

**An *in vivo* and *in ovo* evaluation of the toxicity of
Sibutramine**

By

Ciska van der Schoor

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CISKA VAN DER SCHOOR

SUPERVISOR: Dr HM Oberholzer

CO-SUPERVISOR: Prof MJ Bester

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Abstract

Sibutramine hydrochloride monohydrate is a weight loss agent banned from most global markets due to reports of serious adverse events including death. It is classified as a serotonin-norepinephrine reuptake inhibitor and acts within the central nervous system by inhibiting the reuptake of these neurotransmitters essentially influencing satiety and also energy expenditure. Despite its ban, sibutramine is still available for use in some countries and is often an unlisted ingredient in herbal or natural weight loss products. This leads to the oblivious and unintentional consumption of this compound which is of great concern especially if used by pregnant women. The aim of this study was to investigate the possible toxic and teratogenic effects of sibutramine by using a Sprague Dawley rat and an *in ovo* chick embryo model.

The Sprague Dawley rat was used as *in vivo* model and animals were divided into three experimental groups, each consisting of 12 animals (6 males, 6 females). Rats were administered sibutramine over a 28 day period according to their assigned experimental groups namely control (C; sterile H₂O), Low dose (LD; 1.32 mg/kg) and High Dose (HD; 13.2 mg/kg). On the day of termination blood was collected for various analyses which included biochemical tests for liver and kidney function, hormonal changes as well as the investigation of coagulation profiles. Brain, heart, lung, kidney and liver tissue of each animal was harvested for investigation of tissue and cellular structure.

Rats were weighed daily and this data suggested that sibutramine was well tolerated by all animals, with only the female rats in the HD group showing a significant 8.2% decrease in their rate of weight gain. Biochemical data of liver and kidney function in all groups were normal. Thyroid hormone levels were comparable to control values though cortisol levels were lowered in the HD female group, a finding which correlates with the observed weight loss.

Investigations of the ultrastructural morphology of the platelets and fibrin networks revealed differences between the experimental groups that are consistent with changes associated with a procoagulable state. Brain, kidney and liver tissue morphology appeared normal upon investigation, the latter confirming the biochemical findings. Examination of cardiac tissue revealed a slight increase in collagen deposition between the muscle fibers and ultrastructural analysis of lung tissue showed thickening of the alveolar walls, decreased intra-alveolar space and drastic increases in collagen deposition in the sibutramine-exposed groups. These findings were dosage dependant.

The *in ovo* model was implemented with ephedrine, a known teratogen, as positive control. Three control groups were included to ensure the efficacy of the method. Eight experimental groups, each containing sixteen eggs, were exposed to four different concentrations of each drug (i.e. 0.5, 2.5, 5.0, 10.0 μmol). The brain, heart, liver and kidneys were harvested on embryonic day thirteen for morphological analysis. Macroscopic evaluation revealed that both drugs caused congenital abnormalities which included ventral wall and limb defects as well as growth retardation and increased mortality. Sibutramine was found to have a greater teratogenic potential than ephedrine.

Histological investigation of kidney and brain samples of embryos revealed no morphological differences between the various experimental groups. Livers and hearts of embryos exposed were severely affected by both compounds in a dose dependant manner. Replacement of myocardium with adipose and connective tissue was observed in cardiac tissue, which is characteristic of muscular dystrophy. Severe liver steatosis was also evident.

In conclusion, results from this animal-based study show that, at concentrations which are not toxic to the liver or kidneys, sibutramine administration led to increased coagulation, moderate cardiac and excessive lung fibrosis within the Sprague Dawley rat model. This indicates significant toxicity with the cardiac and respiratory system being more susceptible targets. In addition sibutramine was shown to possess greater teratogenic effects than ephedrine. Both drugs caused cardiac dystrophy and liver steatosis resulting in extensive liver and heart damage. Understanding the underlying mechanisms involved in the pathogenesis of these findings strongly emphasize an important area for future research.

Declaration

I, Ciska van der Schoor, hereby declare that this research dissertation is my own work and has not been presented by me for any degree at this or any other University.

Signed:

Date:

Department of Anatomy, School of Medicine, Faculty of Health Sciences

University of Pretoria

South Africa

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List of Abbreviations, Symbols and Chemical Formulas

%	Percentage
±	Plus or minus
≥	Equal to or more than
°C	Degree centigrade
♀	Female
♂	Male
g	Gram
G	Gravity
D	Day
dL	Decilitre
E	Embryonic day
kg	Kilograms
M	Molar
mg	Milligram
ml	Millilitre
mg/day	Milligrams per day
ml/day	Millilitres per day
mg/kg/day	Milligrams per kilogram per day
mmHg	Millimetres mercury
n	Sample size
µl	Microliter

μmol	Micromole
μmol/egg	Micromole per egg
U	Units
5-HT	Serotonin
5-HT_{2A/2C}	Serotonin receptor subtype 2A and 2C
A1c	Glycated haemoglobin
ACTH	Adenocorticotrophic hormone
AEC	Animal Ethics Committee
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
ARVC/D	Arrhythmogenic right ventricular cardiomyopathy/dysplasia
AST	Aspartate aminotransferase
BMI	Body mass index
BSA	Body surface area
C	Control
CAM	Chorioallantoic membrane
CCl₄	Carbon-tetrachloride
•CCl₃	Trichloromethyl free radical
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein

CYP	Cytochrome P450
DA	Dopamine
EMA	European Medicines Agency
FDA	Food and Drug administration
GA/FA	Glutaraldehyde/formaldehyde
GGT	Gamma-glutamyl transferase
GIT	Gastrointestinal tract
GLM	General linear model
GLDH	Glutamate dehydrogenase
GLOB	Globulin
HD	High dose
HDL	High density lipoprotein
HED	Human equivalent dose
HMDS	Hexamethyldisilazane
HPA	Hypothalamus-pituitary-adrenal
IL	Interleukin
K⁺	Potassium
Km	Conversion factor for dose extrapolation based on body weight and body surface area
LD	Low dose
LDL	Low density lipoprotein
LM	Light Microscopy
M1	Mono-desmethylsibutramine
M2	Di-desmethylsibutramine

MRC	Medical research council
MCC	Medicine control council
mRNA	Mitochondrial ribonucleic acid
Na⁺	Sodium
NAFLD	Non-alcoholic fatty liver disease
NE	Norepinephrine
NET	Norepinephrine transporter
NIH	National Institute of Health
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PRP	Platelet rich plasma
SANS	South African national standard
SCOUT	Sibutramine Cardiovascular OUTcome
SDH	Sorbitol dehydrogenase
SEM	Scanning electron microscopy
SERT	Serotonin transporter
SNRI	Serotonin-norepinephrine reuptake inhibitor
SNS	Sympathetic nervous system
SOT	Society of Toxicology
SSRI	Selective serotonin reuptake inhibitor
TEM	Transmission electron microscopy
TP	Total protein
TSH	Thyroid stimulating hormone
T3	Triiodothyronine

TT3	Total triiodothyronine
T4	Thyroxin
TT4	Total thyroxin
UPBRC	University of Pretoria Biomedical Research Centre
US	United States
USA	United States of America
USFDA	United States Food and Drug Administration

CHAPTER 1: Introduction

The prevalence of obesity has been on the increase globally and comorbidities include type 2 diabetes, hypertension, inflammatory complications and various forms of cancer. Interventions which include lifestyle changes to maintain a healthy bodyweight has been proven successful in some individuals, although recently these interventions are regarded as ineffective in sole treatment of obesity. Anti-obesity drug therapy has been proven to be beneficial in weight loss and weight maintenance when used in combination with such lifestyle modifications (Padwal and Majumdar, 2007) and the popularity of these agents has increased tremendously. Weight loss agents are often recommended for patients who are obese or overweight and present with associated complications (Li and Cheung, 2011) and various remedies are available over the counter to all without much regard or regulation.

Although various weight loss agents promising “quick and effective” weight loss have been withdrawn from the market due to suspected side effects, actual research data related to the tissue and cellular toxicity of these products is limited. One example of such a compound is sibutramine, which has been identified in various herbal or natural weight loss products despite being withdrawn from the market due to various cardiovascular complications including hypertension, arrhythmias, stroke and even death (Wooltorton, 2002; Inchiosa, 2010). Unfortunately, sibutramine use continues unregulated worldwide and often unintentionally. Further information regarding the safety and efficacy of this compound is essential in ensuring the safe use thereof.

Sibutramine was initially used as an antidepressant and acts within the central nervous system (CNS) by inhibiting the reuptake of serotonin (5-HT) and norepinephrine (NE), and to a far lesser extent dopamine (DA), essentially influencing satiety and energy-balance. These neurotransmitters are involved in a large array of normal physiological processes and any dysregulation of such processes could have possible detrimental effects.

Due to the nature of its action sibutramine administration could affect various organ systems and pathways either directly due to metabolism, absorption or excretion of the compound or indirectly by altering the nature of various molecule-receptor interactions. Several questions arise with regards to the safety of this compound as some previous investigations present conflicting data. Very little published data is available on the effects of sibutramine with regards to organ morphology, in which alterations are associated with various pathological processes, as well as its possible effect on development. Histological investigations of tissues and pathways affected by sibutramine's mechanism of action may provide essential information on the process of pathogenesis which eventually leads to the observed adverse events. Furthermore, as this compound is often a hidden ingredient, the risk of prenatal exposure is much greater.

The literature review in this thesis provides essential background information on the current obesity epidemic, the rise and fall of sibutramine as weight loss agent together with its predecessors, and current research on the associated adverse effects of sibutramine.

The aim of this study consisted of two separate research goals, which firstly entailed the investigation of the toxicity of this compound with regards to normal physiology, and secondly with regards to developmental biology. This was achieved by implementing two separate animal models, each suitable to attain these specific goals. These models included:

a) The Sprague Dawley rat model which was implemented to assess the toxic potential of sibutramine. Animals on a normal diet were used to eliminate the possible confounding effects of high fat, high carbohydrate diet on general health. The toxicity of sibutramine on various organ systems was evaluated at concentrations equivalent to the prescribed dose as well as a significantly higher dose over a period of 28 days. Specific organ systems associated with the metabolism and excretion of the compound, as well as possible targets for drug interaction were of interest. These organ systems included the CNS, cardiovascular and respiratory system and these systems were investigated using biochemical and ultrastructural methods.

b) The *in ovo* chick embryo model which was used to evaluate the possible teratogenic effects of sibutramine, in conjunction with ephedrine, which was used as a positive control. Embryos were exposed to a serial concentration range of both compounds during the early stages of development. Organ systems specifically associated with the metabolism, action and excretion of these compounds were evaluated for any significant morphological alterations due to this exposure.

The results obtained from investigative analysis of these objectives are presented in the chapters to follow.

CHAPTER 2: Literature Review

2.1 Introduction

Over the past few decades the incidence of obesity has been on the increase in both developed and developing countries (Philip *et al.*, 2000). The impact it has on general health and well-being is so staggering that obesity has now been classified as a major worldwide epidemic (Philip *et al.*, 2000; Sunyer and Xavier, 2002). Recent statistics from the Medical Research Council (MRC) on the obesity status of South Africans showed that 70% of all women above the age of 35 are either overweight or obese, compared to 40% of all men above the age of 35 years (Child, 2013). It is also estimated that at least one in every five youngsters worldwide are suffering from obesity (Wang and Lobstein, 2006). Body mass index (BMI; kg/m^2) is commonly used as an indicator to classify overweight and obesity in adults. It is defined as the weight of an individual in kilograms (kg) divided by the square of the height of the individual in meters (m). Table 2.1 shows the different classes of obesity as defined by the World Health Organization (WHO; 2006) whereas Table 2.2 shows some of the obesity-related comorbidities and the benefits associated with weight loss.

Table 2.1: BMI based classification of adult overweight and obesity [Additional cut-off points facilitate international comparisons, BMI is age and gender-independent (adapted from WHO, 2006)]

Classification	BMI	
	Principal cut-off points	Additional cut-off points
Normal range	18.50 - 24.99	18.50 - 22.99 23.00 - 24.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 - 29.99	25.00 - 27.49 27.50 - 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 - 34.99	30.00 - 32.49 32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49 37.50 - 39.99
Obese class III	≥40.00	≥40.00

Table 2.2: Co-morbidities related to obesity and concomitant benefits associated with weight loss (adapted from Look, 2010)

	Risk	Weight loss outcome
Cancer	<ul style="list-style-type: none"> Women with breast cancer and BMI ≥30 have an increased risk of all-cancer mortality Obesity 1-5 years before breast cancer diagnosis associated with 2-fold increased risk of cardiovascular mortality Men with prostate cancer with higher baseline BMI have increased risk of cancer specific mortality and poorer overall survival 	<ul style="list-style-type: none"> Decreased incidence of cancer Decreased mortality from all cancers
Insulin Resistance and Type 2 Diabetes Mellitus	<ul style="list-style-type: none"> Impaired fasting glucose; Fasting plasma glucose 110-125 mg/dL 	<ul style="list-style-type: none"> In obese diabetics, 5% weight loss at 1 year results in decreased fasting blood glucose, insulin and haemoglobin A1c concentration ≥30% weight loss results in normalization of blood glucose and haemoglobin A1c

Dyslipidaemia	<ul style="list-style-type: none"> High LDL (≥ 160 mg/dL), borderline high risk LDL (130-159 mg/dL) plus 2 other risk factors conveys higher risk 	<ul style="list-style-type: none"> Decrease in serum low density lipoprotein (LDL)-cholesterol and triglycerides concentrations Increase in serum high density lipoprotein (HDL)-cholesterol
Hypertension	<ul style="list-style-type: none"> Systolic blood pressure ≥ 140 mmHg Diastolic blood pressure ≥ 90 mmHg 	<ul style="list-style-type: none"> Decreased systolic and diastolic blood pressure
Pulmonary Disease	<ul style="list-style-type: none"> Altered pulmonary function resulting in respiratory conditions such as obstructive sleep apnea and obesity hypoventilation syndrome 	<ul style="list-style-type: none"> Improved restrictive and obstructive pulmonary mechanics
Inflammation	<ul style="list-style-type: none"> Increase in inflammatory markers including C-reactive protein (CRP) and cytokines associated with insulin resistance and atherosclerotic events 	<ul style="list-style-type: none"> Significant decrease in inflammatory markers, often to normal levels
Autonomic nervous system dysfunction	<ul style="list-style-type: none"> Cardiac autonomic neuropathy 10% increase in weight associated with decreased parasympathetic tone and increase in heart rate 	<ul style="list-style-type: none"> Weight loss results in increased vagal activity and cardiac parasympathetic activity
Cardiovascular	<ul style="list-style-type: none"> Increase in total blood volume and cardiac output Decrease in peripheral vascular resistance 	<ul style="list-style-type: none"> Marked improvements in cardiac structure and function, including improvements in both left and right ventricular structure

Due to the severity of the associated health risks health professionals are constantly focused on managing the co-morbid conditions which involve physical, cardiovascular, metabolic, reproductive, and psychosocial complications (Philip *et al.*, 2000).

Although making the necessary lifestyle changes for maintaining a healthy bodyweight has been proven successful in some individuals, it is rather uncommon, and lifestyle modification as sole treatment for obesity is regarded as ineffective. Anti-obesity drug therapy has proven to be beneficial in weight loss and weight maintenance when used in combination with lifestyle modifications (Padwal and Majumdar, 2007). As obesity has become such a fast growing epidemic and its associated health risks are extremely hazardous (Sunyer and Xavier, 2002; Inchiosa, 2010), more focus has been directed toward anti-obesity pharmacotherapy. Weightloss agents are usually recommended for patients who are obese or overweight and present with associated complications such as type II diabetes (Li and Cheung, 2011). On the basis of their mechanism of action, anti-obesity drugs are generally classified as either appetite suppressants, inhibitors of fat absorption, or stimulators of thermogenesis and energy expenditure (Li and Cheung, 2009). Some weight loss agents approved for the management of obesity include Orlistat, a gastrointestinal lipase inhibitor with common side effects such as diarrhoea, flatulence, bloating, abdominal pain and dyspepsia (Maahs *et al.*, 2006), as well as Phentermine, a noradrenergic drug, which potentiates the release of norepinephrine (NE) and essentially reduces food intake (Li and Cheung, 2011). In South Africa, Phentermine is available via prescription under the brand name Duromine. Complications that have been reported include headaches, insomnia, irritability, palpitations and nervousness all of which can be associated with its stimulatory effect via β -adrenergic receptor interactions (Bray, 1993). Data on the long term effects of these agents and the associated cardiovascular complications however, is still very limited.

Although many drugs have been approved and marketed for weight loss purposes, numerous agents have been withdrawn due to reported serious adverse events associated with their use. Some examples of such weight loss drugs include

Rimonabant, Fenfluramine and the combination of Phentermine with Fenfluramine, known as Dexfenfluramine (Li and Cheung, 2011). Dexfenfluramine was once commonly used in managing obesity. However, both Fenfluramine and Dexfenfluramine were withdrawn from the market by the United States Food and Drug Administration (USFDA) in 1997 due to identified heart valve damage as a potential side effect associated with its use (Connolly *et al.*, 1997; Li and Cheung, 2011). Pulmonary arterial hypertension was also identified as a related adverse effect (Connolly *et al.*, 1997). The use of Rimonabant, an endocannabinoid shown to play a significant role in controlling food intake, energy balance and lipid and glucose metabolism, was declined by the USFDA and also suspended by the European Medicine Agency (EMA) in 2008. This was based on studies connecting its use with certain psychiatric complications such as anxiety, depression and suicidal ideation (Samat *et al.*, 2008).

Another example of a weight loss agent removed from the market due to serious complications, which will also be discussed in greater detail throughout this literature review, is sibutramine which was the active compound in weight loss drug Simply Slim widely marketed in South Africa (Jobson, 2010; Van Heerden, 2010a). This product was shown to contain far higher levels than the recommended daily dose of 5 to 15 mg of this drug (Müller *et al.*, 2009) without it even being listed as an ingredient. After subsequent analysis it was found to be in a variety of weight loss agents sold over the counter as “natural products” (Jobson, 2010). Suspensions were initially aroused after doctors reported that some patients had developed abdominal pain, high blood pressure and cardiac arrhythmias and were all using sibutramine-containing slimming capsules (Van Heerden, 2010a). Eventually, in October 2010, Abbott Laboratories voluntarily withdrew their sibutramine-containing slimming drugs from the United States of America (USA), Australian, Taiwanese and South African markets (Van Heerden, 2010b).

Despite sibutramine's withdrawal as well as the potential dangers associated with its use, it is still available in many third world countries such as India (Biyani *et al.*, 2011), and accessible worldwide via the internet (Lam *et al.*, 2013). Many herbal products have also been shown to contain far higher concentrations of sibutramine than that which is found in the prescription drug. There is also a lot of controversy surrounding the ban of sibutramine as weight loss agent as some opinions are that the benefits associated with its use far outweigh the "possible" complications, however this may only be true in cases of morbid obesity (Müller *et al.*, 2009). In-depth investigations are required to gain more information on the interactions of this compound within the body especially with regards to key organs specifically involved in the absorption, distribution, metabolism or excretion of sibutramine and the effects that these interactions might have on various physiological pathways. Furthermore, the question that arises is whether this product is harmful when used during pregnancy. Very little published data on the teratogenic effects of sibutramine is available and research directed towards the effects of sibutramine on embryonic development may prove highly beneficial.

2.2 Sibutramine

2.2.1 Discovery of sibutramine as a weight loss agent

Sibutramine hydrochloride monohydrate (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine hydrochloride monohydrate; sibutramine) was originally studied in the 1980's as a possible antidepressant but eventually investigations were directed towards its potential use as a weight loss agent (Luque and Rey, 2002). This change in research could most probably be attributed to the unanticipated weight loss observed in obese patients during the early depression trials. Sibutramine eventually received FDA approval in 1997 for the treatment of

obesity and was subsequently approved in approximately 40 countries around the world. Since its launch in 1998, more than three million prescriptions had been written by 2001 and numerous clinical trials data indicated that sibutramine, in conjunction with a low calorie diet, produced initial and sustained weight loss (Luque and Rey, 2002). More than this, studies also indicated that weight loss induced by sibutramine was of great clinical significance as it also showed improvement in glycaemic control and enhancement of insulin sensitivity, lipid profiles, and significant reductions in total cholesterol (Luque and Rey, 2002; Florentin *et al.*, 2008).

Sibutramine is part of a group of compounds known as serotonin-norepinephrine reuptake inhibitors (SNRIs), (Jackson *et al.*, 1997b) which are a class of antidepressants indicated in the treatment of a variety of conditions including depression, fibromyalgia, anxiety, panic disorder, schizophrenia, Tourette Syndrome, cocaine and alcohol addiction, Parkinson's disease, and epilepsy amongst others (US patent, 2009).

Sibutramine however, has been developed solely for the treatment of obesity. Its therapeutic effects are produced by inhibition of norepinephrine (NE) and serotonin (5-HT) and, to a minor extent, dopamine (DA) reuptake at neuronal synapse sites in the central nervous system (CNS) with a reuptake inhibition rank order of NE>5-HT>DA (Luscombe *et al.*, 1990). Essentially this causes an increase in the synaptic concentrations of these neurotransmitters (Nutt, 2002) which then leads to the subsequent activation of alpha (α)-adrenoceptors, beta (β)-adrenoceptors and serotonin receptor 2A and 2C subtypes (5-HT_{2A/2C}) (Buckett *et al.*, 1988; Heal *et al.*, 1992a; Heal *et al.*, 1992b; Fantino and Souquet, 1995; Jackson *et al.*, 1997a; Jackson *et al.*, 1997b; Stock, 1997). These interactions produce an eventual increase in satiety and energy expenditure, with a subsequent decreased body weight (Luque and Rey, 1999).

5-HT can be found primarily in the gastrointestinal tract (GIT), platelets, and in the CNS of humans and animals (Young, 2007). Serotonergic neurons of the CNS are responsible for the synthesis of some 5-HT, where it functions as neurotransmitter and is involved in the regulation of mood, appetite, sleep and some cognitive functions such as memory and learning (Berger *et al.*, 2009). In the GIT, 5-HT is located in the enterochromaffin cells where it is responsible for regulating intestinal movements. Enterochromaffin 5-HT secretions ultimately end up in the circulating blood where it is then stored in platelets. Here, 5-HT contributes to maintaining haemostasis and blood clotting and also acts as a vasoconstrictor (King, 2012). In certain cell types 5-HT may also serve as a growth factor. As a neurotransmitter in the CNS and sympathetic nervous system (SNS), NE is released from noradrenergic neurons found in the locus coeruleus where it increases arousal and alertness and has an influence on the reward system (Tanaka *et al.*, 2000). NE release from the sympathetic neurons affecting the heart increases the rate of cardiac contractions (Guyton and Hall, 2006). Furthermore, as a stress hormone, along with epinephrine, NE is also responsible for the fight-or-flight response where, when activated under stressful events, heart rate is increased, the release of glucose from energy stores is triggered, blood flow to skeletal muscle is increased and the brain's oxygen supply is subsequently increased (Anawalt, 2013). It is clear that both these neurotransmitters are central to a variety of physiological processes. As sibutramine acts specifically on the receptors involved in these processes, further investigations are warranted regarding the subsequent outcomes of such interactions.

2.2.2 Retraction of sibutramine

Despite sibutramine's success as a weight loss agent, it was removed from the Italian market in 2002. Fifty adverse events had been reported including tachycardia, hypertension and arrhythmias as well as two fatal cardiovascular events. The EMA started comprehensive risk assessments of the drug, while it still remained available on the European market. Between February 1998 and September 2001 the USFDA received reports of 397 adverse events, including 143 cardiac arrhythmias and 29 deaths and in Canada reports of 28 adverse events in patients using sibutramine were received including cases of stroke and eye haemorrhage (Wooltorton, 2002). In January 2010 the EMA withdrew sibutramine from the European market based on the initial findings of the Sibutramine Cardiovascular OUTcome (SCOUT) trial done by the FDA (Inchiosa, 2010). This study aimed to investigate the associated cardiovascular consequences of weight management with and without sibutramine administration. More than 5700 subjects at high risk for cardiovascular events participated in this follow-up trial which extended over a mean period of 3.4 years. Investigators concluded that participants with pre-established cardiovascular complications had an increased risk of non-fatal myocardial infarction and stroke associated with long term sibutramine use (James *et al.*, 2010). Following these findings, Abbott Laboratories voluntarily withdrew their sibutramine-containing slimming drugs from the USA, Australian, Taiwanese and South African market in 2010 after discussions with the Medicines Control Council (MCC) (Van Heerden, 2010b).

2.2.3 Pharmacology of sibutramine

Sibutramine's pharmacological effects can mostly be attributed to its metabolites. In the human GIT, sibutramine is rapidly absorbed after oral administration, with a standard bioavailability of 77% (Luque and Rey, 1999; Bae *et al.*, 2008). After absorption, sibutramine is metabolized by the hepatic cytochrome P450 2B6 enzymes (CYP2B6), which demethylates the parent compound to form its pharmacologically active metabolites, M1 (*N*-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-*N*-methylamine) and, after successive demethylation, M2 (1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl-amine) (Bae *et al.*, 2008). Figure 2.1 shows the chemical structures of sibutramine and its two active metabolites M1 and M2.

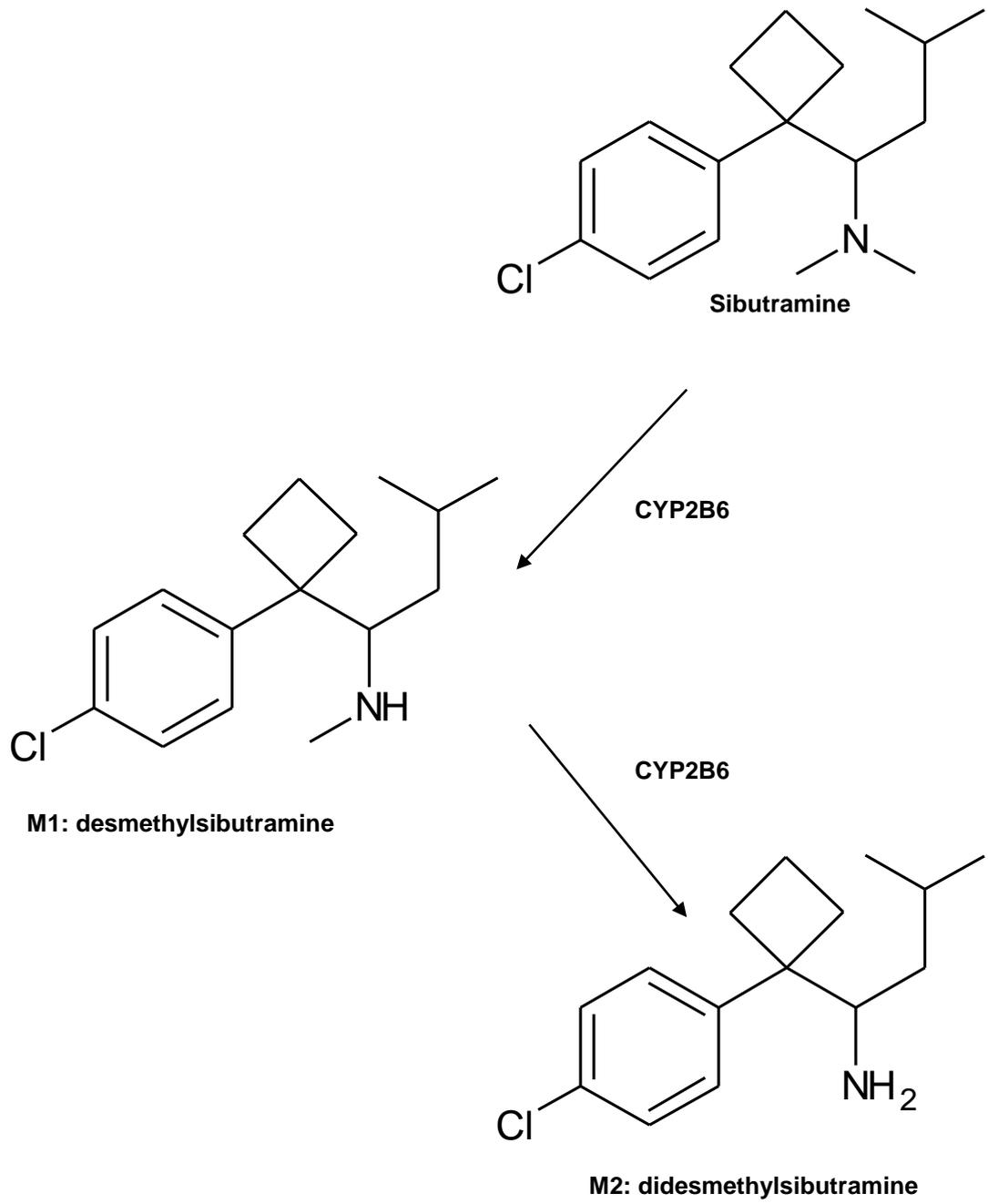


Figure 2.1: Proposed metabolic pathway of sibutramine (parent compound) to its active metabolites M1 and M2 (adapted from Bae *et al.*, 2008)

M1 and M2 have been shown to be even more potent than sibutramine in inhibiting monoamine reuptake. Studies done by Cheetham *et al.*, (1993; 1996) in which they investigated the effectiveness of various antidepressants on inhibiting the reuptake of the NE and 5-HT, including sibutramine and its two active metabolites, showed that the metabolites, M1 and M2, possessed potency comparable to many of the antidepressants currently in use. M1 and M2 are eventually hydroxylated and conjugated to glucuronide to form pharmacologically inactive metabolites M5 and M6. Approximately 85% of a single orally administered dose is excreted in the urine and faeces (Stock, 1997).

2.3 Ephedrine

2.3.1 Ephedrine, common uses and weight loss effects

Ephedrine is one of the principle compounds that form part of the Ephedra alkaloids. This alkaloid group consists of several agents including pseudoephedrine, nor-pseudoephedrine and norephedrine which are all sympathomimetic agents that are derived from the *Ephedra* genus of plants of which more than 40 species are spread throughout Asia, Europe, and the Americas. The chemical structure of ephedrine, as seen in Figure 2.2, is closely related to that of other amphetamines and catecholamines such as NE, epinephrine and DA which possess strong α - and β -adrenergic stimulatory effects and also produce an increase in endogenous catecholamine release, which, after repeated use, could eventually lead to tachyphylaxis (Andrews *et al.*, 2005).

