin C content of the neonate, thereby rendering it more vulnerable to oxidant damage. We could also posit that nicotine intake reduces the vitamin C content of the mother's blood and thus the potential of the mother to protect the offspring against the harmful effects resulting from nicotine. Lower serum vitamin C levels in maternal nicotine exposure could be caused by impaired vitamin C absorption or increased vitamin C breakdown. Maternal vitamin C supplementation during gestation and lactation could help prevent the deleterious effect of nicotine as it will ensure that a reasonable amount of vitamin C is available for development of the fetus and neonate.

Many studies have also shown the effect of maternal antioxidant therapy on the other body organs. In the lung, a previous human trial has suggested that maternal intake of antioxidant (vitamin E) is associated with a reduced risk of respiratory disease (such as wheeze and asthma) of offspring (Devereux *et al*., 2006). It has also reported that administration of vitamin C and E during pregnancy could diminish adiposity in the offspring (Sen and Simmons, 2010). Damage of the fetal brain caused by cell death via apoptosis is due to oxidative stress, and can be reduced by using antioxidants during pregnancy and lactation (Shirpoor *et al*., 2009).

The results from our study show that maternal vitamin C supplementation in rats exposed to nicotine during pregnancy and lactation did not prevent development of hypertension of the male offspring. Contrary to this, vitamin C given to pups after weaning significantly improved the nicotine induced hypertension of male offspring as it leads to a reduction in the blood pressure. Our results also show that supplementation with vitamin C after weaning leads to a reduction of both systolic and diastolic blood pressure (table 4.2). It was observed that systolic blood pressure was reduced more than diastolic blood pressure 30.6±8.8 mmHg and 22±10.4 mmHg, respectively, when compare with the nicotine group. To our knowledge this is the first time that it is shown that hypertension induced by nicotine exposure during pregnancy and lactation, can be attenuated or even reversed.

5.5. Effect of vitamin C supplementation on serum oxidative parameters

Oxidative stress is caused by an imbalance between the antioxidants and the reactive oxygen species, resulting in damage to cells or tissues. Several micronutrients are of vital importance to health due to its antioxidant properties (Mocchegiani *et al*., 2014). It is now widely believed that diet derived antioxidants play a role in the prevention of human disease and deficiency of the dietary derived antioxidants may prejudice fetal and childhood development (Evans and Halliwell, 2001).

The measurement of TBARS is well established method for screening and monitoring lipid peroxidation. MDA is one of the most prevalent byproducts of lipid peroxidation during oxidative stress (Yagi, 1998). SOD is thought to play a very important role in protecting living cells against toxic oxygen derivatives. The enzyme catalyzes the dismutation of two superoxide radicals into O_2 and H_2O_2 (Sun *et al*., 1988). The TAC assay can measure either the combination of both small molecule and protein antioxidants or small molecules antioxidant alone in the presence of protein mask. In this study we thus used a three pronged approach to evaluate of maternal nicotine exposure and vitamin C (an antioxidant) supplementation on the antioxidant capacity of the offspring.

Maternal exposure to nicotine during pregnancy and lactation resulted in an increased oxidative stress in fetal, neonatal, and adult tissues (Wetscher *et al*., 1995; Crowley-Weber *et al*., 2003; Zhao and Reece, 2005). In our result, we did not find any significant difference in serum activity of MDA, SOD or TAC (figure 4.9, 4.11 and 4.13) among the groups at five weeks. The difference in the result might be attributed to the difference in the chosen dose of nicotine in this study compared with other studies where nicotine was proven to induce

oxidative stress. In this study, the dose of nicotine (1mg/kg/day) was very low compared with previous studies (>4 mg/kg/day) (Crowley-Weber *et al*., 2003; Zhao and Reece, 2005). It may also be due to the fact that the studies by Crowley-Weber *et al*., (2003) and Zhao and Reece (2005), evaluated oxidative stress in tissue and not in serum. Our finding however corresponds with a previous study which concluded that the concentration of vitamin C as well as total antioxidant status in nicotine treated rats, were not altered amongst groups, but a significant decrease of vitamin E was observed (Helen *et al*., 2000).

In this study, we used vitamin C as an antioxidant to determine whether taking vitamin C supplements will help or prevent the effects of oxidative stress in the serum, induced by nicotine. Since some offspring received vitamin C during pregnancy and lactation, and others received vitamin C after weaning, total antioxidant capacity assay was tested at 5 weeks and at 5 months. The total antioxidant capacity in the serum of animals at 5 weeks and 5 months of nicotine withdrawal was not significant different between any of the groups. The findings imply that neither nicotine, nor vitamin C supplementation, prompted any change in the antioxidant capacity at 5 weeks or at 5 months. Therefore, and due to the cost of these assays, we did not test the TBARS and SOD assays again at five months. In contrast to this result, previous research from our laboratory showed that vitamin C decreased oxidative status in animal tissue (Daramola, 2015) which is more in line with the results of Crowley-Weber *et al*., (2003) mentioned earlier.

5.6. Histology and immunochemistry

5.6.1. Morphology study

A morphometric study on aorta tissue from hypertensive rats has shown alteration in the architectural layout of the layers (Intengan and Schiffrin, 2001; Zarkovic *et al*., 2015). In hypertension, artery structure changes are mainly of two kinds: (1) inward eutrophic remodelling, in which outer and lumen diameters are decreased, i.e. media/lumen ratio is increased, and cross-sectional area of the media is unaltered; and (2) hypertrophic remodelling, in which the media thickens and encroach on the lumen, resulting in increased media cross-sectional area and media/lumen ratio (Intengan and Schiffrin, 2001).