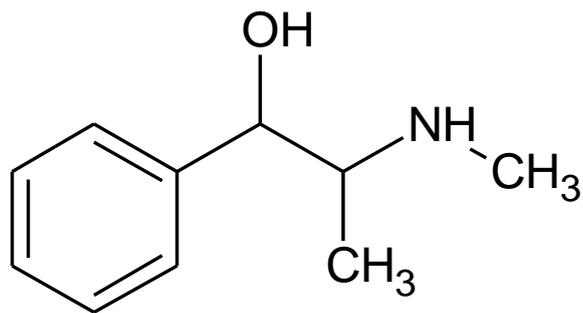


Figure 2.2: Chemical structure of ephedrine

Traditionally ephedrine has been in use in Chinese medicine as folk remedy since 3100 B.C. The dried out branches of the plant were called “mu huang” and it was implemented to improve circulation, reduce coughing and fever and for its mydriatic effects (Andrews *et al.*, 2005). Nowadays however, its purest form has gained many uses in modern medicine. Clinically, ephedrine is indicated in the treatment of allergic disorders such as bronchial asthma acting as bronchodilator and decongestant. It has long been used as pressor agent, particularly during spinal anaesthesia in cases with frequent hypotension occurrences and is also indicated as a CNS stimulant in narcolepsy and depressive states as it stimulates the cerebral cortex and subcortical centres. It is also used in myasthenia gravis, a neuromuscular disorder characterized by muscle weakness, providing a modest increase in motor power (RxList: The internet drug index [Internet], 2009).

Apart from its medicinal value, ephedrine has also been marketed as a weight loss agent and athletic performance enhancer. It is lipophilic and thus able to cross the blood-brain barrier and exert a strong stimulatory effect on the CNS. This is an important characteristic associated with its weight loss properties as it influences appetite and gastric motility (Jonderko and Kucio, 1991; Astrup *et al.*, 1995; Shekelle *et al.*, 2003). Various studies have shown that ephedrine has produced significant weight loss in treated individuals when compared to placebo groups. Its performance enhancement properties are attributed to the “energy boost” associated with its use.

This is due to the stimulation of adrenergic pathways within the CNS which are similar to those stimulated by amphetamines (Andrews *et al.*, 2005).

2.3.2 Pharmacology of ephedrine

After ephedrine administration 100% of the dose is absorbed through the GIT within 2-4 hours. After absorption the effects of ephedrine last for approximately 1 hour and it is eventually excreted by the kidneys as mostly unchanged drug. Apart from the renal excretion, ephedrine has also been detected in breast milk and is also capable of crossing the placenta posing a possible risk to the developing foetus (Andrews *et al.*, 2005).

2.3.3 Ephedrine's adverse effects

Despite ephedrine's therapeutic effects, many serious adverse events have been reported associated with its use. Reported side effects include nervousness, insomnia, vertigo, and headaches and prolonged abuse of ephedrine can lead to symptoms of paranoid schizophrenia and psychosis (Herridge and A'brook, 1968; RxList: The internet drug index [Internet], 2009). Cardiovascular complications reported include hypertension, palpitations, tachycardia, arrhythmia, myocardial infarction and cardiac arrest or sudden death (Andrews *et al.*, 2005; RxList: The internet drug index [Internet], 2009). Other CNS complications include stroke, transient ischaemic attack and seizures (Andrews *et al.*, 2005). In 2004, due to the associated risks, ephedrine alkaloids that were marketed for reasons other than asthma, colds, allergy treatments or traditional Asian uses were banned by the FDA (US Department of Health and Human services, 2008). In South Africa ephedrine was rescheduled to Schedule 6 in May 2008, which makes the substance legal to possess but available via a prescription only (West Virginia Asthma Education and Prevention Program, 2010).

2.4 Toxicological investigations

2.4.1 Toxicity of sibutramine

Despite the variety of complications associated with sibutramine administration, as well as the reported mortalities, the exact mechanism or systemic interaction by which sibutramine leads to possible complications are not known. Various research involving different aspects of sibutramine's mechanism of action have provided evidence which warrant further investigations. Dees *et al.* (2011) linked elevated 5-HT levels to vascular disease and tissue fibrosis due to its ability to stimulate extracellular matrix production in both sclerotic and healthy fibroblast cultures and, in a clinical study involving patients, Rumantir *et al.* (2000) demonstrated that when the neuronal reuptake of NE is impaired due to dysfunction of the NE transporter it could lead to the development of hypertension. A suitable model is crucial when investigating the possible toxic properties of a compound. Cell culture systems cannot compensate for the possible interactions and transformation that could take place as the drug or compounds are absorbed, distributed, metabolized and eventually excreted. Furthermore these processes often involve various organ systems. Of specific interest in the case of sibutramine are the liver and kidneys, as these organs are involved in the metabolism and excretion of this compound. Also, organs which express drug related receptors provide further targets for research.

Therefore, investigations of the morphology and cellular arrangement of liver, kidneys and target organs such as the brain, lungs and cardiovascular system may provide the necessary information required to better describe the toxicity of sibutramine.

2.4.2 Animal models in toxicology

According to the Society of Toxicology (SOT) Animals in Research Public Policy, it is stated that in the absence of human data, research done with experimental animals remains one of the most reliable approaches for evaluating the significant toxic properties of chemical substances and for further estimating what risks these compounds could hold with regards to human and environmental health. When evaluating the toxic properties of any given compound a systemic approach is essential and cannot be duplicated in any cell culture or non-living system (Society of Toxicology, 2006).

Animal models of toxicology range from rodents including rats, mice and guinea pigs, to dogs, pigs and primates, and also include various strains of animals, all of which have been successfully implemented to accurately predict the possible toxic interactions of compounds within the human body (Gad, 2006). When deciding on which animal model to employ in such studies, it is important that a model from the lowest phylogenetic order possible which will yield the most relevant data is chosen (Silverman, 2008). The replacement, refinement, and reduction of animals in research (the 3R's) is a well-established concept, originally described in 1959 by Russell and Burch to ensure animal welfare. When planning toxicity studies it is of utmost importance that this 3R's principle is considered at all times and key mechanisms often considered when adhering to this concept include adequate preceding *in vitro* analysis. This could improve the predictive value of *in vivo* studies by supporting the selection of the most suitable animal species, ensuring that the chosen model does not lack human relevance. Making use of appropriate statistical analysis which could also result in reductions in animal use and the generation of better data from smaller samples further contribute to reducing animal numbers. Finally, upon implementing the animal model the study must be designed and

conducted in such a way that as much data as possible can be generated from the given model (Chapman *et al.*, 2013).

2.4.3 The Sprague Dawley rat model

The Sprague Dawley rat model has been used with great success as a general model in human toxicology, pharmacology, reproduction, and behavioural studies. One of the advantages of using these rats also include their calmness and ease of handling which could eliminate any unnecessary stress to the animal (Carly, 2012). Various studies involving sibutramine have been performed by implementing Sprague Dawley rats. One of these studies conducted by Jackson *et al.* (1997a) focused on evaluating the sibutramine-induced hypophagic effect by identifying the key adrenoceptors and serotonergic receptors involved in its pharmacological mechanism by using monoamine receptor antagonists. Activation of these receptors in both animals and humans induces hypophagic effects highlighting a similar mechanism of action as an advantage to using this animal model.

Another recent morphological study conducted with male Sprague Dawley rats by Oberholzer *et al.* (2013) showed that sibutramine administration together with a high energy diet that did not produce significant weight changes, led to the development of hepatic fibrosis with fat accumulation. These reported findings were based on ultrastructural evaluation. These animal studies further substantiate the successful use of the Sprague Dawley rat model in these investigations.

Research has shown that sex related differences exist within the Sprague Dawley rat model with regards to metabolism of compounds (Mezey *et al.*, 1992), the reaction to metabolites (Gautier *et al.*, 2014) and behaviour and stress responses (Falconer and Galea, 2003) and have highlighted the importance of more studies which employ animals of both sexes to define various parameters.

2.4.4 Biochemical investigations

In a recent study by Saleh *et al.* (2010) the metabolic effects and possible hepatic alterations in biochemical markers associated with sibutramine use were investigated. The results obtained indicated that long term use of sibutramine induced significant hormonal and hepatic enzyme alterations. The hormonal results showed significant increases in the serum levels of total Triiodothyronine (T3, TT3) and cortisol hormone which was attributed to possible defects in the thyroid and adrenal glands, which could result in various complications with varying degrees of severity. Apart from these increases, a significant decrease was recorded for total Thyroxin (T4, TT4) which the authors further attributed to possible histopathological alterations in the thyroid gland. However, previous findings reported by Keskin *et al.* (2006) indicated that patients treated daily with sibutramine (15 mg) for a period of three months did not show any significant changes in the serum levels of T3 and T4 hormones. Keskin and colleagues (2006) further stated that the degree of obesity may in itself affect the thyroid hormone concentrations further casting doubt on sibutramine's involvement in the findings recorded in these studies.

Saleh *et al.* (2010) further reported significant increases in the liver enzymes [Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP)] and bilirubin levels due to sibutramine treatment. These alterations are usually associated with altered liver functions contributed to cellular damage. Due to the differences in these findings further research is necessary to investigate a possible mechanism for these mentioned alterations which could attribute to eventual conclusions. Research also suggests a positive relationship between Thyroid Stimulating Hormone (TSH) and cortisol levels (Walter *et al.*, 2012), thus cortisol biochemical analysis together with TT3 and TT4 tests could provide essential information on thyroid function. Serum analysis together with histological

studies could provide further information on the safety and efficacy of sibutramine use.

2.5 Teratogenic evaluations

2.5.1 Sibutramine teratogenicity

Very little published data on the outcomes of sibutramine exposure during pregnancy based on human studies are available and various animal studies involving rat and rabbit models have been found to be inconclusive (Einarson *et al.*, 2004).

In a Motherisk study conducted in 2004, the outcome of the pregnancies of 10 women who were exposed to sibutramine was observed. No malformations were observed, however 2 of the 10 pregnancies resulted in spontaneous abortion (Einarson *et al.*, 2004). In a subsequent study involving 52 pregnant women exposed to sibutramine during the first trimester, the incidence of premature deliveries and hypertensive complications were more prevalent in the treated group than in the control group (De Santis *et al.*, 2006). The authors of the study acknowledged, however, that there were some limitations to this study.

Previous studies investigating the effects of other SNRIs during pregnancy on various parameters including foetal and neonatal weights as well as postnatal growth in rat offspring have reported significant dose related decreases in these parameters. These results also displayed a strong, dose dependant pattern of body weight reduction in both the pre- and post- weaning period, which suggests a drug-induced effect and dismisses nutritional influences (Da-Silva *et al.*, 1999; Singh and Singh, 2013).

The available information on sibutramine as teratogen is very limited and although no major malformations have been reported (Garcia-Bournissen, 2007) the lack of published data on the use of this compound during gestation is a major limiting factor in reaching a definite conclusion regarding its safe use. Both 5-HT and NE have been shown to possess some teratogenic potential and other SNRIs have been described as teratogens (Hirsch and Fritz, 1981; Reddy *et al.*, 1963) therefore further investigations are essential.

2.5.2 Ephedrine teratogenicity

Ephedrine is one agent which has been well evaluated for teratogenic properties using the *in ovo* method (Nishikawa *et al.*, 1985a; 1985b). One such study by Nishikawa *et al.* (1985a) found cardiovascular malformations in 29% of treated embryos. These malformations were induced by Ephedrine at a dose as low as 1 $\mu\text{mol/egg}$. Using ephedrine as a positive control for malformations in the developing embryo, the *in ovo* method can be applied to evaluate sibutramine's potential teratogenic effects.

2.5.3 Chick embryo's: an in ovo model for teratogenic studies

Chicken (*Gallus gallus*) embryos have been used with exceptional success as tools for research in developmental biology, experimental embryology and teratology. The development of the chick embryo has also been studied and described extensively and this information is freely available and easily obtainable (Hamburger and Hamilton, 1951). Various genetic factors have also been identified in the developing chick that resembles the general expression patterns and functions of those in human and rodent development (Drake *et al.*, 2006).

Some of the key advantages of using this embryonic model in teratology and experimental biology is that the embryos can be viewed and examined should it be required and precise developmental stages can be targeted for exposure. Overall it provides an experimental tool that allows one to ascertain how a teratogen would interfere with specific mechanisms that underlie organogenesis and morphogenesis (Drake *et al.*, 2006).

During the experimental procedure however, the manner in which the teratogen is delivered does play a critical role in the experimental outcome. Various studies have demonstrated that vehicle treatments alone have the ability to bring about developmental abnormalities (Mann and Persaud, 1978; Fineman *et al.*, 1986). *In ovo* injection methods have been found to minimize distress of young embryos and may be one of the most appropriate and minimally invasive methods for teratogen delivery (Drake *et al.*, 2006).

Further toxicological investigations of sibutramine are essential at both the prescribed dose as well as a significantly higher dose so that the exact parameters of toxicity, if any, can be well defined. Histological examination of the various organ systems involved in the mechanism of action together with blood analysis identifying various markers of disease might provide valuable information on the involvement of sibutramine in the development of a variety of underlying pathological conditions, such as fibrosis, associated with its use. Furthermore, investigation of the possible teratogenic effects of sibutramine administration using the *in ovo* model may provide much needed information on the effects of sibutramine on growth and development, should exposure occur during gestation.

2.6 Aim and Objectives

This study was aimed firstly at investigating the effect of sibutramine administration, at both the pharmacological dose and a significantly higher dose, on blood coagulation and organ function as well as morphology in the Sprague Dawley rat model and to biochemically evaluate possible alterations in hormone levels and liver function parameters associated with its use, compared to control animals. Secondly, this study was aimed at investigating the teratogenic potential of sibutramine at an embryological level in comparison with the positive control ephedrine, by implementing the *in ovo* chick embryo model.

The aim of this study is embedded in the following research objectives:

1. To implement the Sprague Dawley rat model in which the tolerance of sibutramine administration can be investigated and changes in weight can be monitored over the experimental period as an indicator of possible toxicity;
2. To evaluate blood samples obtained from the rat model for possible markers of liver and kidney toxicity and for possible changes in plasma TT3, TT4 and cortisol;
3. To investigate the effect of sibutramine on the coagulation profile of Sprague Dawley rats by evaluating platelet ultrastructure and fibrin networks using scanning electron microscopy (SEM);
4. To investigate the ultrastructural effects of sibutramine on target organs including the heart, lungs, liver, kidneys and brain of Sprague Dawley rats by using light (LM) and transmission electron microscopy (TEM);

5. To implement the *in ovo* chick embryo model to investigate the effects of sibutramine exposure on growth and development at different concentrations compared to the positive control ephedrine;
6. To investigate the macroscopic and ultrastructural effects of sibutramine on target organs including the heart, liver, kidneys and brain of chick embryos compared to ephedrine by using LM and TEM.

THE SPRAGUE DAWLEY RAT MODEL

CHAPTER 3: The Sprague Dawley rat as a model to investigate the tissue and cellular toxicity of sibutramine

3.1 Introduction

Sprague Dawley rats have been used extensively with great success as general model in human toxicology, pharmacology, reproduction, and behavioural studies, some of which involved sibutramine. One such study conducted by Jackson *et al.* (1997a) focused on evaluating the sibutramine-induced hypophagic effect by identifying the key receptors involved in its pharmacological mechanism consequently highlighting a similar mechanism of action as in this model. Oberholzer *et al.* (2013) showed that sibutramine administration together with a high energy diet that did not produce significant weight changes, led to the development of hepatic fibrosis with fat accumulation, based on ultrastructural evaluation, in male Sprague Dawley rats. These findings indicate that various physiological and biochemical interactions or pathways are influenced by sibutramine administration and provides incentive for further analysis of the full extent of these interactions in a wider array of organ systems.

It is also important to determine if sex-related differences exist with regards to these physiological and biochemical interactions as such differences have been described previously (Mezey *et al.*, 1992; Falconer and Galea, 2003; Gautier *et al.*, 2014). In experimental toxicity studies male rats are used more often than their female counterparts. This is worrying as clear sex-related differences in the toxicity of various drugs and chemicals have been reported which are mostly attributed to sex-related variances in hepatic drug metabolism (Kato and Yamazoe, 1992).

Weight studies employing animal models have been reported on by many researchers and many of these entail the use of genetically- or diet-induced obese animals. An important step in evaluating the toxicity of sibutramine is to determine its effect in normal, unaltered animals. This also allows for deductions on the possible sex-related differences in the metabolism of sibutramine which could exist.

Investigations conducted by Brown *et al.* (2001) reported that daily sibutramine treatment (3 mg/kg) led to a significant, progressive drop in weight gain in diet induced obese (DIO) Wistar rats, with a final weight reduction of 9% when compared with DIO controls. The same was reported for treated lean rats with the final body weights being 7% below that of lean controls. These findings have also been reported by Stricker-Krongrad *et al.* (1995) who reported that sibutramine noticeably influenced the feeding behaviour in lean and genetically obese rodents and is effective in promoting weight loss.

Another study by Tallett *et al.* (2008) reported that although sibutramine did not significantly alter absolute weight gain, a clear dose-dependent (0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg) trend towards suppression was evident. In contrast to these findings, Oberholzer and colleagues (2013) found no significant differences between the weights of control animals and rats administered sibutramine following consumption of a high energy diet.

These inconsistencies within the literature warrant further research especially in normal, healthy animals so that the effects of this compound can be defined for control situations as many studies make use of genetically modified or diet induced obese animal models. Also, using animals of both sexes would be more comprehensive with regards to the extent of sibutramine interactions within the body. Ultrastructural investigations of target organs such as the central nervous system (CNS), and liver and kidneys which are directly involved in the metabolism and

excretion of sibutramine may provide essential information on how sibutramine administration may lead to adverse events in patients. As cardiovascular complications are predominantly reported with sibutramine use, investigations on various aspects thereof are essential, including morphological investigations of heart and pulmonary tissue, as well as coagulatory processes. Additionally, biochemical analysis of key markers of organ function could further substantiate certain pathogenesis attributed to sibutramine use. In the current study, the possible toxic effects of sibutramine on organ ultrastructure, coagulation and various biochemical parameters were investigated *in vivo* by employing the Sprague Dawley rat model. Changes in weight were also evaluated statistically over the 28-day experimental period as a means of monitoring how the drug was tolerated compared to control animals.

3.2 Materials and methods

3.2.1 Implementing the Sprague Dawley rat model

Six week old male and female Sprague Dawley rats (average weights of 250 g - 300 g) were used and were maintained at the University of Pretoria Biomedical Research Centre (UPBRC). Standard irradiated commercial Epol rat pellets and municipal water was provided *ad libitum*. The animals were housed conventionally in cages complying with the sizes laid down in the SANS 10386:2008 recommendations. A room temperature of 22°C (± 2); relative humidity of 50% (± 20) and a 12 hr light/dark cycle was maintained. A total of 36 animals was obtained for this study. Two animals were housed per cage and autoclaved pinewood shavings were used as bedding material. White facial tissue paper was also added for enrichment according to standard procedures at the UPBRC. All experimental protocols complied with the requirements of the University of Pretoria's Animal Ethics Committee (AEC). Ethical

clearance number: [h003-13]. Rats were randomly divided into the following experimental groups indicated in Table 3.1.

Table 3.1: *In vivo* experimental design

Experimental group	Number of animals	Gender
Control (C)	12	6 ♀ and 6 ♂
Low dose (LD)	12	6 ♀ and 6 ♂
High dose (HD)	12	6 ♀ and 6 ♂

Animals were allowed to acclimatise for 7 days prior to project commencement which was conducted over the following 28 days after acclimatisation and thus were housed for a total period of 35 days.

3.2.2 *Sibutramine administration*

Sibutramine hydrochloride monohydrate (BIOCOM Biotech; Clubview, SA) solution was prepared by dissolving the white powder in sterile water. The dosages to which the animals were exposed were extrapolated from the equivalent prescribed human dose (i.e 15mg/day) using the formula for dose translation based on Body Surface Area (BSA) as described by Reagan-Shaw *et al.* (2007). Figure 3.1 shows the formula used for dose extrapolation.

**Formula for Dose translation based on BSA
(Body Surface Area)**

$$\text{HED (mg/kg)} = \text{animal dose (mg/kg)} \times \frac{\text{Animal } Km}{\text{Human } Km}$$

Figure 3.1: Formula for dose translation based on BSA adapted from Reagan-Shaw *et al.* (2007). HED is the human equivalent dose and the Km factor is determined by body weight (kg) divided by BSA (m²). Km values are available as standard values for both rats and humans

Animals were also treated with a ten times higher dose than the extrapolated dose so that the full extent of the potential sibutramine toxicity could be evaluated. The average weights of animals were used in calculating amounts required for preparing the sibutramine solution on a weekly basis for each experimental group. Table 3.2 outlines the manner in which sibutramine administration proceeded.

Table 3.2: Sibutramine administration

Experimental group	Treatment	Days
Control (C)	0.5 ml/day sterile H ₂ O	28 days
Low dose (LD)	0.5 ml/day Sibutramine (1.32 mg/kg)	28 days
High Dose (HD)	0.5 ml/day Sibutramine (13.2 mg/kg)	28 days

Animals were treated daily via oral gavage and were at all times restrained by proper handling method as required for oral gavage and this was done by qualified personnel at the UPBRC. Therefore no chemical or mechanical restraint was required.

3.2.3 Observations during experimental period

a) *Weighing of animals*

Animals were weighed daily to monitor weight loss and to statistically evaluate changes in weight. Weekly average weights were also used in calculations for solution preparations.

i) Statistical analysis

Following the conclusion of the experimental procedures the changes in weights of the rats in the different experimental groups were analysed and categorized according to the different experimental groups [i.e C, LD and HD]. Changes in the weights recorded on day (D) 1 and D 28 of the experimental period, was determined. Following analysis on the distribution of the data, a General Linear Model (GLM) was selected for statistical analysis. Analysis of Variance (ANOVA) was used to analyse the data with regards to sex (i.e. male and female) and dosage groups using the GLM procedure in SAS v9.3. Where the data showed that differences existed between the dosage groups, Scheffe's post hoc test (pairwise test) was used to identify these groups. Male and female weight data obtained was analysed separately as the male weights were considerably higher throughout the 28 day weighing period.

b) Behaviour and form

Animals were monitored daily for any changes in normal behaviour. They were also monitored for any changes in coat or skin conditions and general well-being.

3.2.4 Termination

Rats were terminated via Isoflurane overdose, according to standard methods employed by the UPBRC.

3.2.5 Sample collection

On the day of termination, blood samples were collected via cardiac puncture in both plasma tubes and citrate tubes for subsequent biochemical and coagulation studies. The heart, brain, liver, kidneys and lungs were dissected from each animal and these tissue samples were then prepared for light (LM)-, transmission- (TEM) and scanning electron microscopy (SEM) which will be discussed in the relevant chapters to follow.

3.3 Results

Following the initial data processing, the change in weight from D1-D28 for both male and female animals was determined. This data was then categorized according to the different experimental groups. Figure 3.2 shows the distribution of the data for weight change as determined for each group.

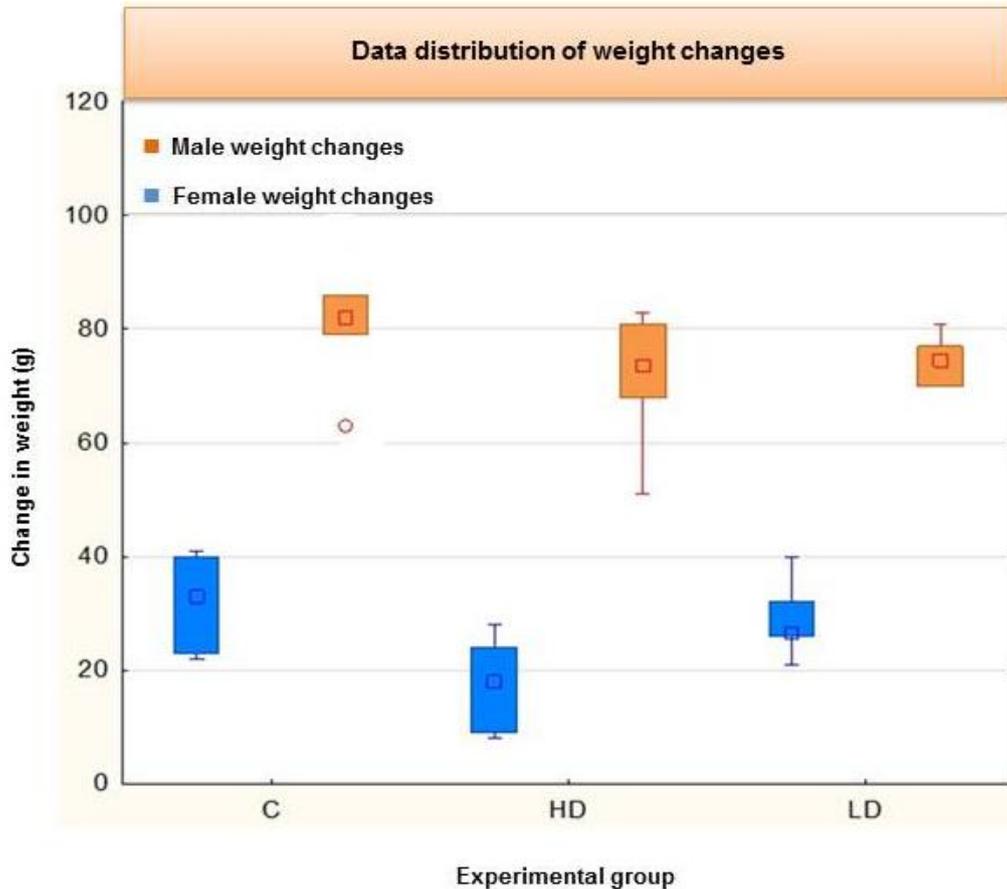


Figure 3.2: Box plot indicating the data distribution of weight change for the different experimental groups in the male and female rat population

3.3.1 Female weight changes

For the female rat population ANOVA was done by employing GLM procedures which indicated that there were significant differences in weight changes between the groups over the 28-day experimental period with a P-value of 0.0159 (significance was set at 0.05). Scheffe's post hoc test was used to determine where the significance lies. Figure 3.3 shows the weight gain within the female rat population over the experimental period according to the different experimental groups. Table 3.3 provides the average weights per experimental group for each day, with standard deviation (SD).

Weights of Female Rats

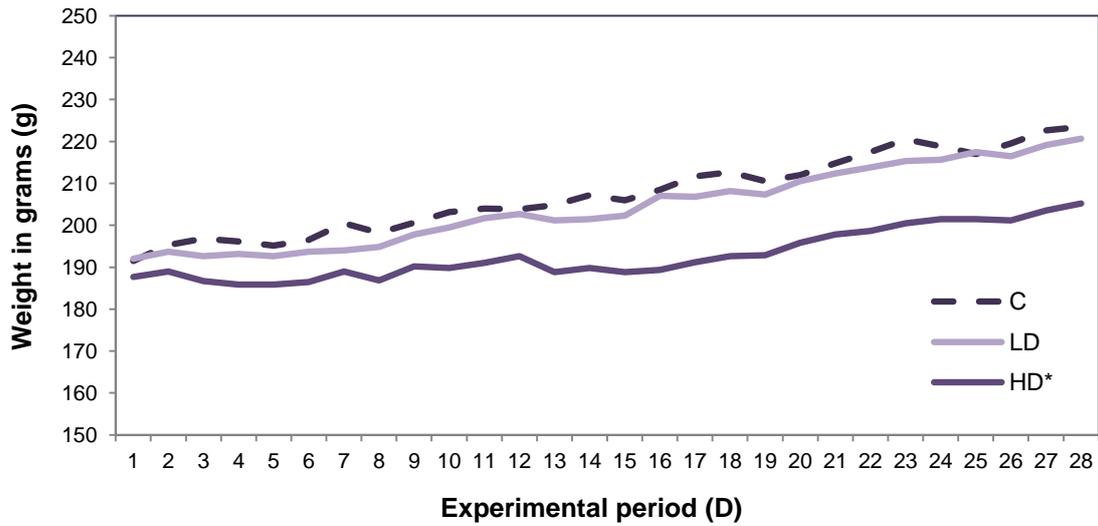


Figure 3.3: The average weight gain of the three experimental groups over the experimental period within the female population. The HD data differed significantly from both the LD and C groups as indicated by the *; (P=0.0159)

Table 3.3: Female weights (g) with SD values for experimental period D1-D28

	1	2	3	4	5	6	7
Control	191±10.52	195±9.35	197±6.56	196±7.73	195±8.57	197±7.41	201±6.47
LD	192±10.76	194±10.06	193±6.60	193±7.09	193±7.44	194±8.88	194±8.79
HD	188±14.56	189±12.61	187±12.68	186±11.83	186±13.64	187±11.39	189±13.07
	8	9	10	11	12	13	14
Control	198±6.69	201±6.79	203±6.38	204±6.74	204±8.29	205±7.08	207±7.25
LD	195±6.06	198±4.22	200±8.58	202±7.64	203±9.31	201±6.61	202±6.82
HD	187±12.21	190±10.76	190±10.91	191±12.89	193±11.85	189±11.64	190±9.41
	15	16	17	18	19	20	21
Control	206±6.24	209±4.69	212±6.83	213±8.91	211±8.21	212±7.99	215±8.02
LD	202±5.81	207±6.50	207±7.99	208±5.13	207±7.66	211±7.36	212±9.42
HD	189±8.93	189±8.95	191±11.33	193±10.70	193±9.61	196±10.23	198±9.54
	22	23	24	25	26	27	28
Control	218±8.47	221±8.80	219±9.44	217±9.83	220±8.51	223±10.07	224±10.23
LD	214±5.67	215±5.96	216±7.23	218±6.38	217±7.63	219±8.46	221±6.14
HD	199±8.86	201±9.18	202±10.17	202±9.89	201±9.97	204±8.52	205±8.02

Table 3.4 provides the results obtained from the Scheffe's post hoc test after it was determined that significance existed within the female population. As this data indicates, the female animals treated with the higher dose of sibutramine (13.2 mg/kg/day) showed significantly less progressive weight gain or change in weight when compared to that of the female rats from both the C and LD groups. These findings are clearly illustrated in Fig 3.3.

Table 3.4: Data generated by Scheffe's test for female changes in weight

Scheffe Grouping	Mean	N	Group
A	32	6	C
A	28.66	6	LD
B*	17.5	6	HD

Means with the same letter are not significantly different

* indicates statistically significant difference

3.3.2 Male weight changes

For the male population, the same data analysis methods were employed in the interpretation of the data as implemented with the female animals. The GLM procedure showed that no statistically significant differences existed between the three experimental groups within the male population. Figure 3.4 clearly illustrates the changes in weights for male animals over the experimental period. Although the data tends to differ in a dose related manner, a p-value of 0.1442 was obtained indicating that no significant decreases in the rate of weight gain between the different experimental groups existed (significance was set at 0.05).

Weights of Male Rats

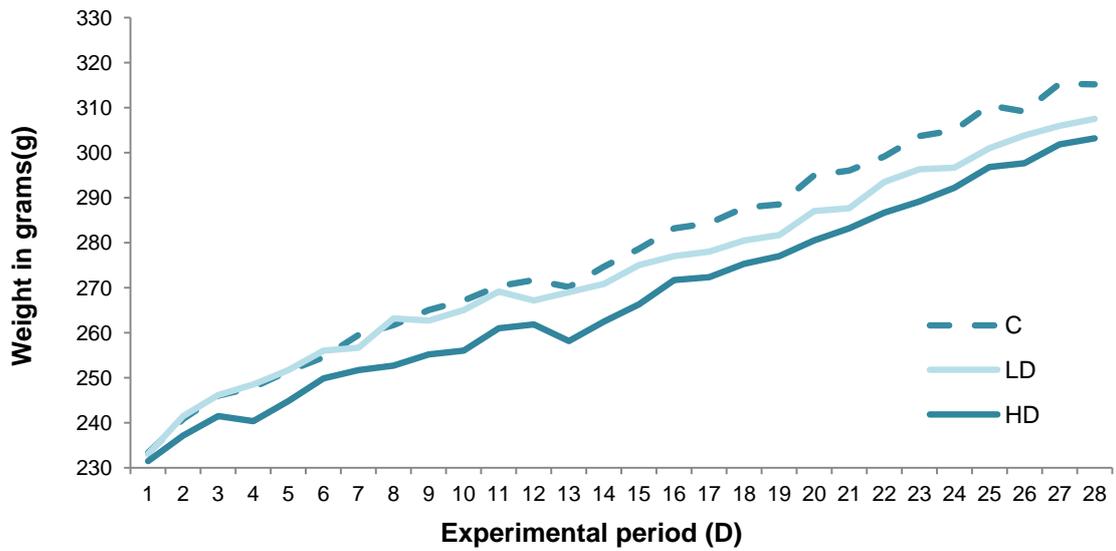


Figure 3.4: The average weight gain of the three experimental groups over the experimental period within the male population. Analysis showed that no significance existed within this population

Table 3.5: Male weights (g) with SD values for experimental period D1-D28

	1	2	3	4	5	6	7
Control	233±10.50	241±11.53	246±10.43	248±8.47	252±9.12	255±7.69	260±9.67
LD	233±4.20	242±7.56	246±4.17	249±2.66	252±3.27	256±2.90	257±4.23
HD	232±9.33	237±9.56	242±9.57	240±7.63	245±10.17	250±8.98	252±10.97
	8	9	10	11	12	13	14
Control	262±11.78	265±7.80	267±8.38	270±6.83	272±6.31	270±6.88	275±6.59
LD	263±5.64	262±5.20	265±2.10	269±3.82	267±2.79	269±5.02	271±6.65
HD	253±10.48	255±11.00	256±9.98	261±9.94	262±7.63	258±8.47	263±9.40
	15	16	17	18	19	20	21
Control	279±7.06	283±4.96	284±5.75	288±4.62	289±4.64	295±5.73	296±3.79
LD	275±4.47	277±5.83	278±7.07	281±7.40	282±8.19	287±6.54	288±7.45
HD	266±9.46	272±7.87	272±8.69	275±8.12	277±7.77	281±8.02	283±9.75
	22	23	24	25	26	27	28
Control	299±5.12	304±5.13	305±4.43	311±5.32	309±5.42	315±6.71	315±5.15
LD	294±8.17	296±9.35	297±7.12	301±6.84	304±8.06	306±6.81	308±4.76
HD	287±8.89	289±10.74	292±11.62	296±10.68	297±12.29	302±11.43	303±11.58

3.4 Discussion

Results obtained from this study revealed that female rats showed a significant decrease in their progressive weight gain at doses far higher than the effective dose which is prescribed for obese individuals (15 mg/day). Despite the animals in the HD group receiving a dose ten times higher (13.2 mg/kg/day) than that of the prescribed dose, a 8.2% decrease in body mass, compared to control values on D28, was achieved. In comparison with individuals from the LD groups, a body mass decrease of 6.94% was achieved in animals receiving the higher dose of sibutramine. Animals in the LD group received a dose that correlated with that of the equivalent human dose of 15 mg/day. The extrapolated dose for the rats within this experimental group was determined according to methods previously described by Reagan-Shaw *et al.* (2007) and was calculated to be 1.32 mg/kg/day. These animals presented no significant weight loss when compared to control values, with a mere 1.27% reduction in body mass over the experimental period.

Over the entire course of the experimental period, male rats gained more weight than female rats which can be attributed to the influence of sex hormones on body growth and development. Previous studies using male rats and both ovariectomized and gonado-intact control female rats showed that the ovariectomized female group weighed significantly more despite both female groups consuming similar amounts of calories (El-Mas and Abdel-Rahman, 2000; Thawornkaiwong *et al.*, 2003). This specifically implicates the sex-hormones in this observed weight difference between male and female rats and is further substantiated by the observable increase in body mass and body fat distribution associated with menopause (Ley *et al.*, 1992).

In both studies previously referred to done by Brown *et al.*, (2001) and Tallett *et al.* (2008) male rats were used during experimentation. Although Brown *et al.* reported significant reductions in the rate at which the rats gained weight over the 21 day experimental period, Tallett *et al.* found no significant weight differences between the experimental groups ($p > 0.05$) although the data showed a tendency towards weight suppression. These results are similar to the results obtained in the current study with regards to the male population data. No significant differences existed between the different groups although a possible dose related trend could exist, which is evident upon examination of the data presented in Figure 3.4 (D28 values; control: 315.17 ± 5.15 g; LD: 307.50 ± 4.76 g; HD: 303.17 ± 11.58 g).

In another study done by Strack *et al.* (2002) the effects of sibutramine on body weight and carcass composition was investigated. The study employed younger animals with a fat percentage of 15%, and older animals with a fat percentage of 25%, which were treated with sibutramine (0.6 and 2 mg/kg respectively) for a period of 14 days. Results showed that sibutramine demonstrated efficacy in decreasing body fat in both groups of rats although sibutramine showed greater efficacy in preventing weight gain in older rats with a higher body fat percentage. This data could propose an action of sibutramine specific to adipose tissue (Strack *et al.*, 2002) as such precedence has previously been described for anti-obesity agents, showing greater efficacy in animals with increased adiposity (Blundell and Hill, 1985; Fantino *et al.*, 1986; Rowland and Carlton, 1988; Blundell and Lawton, 1995; Thibault and Booth, 1999).

Possible reasons for the differences in the weight changes reported in these studies as well as the current results could be a resultant increase in metabolic rate contribution in the response to weight alterations (Leibel *et al.*, 1995). Previous studies have reported an observed decrease in sibutramine action over the

experimental period which could be due to regulatory pathways which counter the anorectic effects of sibutramine to ensure the maintenance of the caloric supply and the subsequent maintenance of body weight. Together with the normalization of neuropeptide Y and pro-opiomelanocortin mRNA expression in the hypothalamic arcuate nucleus, persistent sibutramine treatment decreases the protected body weight (Levin and Routh, 1996; Levin and Dunn-Meynell, 2000). This further suggests and substantiates the idea that such neurochemical adjustments could possibly contribute to the activation of these compensatory mechanisms and essentially decrease the efficacy of chronic sibutramine treatment.

3.5 Conclusion

The Sprague Dawley rat model has been used extensively with great success within a laboratory setting. In this study, the Sprague Dawley rat model was established over a period of 28 days and proved to be a versatile and highly reliable model for toxicological analysis throughout the experimental procedure. The changes in weight of the animals in the different experimental groups exposed to 1.32 mg/kg and 13.2 mg/kg doses of sibutramine respectively, were analysed and compared to determine if any possible significant differences existed throughout the experimental period. The results obtained indicated that the weights of female animals in the HD group differed significantly from those in both the C and LD groups, demonstrating a less progressive weight gain in animals within this group. Evaluation of the data obtained from the male rats indicated that no statistical significant difference between any of the experimental groups existed. Throughout the duration of the experimental procedure, no animals presented with behavioural or physiological complications which would result in withdrawal from the study or possible sacrifice. This, together with the findings of this chapter, suggest that sibutramine was well tolerated in the Sprague Dawley model.

CHAPTER 4: The effect of sibutramine on liver and kidney function as well as plasma cortisol, TT3 and TT4 levels in Sprague Dawley rats

4.1 Introduction

Previous studies aimed at investigating the metabolic effects and possible hepatic alterations in biochemical markers associated with sibutramine use, indicated that long term use of this compound induced substantial change in plasma concentrations of cortisol and thyroid hormones Triiodothyronine (T3) and Thyroxine (T4) (Saleh and Bisher, 2010). Significant alterations in liver enzymes alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin levels have also been reported which are usually associated with changes in liver functions attributed to hepatocyte damage (Saleh and Bisher, 2010). Discrepancies do however exist between different studies with regards to the concentrations of these molecules being increased or decreased as a result of sibutramine use.

Both cortisol and the thyroid hormones play essential roles in normal bodily function and are involved in cellular secretions, metabolism of carbohydrates, lipids and proteins, blood pressure and circulation, and reproduction (Meyer *et al.*, 2002). Cortisol, a glucocorticoid secreted from the adrenal cortex via corticotrophin stimulation, is one hormone that is essential in maintaining life as it plays a vital role in normal physiological pathways associated with the immune, nervous, digestive, circulatory and haematopoietic systems. Disturbances in normal cortisol blood concentrations could lead to a variety of pathological conditions such as diabetes mellitus, osteoporosis, hypertension and kidney stones (Meyer *et al.*, 2002; Faggiano *et al.*, 2003). The iodine-containing hormones T3 and T4 which are

responsible for the maintenance of metabolism of most body cells are secreted by the thyroid gland. These hormones also have important functions in the cardiovascular and nervous system, as well as growth and differentiation (Meyer *et al.*, 2002).

Plasma cortisol, total T3 and TT4 (TT3 and TT4) levels were investigated in a study conducted by Saleh and Bisher (2010) using male Wistar rats, in which the effect of sibutramine administration on these parameters were of interest. Results showed significant changes in serum levels of these hormones which the authors attributed to possible defects in the thyroid and adrenal glands, which could result in a variety of complications with varying degrees of severity. A significant decrease was reported for TT4 levels which the authors further attributed to possible histopathological alterations in the thyroid gland. However, previous findings reported by Keskin *et al.* (2006) indicated that patients treated daily with sibutramine (15 mg) for a period of three months did not show any significant changes in the serum levels of TT3 and TT4 hormones.

Based on their findings, Keskin and colleagues (2006) suggested that the degree of obesity may in itself affect the thyroid hormone concentrations further casting doubt on sibutramine's involvement in the observations reported from these studies. This further highlights the importance of using a suitable model in which the possible effects of obesity itself on hormone and enzyme activities can be ruled out.

Due to the differences in research findings further research is necessary to investigate a possible mechanism for these reported alterations by using a suitable model which could rule out the possible effects of body weight on these parameters. Research also suggests a positive relationship between thyroid stimulating hormone (TSH) and cortisol levels (Walter *et al.*, 2012), thus cortisol biochemical analysis in

conjunction with TT3 and TT4 tests could provide essential information on thyroid function.

Vitally important and central to metabolism, is the liver. This organ performs a variety of essential independent tasks such as the metabolism of proteins, fats and carbohydrates, the storage of vital nutrients, detoxification, and the secretion of bile (Meyer *et al.*, 2002). Despite some liver functions being vague or not fully comprehended, and the difficulty in obtaining accurate measurements, numerous tests currently exist for liver function analysis which are used routinely to diagnose diseases, define prognosis, measure disease progression and also to direct and measure an individual's response to therapy (Sallie *et al.*, 1991).

Previous studies have presented data which suggests that sibutramine administration could lead to hepatic fibrosis. These findings were based on morphological investigations employing transmission electron (TEM) and light microscopy (LM) analysis. The authors did however mention that the lack of biochemical analysis of markers for liver disease was one limitation of the study (Oberholzer *et al.*, 2013). Following administration of sibutramine, the compound is metabolised to its active metabolites M1 and M2 via CYP2B6 enzymes within hepatocytes. Due to this biochemical conversion within the liver, possible damage could occur as consequence.

Serum analysis together with histological studies could further provide critical information on possible toxicity associated with sibutramine use. Therefore, in the current chapter, blood samples collected from Sprague Dawley rats following sibutramine administration were used for analysis of plasma hormone levels (cortisol, T3 and T4) as well as kidney and liver function analysis.

4.2 Materials and methods

4.2.1 *Sprague Dawley rat model*

Blood samples were obtained from the Sprague Dawley rat model as described in Chapter 3 of this dissertation.

4.2.2 *Blood collection*

On experimental day 28, 5-10 ml of blood was collected from each rat via cardiac puncture under isoflurane anaesthesia. These samples were collected in separate plasma tubes and liver function tests were performed as well as hormone tests to determine the plasma levels of free cortisol, TT3 and TT4. Following blood collection, the animals were terminated via Isoflurane overdose, according to standard methods employed by the UPBRC.

4.2.3 *Biochemical analysis*

Blood samples were subject to liver function analysis in which Total Protein (TP), Albumin (Alb), Globulin (Glob), ALT, ALP, AST, Gamma-Glutamyl Transferase (GGT), Sodium (Na⁺), Potassium (K⁺), urea and creatinine levels were determined according to standard biochemical methods employed by the Section of Clinical Pathology of the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, South Africa.

4.2.4 Hormonal analysis

For the analysis of cortisol, TT3 and TT4 levels, blood samples were sent to the Section of Reproduction at the Veterinary Hormone Laboratory, Faculty of Veterinary Sciences, University of Pretoria, South Africa and the plasma concentrations of these hormones were determined in routine methods by means of Enzyme-Linked Immunosorbent Assay (ELISA) kits specific for the different molecules tested which were obtained through the Section of Reproduction.

4.2.5 Statistical analysis

The sample consisted of 36 Sprague Dawley rats which were divided into three experimental groups of equal size (n=12 animals/group, each comprised of 6 males and 6 females) namely a Control or C, Low Dose (LD), which received 1.32 mg/kg sibutramine daily and a High Dose (HD) group which received 13.2 mg/kg sibutramine daily for a period of 28 days. The data analysis consisted of descriptive statistics to describe and investigate the distribution of raw data. Comparisons were made between control and experimental groups. In the case of the hormone results data following a normal distribution were subject to GLM analysis. The non-parametric Kruskal-Wallis test was used where the data distribution warranted, i.e. where the distribution of data was skewed.

The liver function data obtained from the Clinical Pathology laboratory were subjected to statistical analysis to determine whether or not significant differences existed between male and female samples for the various parameters and if so, where within the population this difference existed. Two-factor ANOVA with interaction (two-way interaction) was employed to test for the effects of sex and dosage group and the two-way interaction between sex and dosage group. P-values were calculated for all three categories to establish which categories differed. To

further asses where possible interaction existed, two-factor ANOVA was used with main effects to evaluate the effect of only sex and group on the given parameters. Again, p-values were calculated. One-factor ANOVA was then used to test for effects of sex or group on the given parameters. In some cases where the interaction effect was significant, two separate one-factor ANOVA's were done to test for the effect of group, males and females. When the group category showed statistically significant effects, the Scheffe's post hoc (or pairwise) test was used to determine which of the 3 groups (i.e. C, LD, HD) differed from each other.

4.3 Results

To determine if sibutramine administration has an effect on plasma cortisol levels as well as TT3 and TT4, blood samples from C, LD and HD animals were subject to ELISA methods to determine the plasma concentrations of these hormones.

4.3.1 Plasma cortisol

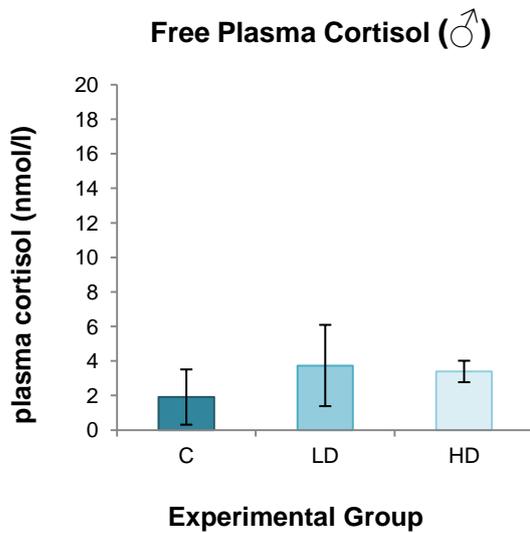


Table 4.1: Mean and SD values obtained for male experimental groups

Variable	Group	Mean	SD
Cortisol	C	1.91	0.74
	LD	3.74	1.90
	HD	3.39	0.20

Figure 4.1: Changes in plasma cortisol levels between the different male experimental groups. Error bars indicate standard deviations and * indicates significance

Table 4.2: Multiple Comparisons p-values (2-tailed) for male experimental groups

	C	LD	HD
C	-	0.52	0.82
LD	0.52	-	1.00
HD	0.82	1.00	-

* Significance

No significance exists within male population

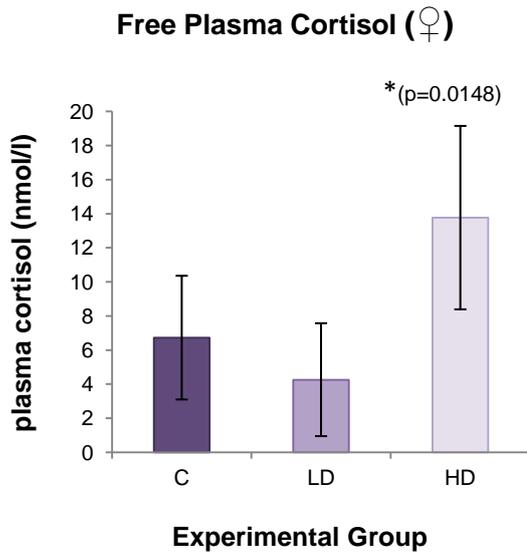


Table 4.3: Mean and SD values obtained for female experimental groups

Variable	Group	Mean	SD
Cortisol	C	6.73	2.26
	LD	4.26	1.835
	HD	13.77	5.28

Figure 4.2: Changes in plasma cortisol levels between the different female experimental groups. Error bars indicate standard deviations and * indicates significance

Table 4.4: Multiple Comparisons p-values (2-tailed) for female experimental groups

	C	LD	HD
C	-	0.10	0.18
LD	1.00	-	0.01*
HD	0.18	0.01*	-

* Significance exists between HD and LD experimental groups

Exploration of the data distribution of the results obtained from the plasma cortisol analysis indicated a skewed distribution and therefore the non-parametric Kruskal-Wallis test was used to analyse the data. Subsequent analysis was done separately for male and female experimental groups. P-values showed no statistically significant differences between the C, LD or HD groups within the male population as indicated in Table 4.2. Figures 4.1 and 4.2 further illustrate the mean values obtained for the different experimental groups within the male and female populations respectively. Tables 4.1 and 4.3 also provide the mean values together with the standard deviations obtained from the data received for the different experimental groups. Multiple comparisons of the different groups in the female population did however show that there were significant differences within the population ($p=0.0164$). Further analysis showed significant differences exist between the LD and HD groups specifically as indicated in Table 4.4.

4.3.2 Total plasma T3 (TT3)

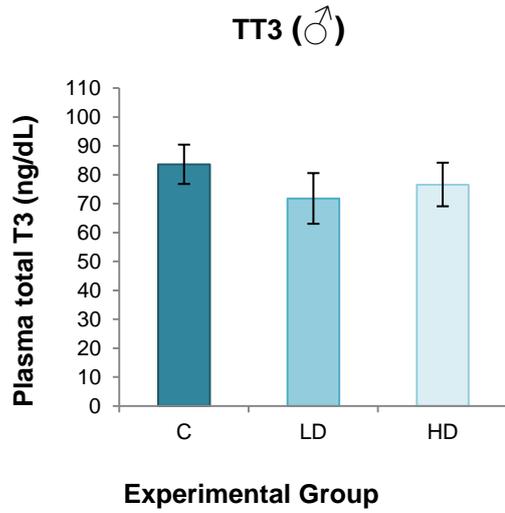


Table 4.5: Mean and SD values obtained for male experimental groups

Variable	Group	Mean	SD
TT3	C	83.60	6.81
	LD	71.77	8.77
	HD	76.60	7.55

Figure 4.3: Changes in plasma TT3 levels between the different male experimental groups. Error bars indicate standard deviations and * indicates significance

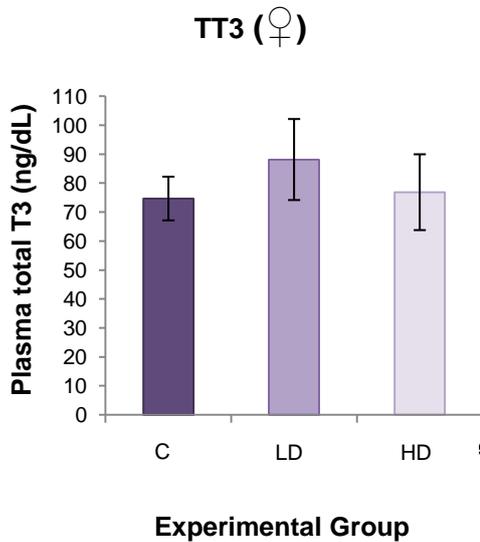


Table 4.6: Mean and SD values obtained for female experimental groups

Variable	Group	Mean	SD
TT3	C	74.63	7.54
	LD	88.11	13.99
	HD	76.83	13.09

Figure 4.4: Changes in plasma TT3 levels between the different female experimental groups. Error bars indicate standard deviations and * indicates significance

4.3.3 Total plasma T4 (TT4)

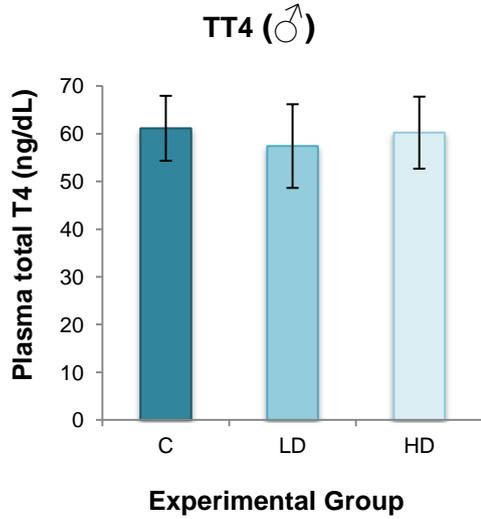


Table 4.7: Mean and SD values obtained for male experimental groups

Variable	Group	Mean	SD
TT4	C	61.15	6.81
	LD	57.42	8.77
	HD	60.25	7.55

Figure 4.5: Changes in plasma TT4 levels between the different male experimental groups. Error bars indicate standard deviations and * indicates significance

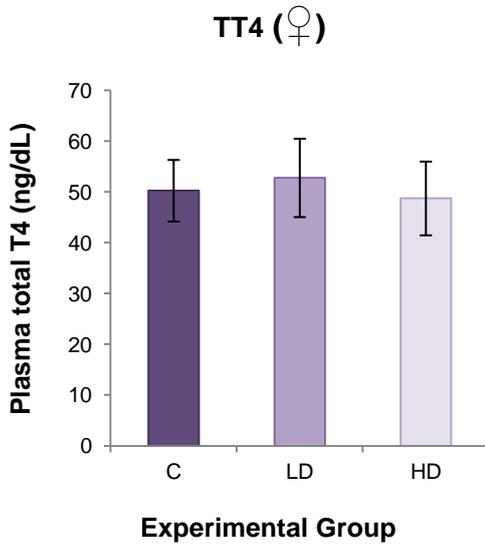


Table 4.8: Mean and SD values obtained for female experimental groups

Variable	Group	Mean	SD
TT4	C	50.25	6.06
	LD	52.77	7.72
	HD	48.72	7.26

Figure 4.6: Changes in plasma TT4 levels between the different female experimental groups. Error bars indicate standard deviations and * indicates significance

Analysis of the data obtained from the different experimental animals regarding the thyroid hormones showed that no significant differences existed for both male and female populations. Analysis did however show that differences do exist between male and female animals for serum T4 levels ($p=0.0006$). Analysis of T3 values showed a tendency for an interaction to exist between different experimental groups and sexes ($p=0.0502$). This is clearly demonstrated in Figures 4.3 and 4.4 where TT3 concentrations were lowered in the LD Male group and increased in the LD Female groups. Although the overall changes were not significant this could indicate a possible difference in response to the drug between the male and female animals.

4.3.4 Liver function results

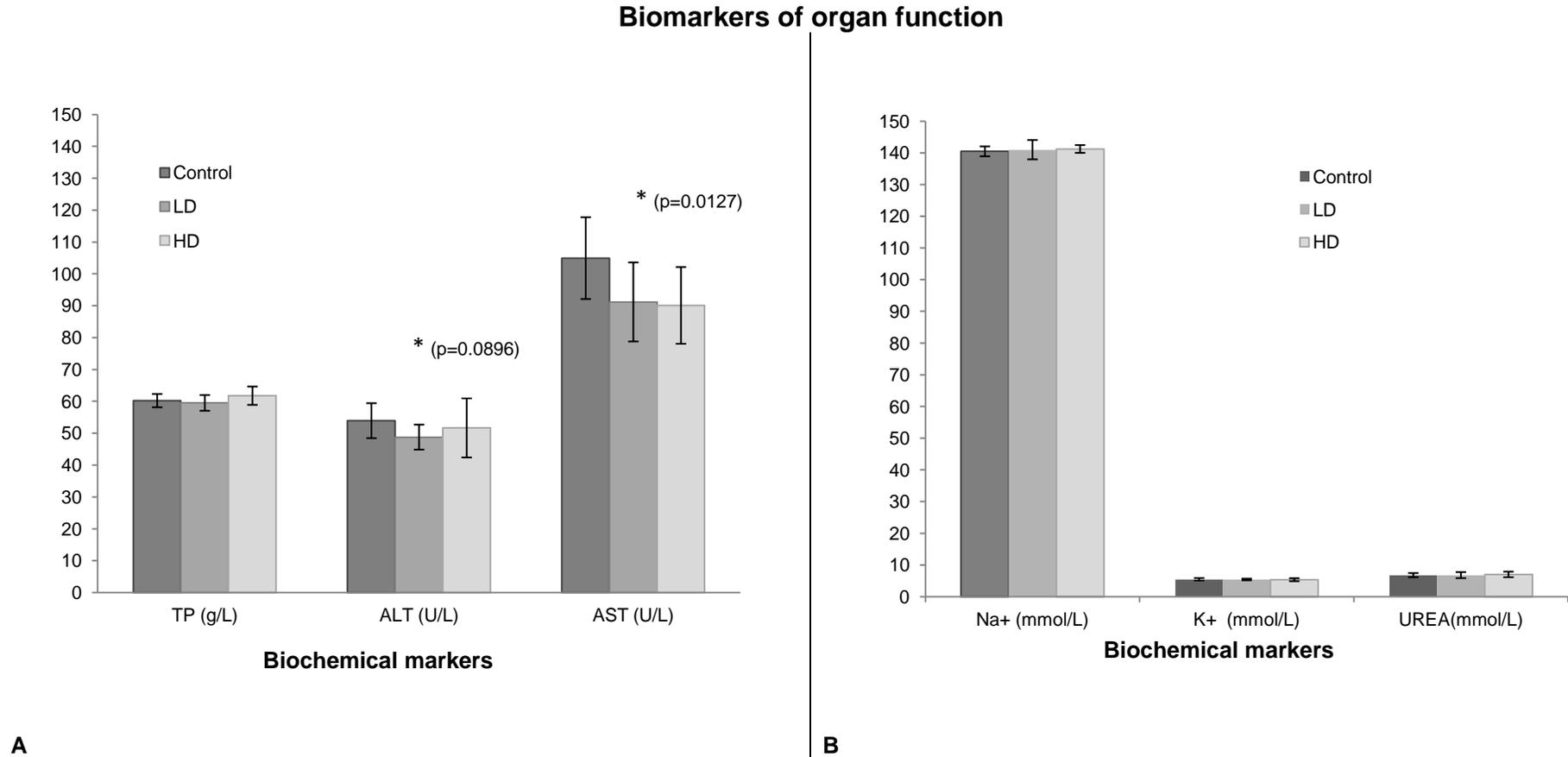
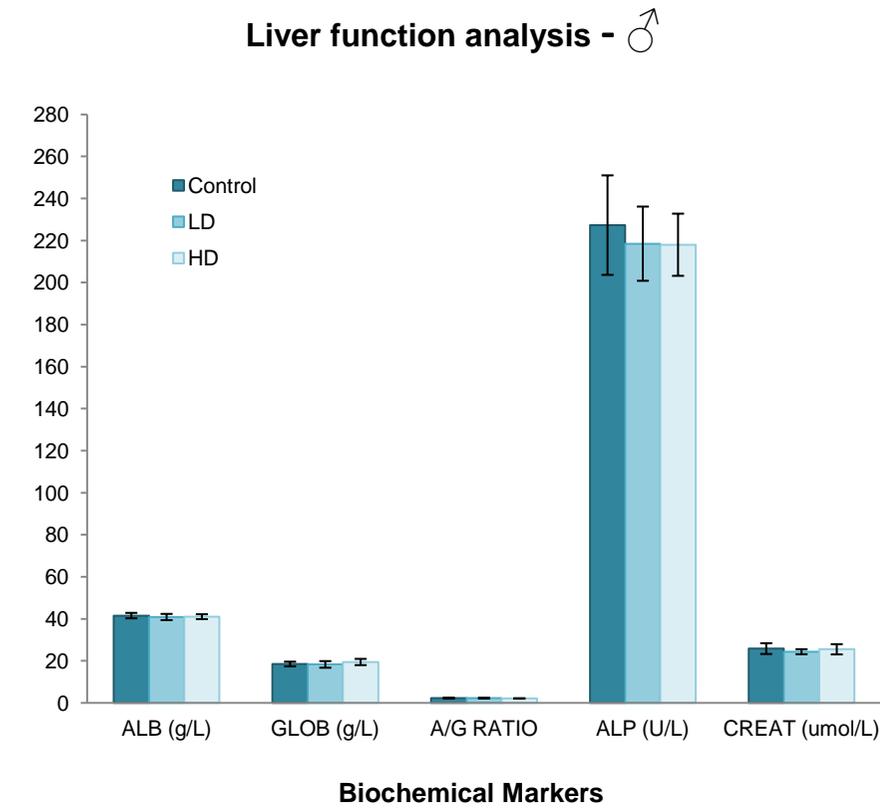
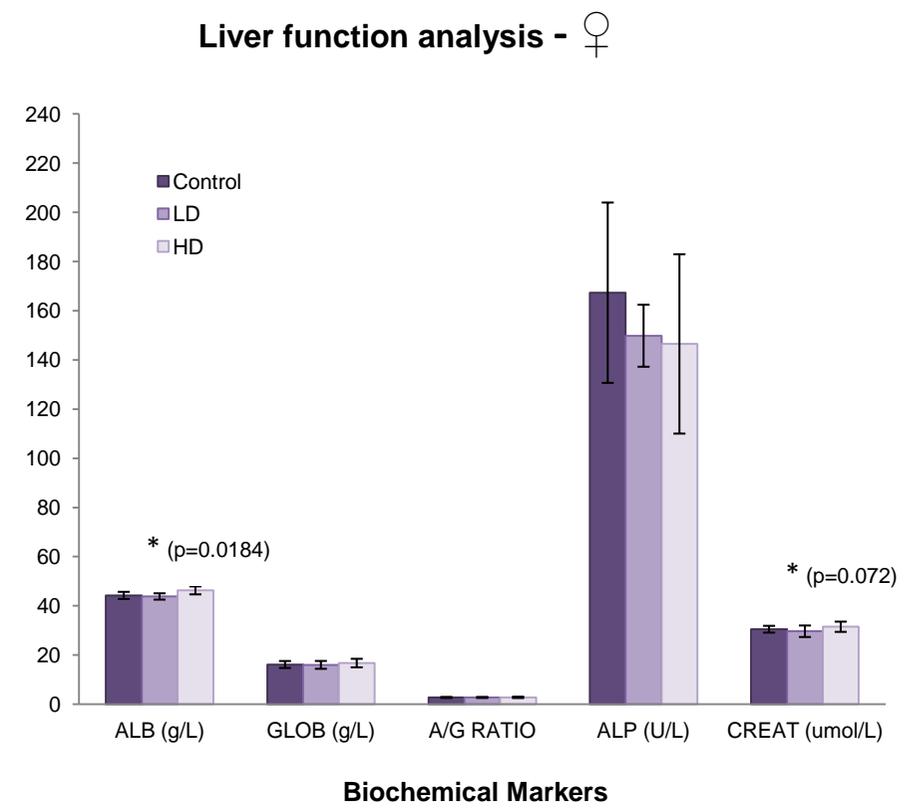


Figure 4.7: Liver function test results according to dosage groups (n=36). **A:** Markers associated specifically with liver function; **B:** Markers associated with general liver and kidney function. Biochemical markers which showed significant differences between sexes are represented separately. Error bars indicate standard deviations and * indicates significance (set at $p < 0.05$), see text for further discussion



A



B

Figure 4.8: Biomarkers of liver function in which significant differences were identified between sexes. **A:** Data obtained from the male population; **B:** Data obtained from female rats. Error bars indicate standard deviations and * indicates significance (set at $p < 0.05$), see text for further discussion

Levels of TP, urea, Na⁺, K⁺ and creatinine revealed no significant differences between males and females nor did concentrations differ between experimental groups. Although ALT levels did not differ significantly between sexes, concentrations showed a tendency to differ significantly between animals in the control group and animals receiving the lower sibutramine dose ($p=0.0896$). This tendency is clearly illustrated in Figure 4.7A, which also represents biomarkers specifically associated with liver function. Initial analysis of AST values showed that significance existed for this data. Upon further investigation the significance was found to exist between the control individuals and experimental groups ($p=0.0127$), this can also be seen in Figure 4.7A. Figure 4.7B shows results obtained for more general biomarkers which can be used to assess both kidney and liver function. Again, analysis revealed no differences in data between sexes existed and therefore male and female values were grouped together. The same could not be said for all parameters however, as ALB, GLOB, A/G Ratio, ALP and creatinine analysis showed that the data obtained differed significantly between sexes and the data was consequently analysed separately. Upon further analysis significant differences were detected in ALB levels for LD and HD female animals ($p=0.0284$). This can be seen in Figure 4.8A, which shows all parameters in which sex showed an effect (Figure 4.8A and B). The same is also true for creatinine which shows a tendency to differ between female animals receiving the high and low doses of sibutramine. Tables 4.9, 4.10 and 4.11 provide the values of the various markers, correlated to Figure 4.7 and 4.8, and also indicate where exact significance exists.