The results of a study conducted by Intengan and Schiffrin (2001) is in agreement with our study which indicates that fetus and neonatal nicotine exposure leads to increased blood pressure and morphological alterations in the layers of the aorta, characterized by irregular and abnormal alignment of the aortic wall. The alteration in the layers of the aorta tissue in the group that received the combination of nicotine and vitamin C during pregnancy and lactation, and the group that received vitamin C after weaning, was similar to the alteration of the nicotine group (figure 4.14). This indicates that maternal vitamin C supplementation during gestation and lactation or, treating the offspring after weaning, did not prevent the effects of maternal nicotine exposure on the aorta tissue of the offspring. In contrast to our study, Maritz (1993) illustrated that maternal vitamin C supplementation during pregnancy and lactation prevented the structural changes in the lung of the offspring induced by maternal nicotine exposure (Maritz, 1993).

It is interesting that, although the alteration in aortic structure persisted, supplementation with vitamin C did reduce nicotine induced hypertension in our study. Grossman *et al* (2001), suggested that vitamin C modifies the redox state of soluble guanylyl cyclase, activating cyclic GMP–dependent K-channels that hyperpolarize VSMC, inducing vasodilation. In view of the fact that the structure of the aortic wall in the nicotine + vitamin C group resembles that of the nicotine group, it will be of interest to see how vascular function is affected in the nicotine + vitamin C group when the animals become older. The vitamin C induced protection can perhaps be attributed to the fact that vitamin C is water soluble while nicotine is water as well as lipid soluble (Yildiz, 2004). This may reduce the capacity of vitamin C to protect the aorta against the oxidant effects of nicotine in aorta wall. Thus, a combination of vitamin C and fat soluble vitamins with anti-oxidant properties, such as vitamin E or vitamin A, is recommended.

5.6.2. Immunohistochemistry study

AGEs elicit oxidative stress generation and subsequently cause inflammatory in various types of cells via interaction with a receptor for AGEs (RAGE), thereby being involved in vascular complications. In diabetes, it was suggested that pathophysiological crosstalk between the AGE–RAGE axis and the renin–angiotensin system (RAS) could contribute to the progression of vascular damage (Yamagishi *et al*., 2012).

In this study, anti-AGE antibody which is specific for AGE was used for immunohistochemical staining of the aorta. AGE staining helps to reveal the extracellular

deposition and intracellular accumulation of AGEs in the atherosclerosis lesions of rat aorta (Sano *et al*., 1999).

From our result, it was observed that there was AGE accumulation among the groups treated with nicotine. Taken together, we could infer that accumulation of AGE which is related to atherosclerosis progression might be as a result of the relationship between extracellular and intracellular AGE accumulation and hypertension. These findings correlate to studies conducted by Treweek *et al*., (2009) who reported that accumulation of AGE was increased in nicotine treated rats. Treweek *et al*., (2009) also hypothesized that nornicotine, a minor metabolite of nicotine, induces the advanced glycation end products in the serum samples of smokers.

The accumulation of AGE in nicotine + vitamin C group and the group that received vitamin C after weaning was similar to nicotine treated rats, and is similar to our morphological results. As discussed previously, vitamin C may exert its protective effects via modification of the soluble guanylyl cyclase activity despite the structural alterations, at least in the beginning. It will thus be of interest to measure blood pressure in the animals at a later age.

CHAPTER SIX

CONCLUSION

In this project I studied the effect of low dose maternal nicotine exposure on the BP of the male offspring at five months old. We were particularly interested to determine whether hypertension induced by maternal nicotine exposure can be prevented or attenuated by vitamin C (antioxidant) supplementation during the perinatal period or after weaning. Since we expected the offspring to become hypertensive and hoped that our interventions will attenuate the hypertension which developed due to maternal nicotine exposure, we also evaluated the serum antioxidant capacity, aorta morphology and AGE expression in the aorta.

The results show maternal exposure to 1mg/kg/day nicotine during pregnancy and lactation caused the male, but not the female, offspring to develop hypertension. The male offspring were not hypertensive at weaning but were hypertensive at three months. Maternal vitamin C co-administration during pregnancy and lactation did not prevent the male offspring from becoming hypertensive. If the offspring were however given vitamin C after weaning, they did not develop hypertension. To our knowledge it is the first time that it has been showed that hypertension which develops due to maternal nicotine exposure can be reversed. Serum antioxidant capacity, as evaluated by the TAC, SOD activity and TBARS assay showed no significant difference between groups. Our results suggests that the stimulus/trigger for development of hypertensionis probably during pregnancy, and perhaps also lactation, but

that therapeutic intervention must be when the offspring develops hypertension. Vitamin C thus did not neutralise the trigger, but prevented development of the disease.

Maternal nicotine exposure induced morphological changes in the structure of the aorta and increased the expression of AGE, which were not prevented by vitamin C administration.

***Future perspectives*:**

To determine whether hypertension which develops as a result of maternal nicotine exposure is truly a phenomenon of fetal programming future experiments should include experiments to determine whether maternal nicotine exposure during pregnancy only, or during lactation only will cause hypertension in the offspring. It will also be of interest to determine whether epigenetic changes occurred.

It will be important to establish whether vitamin C administration, when the animals are already hypertensive (e.g. at 3 or 4 months) will attenuate hypertension. The timing of vitamin C administration may be crucial.

In order to further investigate the mechanism by which vitamin C prevented development of hypertension in our study, it will be necessary to evaluate oxidative stress in aortic tissue. It will also be useful to evaluate the effect of nicotine on vascular reactivity in the presence or absence of vitamin C.

CHAPTER SEVEN

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