Table 4.9: Values presenting the effects of sibutramine administration on serum biological markers in rats

Treatment	TP (g/L)	ALT (U/L)	AST (U/L)	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	Urea (mmol/L)
Control	60.21 ± 0.6	53.92 ± 1.6	104.91 ± 3.7	140.46 ± 0.4	5.46 ± 0.1	6.76 ± 0.2
LD (1.32 mg/kg Sib)	59.48 ± 0.7	48.75 ± 1.1**	91.17 ± 3.6*	140.98 ± 0.9	5.41 ± 0.07	6.77 ± 0.3
HD (13.2 mg/kg Sib)	61.74 ± 0.8	51.67 ± 2.7	90.08 ± 3.5*	141.22 ± 0.4	5.32 ± 0.1	7.00 ± 0.3

Values represent mean ± SE, n = 12.

* P < 0.05 compared to corresponding value of control group

** P < 0.1 compared to corresponding value of control group, showing tendency to differ significantly

Table 4.10: Values presenting the effects of sibutramine administration on serum biological markers for male rats

Treatment	Alb (g/L)	Glob (g/L)	A/G Ratio	ALP (U/L)	Creatinine (µmol/L)
Control	41.53 ± 0.5	18.48 ± 0.5	2.26 ± 0.05	227.33 ± 9.7	25.83 ± 0.6
LD (1.32 mg/kg Sib)	40.85 ± 0.6	18.3 ± 0.6	2.25 ± 0.08	218.5 ± 7.2	24.33 ± 1.0
HD (13.2 mg/kg Sib)	41.02 ± 0.5	19.45 ± 0.6	2.11 ± 0.04	218 ± 6.0	25.5 ± 1.0

Values represent mean ± SE, n = 06.

Table 4.11: Values presenting effects of sibutramine administration on serum biochemical markers for female rats

Treatment	Alb (g/L)	Glob (g/L)	A/G Ratio	ALP (U/L)	Creatinine (µmol/L)
Control	44.25 ± 0.6	16.5 ± 0.6	2.75 ± 0.08	167.33 ± 15.0	30.5 ± 0.5
LD (1.32 mg/kg Sib)	43.82 ± 0.5	16 ± 0.7	2.75 ± 0.08	149.83 ± 5.1	29.67 ± 1.0
HD (13.2 mg/kg Sib)	46.28 ± 0.7***	16.73 ± 0.7	2.79 ± 0.1	146.4 ± 14.9	31.5 ± 0.8***

Values represent mean ± SE, n = 06.

*** P < 0.05 compared to corresponding value of LD group

4.4 Discussion

In this study the effects of sibutramine administration on both hormone levels and biochemical serum markers were investigated as some inconsistencies exist within the literature. Findings based on morphological investigations have also associated sibutramine use with liver damage although the biochemical data supporting these results are not available (Oberholzer *et al.*, 2013).

Changes in cortisol levels as well as TT3 and TT4 were investigated to determine if sibutramine administration had an effect on the concentrations as all three these hormones are essential and deviations from normal physiological concentrations could have serious consequences. The results obtained revealed that female rats administered high doses of sibutramine (13.2 mg/kg/day) had significant increases in plasma cortisol levels; almost double that of the control and LD animals. This could be due to the fact that the female HD animals also showed a significantly decreased rate of weight gain when compared to control and LD groups. Male rat samples however did not show any significant changes in serum cortisol concentrations.

These findings coincide with a study conducted by Wabitsch *et al.* (1995) in which ninety-two obese adolescent girls partook in a 6-week intervention study to examine the effects of body fat distribution on steroid hormone serum concentrations before and after weight reduction. Data showed that, after a mean weight loss of 8.3 ± 2.6 kg, free cortisol levels increased significantly.

Neuropeptide Y is a member of the pancreatic polypeptide family and is responsible for stimulating food intake (Szenas and Pattee, 1959, Strain *et al.*, 1980) and lowering energy expenditure (Lottenberg *et al.*, 1998). Furthermore neuropeptide Y acts in the hypothalamus to favour the synthesis and storage of fat. In rodents, weight loss essentially stimulates the release of this peptide (Rosmond *et al.*, 2000). Increased

concentrations of glucocorticoids such as cortisol stimulate neuropeptide Y release while inhibiting corticotropin-releasing hormone (CRH) and this combination of effects should promote weight gain. Activation of the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) in this manner, as an adaptive response to weight loss, may enable the mobilization of fat from peripheral tissues and activation of central nervous system responses which promote the restoration of adipose stores in both animals (Galvař-Teles *et al.*, 1976) and humans (McEwen, 1998).

The thyroid hormones concentrations did not show any significant differences for either male or female animals. Previous research does however suggest that interventions such as weight loss did not result in any significant or clinically important changes in thyroid form or function (Ramazan *et al.*, 2003). Keskin *et al.* (2006) also reported that weight loss due to sibutramine administration did not result in changes in the concentration of any thyroid hormones and our results correspond with these findings despite some studies stating otherwise (Saleh and Bisher *et al.*, 2010).

Samples were also subject to biochemical analysis in which the plasma concentrations of various biological markers of organ function (TP, ALB, GLOB, ALT, ALP, AST, GGT, Na⁺, K⁺, urea, creatinine) were analysed. The total serum protein content provides information regarding the general status of organ function and individual health, while fractioning this data provides more significant information. Albumin makes up most of the total protein contents in plasma and globulins the rest. The concentrations of both albumin and globulin are subsequently determined in routine functional tests together with the ratio in which these proteins co-exist within serum. Albumin makes up about half of the serum protein content whereas the globulin portion constitutes hundreds of different serum proteins such as enzymes, carrier proteins and immunoglobulins. Most of these proteins are synthesized by the

liver and alterations in these concentrations and the A/G ratio could indicate liver damage or even nephritic syndrome due to protein loss. The TP, Alb, Glob and A/G ratio values are collectively studied when evaluating diseases parameters (Busher, 1990). Although statistical analysis showed albumin levels were significantly increased in HD females, the clinical significance thereof is less valuable as TP and Glob levels were unchanged and more specific markers of liver function did not show significant changes.

The possible leakage of hepatocellular enzymes from hepatocyte cytoplasm into circulation is also investigated during liver function tests and this entails analysis of serum ALT and AST and also sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH) levels. Increases in the serum concentrations of these enzymes are often due to severe liver injury such as hepatitis and necrosis or alterations in membrane permeability in certain cases such as lipid accumulation. Decreases in ALT and ASP are rarely seen and therefore not good indicators of cellular damage. GGT and ALP are cholestatic induction enzymes of which the synthesis is increased in cases of impaired bile flow, which result in their release into the circulation. Increases in levels of ALP is often associated with drug administration although in rats increased ALP has been noted following food intake. These levels also decrease as body weight decreases and food intake is reduced. GGT levels increase in cases of bile duct hyperplasia making it a less sensitive but more specific marker of cholestasis (Ramaiah, 2007).

The incidence of renal dysfunction in cases of liver disease is also quite high and biomarkers of kidney function are included in routine liver function tests which provide information on both these organ systems. Creatinine and urea are both markers of renal excretion and metabolites formed in the liver and electrolyte values

also relate to the functional status of both these organs with regard to electrolyte balance (Slacket *et al.*, 2010).

Biochemical analysis showed significant differences in some markers. ALT and AST levels both decreased in experimental animals when compared to control animals whereas albumin and creatinine differed significantly between female rats in the HD and LD groups respectively. Decreases in ALT and AST levels could also be associated with weight loss as BMI and physical activity have been shown to influence the concentrations of these enzymes (Robinson and Whitehead, 1989). Although these observed changes were significant, the question is whether it is clinically important as these changes were very small, mostly within a 1-10 unit range.

Carbon-tetrachloride (CCl₄) is an agent well known for its use as an inducer of liver damage in experimental animal models and is often used as positive control when screening agents for possible hepatoprotective properties (Czaja *et al.*, 1995; Naziroglu *et al.*, 1999). These observed damages are attributable to the CCl₄ metabolite, trichloromethyl free radical ($\cdot\text{CCl}_3$) which damages cell membranes essentially leading to liver-injury processes such as steatosis (Wang *et al.*, 2008). This mechanism of drug-induced liver damage results in liver function tests with enzyme levels far higher than control animals, almost doubling in value (Wang *et al.*, 2008) and this also correlates with observable histological lesions or pathologies.

Data obtained from this study showed that liver enzyme levels were decreased in experimental animals when compared to control animals. Decreases in liver enzymes are not commonly reported in normal conditions. Various studies involving patients with existing liver conditions such as non-alcoholic fatty liver disease (NAFLD) or hepatitis C, show that weight loss results in improvements or decreases in liver

enzyme levels, especially in overweight and obese patients which are risk factors for progression of fibrosis (Hickman *et al.*, 2004; Mattar *et al.*, 2005). However, these values are compared to enzyme levels which are associated with disease.

4.5 Conclusion

Despite previous reports that sibutramine administration leads to increases in cortisol and thyroid hormone activity, which could point to thyroid or adrenal gland dysfunction, our current findings showed that sibutramine did not alter these hormone concentrations significantly despite the use of concentrations ten times higher than prescribed doses. The cortisol concentration however differed significantly between female HD and LD animals but this could most probably be attributed to the weight changes observed in the HD experimental group. Liver function data did not show any clinically significant changes in liver biochemistry. This confirms that sibutramine at the concentrations used in this study does not cause liver or kidney damage or alter thyroid and adrenal gland function.

CHAPTER 5: The effect of sibutramine on platelet and fibrin network morphology of male Sprague Dawley rats

5.1 Introduction

Sibutramine is a serotonin-norepinephrine reuptake inhibitor (SNRI) of which the therapeutic effects are mostly attributed to the potent inhibition of serotonin (5-HT) and norepinephrine (NE) transporters (SERT and NET, respectively) by its metabolites M1 and M2 at neuronal synapse sites centrally (CNS) and peripherally (Cheetham *et al.*, 1996; Jackson *et al.*, 1997a; Carek and Dickerson, 1999; Maurer-Spurej, 2005; Lechin *et al.*, 2006). This inhibition ultimately leads to an increase in satiety and increase in energy expenditure which is mediated centrally by the beta (β)1-adrenergic and serotonin receptor 2A and 2C subtypes (5-HT_{2A/2C}), and peripherally by enhancing the function of NE through β 3-adrenoceptors (Stricker-Krongrad *et al.*, 1996; Jackson *et al.*, 1997a; Jackson *et al.*, 1997b; Luque and Rey; 1999).

Several studies have reported bleeding complications associated with the use of selective serotonin reuptake inhibitors (SSRIs) (Barbui *et al.*, 2009; Dalton *et al.*, 2003; Opatrny *et al.*, 2008; Targownik *et al.*, 2009). In contrast, SNRIs with a similar mechanism of action as sibutramine, generally are not associated with increased bleeding risks. However, ischaemic events after treatment with these drugs have been reported, indicating an increased cardiovascular disease and stroke risk in this patient population (Godkar *et al.*, 2009; Reznik *et al.*, 1999). Previous studies involving sibutramine have shown that extracellular NE concentrations are increased and this effect is gradual and sustained. It has been reported that this gradual increase in extracellular NE induced by sibutramine, is mediated by noradrenergic

activation of alpha (α)₂-adrenoceptors (Wortley *et al.*, 1999). Platelets also express α ₂-adrenergic receptors (Eriksson and Whiss, 2008) and increased expression of these receptors is associated with increased platelet activation and possible increased risk for thrombosis.

As sibutramine is still commonly found as additive in slimming medications available on the internet or over-the-counter (Lam *et al.*, 2013) despite the retraction of FDA approval, this study was aimed at determining the effects of sibutramine on blood coagulation and the consequent role this could play in cardiovascular complications often observed with prolonged sibutramine use. The coagulatory effects of this compound were investigated in particular on platelets and fibrin networks of male Sprague Dawley rats at a prescribed dose and higher toxicological dosage.

5.2 Materials and methods

5.2.1 Implementation of the Sprague Dawley rat model

The Sprague Dawley rat model was implemented as described in Chapter 3. On day 28 the rats were terminated and blood samples were collected. Only samples from male rats were used for this part of the research project as female platelet reactivity and coagulation has been shown to vary during the oestrus cycle due to the influences of oestrogen and progestogens (Oliver, 1959; Emms and Lewis, 1985).

5.2.2 *Blood collection*

On the day of termination, five to ten ml of blood was collected from each rat via cardiac puncture under Isoflurane anaesthesia. All samples were collected in separate sodium citrate tubes which inhibit coagulation. Following blood collection, the animals were terminated via Isoflurane overdose, according to standard methods employed by the UPBRC.

5.2.3 *Platelet Rich Plasma (PRP) preparation*

Blood samples were centrifuged at 300 x g for two minutes to obtain PRP. PRP aliquots of 10 μ l were used to prepare smears on round glass cover slips (10 mm diameter). The cover slips were allowed to dry and were then washed in a phosphate buffered saline (PBS, pH 7.4) solution on a shaker for 20 minutes. The samples were then fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution in 0.075M PBS for 30 minutes and then washed three times with PBS (pH 7.4) allowing 3 minutes per wash. This was followed by secondary fixation in osmium tetroxide for 30 minutes and the samples were washed again as described above. The samples were then serially dehydrated in 30%, 50%, 70% and 90% ethanol, followed by three changes of absolute ethanol (three minutes each) and then dried using hexamethyldisilazane (HMDS). The cover slips were then mounted on aluminium stubs, coated with carbon and viewed with a Zeiss Ultra Plus FEG SEM.

5.2.4 *Fibrin networks*

Thrombin (provided by the South African National Blood Services) was used to prepare fibrin clots. The thrombin was prepared in a biological buffer containing 0.2% human serum albumin at a concentration of 20 U/ml. A volume of 10 μ l of thrombin was added to 10 μ l of rat PRP causing the conversion of fibrinogen to fibrin and the release of intracellular platelet components, such as transforming growth factor,

platelet derived growth factor and fibroblastic growth factor, into the coagulum. The PRP/thrombin mix was immediately prepared on a round glass cover slip to form the fibrin coagulum simulating the coagulation process in the body. The cover slips were then placed in a petri dish and kept at 37°C for 10 minutes. Finally the cover slips with the coagula were placed in PBS (pH 7.4) and washed for 20 minutes on a shaker. This washing step was necessary to remove any blood proteins possibly trapped within the fibrin network.

Again, these samples were fixed in 2.5% GA/FA in 0.075M PBS (pH 7.4) for 30 minutes after which sample processing was done as described for PRP samples. The cover slips were dried using hexamethyldisilazane (HMDS), after which the coverslips were mounted on aluminium stubs and coated with carbon. The samples were viewed with a Zeiss Ultra Plus FEG SEM.

All chemicals and reagents were obtained from Merck Pty (Ltd) (Modderfontein, South Africa) and were of analytical grade.

5.3 Results

Figure 5.1 shows images acquired from smears prepared from the PRP of animals in the control group as well as LD and HD which represent the groups exposed to low and high doses of sibutramine respectively. Figure 5.1A-C depicts platelets of all three groups at low magnification to show general morphology whereas Figure 5.1D-F are micrographs of platelets at high magnification to study changes in morphology of the platelet membrane. Figure 5.1A shows a typical inert control platelet with a single pseudopod extending from the platelet membrane. At higher magnification (Figure 5.1D) the platelet membrane appears to be intact with a smooth surface and open canalicular pores are visible (indicated by white arrows).

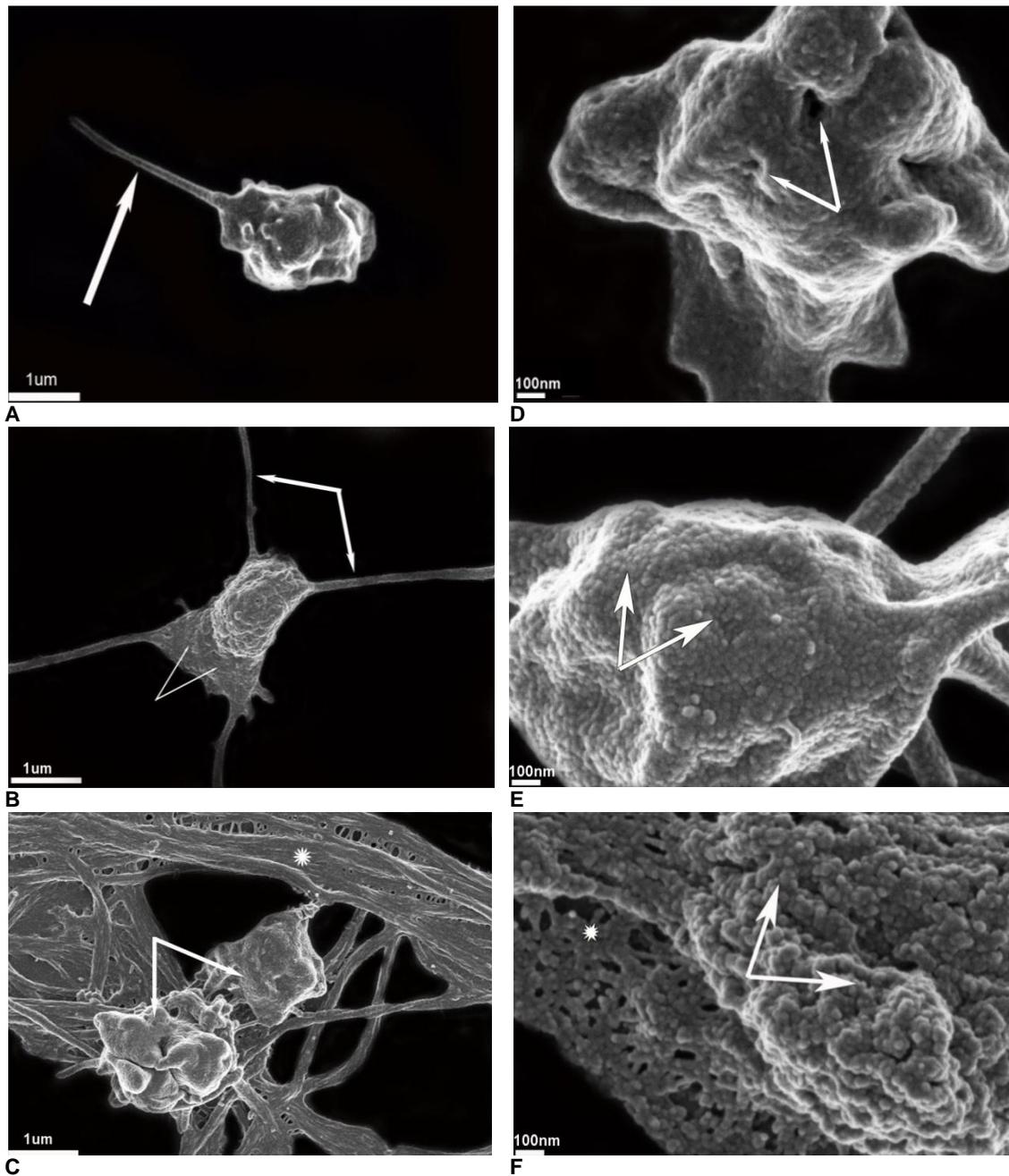


Figure 5.1: Platelets from animals in the three experimental groups. **A and D:** low and high magnification respectively of a typical platelet structure from control rats. (Arrow in **A** indicates a pseudopod and the arrow in **D** shows the open canalicular pores); **B and E:** High and low magnification respectively of platelets of LD rats. (Thick arrows show multiple pseudopodia and thin arrows show membrane spreading in **B**; Arrows in **E** indicate the granular appearance of the membrane); **C and F:** Platelets from HD rats, low and high magnification respectively (arrows indicate platelet interaction and the star indicates spontaneous fibrin formation in **C**; arrows in **F** indicate the necrotic appearance of the membrane and the star shows membrane ruptures)

Figures 5.1B and E are representative of animals from the LD group. Multiple pseudopodia (thick white arrows) and membrane spreading (thin white arrow) can be seen. When the different platelets were viewed under higher magnification the membranes appeared granular. Figures 5.1C and F show platelets from rats exposed to the higher dose of sibutramine. In Figure 5.1C a platelet with numerous pseudopodia is shown, with spontaneous formation of matted thick fibres associated with fibrin formation (white star). This was seen in 25% percent of the images acquired from HD samples but was not observed in any of the other experimental groups. Also, platelet interaction is apparent and when platelets were examined using higher magnification the membranes appeared necrotic [white arrows (52% of acquired images)] (Pretorius *et al.*, 2012). In some areas, membrane ruptures are also visible [(38% of acquired images) white star]. These percentages are related to samples within the HD experimental group only.

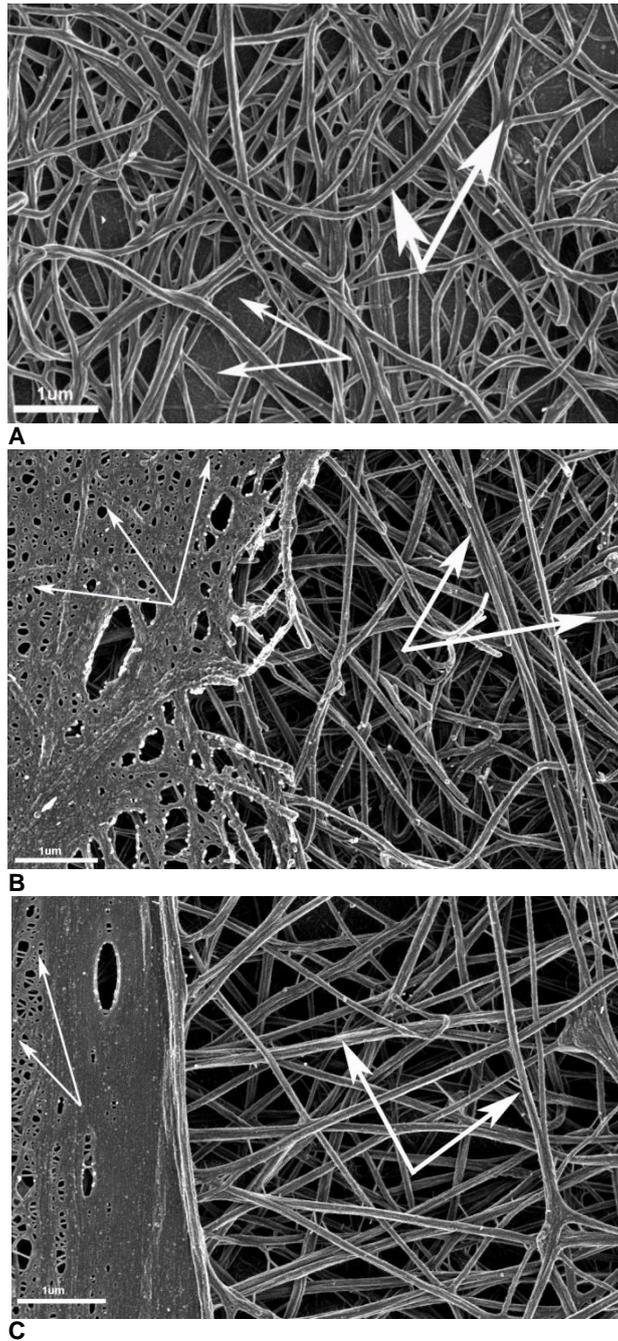


Figure 5.2: Fibrin networks of animals in the different experimental groups. **A:** Control network with major thick (thick arrows) and minor thin (thin arrows) fibres; **B:** LD rats showing fused thick fibres (thick arrows) and minor fibres forming a net-like structure (thin arrows); **C:** HD rats showing minor fibres (thin arrows) covering the thick fibres (thick arrows)

Figures 5.2A-C are representative of the fibrin networks of the animals in the control and two experimental groups. Figure 5.2A shows a typical fibrin network of control animals where major thick fibres (thick white arrows) and minor thin fibres (thin white arrows) are present. The major, thick fibres are more abundant than the thin fibres, which is characteristic of control individuals (Pretorius, 2007). Figure 5.2B represents fibrin networks of LD rats, which were exposed to the low dose of sibutramine. Major thick fibres are present but appear fused as indicated by the thick white arrows. Also, the minor thin fibres form a net-like structure (thin arrows) covering large parts of the thick fibres and therefore the clot. A similar morphological appearance was observed in samples obtained from HD rats as shown in Fig 5.2C. Major thick fibres are visible (thick arrows) with thin fibres (thin arrows), covering the thick fibres as seen in Figure 5.2B. In some areas the thin fibres are arranged in a denser net-like structure covering the thick fibres. This was not seen in the control group. Also, fused major thick fibres are present in both experimental groups (LD and HD) but this appearance was not observed in the control group.

5.4 Discussion

Haemostasis involves the highly coordinated processes of platelet activation and blood clotting with the ultimate goal of vascular repair. The coagulation cascade is the vital component that maintains the balance required for haemostasis by activating platelets and forming a haemostatic platelet plug as well as stabilizing fibrin networks, contributing to blood clot formation (Gailani and Renné, 2007a; Versteeg *et al.*, 2013).

The coagulation process is subdivided into initiation, amplification, and propagation phases and entails highly specific interactions between cell surfaces, plasma-derived zymogens and cofactors. Given the appropriate stimulus, a complex series of events are initiated and coagulation factors IX and X are eventually activated. The activated factor Xa initiates the generation of moderate amounts of thrombin. This is known as the amplification phase (Wolberg *et al.*, 2008). Thrombin is responsible for the activation of cofactors V, VIII and platelets. Once activated, platelets expose phosphatidylserine which provides binding sites for various procoagulant zymogens and enzymes, including factors II, Va, VIIIa, IXa, X, and Xa, (Tracy *et al.*, 1978; Nesheim *et al.*, 1988; Ahmad *et al.*, 1989; Rawala-Sheikh *et al.*, 1990 Wolberg *et al.*, 2008). Finally, intrinsic reactions which are supported by the activated platelet surface leads to the production of a large amount of thrombin during the propagation phase which is responsible for the conversion of fibrinogen to fibrin (Hoffman *et al.*, 1995; Monroe *et al.*, 1996). The produced thrombin further leads to the formation of eventual cross linkages between fibrin fibres, which increase the elasticity of individual fibres as well as the viscoelasticity of the entire clot (Liu *et al.*, 2006; Tran *et al.*, 2013). Defects or dysregulation of this process could lead to severe cases of haemophilia or prothrombotic complications.

Laboratory based investigations of fibrin structures, platelet morphology and the fibrin clot in general has become a popular and powerful research tool as these structural findings could differ in and provide essential information on various diseased states possibly contributing to the discovery of new therapeutic targets (Adams *et al.*, 2007; Pretorius *et al.*, 2013).

The results obtained in this study showed different degrees of platelet activation between the control and experimental groups. Treatment of rats with sibutramine shows an increased degree of platelet activation characterised by the presence of more pseudopodia in conjunction with platelet spreading in the LD group while spontaneous fibrin formation can be seen in the HD group. In this animal model, indications are that sibutramine causes the activation of platelets thereby increasing the thrombotic tendency. The fibrin networks also differ between groups. When comparing the control fibrin network to that of the sibutramine groups, a dramatic difference in fibrin structure can be observed. Where the major fibres are more abundant in control individuals, minor fibres appear to become more prevalent in LD and increased in HD groups. Generally, increased thrombin concentrations are associated with the formation of stiffer clots and increased thrombin activity on platelet surfaces also plays a role in the architecture of the fibrin clot (Wolberg *et al.*, 2008, Pretorius *et al.*, 2011) In both experimental groups the fibrin clots also presented with a matted, layered morphology and thicker, fused fibres. These characteristics are not observed in control individuals.

The fibrin morphology as described in this study strongly correlates with that seen in thromboembolic ischaemic stroke, described by Pretorius *et al.* (2012), which is associated with a hypercoagulable state (Takano *et al.*, 1990; Sacco, 1995, Pretorius 2012). Reports by Wolberg (2008) show that increased thrombin levels produce thinner fibrin fibres that are more tightly packed. This can be seen in the matted, net-like arrangement of fibres in experimental animals. This suggests, as described by Pretorius *et al.* (2012), that the formation of this observed atypical fibrin morphology is due to elevated concentrations of the responsible coagulation factors and this may be a consequence of prolonged sibutramine usage.

Between the LD and HD groups, elevated levels of coagulation factors could also explain the observed differences in platelet morphology. PRP smears prepared from samples collected from animals administered high doses of sibutramine, showed membrane blebbing and membrane ruptures, which is characteristic of cells undergoing necrosis. Again, a similar morphology has been described for platelets in procoagulant conditions such as stroke (Pretorius, 2012).

β -adrenergic stimulation also leads to the release of von Willebrand Factor (vWF) from endothelial cells, which could further contribute to increased cardiovascular risk (Von Känel *et al.*, 2003). The vWF enhances both the integration of platelets into polymerizing fibrin (Loscalzo *et al.*, 1986; Ruggeri and Ware, 1993) as well as the adhesion of platelets to fibrin clots which is a function essential in high shear stress conditions. It has previously been demonstrated in the Sprague Dawley rat model, that sibutramine administration produces substantial tachycardic and pressor effects that are inhibited by adrenoceptor antagonists such as propranolol and phentolamine. Results such as these suggest that despite sibutramine's central inhibitory effects, the described events are mostly attributed to a predominant inhibition of peripheral NE reuptake (Woolard *et al.*, 2004).

As sibutramine has been known to increase heart rate and blood pressure, effects which were completely reversed when β -adrenoreceptors are blocked (Birkenfeld *et al.*, 2002), this increased pressure and shear on vessel walls could contribute to the procoagulant morphology observed in this study.

SNRIs, such as sibutramine, have been associated with increased risk of bleeding to a much lesser extent than SSRIs. Additionally, various case reports have been published reporting ischaemic events, including infarction (Reznik *et al.*, 1999; Godkar *et al.*, 2009). Adrenergic receptors expressed on the surface of platelets

increase platelet activation (Eriksson and Whiss, 2008), and in a study conducted by Hallbäck *et al.* (2012) results showed that SNRIs, such as Venlafaxine, increased adhesion by possibly acting on these receptors, perhaps providing an explanation for the alterations in platelet morphology as observed in this study.

NE has extensively been described as a trigger for the induction of human platelet aggregation (O'Brien, 1963; Mills and Roberts, 1967; Bygdeman and Johnson, 1969; Newman *et al.*, 1978). In a study conducted in 2002 by Von Känel *et al.* it was also shown that acute mental stress, during which NE levels are significantly increased, increases thrombin activity and fibrin turnover reliant on β 2-adrenergic receptor functioning and associated catecholamine activity.

The active metabolites of sibutramine, M1 and M2 possess potencies for the inhibition of reuptake of 5-HT and NE that are relatively similar to the existing SSRI's and SNRI's such as fluoxetine and desipramine respectively (Luque and Rey, 2002). To assess the potential of certain drugs to inhibit the reuptake of NE and 5-HT, Cheetham *et al.* (1993, 1996) conducted two studies, which included sibutramine and its two metabolites. Results showed that the active metabolites M1 and M2 were as potent as many of the antidepressants currently in use. It is believed that through the inhibition of the cellular reuptake of NE and 5-HT, the extracellular synaptic concentrations are consequently increased, and the subsequent activation of α -adrenoceptors, β -adrenoceptors and 5-HT_{2A/2C} receptors occurs (Buckett *et al.*, 1988; Heal *et al.*, 1992a, 1992b; Fantino and Souquet, 1995; Jackson *et al.*, 1997a, 1997b; Stock, 1997). Reports based on animal research have also showed that sibutramine administration leads to the down regulation of pre- and post-synaptic adreno- and 5HT receptors (Buckett *et al.*, 1988; Luscombe *et al.*, 1989; Heal *et al.*, 1989; Heal *et al.*, 1991a, 1992b; Martin *et al.*, 1992) which further substantiate theories that

sibutramine exerts its therapeutic effects by incidentally increasing the activity of NE and 5-HT at each particular receptor site.

5.5 Conclusion

Cardiovascular complications such as hypertension, arrhythmias and tachycardia are adverse events associated with sibutramine use. In addition this present study has shown that sibutramine increases the coagulation potential of male rats exposed to sibutramine. It is therefore proposed that administration of sibutramine at concentrations that do not necessarily cause alterations in biochemical parameters associated with toxicity, could lead to a procoagulant state possibly increasing the likelihood of stroke and myocardial infarction.

CHAPTER 6: The effect of sibutramine on organ ultrastructure of Sprague Dawley rats

6.1 Introduction

Available research presents with several inconsistencies regarding the toxicity of sibutramine. In rats it has been shown by our laboratory that liver fibrosis with steatosis can be associated with concomitant high-fat diets and sibutramine use (Oberholzer *et al.*, 2013). In contrast other studies reported improvements in obesity and hyperlipidaemia steatohepatitis in rats following sibutramine administration (Xing *et al.*, 2004).

In the previously mentioned study in which rats on a high energy diet were exposed to sibutramine (Oberholzer *et al.*, 2013), it was shown that fibrosis developed with hepatic steatosis although it was not possible to ascertain whether or not this was directly due to sibutramine administration or the combination of both the compound and high-fat diet. The limitations of this study were therefore addressed in the current research, which was aimed at investigating the effects of sibutramine alone on liver tissue and cellular structure. In addition this study will also provide important information on the toxicity of sibutramine in other organ systems.

For several organ systems the plasma markers of toxicity are well defined such as the measurement of hepatic enzyme activity for liver function and the measurement of creatine levels and clearance for kidney function. Nevertheless small changes in tissue morphology and cellular structure can occur prior to changes in marker levels. In this study the effect of sibutramine on the ultrastructure of the liver and kidney was determined. In contrast to the liver and kidneys no specific blood tests exists which can be used to evaluate lung function. Common tests often used when evaluating

pulmonary function usually involves volumetric or spirometric tests. Such procedures are impossible to perform on rats thus the effects of sibutramine on the tissue and cellular structure of the lungs was also determined. To obtain an overall picture of the toxicity of sibutramine other organs such as the heart and brain were included.

This study was aimed at evaluating the solitary effects of sibutramine on organ systems, independent of possible diet interactions, so as to add to the toxic profile of this compound. These include organ systems associated with metabolism and excretion which are the liver and kidneys. The brain, lungs and hearts were also collected for ultrastructural evaluation from animals on an unaltered diet to determine the possible adverse effects, if any, of sibutramine on the morphology of these organs.

6.2 Materials and methods

6.2.1 Animal model

The Sprague Dawley rat model was implemented as described in Chapter 3 of this dissertation.

6.2.2 Sample collection

On experimental day 28, rats were terminated following blood collection, via Isoflurane overdose, according to standard methods employed by the UPBRC. Liver, lung, brain, heart and kidney sections were then collected for histological investigation.

6.2.3 Tissue preparation for light microscopy (LM)

Samples were fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution overnight at 4°C. The tissue was then removed from the fixative, rinsed three times in 0.075 M sodium potassium phosphate buffer (PBS; pH 7.4) and serially dehydrated in 30%, 50%, 70% and 90% ethanol, followed by three changes of absolute ethanol for 15 min/change. Tissue samples were then embedded in paraffin wax by implementing the Leica TP1020 Automatic Tissue Processor (Scientific Group, Johannesburg, SA). Following dehydration the samples were placed in a 1:1 xylene:ethanol solution for 1 hour followed by one change of 100% xylene for 2 hours. Finally, the samples were placed in 100% paraffin wax for 2 hours followed by a final change of 100% Paraffin for 4 hours. Sections were then made using a Leica RM 2255 wax microtome. Sections were mounted on glass slides and stained with the stain. Slides were cleared of paraffin wax by submersion in 2 changes of xylene for 5 min each, and serially dehydrated in 2 changes of 100% (2 min), 90% (1 min) and 70% (1 min) ethanol, followed by a rinsing step in dH₂O (1 min). Slides were placed in the Haematoxylin solution (1 g haematoxylin powder, 0.2g sodium iodate, 50 g potassium aluminium sulphate, 1 g citric acid and 50 g chloral hydrate in 1L dH₂O) for 5-7 min after which the stain was allowed to differentiate for 5-7 min using Scott's Buffer (2 g potassium bicarbonate, 20 g magnesium sulphate in 1 L dH₂O) as blueing agent. Sections were then counterstained by submersion in Eosin (2 g eosin powder in 200 ml dH₂O) for 1-2 min. Following counterstaining, slides were rinsed in dH₂O for 1 min and sequentially dipped in 70%, 90% and 100% ethanol followed by two xylene dips. Slides were then mounted using entellan and after drying was viewed using a Nikon Optiphod transmitted light microscope (Nikon Instech Co., Kanagawa, Japan) to investigate any histological changes in above mentioned organs.

6.2.4 Tissue preparation for transmission electron microscopy (TEM)

Tissue samples were fixed in 2.5% GA/FA in 0.075M phosphate buffer for 1 hour and rinsed three times in 0.075M PBS (pH 7.4) for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide, for 1 hour. Following fixation, the samples were rinsed again as described above. The samples were dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol and were then embedded in Quetol epoxy resin. Ultra-thin sections (70-100 nm), cut with a diamond knife using an Reichert-Jung Ultracut E ultramicrotome, were then made and were contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate. Finally the samples were allowed to dry for a few minutes before examination with the JEOL TEM (JEM 2100F).

All chemicals and reagents were obtained from Merck Pty (Ltd) (Modderfontein, South Africa) and were of analytical grade.

6.3 Results

In this part of the study, the possible effects of sibutramine administration on tissue and cellular structure of liver, kidney, brain, heart and lung samples from the control, low and high dose (LD and HD) experimental groups were evaluated. The results are presented in Figures 6.1 to 6.5.

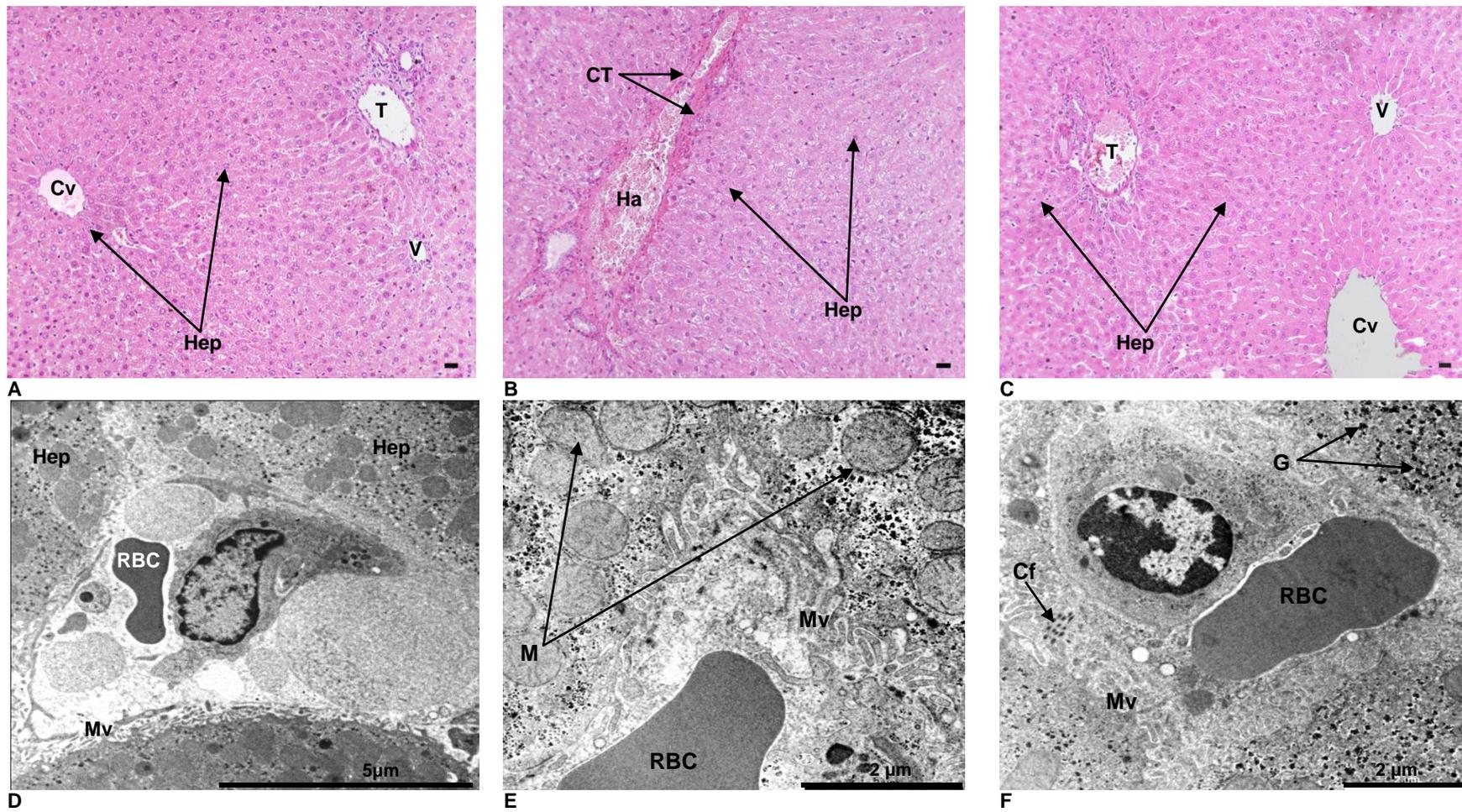


Figure 6.1: LM and TEM micrographs of liver samples collected from animals in the different experimental groups. **A and D:** Control LM and TEM respectively; **B and E:** LD sibutramine LM and TEM respectively; **C and F:** HD sibutramine LM and TEM respectively. LM scale bar=10 μm. (Cf=collagen fibres; Cv=central vein; CT=connective tissue; G=glycogen granules; Ha=hepatic artery; Hep=hepatocyte/s; V=portal venule; T=portal tracts; RBC=red blood cell; M=mitochondria; Mv=microvilli)

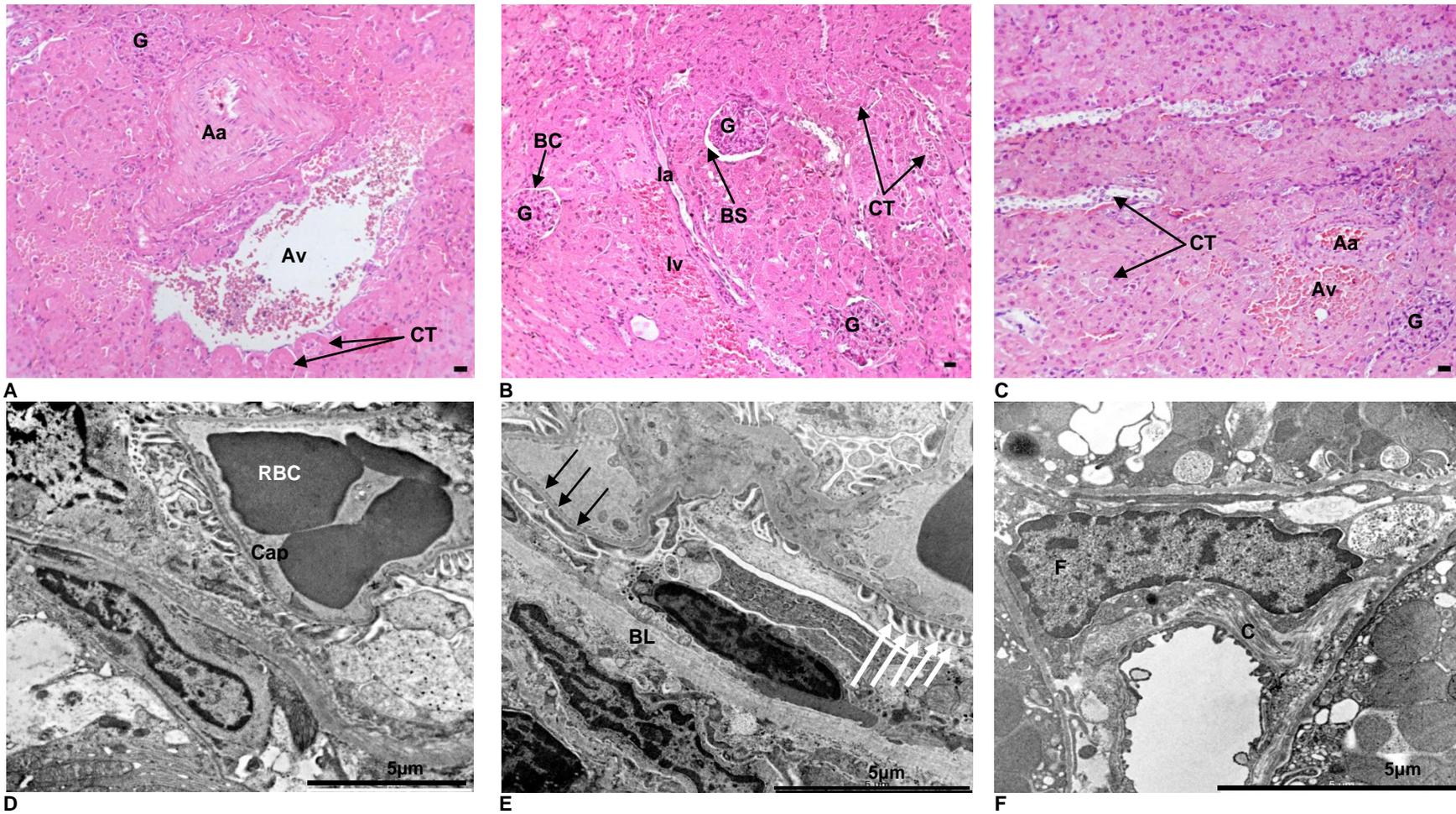


Figure 6.2: LM and TEM micrographs of kidney samples collected from animals in the different experimental groups. **A and D:** Control LM and TEM respectively; **B and E:** LD sibutramine LM and TEM respectively; **C and F:** HD sibutramine LM and TEM respectively. LM scale bar=10 μ m. (Aa=Arcuate artery; Av=Arcuate vein; BC=Bowman's capsule; BS=Bowman's space; BL=basal lamina; C=collagen; Cap=capillary; CT=collecting tubule; G=glomerulus; Ia and v=interlobular artery and vein; RBC=red blood cell; Black arrows indicate filtration slits; White arrows indicate podocyte pedicles)

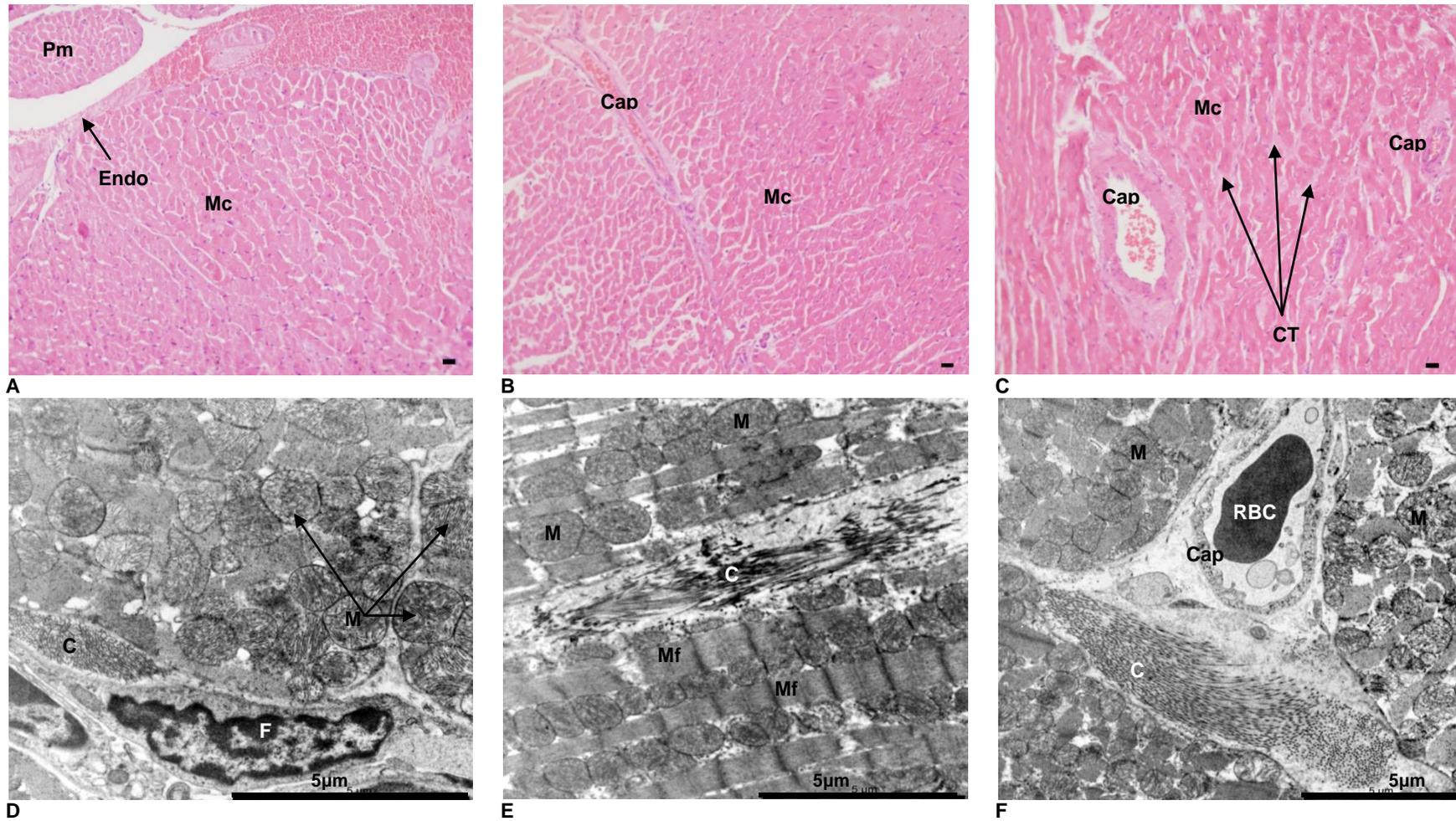


Figure 6.3: LM and TEM micrographs of heart samples collected from animals in the different experimental groups. **A and D:** Control LM and TEM respectively; **B and E:** LD sibutramine LM and TEM respectively; **C and F:** HD sibutramine LM and TEM respectively. LM scale bar=10 μm. (C=collagen; Cap=capillaries; CT=connective tissue; Endo=endocardium; F=fibroblast; M=mitochondria; Mc=cardiac muscle; Mf=myofibrils; Pm=papillary muscle)

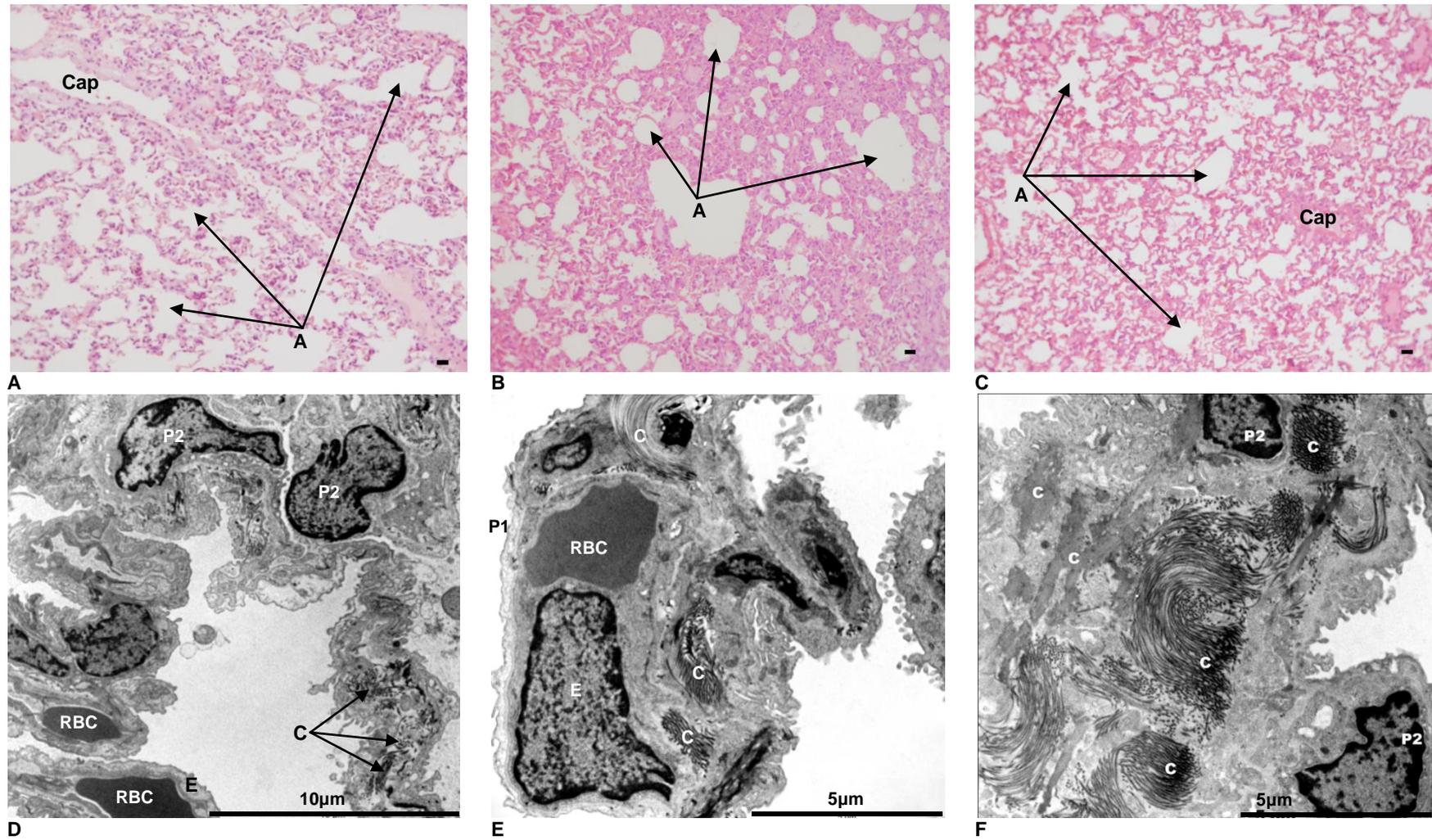


Figure 6.4: LM and TEM micrographs of lung samples collected from animals in the different experimental groups. **A and D:** Control LM and TEM respectively; **B and E:** LD sibutramine LM and TEM respectively; **C and F:** HD sibutramine LM and TEM respectively. LM scale bar=10 µm. (A=Alveolar spaces; C=Collagen; Cap=capillary; E=endothelium; P1=type I pneumocyte; P2=type II pneumocyte; RBC=red blood cell)

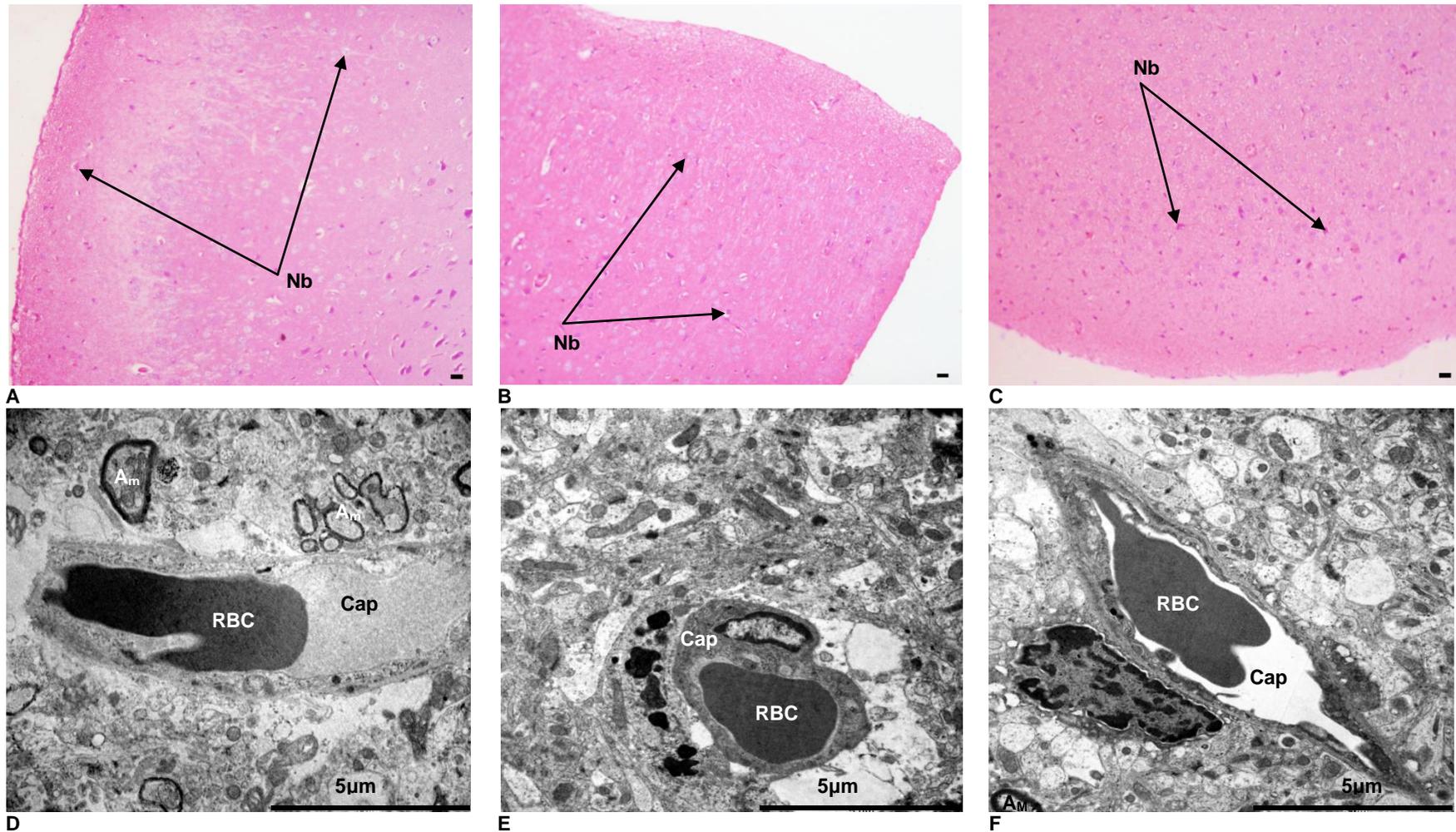


Figure 6.5: LM and TEM micrographs of brain samples collected from animals in the different experimental groups. **A and D:** Control LM and TEM respectively; **B and E:** LD sibutramine LM and TEM respectively; **C and F:** HD sibutramine LM and TEM respectively. LM scale bar=10 μ m. (Am=myelinated axon; Cap=capillary; Nb=Neuron cell bodies; RBC=red blood cell)

Figures 6.1A-F are micrographs of liver samples collected from animals in the control, LD and HD groups respectively. Figure 6.1A shows the normal histology of the liver typical of control individuals. The liver is a dense organ composed of the tightly packed plate-like arrangement of hepatocytes, which are visible in Figure 6.1A as indicated by the arrows (Hep). Normally, sinusoids are distinguishable between these plates as the pale staining spaces which can be observed. Most of the connective tissue found in the liver is associated with the portal tracts which contain the main blood vessels of the liver, delineated by T in Figure 6.1 as well as some central veins (C_v) and portal venules (V) which are generally less conspicuous than the portal tracts and are tributaries of the hepatic vein. Figures 6.1B and C represent histological sections obtained from LD and HD animals respectively. Tissue samples show normal morphology with no deviation from control animals. Figures 6.1D-F are TEM micrographs from the different experimental groups. Figure 6.1D shows the ultrastructural arrangement of the hepatic sinusoid with microvilli (Mv) in the space of Disse and numerous mitochondria (M) in the surrounding, highly metabolic hepatocytes. Glycogen granules (G) are also evident and the same structural arrangement can subsequently be seen in the LD and HD groups (Figure 6.1E and F respectively) where the sparse distribution of collagen fibres (Cf) throughout the tissue can be seen.

Figures 6.2A-F are LM and TEM micrographs prepared from the collected kidney samples from the different experimental animals. Figure 6.2A shows the histology of the kidney of control animals and together with Figure 6.2B and C show sections through the renal cortex as renal corpuscles can be seen. Typically the renal corpuscles are dense rounded structures consisting of the glomeruli (G) surrounded by the Bowman's capsule and the bulk of the renal cortex is filled by collecting tubules, predominantly proximal convoluted tubules. This normal arrangement of renal tissue can be seen in Figures 6.2A-C in which glomeruli (G) and collecting

tubules (Ct) are visible as well as the arcuate artery (Aa) and vein (Av) which usually demarcate the cortico-medullary junction. Samples obtained from both the LD (Figure 6.2B) and HD (Figure 6.2C) experimental groups showed little to no morphological differences when compared to the control animals. Glomeruli (G) are clearly distinguishable with visible Bowman's spaces (BS) and capsules (BS) (Figure 6.2B) and the bulk of the parenchyma between these renal corpuscles is filled by the collecting tubules. Interlobular vessels are also visible (Ia and Iv). TEM micrographs further show the normal ultrastructural arrangement of the tissue. In Figure 6.2D components of the renal corpuscle can be identified as capillaries (Cap) containing red blood cells (RBC). Podocyte secondary processes (white arrows) as well as filtration slits (black arrows) can be seen, together with a clear basal lamina (BL) (Figure 6.2E). Fibroblasts (F) can also be identified with collagen fibres (C) dispersed within the interstitium (Figure 6.2F). This can also be seen in TEM images from C, LD and HD samples in which no deviations from normal renal tissue was noted.

Figures 6.3A-F are representative of heart samples obtained from the different experimental groups. Images show sections through the myocardium, which is the muscular component of the heart wall and is made up of cardiac type muscle. Extensions of the myocardium are often visible, which form the papillary muscles (Pm). In Figure 6.3A the endocardium (Endo), the inner lining of the heart wall similar to the tunica intima found in large blood vessels, can also be seen. Compared to control animals, histology micrographs revealed no significant differences between the experimental groups although connective tissue (CT) seemed to increase slightly between muscle fibres (Mf). However, the acquired images were not conclusive. Capillaries (Cap) can also be seen throughout the muscle tissue. The acquired TEM micrographs (Figure 6.3D-F) clearly indicate the arrangement of the cardiac muscle myofibrils. Mitochondria are clearly visible and in Figure 6.3D a fibroblast (F) can be seen with some collagen fibres (C). Figure 6.3E also shows some collagen fibres

situated between the longitudinally arranged myofibrils in which the alternating light and dark bands are clearly visible. In Figure 6.3F a capillary (Cap) is also visible containing red blood cells (RBC). Collagen fibres (C) are visible between the cardiac muscle. Based upon these morphological investigations, collagen fibres appeared to be slightly increased in the HD group, however, due to the descriptive nature of this study, no quantitative analysis was done to determine the significance thereof.

Figures 6.4A-F are LM and TEM micrographs of the collected lung samples. The LM images acquired from the collected samples show sections through the respiratory portion of the bronchial tree. The normal arrangement of this region consists of numerous alveoli, their walls formed by alveolar epithelium. Numerous capillaries containing single erythrocytes are found within the alveolar walls. LM micrographs show clear differences in the general morphology of these samples when comparisons are made between the experimental groups. Control samples (Figure 6.4A) reveal normal lung alveoli (A) with thin walls and large open spaces. The control TEM micrograph (Figure 6.4D) consequently shows the ultrastructural arrangement of the different elements which make up the alveolar wall. Type II pneumocytes can be seen as well as the endothelial cells which form the capillary walls containing red blood cells. Some collagen fibres (C) are also visible. LM micrographs (Figure 6.4B and C) representative of the LD and HD animals show thickened alveolar walls and a general decrease in open spaces. The corresponding TEM micrographs (Figure 6.4E and F) show dramatic increases in collagen deposition in a dose related manner and thick banding can also be seen in Figure 6.4F. This was not present in the control group.

Figures 6.5A-F are images acquired from the collected brain samples. The neurons of the cerebral cortex are usually arranged in several layers and pyramidal and stellate cells are the most prominent but support neuroglia cells, such as the

astrocytes, are also present. Due to the magnification of the images produced, this layered appearance of the cerebral cortex is not clearly visible but no morphological differences were observed between the experimental groups. Figures 6.5A-C show histology sections in which various neuronal cell bodies (Nb) can be seen. TEM (Figure 6.5D-F) micrographs show myelinated axons (Am) and dendrites (D). Capillaries (Cap) containing red blood cells (RBC) can also be seen.

6.4 Discussion

Available data involving histological investigations on the effects of sibutramine on organ ultrastructure is very sparse. Despite the associated complications and known risks, very little research has been done to validate or refute the sibutramine associated claims. One of the major research objectives of this study was to assess the possibility of morphological alterations in vital tissues associated with sibutramine use as an exclusive variable.

When considering sibutramine action, the liver and kidneys are important target organs with regards to the metabolism and excretion of this compound. These organs are often of interest in toxicological studies and models have been established in which various physiological parameters can be assessed such as the CCl₄-induced liver fibrosis model (Kovaloviche*t al.*, 2000). In this study the histological and ultrastructural arrangement of the collected liver and kidney samples within the experimental groups did not deviate from control samples coinciding with the biochemical findings reported in Chapter 4 which showed no significant alterations in liver function analysis (ALT, ASP ALK and bilirubin) or parameters of kidney function such as creatinine, urea and electrolyte levels.

Lung sections prepared from samples obtained from the exposed animals showed considerable differences between experimental groups. Alveolar walls were noticeably thickened and TEM images revealed increases in collagen deposition in a dose related manner when compared to control animals. This also aligns with previous research within our unit in which sampled lung tissue collected from animals on a high energy diet and exposed to sibutramine showed altered lung morphology upon analysis. Our findings therefore show that this observed fibrosis is a result of sibutramine administration and not necessarily due to diet. It is suggested that these noticeable increases in connective tissue could be due to fibrotic processes associated with increases in inflammatory products as pulmonary fibrosis is characterized by variable degrees of inflammation and fibrosis within lung tissue (Crystal *et al.*, 1976; Turner-Warwick, *et al.*, 1980; Raghu, 1987).

One feature present in most fibrotic cases is a constant irritant that could lead to uncontrollable healing responses which progresses into a pathogenic fibrotic condition (Wilson and Wynn, 2009). Various chemicals have been identified as such irritants which are able to disrupt the delicate structure of the lung and increase the production of pulmonary connective tissue when regulatory checkpoints are overlooked and chronic inflammation develops as a result.

In a study conducted by Choi *et al.*, (2011) a comprehensive proteomic analysis of protein expression patterns in plasma of control and sibutramine-treated rats was done. The authors reported an unexpected increase in the C-reactive protein (CRP) precursor in response to sibutramine treatment although this observed increase could not be explained as the study focused on markers of cardiovascular disease specifically. CRP is a positive acute-phase protein, which is increased by adipocyte-produced pro-inflammatory cytokine interleukin 6 (IL-6) (Lau *et al.*, 2005). This observed increase in CRP due to sibutramine administration could implicate an

underlying inflammatory process or condition associated with this compound, which could lead to the eventual development of fibrosis. Whether this described increase is due to decreased pulmonary function or results in a persistent inflammatory state and eventual fibrotic processes, remains a topic for further research. It must be mentioned though that a converse linear relationship has been described to exist between CRP concentrations and pulmonary function measurements independent of factors such as smoking, fitness or possible metabolic syndrome symptoms which are all known to influence CRP levels (Aronson *et al.*, 2006).

Another interesting finding is that within several pulmonary fibrotic conditions, thrombin, the enzyme responsible for the conversion of fibrinogen into fibrin, has been detected within the lung and intra-alveolar spaces (Hernandez-Rodriguez *et al.*, 1995; Gabazza *et al.*, 1999; Dik *et al.*, 2003). Thrombin has been shown to directly influence fibroblasts (Chen and Buchanan, 1975), resulting in their differentiation into collagen-producing myofibroblasts as well as an increase in proliferation (Chambers *et al.*, 1998; Bogatkevich *et al.*, 2001). Findings in Chapter 5 show that possible increases in thrombin levels, which can be associated with the observed procoagulant morphology, could contribute to the development of pulmonary fibrosis. As results obtained during coagulation studies within our research coincide with possible increases in thrombin concentrations, this link between sibutramine administration and the development of pulmonary fibrosis could also exist. It is proposed that coagulatory factors such as thrombin are increased owing to increases in shear stress due to peripheral adrenergic stimulation as sibutramine has known pressor and tachycardic effects (Woolard *et al.*, 2004). Thrombin is known to increase the synthesis of platelet-derived growth factor (PDGF) together with other peripheral and internal stimuli (Harlan *et al.*, 1986; Daniel *et al.*, 1986; Kourembanas *et al.*, 1997) and PDGF has been implicated in the development of various fibrotic conditions including idiopathic and other forms of pulmonary fibrosis (Heldin and

Westermarck, 1999). It is also involved in the normal development of alveoli and if over expressed often leads to increases and rearrangement of matrix components.

Alveolar epithelium has also been implicated in the development of lung diseases as these cells constantly interact with mesenchymal and vascular cells to control the pulmonary homeostasis in response to environmental alterations. Situations which promote chronic epithelial stress could impair various epithelial interactions ultimately leading to fibroproliferation (Selman and Pardo, 2006).

Despite various laboratory tests available for assessing the functionality of different organ systems, adequate biochemical markers of pulmonary function are scarce and to assess this parameter volumetric tests such as spirometry, are often employed. As specific biological markers of pulmonary fibrosis have not been assessed as of yet, our research suggests that the possibility of the development of pulmonary fibrosis due to sibutramine administration is a very real risk. Although further investigations within this field are needed to conclusively attribute these observations to a disease process, our findings show that this must be taken into consideration.

Apart from the morphological changes observed in the lung samples, heart tissue also showed some variations between the different experimental groups with regards to structural arrangement of muscle fibres as well as collagen deposition. Although these differences were not as prominent as in the pulmonary tissue, further biological and functional assessment of cardiovascular tissue could provide some much needed information on sibutramine-associated side effects which are known to predominantly effect the cardiovascular system. Fenfluramine, a weight loss agent which also exerts its anorectic action by influencing serotonergic pathways in the brain, has been associated with valvular heart disease when taken in combination with phentermine. Phentermine administration has been associated with primary pulmonary hypertension which could be due to its interference with the pulmonary

clearance of serotonin (5-HT) (Mitchell and Smythe, 1990). It has been hypothesised that the combined administration of fenfluramine and phentermine may have an additive effect on circulating 5-HT and could lead to the development of valve damage (Morita and Mehendale, 1983). This could also be a possible consequence of sibutramine administration as it affects serotonergic pathways and our histological findings show significant alterations in lung tissue morphology and slight changes in cardiovascular tissue. This presents a topic for further investigations.

One aspect that has now become clear is that in depth inflammatory investigations following sibutramine administration should be considered as this could not only lead to pulmonary fibrosis but also affect other essential physiological processes. Specific blood based markers of fibrosis are not widely available and as a result lung fibrosis is often overlooked as possible consequence of toxicity.

6.5 Conclusion

Morphological analysis of the ultrastructure of the liver and kidney tissue samples correlates with the findings of blood analysis of liver and kidney function. Based on these results it is suggested that sibutramine has little to no effect on the normal physiology of these organs. Likewise sibutramine did not cause morphologically noticeable neurological damage. In contrast, mild increases in collagen deposition was found in the heart while extensive fibrosis was observed in pulmonary tissues from animals receiving low- and high doses of sibutramine. Further research should focus on the identification of lung specific markers of fibrosis. This would be of value for animal based studies where it is difficult to measure lung functions. Based on these morphological findings sibutramine is toxic and causes fibrosis specifically of the lungs and possibly the heart.

THE CHICK EMBRYO MODEL

CHAPTER 7: The teratogenic effects of sibutramine compared to ephedrine in the chick embryo model

7.1 Introduction

Several substances, including sibutramine, are indicated in the treatment of obesity, both in non-pregnant women and also during pregnancy (Francia-Farje *et al.*, 2010). A major factor which remains a concern in all compound administration is the potential it has to affect the developing foetus should exposure accidentally or unknowingly occur. This teratogenic potential of compounds has received much attention in research especially after the thalidomide disaster of the 1950's and 60's (Froster and Baird, 1993; Dally, 1998). Although patients are generally advised to avoid the use of drugs during pregnancy (Doering *et al.*, 2002), exposure of the developing foetus to various compounds could occur long before a patient realizes she is pregnant. The evaluation of the teratogenic potential of compounds and strict control thereof is therefore essential. In this study, the *in ovo* model was implemented to evaluate the possible teratogenic effects of sibutramine.

Chicken (*Gallus gallus*) embryos have been used with exceptional success as tools for research in developmental biology, experimental embryology and teratology. The development of the chick embryo has been studied and described extensively and this information is freely and easily obtainable (Hamburger and Hamilton, 1951). Various genetic factors have also been identified in the developing chick that resembles the general expression patterns and functions of those in human and rodent development (Drake *et al.*, 2006).

Some of the key advantages of using this embryonic model in teratology and experimental biology is that the embryos can be viewed and examined should it be required and precise developmental stages can be targeted for exposure. Overall it provides an experimental tool that allows one to ascertain how a teratogen would interfere with specific mechanisms that underlie organogenesis and morphogenesis (Drake *et al.*, 2006).

In this study the effect of sibutramine on the development of the chick embryo was evaluated. Like sibutramine, ephedrine has also been marketed as a weight loss agent as well as athletic performance enhancer due to its stimulatory effect within the central nervous system (CNS). Ephedrine's structure is closely related to that of other amphetamines and catecholamines such as norepinephrine (NE) and possess strong alpha (α)- and beta (β)-adrenergic stimulatory effects (Andrews *et al.*, 2005). In this study ephedrine was used as positive control as it has been shown to induce cardiovascular malformations in chicken embryos using the *in ovo* method.

7.2 Materials and Methods

7.2.1 Implementing the *in ovo* model

Fertilized Broiler Hatching eggs were obtained from Swanepoel Boerdery (Bronkhorstspuit, Gauteng, South Africa). Upon delivery the fertilized eggs were incubated in a Heraeus Function Line Incubator at 37.5°C in humidified air for the duration of 13 days. The day of delivery was recorded as day 0. Ethical clearance was obtained from the AEC (ethical clearance number h005-13) for the use of 180 eggs. These exposure experiments were conducted over two experimental procedures in order to facilitate the amount of eggs used.

7.2.2 Inoculation of the eggs

On embryonic day 3 (E3; Hamburger-Hamilton stage 19/20) the eggs were removed from the incubator and swabbed with 70% ethanol. Figure 7.1 shows the chicken embryo at stages 19 and 20 of development (hours 68-72). Once removed from the incubator holes were drilled into the blunt end of the eggs using a sterile needle as shown in Figure 7.2 A and B, during which extreme care was taken not to puncture the underlying chorioallantoic membrane (CAM). Sibutramine and ephedrine was then administered through the drilled holes at various concentrations in such a way that it is placed through the air sac onto the CAM (Figure 7.2 C and D). Three control groups were used to assure that the methods employed do not interfere with the results obtained. Figure 7.2 provides detailed images of the various steps implemented in the *in ovo* procedure and Table 7.1 summarizes the experimental groups as well as the treatment regime of each group.

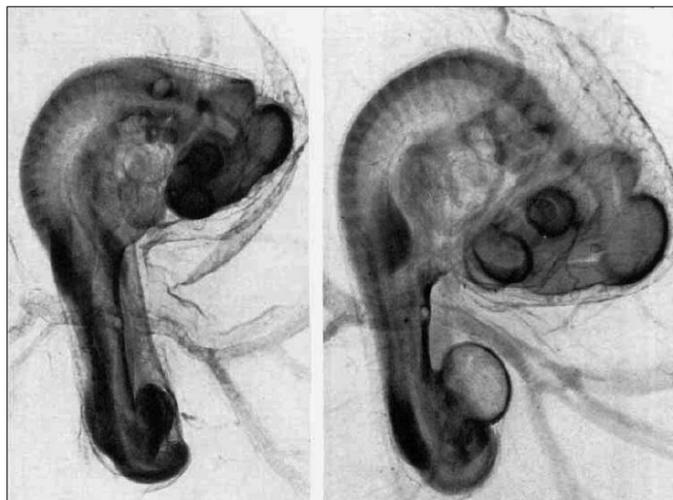


Figure 7.1: Development of chick embryos at stage 19 (A) and 20 (B). **A:** Limb-buds enlarged, symmetrical, Leg-buds slightly larger, 37-40 somites extend into tail, medulla forms an acute angle with the axis of the trunk, contour of the posterior part of the trunk is straight to the base of the tail; **B:** Limb-buds enlarged, leg buds slightly asymmetrical, 40-43 somites, tip of tail still unsegmented, bend in the tail-region extended forward into the lumbo-sacral region, contour of mid-trunk a straight line, slight eye pigmentation. (Hamburger and Hamilton, 1951)

Table 7.1: *In ovo* experimental design

Experimental group	Exposure and dosages	Sample size (n)
Control group 1 (Control)	None	16
Control group 2 (C:Hole)	Drilled hole, no exposure	16
Control group 3 (C:H₂O)	100 µl sterile water	16
Sibutramine (Sib0.5)	0.5 µmol/01.67 mg/ml	16
Sibutramine (Sib2.5)	2.5 µmol/08.34 mg/ml	16
Sibutramine (Sib5.0)	5.0 µmol/ 16.72mg/ml	16
Sibutramine (Sib10)	10 µmol/ 33.43 mg/ml	16
Ephedrine (Eph0.5)	0.5 µmol/01.01 mg/ml	16
Ephedrine (Eph2.5)	2.5 µmol/05.04 mg/ml	16
Ephedrine (Eph5.0)	5.0 µmol/10.08 mg/ml	16
Ephedrine (Eph10)	10 µmol/ 20.17 mg/ml	16

Following exposure, the injection site was swabbed with 70% ethanol and carefully sealed with paraffin wax (Thermo Scientific) and returned to the incubator (Figure 7.2 E and F). All manipulations were performed using sterile technique within a laminar flow hood.

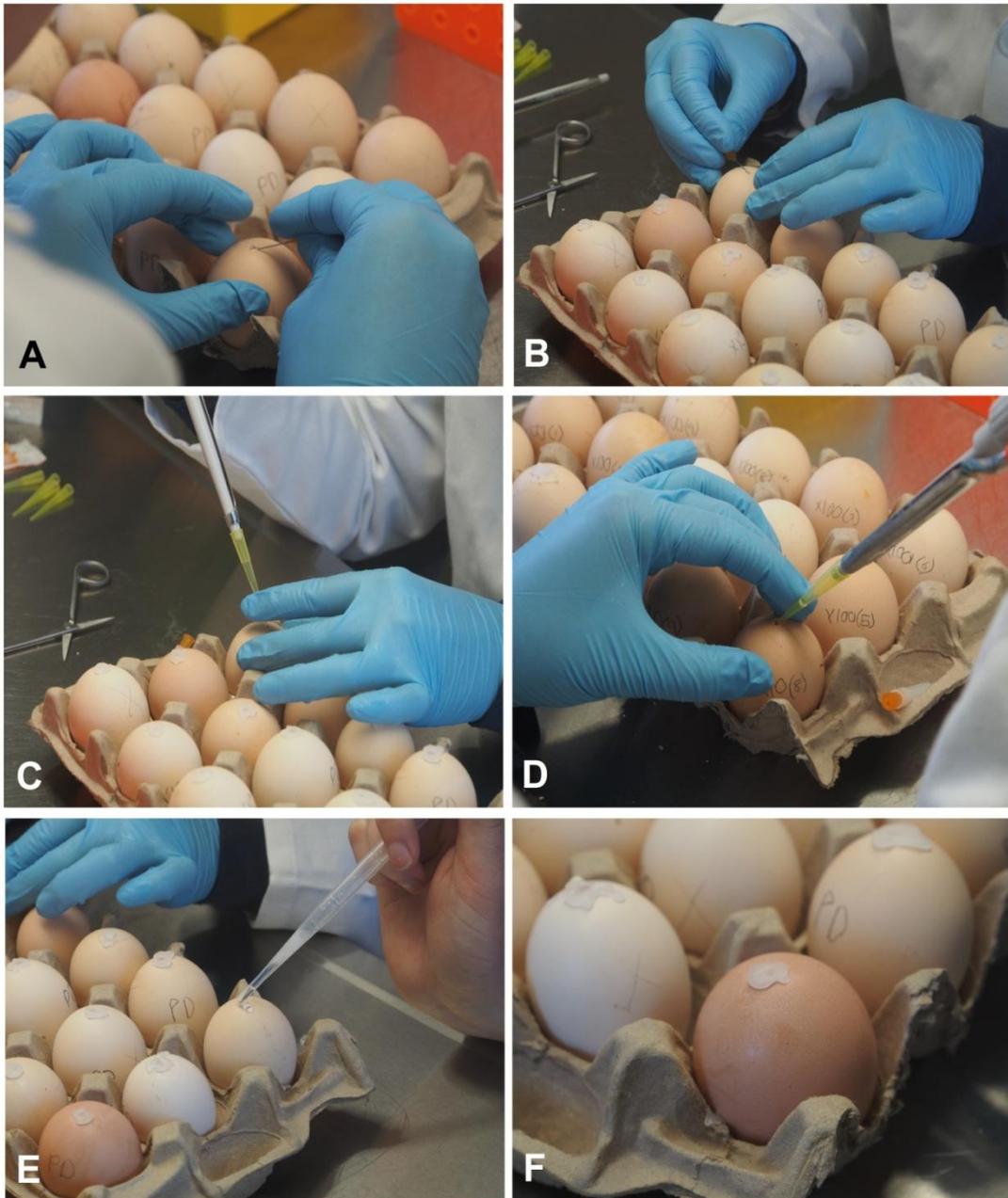


Figure 7.2: Steps implemented in the *in ovo* method. **A and B:** Holes drilled into the blunt end of the eggs using a sterile needle; **C and D:** Exposure to test compounds or sterile water; **E and F:** sealing the holes with paraffin wax

7.2.3 Termination

On E13 the eggs were taken from the incubator and the embryos were removed from the eggs by carefully opening the blunt end of the egg (Figure 7.3A) and peeling away the CAM with forceps. The embryos were then lifted out of the eggs with a spatula as seen in Figure 7.3B, and placed in a petri dish containing PBS (pH 7.4) to prevent unnecessary dehydration. The embryos were examined for any macroscopic variations such as limb abnormalities, organ anomalies and variations in the size of the embryos. The weight for each embryo was also recorded.

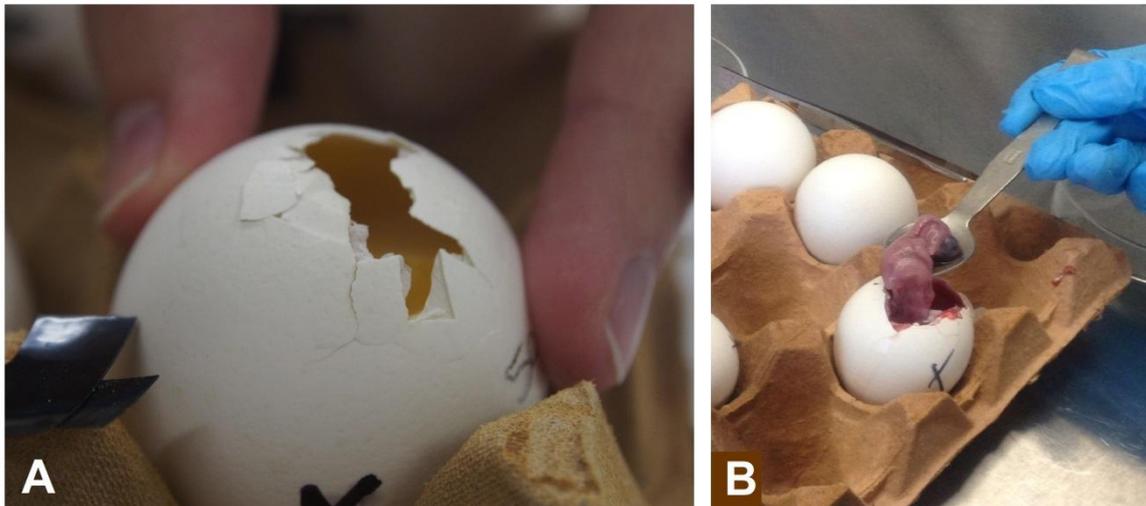


Figure 7.3: Termination and removal of embryos. **A:** Opening of the blunt end of the egg; **B:** Removal of the embryo

7.2.4 Sample collection

The heart, brain, liver and kidneys were harvested from each chick embryo and these tissue samples were then processed for light (LM) and transmission electron microscopy (TEM) as discussed in Chapter 6.

7.3 Results

7.3.1 Macroscopic evaluations

As described by Hamburger and Hamilton (1951), on the 13th day following fertilization, embryos are at stage 39 of development. At this stage scales overlap on the superior surface of the legs and the major phalangeal pads are covered with papillae. The mandible and maxilla are cornified and appear opaque and the channel of the auditory meatus can be seen only at the posterior edge of its external opening. Feather-germs are greatly increased in length and about four to five rows of feather-germs are visible at the edge of the lower eyelid, of which the opening between the lids is reduced to a thin crescent. Upon removal from the eggs any significant malformations observed or noteworthy differences were annotated. Incidences of underdeveloped or non-viable embryos were also recorded and these findings are summarized in Table 7.2. The experimental design included three control groups to ensure the efficacy of the procedure, and these were:

- a normal control group, in which eggs were unaltered and allowed to develop;
- a group of eggs in which the shells were perforated and resealed with no compound administration; and
- a control group comprised of eggs in which holes were made in the shells and sterile H₂O was administered after which the holes were resealed.

Although there were slight differences in the development rates of the control groups, none of the embryos presented with any malformation or growth retardation. On average, the development rate for the entire control population was 73.7%. Table 7.2 provides a summary of all the macroscopic observations including abnormalities that were noted, the weights of the embryo's as well as developmental success rates.

Compared to control embryos, the percentage development of groups exposed to ephedrine did not show considerable differences although more abnormalities were noted which could influence the viability of embryos. In the Eph0.5 experimental group 18% of the developed embryos presented with abnormalities and these are provided in Table 7.2. While embryos within the Eph2.5 experimental group presented with no abnormalities the percentage development coincided with those in the Eph5.0 experimental group (75% developed). Of embryos in this experimental group (Eph5.0), 16% presented with abnormalities upon macroscopic examination and within the Eph10 experimental group 22% of the developed embryos (62.50%) presented with abnormalities. These abnormalities are also summarized in Table 7.2. These findings are modest when compared to those seen in the sibutramine groups. In the Sib0.5 experimental group, which was the lowest dose administered to the chick embryos, 9% of the given population showed developmental abnormalities. In both the Sib2.5 and Sib5.0 groups only 25% of the embryos developed and in the Sib5.0 group 50% of developed embryos presented with abnormalities. Finally, of the embryos exposed to the highest sibutramine dose (10 μ mol), none were viable. These findings suggest that sibutramine is a developmental toxin as it either prevents growth or causes developmental defects such as limb abnormalities or ventral body wall defects.

Table 7.2: Chick development rates, weights and observed abnormalities noted within the different experimental groups

Experimental Group	% Developed	Average weights (g)	Observed abnormalities
Control	75.00	9.27	-
C:Hole	84.60	8.75	-
C:H ₂ O	61.50	8.53	-
Eph0.5	68.75	7.80	9 embryos with no observable anomalies, 1 with limb abnormality, gastroschisis, 1 smaller, underdeveloped
Eph2.5	75.00	9.47	-
Eph5.0	75.00	7.07	10 embryos with no observable anomalies, 1 tissue transparency with fluid build-up, 1 with haemorrhaging, gastroschisis
Eph10	62.50	6.65	7 embryos with no observable anomalies, 2 underdeveloped
Sib0.5	75.00	9.24	11 embryos with no observable anomalies, 1 with gastroschisis
Sib2.5	25.00	7.39	-
Sib5.0	25.00	6.86	2 embryos with no observable anomalies, 1 underdeveloped, limb malformation, haemorrhaging; 1 with gastroschisis, absence of upper limb
Sib10	06.25	1.63	Severe haemorrhage, retarded development, gastroschisis

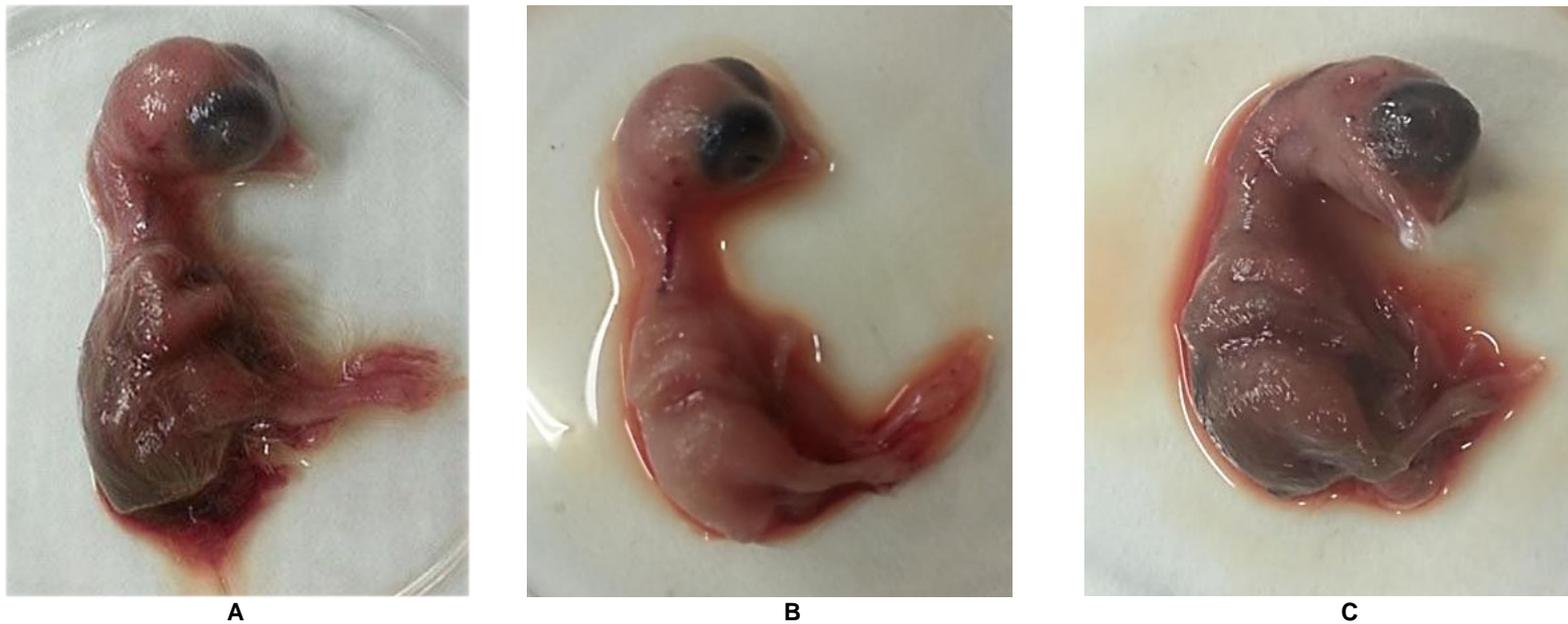


Figure 7.4: Embryo's from control groups. **A:** Embryo from normal control group, **B:** Embryo from C:Hole group, **C:** Embryo from control group administered sterile H₂O (C:H₂O).

Figure 7.4 shows embryos collected from the control population. These images demonstrate the normal development of embryos and it is evident that no macroscopic differences exist between the different control groups. No variations or malformations were noted within the entire viable control population.

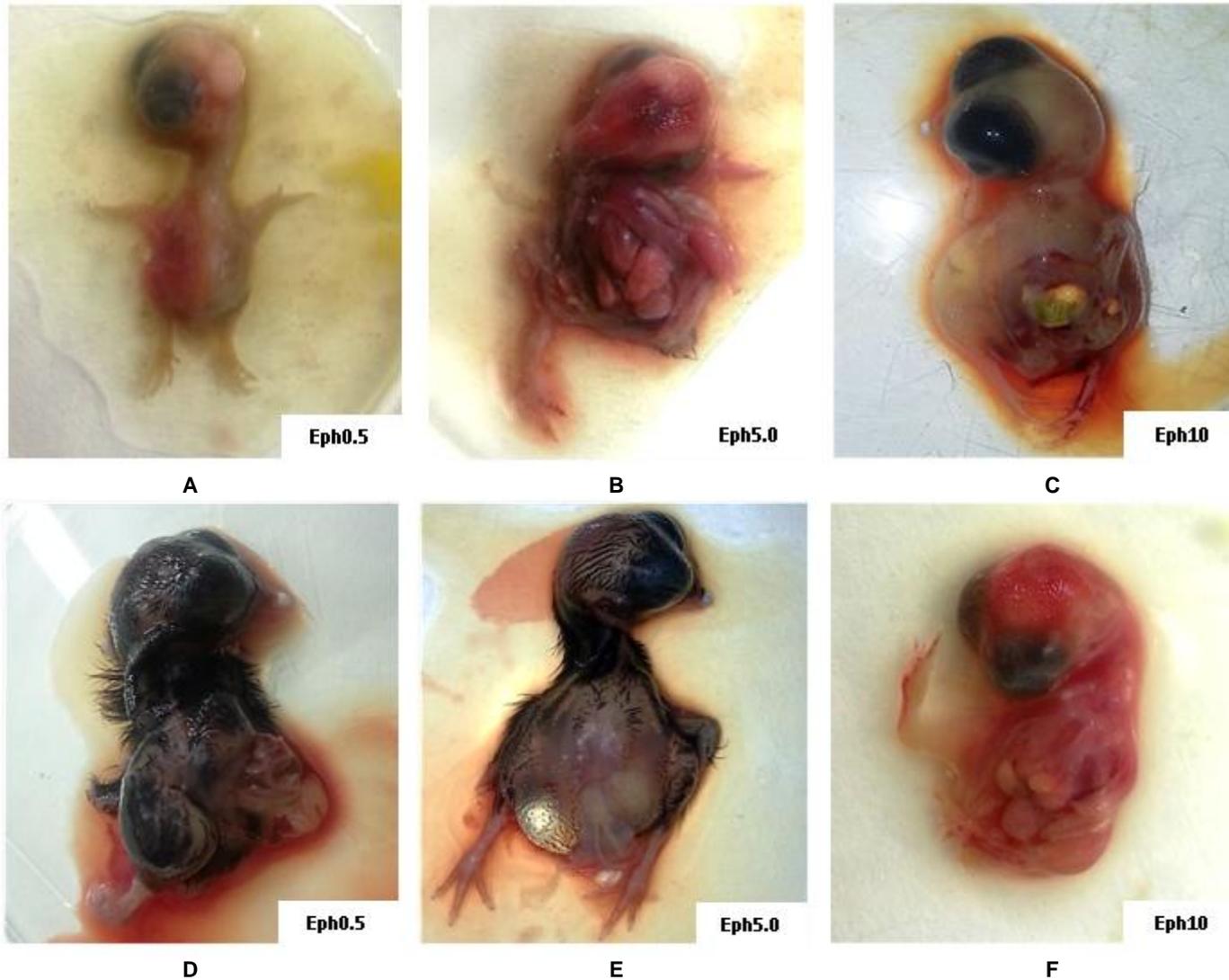


Figure 7.5: Embryos exposed to different concentrations of ephedrine.

A and D:
Underdeveloped chick (A) and ventral wall defect (D) in the Eph0.5 group; limb abnormalities (D);

B and E: Embryos from Eph5.0 group; Underdeveloped embryo with hemorrhaging and ventral wall defect (B); Ventral wall defect and subcutaneous fluid build-up (E);

C and F: Embryos from Eph10 group. Ventral wall defects and retarded growth (C and F); Subcutaneous fluid build-up (C); F: haemorrhaging

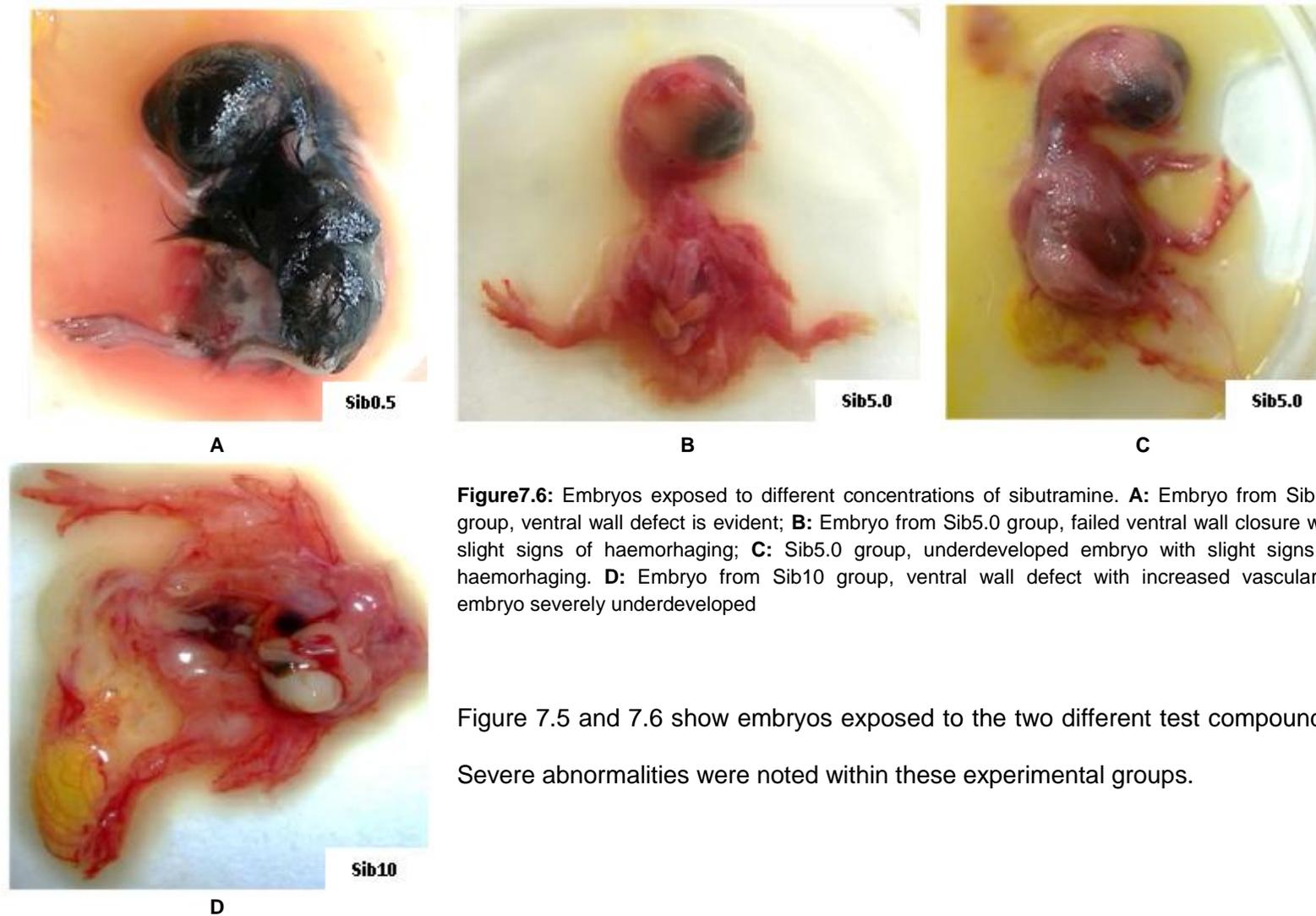


Figure 7.6: Embryos exposed to different concentrations of sibutramine. **A:** Embryo from Sib0.5 group, ventral wall defect is evident; **B:** Embryo from Sib5.0 group, failed ventral wall closure with slight signs of haemorrhaging; **C:** Sib5.0 group, underdeveloped embryo with slight signs of haemorrhaging. **D:** Embryo from Sib10 group, ventral wall defect with increased vascularity, embryo severely underdeveloped

Figure 7.5 and 7.6 show embryos exposed to the two different test compounds. Severe abnormalities were noted within these experimental groups.

7.4 Discussion

It is noteworthy that, before initiation of histological investigations, the percentage of successful development of the embryos in the different experimental groups differed greatly, as is shown in Table 7.2. Control eggs showed a development rate of about 74%. Ephedrine developmental rates did not necessarily differ from controls although some malformations were noted upon macroscopic examination. Experimental groups exposed to different concentrations of sibutramine however revealed drastic increases in mortality. Only at the lowest concentration of sibutramine (0.5 μ mol) did eggs present an average percentage of development comparable to both controls and ephedrine, indicating that sibutramine is more toxic than ephedrine with regards to teratogenicity.

Despite the malformations observed, the different control groups showed very high, successful developmental rates which further confirms the efficacy of this model and reinstates claims that the *in ovo* injection methods minimize distress of young embryos and may be one of the most appropriate and minimally invasive methods for teratogen delivery (Drake *et al.*, 2006).

In a study done by Francia-Farje *et al.* (2010) in which pregnant rats were treated with sibutramine, the number of live foetuses was significantly reduced and the post implantation loss percentages were increased when compared to control animals. Overweight animals were also implemented in this study and potentiating effects were not reported with regards to the weights of the animals owing these effects solely to sibutramine administration.

In the current study gastroschisis was observed in embryos exposed to the low and higher concentrations of sibutramine, as well as ephedrine. This is characterized by evisceration of abdominal organs through a defect in the abdominal wall without a membranous covering and usually involves the small intestines but may include other structures. Various compounds have been reported which are able to induce this malformation (Van Dorp *et al.*, 2009). Examples include mercury, cadmium, and retinoic acid. Ventral body wall defects are one of the leading types of birth defects and, among conditions within this class of malformations, the incidence of gastroschisis has been on the increase, rendering it a particularly important body wall closure defect. The molecular and cellular interactions which lead to the development of these relatively common defects however remain elusive (Williams, 2008).

As previously stated, ephedrine, in conjunction with other sympathomimetic agents such as phenylephrine and epinephrine, has also been evaluated for teratogenic effects using chick embryos at different stages of development. High rates of cardiac anomalies have been reported (Werler, 2006) and limb abnormalities have also been described in combination with theophylline and phenobarbital. This prompted further investigations in which pregnant rabbits were exposed to ephedrine and again, cardiovascular and limb malformations were seen as well as exencephaly and ventral wall anomalies (Gilbert-Barness and Drut, 2000).

In a study by Zhang *et al.*, (1998) the teratogenic effects of cocaine were evaluated by use of the same methods implemented in the current study. Cocaine, which possesses well defined local anaesthetic properties, also blocks the reuptake of neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5-HT), lending to its sympathomimetic and psychoactive properties. Zhang and co-workers specifically evaluated a 5HT-mediated vasoconstrictive cause of cocaine teratogenicity as alterations in haemodynamic processes could result in ischaemia

and eventual differences in oxygen balances (Konzen *et al.*, 1995; Martinez *et al.*, 1996; Zhang *et al.*, 1998). This resultant oxygen imbalance together with deficiencies in nutrient supply and metabolic waste removal due to impaired circulation could result in embryonic developmental disturbances (Rosenak *et al.*, 1990). These deviations in physiological processes have been attributed to adrenergic activation as the primary source for the pathogenesis and numerous studies employing both human and non-human observations and methods have demonstrated an indirect activation of the adrenoceptors due to cocaine administration (Schindler *et al.*, 1995; Gillis *et al.*, 1995). Results obtained by Zhang *et al.* (1998) showed an increase in observed herniated umbilici, a ventral wall defect, which was attributed to a possible indirect consequence of umbilical ischemia due to 5-HT- induced vasoconstriction.

Sibutramine shares an almost similar mechanism of action when compared to cocaine as it inhibits the presynaptic neuronal reuptake of 5HT and NE, whereas ephedrine increases the action of postsynaptic noradrenergic activity directly by acting on α and β noradrenergic receptors. Both 5HT and NE possess haemodynamic properties which could affect development as described previously. The increased severity of sibutramine teratogenicity compared to ephedrine could be further attributed to the additional alterations in serotonergic pathways, as this has been described to have an effect on embryonic growth.

Furthermore, previous studies have shown that prenatal administration of various selective serotonin reuptake inhibitors (SSRIs) may result in foetal death, defects, or growth suppression (Mello *et al.*, 2005; Ofori *et al.*, 2007; Pendersen *et al.*, 2009). All members of SSRI and serotonin-norepinephrine reuptake inhibitor (SNRI) drug classes have also been shown to cross the placenta and are also excreted in small amounts into breast milk (Rosenbaum *et al.*, 1998; Newport *et al.*, 2002).

Limb abnormalities often appear in conjunction with body wall defects, collectively referred to as the limb-body wall complex. Previous data from animal models suggest that hemodynamic disruption could possibly give rise to limb-body wall complex. Again, cocaine abuse has been implicated in these processes due to its vasoconstrictive effects which could affect uteroplacental blood flow during significant stages of development (Viscarello *et al.*, 1992).

7.5 Conclusion

The *in ovo* embryonic model has been used extensively with great success within a laboratory setting. In this study, the *in ovo* model was used to evaluate the teratogenic effects of sibutramine compared to epinephrine. Both drugs caused congenital abnormalities which included growth retardation, ventral wall defects and increased mortality. Based on these findings it can be concluded that sibutramine is more toxic than epinephrine in the *in ovo* model.

CHAPTER 8: The effect of sibutramine on organ ultrastructure of the chick embryo

8.1 Introduction

The teratogenicity of sibutramine, a serotonin-norepinephrine reuptake inhibitor (SNRI) indicated for the treatment of obesity, has not been well studied. A major concern is the unregulated availability and use of this compound despite its withdrawal from most global markets. This complication has led to further uninformed use of this compound as physicians are not consulted in obtaining a prescription. The lack of published data on the use of this compound during gestation is a key limiting factor in making conclusions on its safe use. Both serotonin (5-HT) and norepinephrine (NE) have been described as teratogens and other SNRIs have also been implicated (Reddy *et al.*, 1963; Hirsch and Fritz, 1981), therefore further investigations are essential as these compounds share similar mechanisms of action.

In this study, ephedrine was used as positive control in the *in ovo* model. The teratogenic effects of ephedrine have been previously described in studies implementing similar methods (Nishikawa *et al.*, 1985a; 1985b) which reported various cardiovascular malformations induced by ephedrine at a dose as low as 1 $\mu\text{mol/egg}$.

Given the macroscopic defects that were observed due to both sibutramine and ephedrine administration another question that arises is whether embryonic exposure to sibutramine could lead to ultrastructural changes that might prove significant later in development or in adulthood, resulting in eventual disease. In the current chapter, the effect of sibutramine and positive control epinephrine on the tissue and cellular structure of the liver, kidney, heart and brain of chicken embryos was evaluated.

8.2 Materials and methods

8.2.1 Animal model

The *in ovo* model was implemented as described in Chapter 7 of this dissertation.

8.2.2 Sample collection

On E13 the eggs were taken from the incubator and the embryos were removed from the eggs. Heart, brain, liver and kidney samples were then dissected from the embryos and were processed for light (LM)- and transmission electron microscopy (TEM).

8.2.3 Tissue preparation for LM and TEM

Tissue samples for both LM and TEM analysis were processed and prepared similar to the methods employed for samples collected from the Sprague Dawley rat model previously described in Chapter 3 of this dissertation.

8.3 Results

In this part of the study, the possible effects of sibutramine administration on tissue and cellular structure of liver, kidney, brain, and heart samples from the different *in ovo* experimental groups were evaluated. The results are presented in Figures 8.1 to 8.13.

8.3.1 Liver

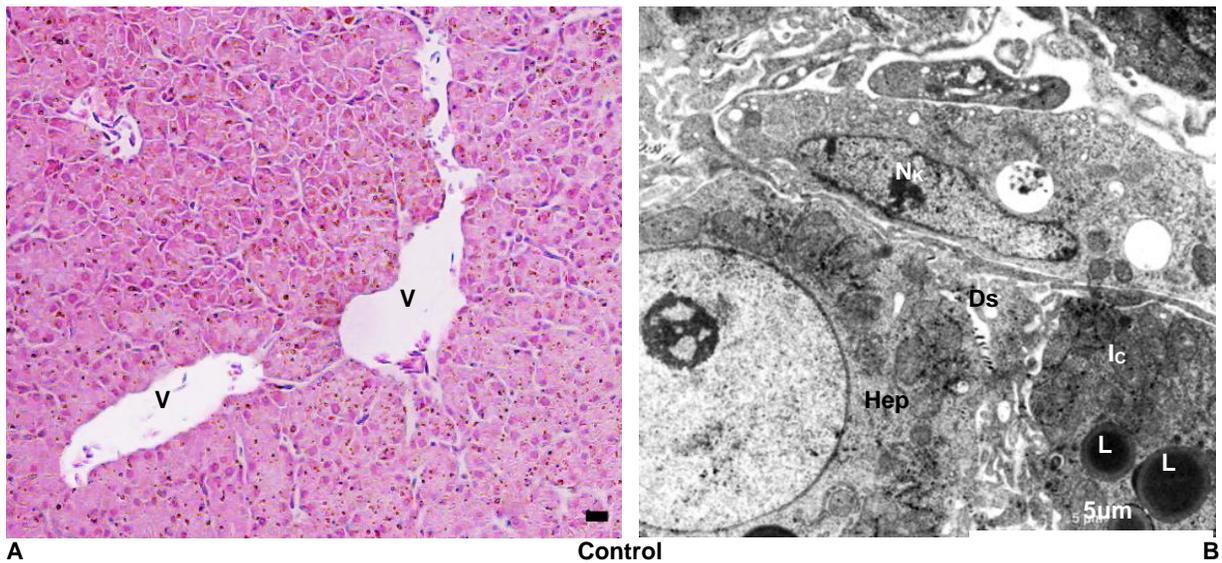
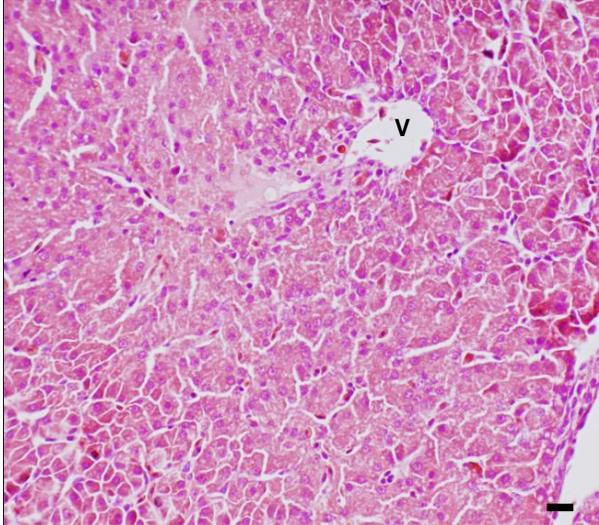


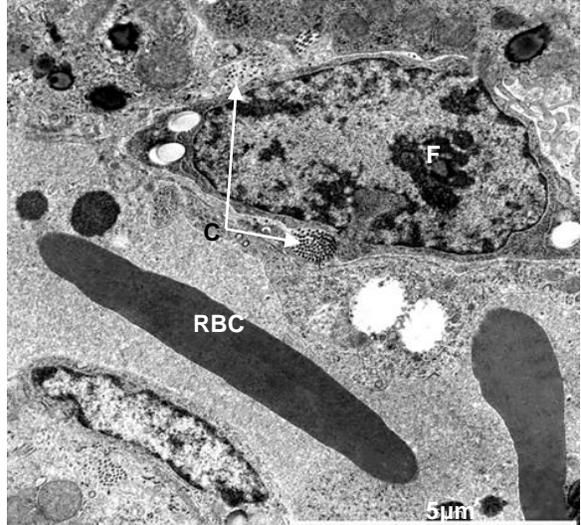
Figure 8.1: Liver samples collected from the control groups. **A and B:** Control LM and TEM respectively. LM scale bar=10 µm, TEM=5 µm. (Ds=Space of Disse; Hep=Hepatocyte; Ic=ITO cell; L=Lipids; N_K=Kupffer cell nucleus; V=venules)

Figures 8.1A and B show LM and TEM micrographs prepared from the collected liver samples from the control embryos. Figure 8.1A shows the normal histology of the control embryos where the normal plate-like arrangement of hepatocytes can be observed as well as some hepatic venules (V) visualised with the H&E stain. Fig 8.1B shows the normal ultrastructural arrangement typical of control samples. The space of Disse (Ds) is visible as well as a Kupffer cell (N_K) within the sinusoid, surrounded by hepatocytes (Hep). A hepatic ITO or stellate cell (I_C) is also visible containing lipid droplets (L).

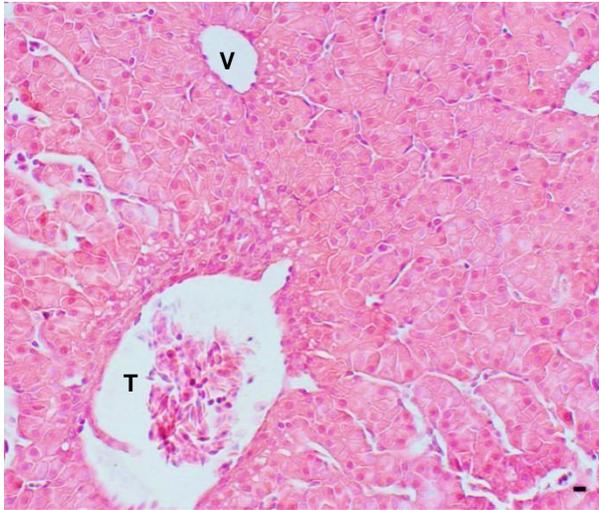


A

Eph0.5

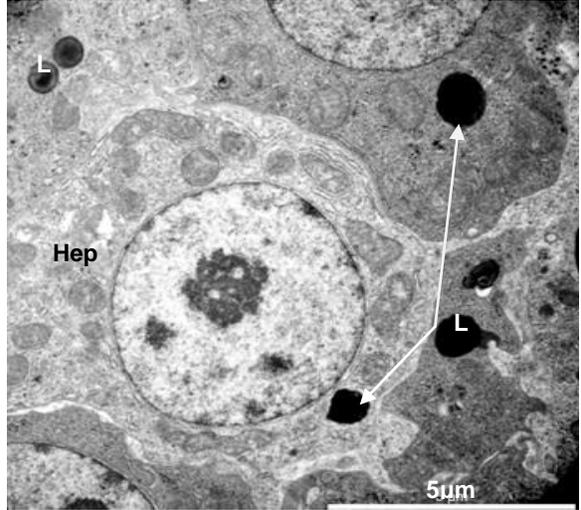


B

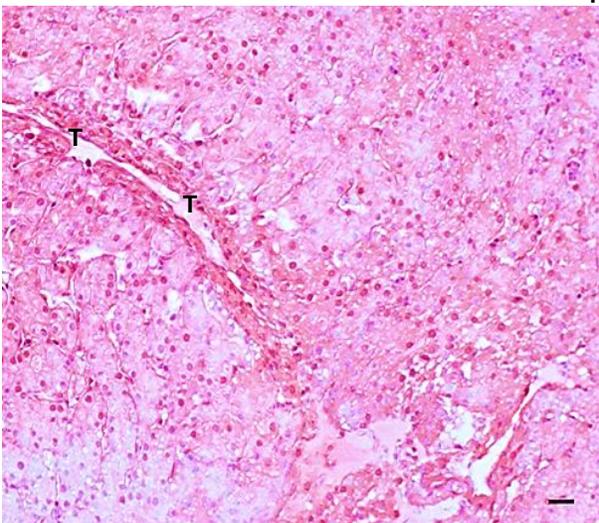


C

Eph2.5

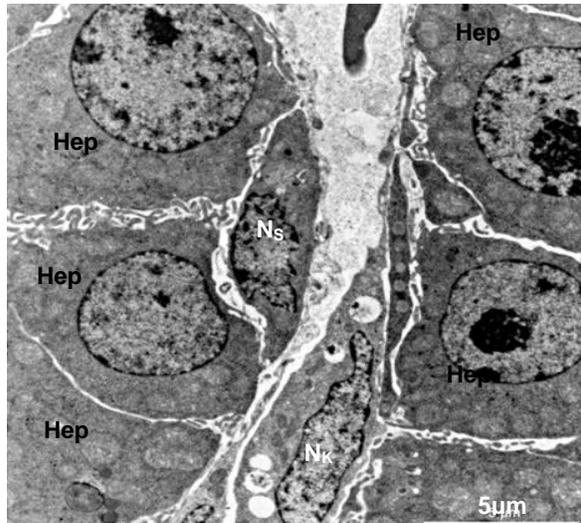


D



E

Eph5.0



F

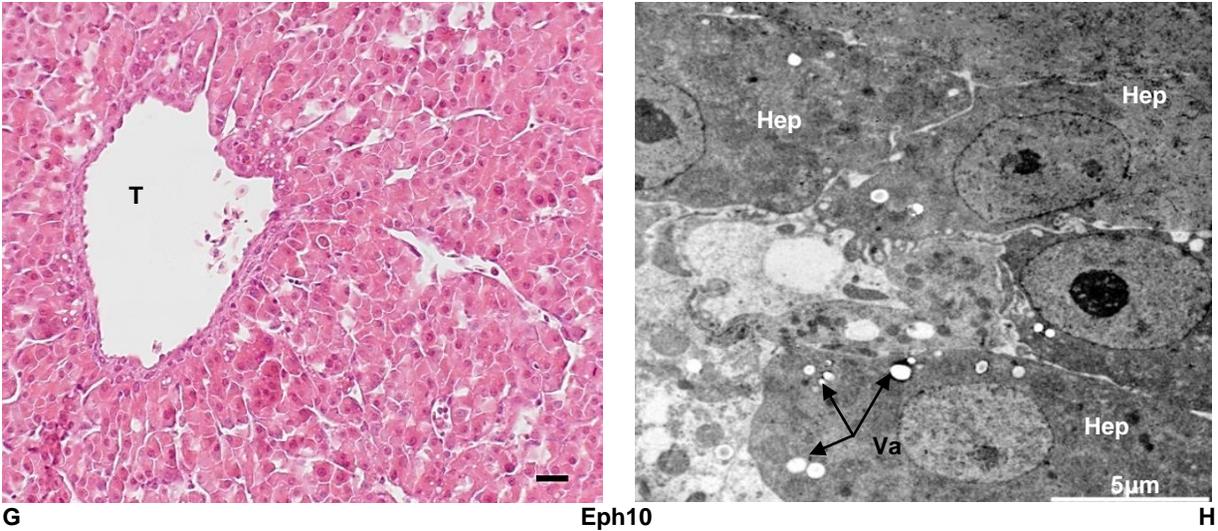
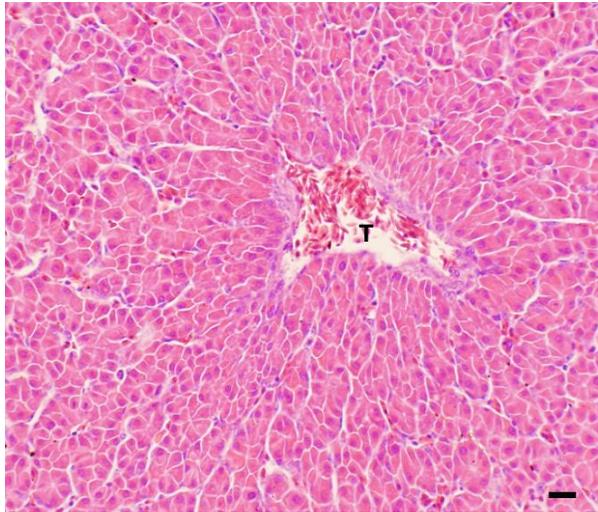


Figure 8.2: Liver samples collected from the ephedrine experimental groups. **A and B:** Eph0.5 LM and TEM respectively; **C and D:** Eph2.5 LM and TEM respectively; **E and F:** Eph5.0 LM and TEM respectively; **G and H:** Eph10 LM and TEM respectively. LM scale bar=10 μ m, TEM=5 μ m. (C=collagen fibres; F=fibroblast; Hep=hepatocyte; L=lipids; N_S=nucleus of sinusoidal lining cell; N_K=nucleus of Kupffer cell; T=portal tracts; V=venules; Va=cytoplasmic vacuoles)

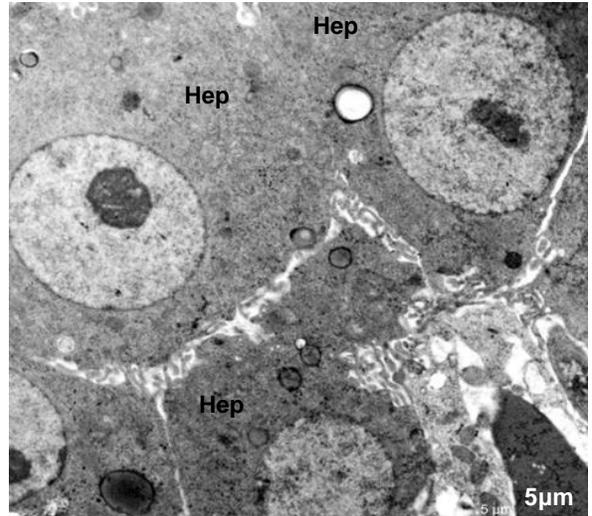
Figure 8.2 shows LM and TEM micrographs acquired from embryo's exposed to different doses of ephedrine. Figure 8.2A and B are images acquired from embryos administered 0.5 μ mol ephedrine (LM and TEM respectively) showing little to no morphological differences when compared to the control embryos (Figure 8.1A and B) and again hepatic venules (V) are visible in Figure 8.2A as well as sinusoidal structures including a fibroblast (F) surrounded by collagen fibres (C). Red blood cells (RBC) are also visible. LM images from embryos in the Eph2.5 and Eph5.0 groups (Figure 8.2C and E) show slight tendencies towards tissue degeneration although TEM images (Figure 8.2D and E) do not differ from the control group indicating normal hepatocyte structure (Figure 8.2D). Sinusoids with visible microvilli and phagocytic cells (Kupffer cells, N_K) as well as sinusoidal lining cells (N_S) can be seen. As the dosage increased to 10 μ mol ephedrine/egg a slight loss in tissue integrity could be observed upon examination of liver parenchyma (Figure 8.2F) as sinusoidal spaces appear larger than that seen in the control images. TEM micrographs from this experimental group (Figure 8.2G) also show

lipid depositions and vacuolisation (Va) within the hepatic cytoplasm and general disruption of sinusoidal organisation.

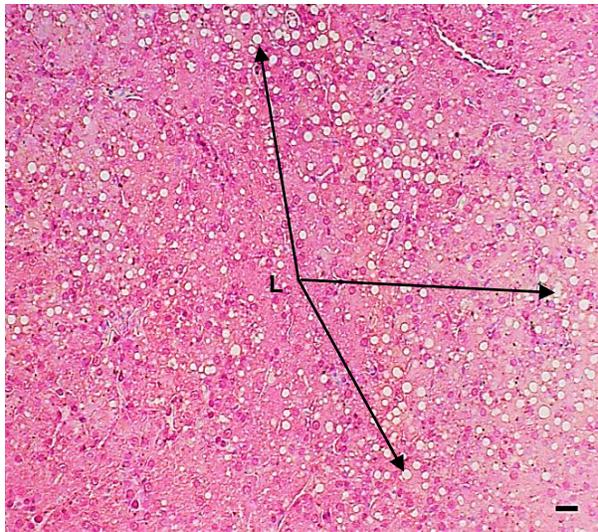


A

Sib0.5

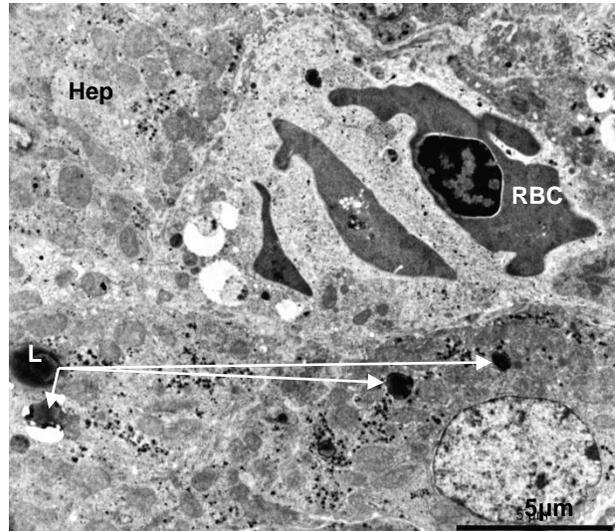


B



C

Sib2.5



D

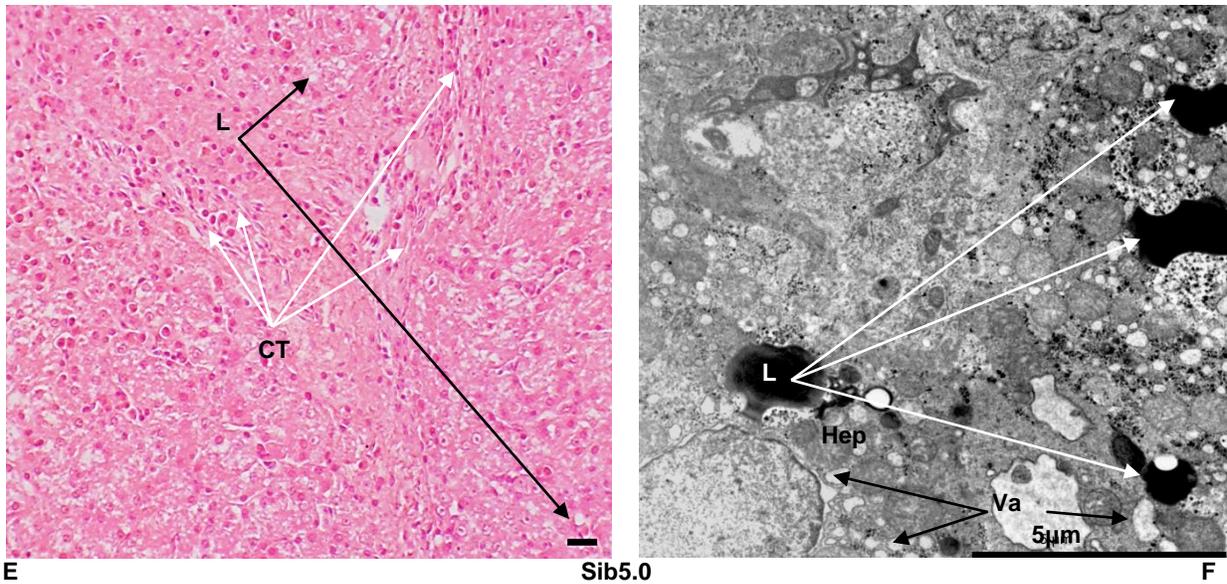


Figure 8.3: Liver samples collected from the sibutramine experimental groups. **A and B:** Sib0.5 LM and TEM respectively; **C and D:** Sib2.5 LM and TEM respectively; **E and F:** Sib5.0 LM and TEM respectively. LM scale bar=10 µm, TEM=5 µm. (CT=connective tissue; Hep=hepatocytes; L=lipids; RBC= red blood cells; T=portal tracts; Va=cytoplasmic vacuoles)

Liver LM and TEM images obtained from embryos exposed to different doses of sibutramine are shown in Figure 8.3A-F. Embryos administered the 0.5 µmol dose of sibutramine show no morphological differences when compared to the control embryos. The plate-like arrangement of hepatocytes can be observed with portal tracts (T) distinguishable (Figure 8.3A) and the corresponding TEM image (Figure 8.3B) also shows normal ultrastructural arrangement of hepatocytes (Hep). Figure 8.3C acquired from the Sib2.5 group shows signs of hepatic steatosis, i.e. lipid accumulation (L) within liver tissue which is a sign of liver damage. The corresponding TEM image (Figure 8.3D) further revealed a less organised arrangement of sinusoidal structures with increased lipid accumulation (L) as well as decreased structural integrity of the space of Disse. This is also seen to a greater extent in Figure 8.3F which was acquired from chicks exposed to 5 µmol of sibutramine. In this image the formation of cytoplasmic vacuoles (Va) can be observed. The corresponding LM image (Figure 8.3E) showed increased connective tissue spread throughout the liver tissue and a general degenerated appearance of liver parenchyma.

8.3.2 Kidney

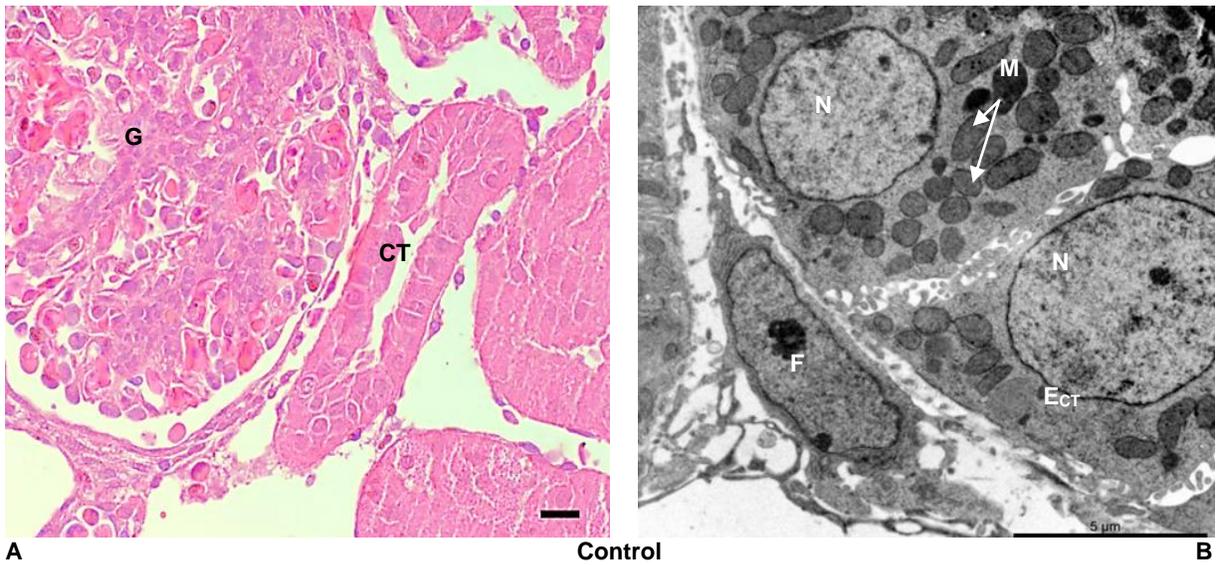
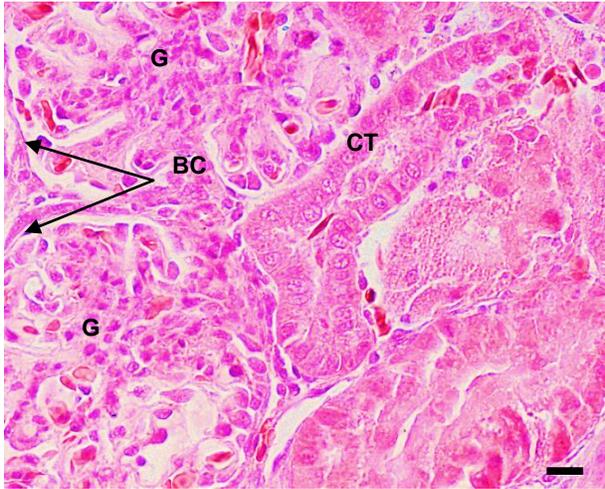
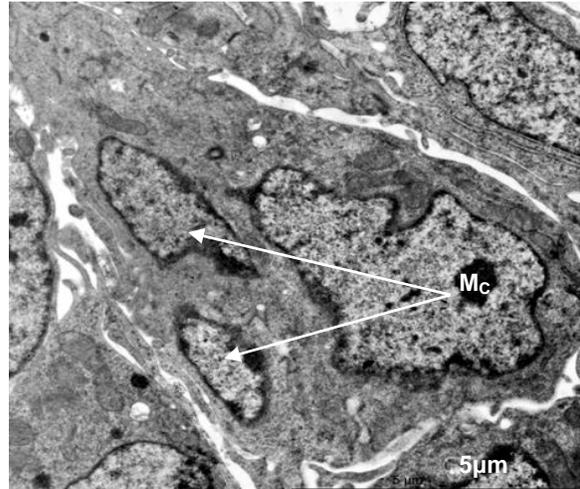


Figure 8.4: Kidney samples collected from the control group. **A and B:** Control LM and TEM respectively. LM scale bar=40 μm, TEM=5 μm. (CT=Collecting tubules; E_{CT}=cuboidal epithelium of collecting tubules; F=Fibroblast; G=Glomerulus; M=mitochondria; N=Nucleus)

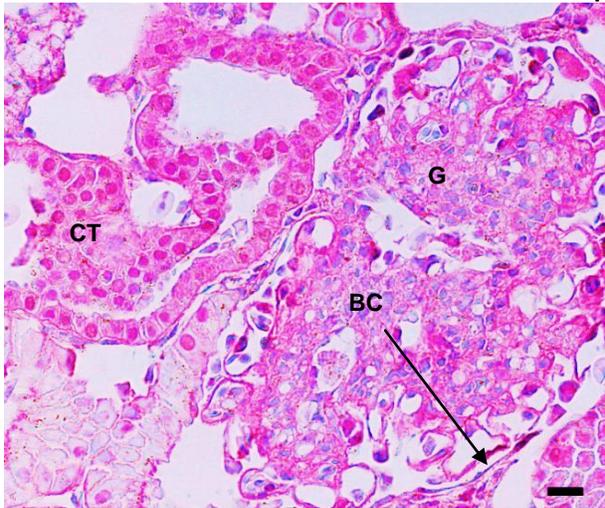
Figures 8.4A and B are LM and TEM micrographs prepared from the collected kidney samples from the different experimental groups. Figure 8.4A shows the histology of the kidney of control embryos, in which glomeruli (G) are clearly distinguishable as well as collecting tubules (CT). The TEM image acquired from the control groups (Fig 8.4B) shows the cuboidal epithelium of the collecting tubules (E_{CT}). Numerous mitochondria (M) can be distinguished and cell nuclei are also visible (N). Fibroblasts can also be identified within this section (F).



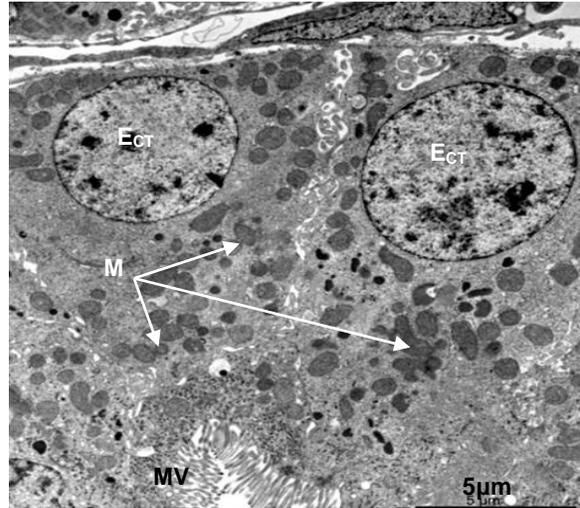
A Eph0.5



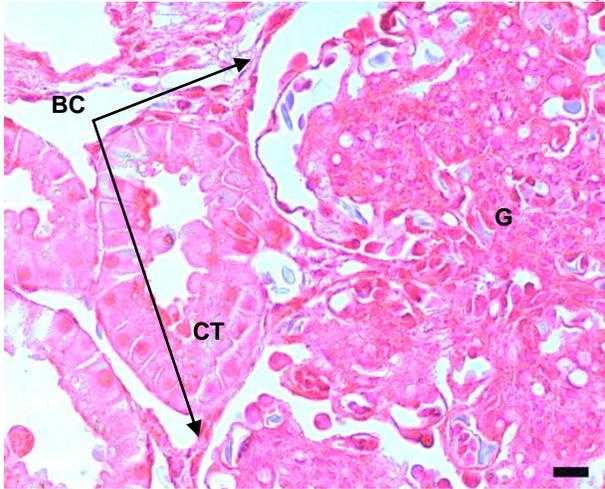
B



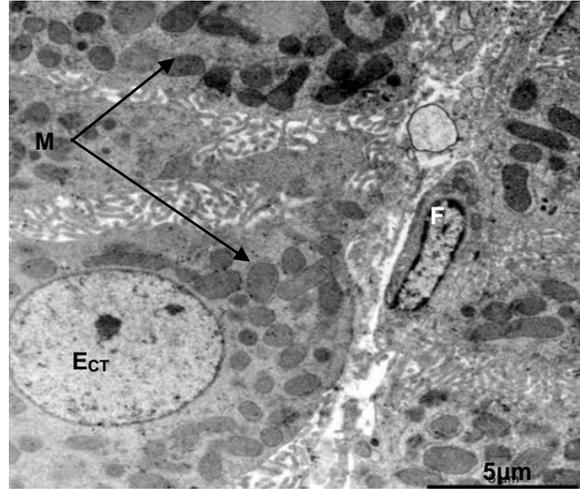
C Eph2.5



D



E Eph5.0



F

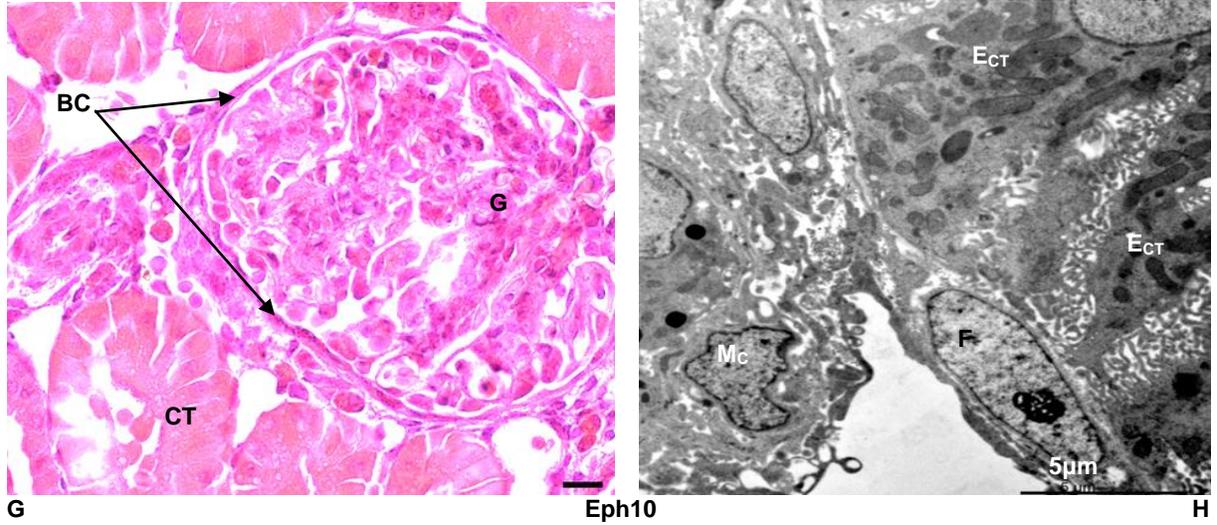


Figure 8.5: Kidney samples collected from the ephedrine experimental groups. **A and B:** Eph0.5 LM and TEM respectively; **C and D:** Eph2.5 LM and TEM respectively; **E and F:** Eph5.0 LM and TEM respectively; **G and H:** Eph10 LM and TEM respectively. LM scale bar=40 µm, TEM=5 µm. (BC=Bowman's capsule; CT=collecting tubule; F=fibroblast; G=glomeruli; M=mitochondria; Mc=mesangial cells)

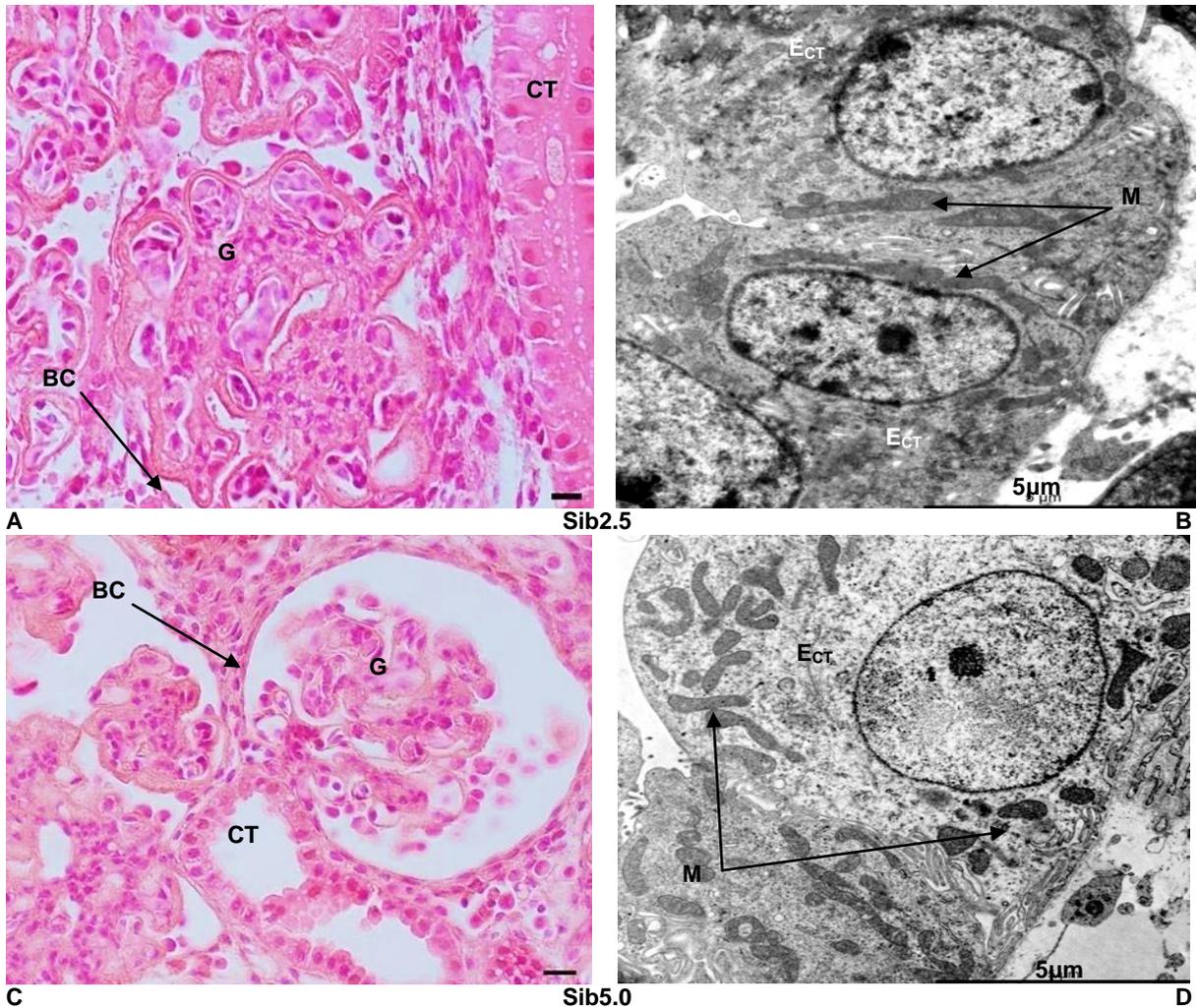


Figure 8.6: Kidney samples collected from the sibutramine experimental groups. **A and B:** Sib2.5 LM and TEM respectively; **C and D:** Sib5.0 LM and TEM respectively. LM scale bar=40 μm, TEM=5 μm. (BC=Bowman's capsule; E_{CT}=epithelium of collecting tubule; G=glomeruli; M=mitochondria)

Figures 8.5A-H and 8.6A-D are LM and TEM micrographs prepared from the collected kidney samples from the different experimental groups. These images show little to no morphological differences when compared to control images. In Figure 8.5A, C, G and Figure 8.6A glomeruli (G) are clearly distinguishable with visible Bowman's spaces and capsules (BC) and the bulk of the parenchyma between these renal corpuscles is filled with the collecting tubules (CT). In both Figure 8.5E and Figure 8.6C signs of glomerular collapse are evident as Bowman's spaces appear to have increased and the glomeruli appear smaller relative to the Bowman's capsule. TEM micrographs however revealed

normal ultrastructural arrangement of the tissue in which mesangial cells (M_C ; Figure 8.5B), and cuboidal epithelial cells (E_{CT}) from collecting tubules can be seen (Figure 8.5D, F, H; Figure 8.6B, D) with clear microvilli (MV) and numerous mitochondria (M). Fibroblasts are also identifiable (Figure 8.5H). More quantitative data is required to determine whether sibutramine administration alters renal function.

8.3.3 Heart

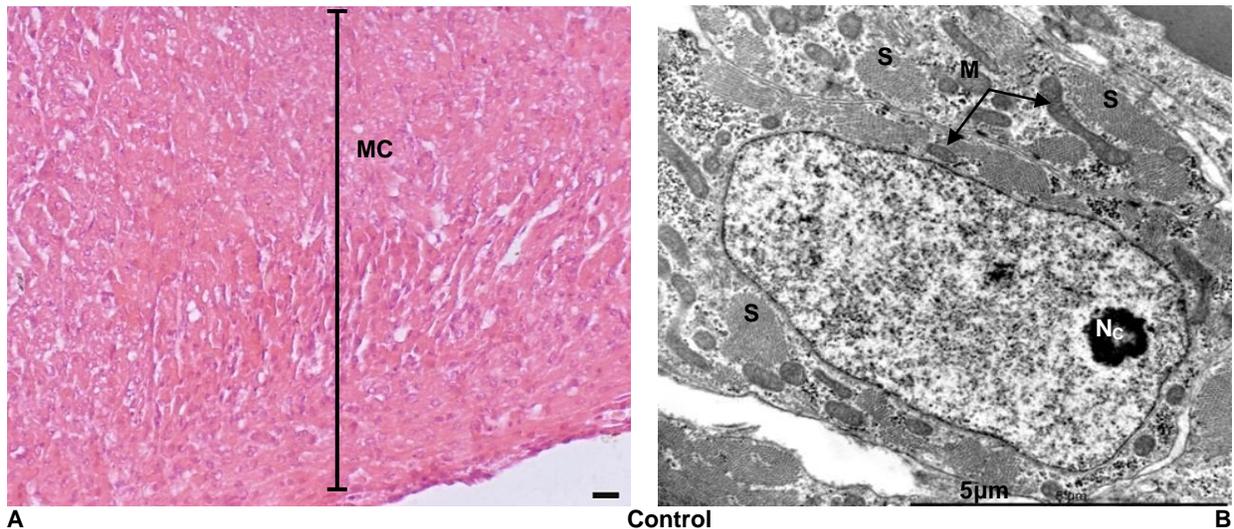
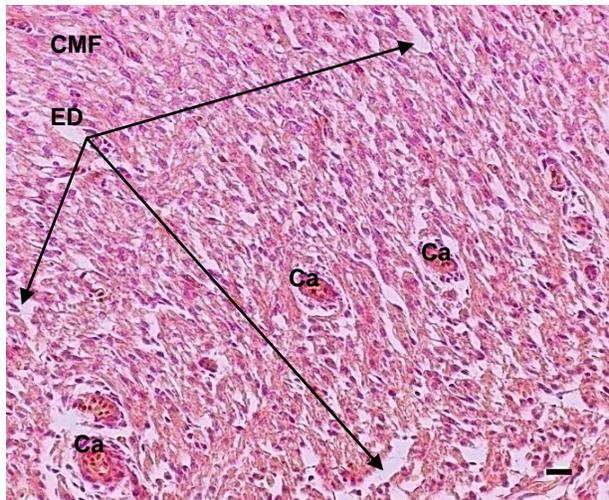


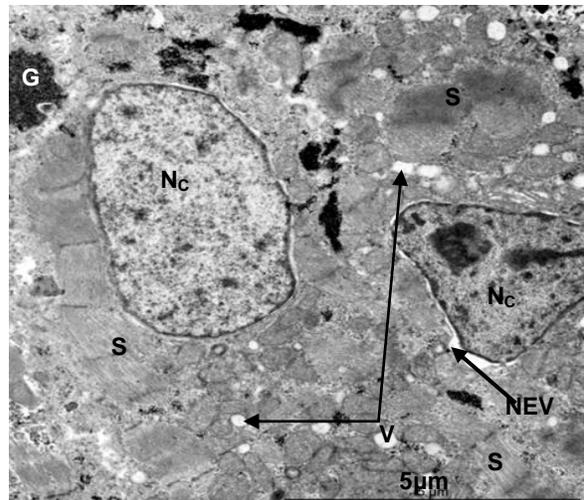
Figure 8.7: Heart samples collected from the control groups. **A and B:** Control LM and TEM respectively. LM scale bar=20 µm, TEM=5 µm. (M=mitochondria; MC=myocardium; Nc=nucleus of cardiac muscle cell; S=sarcomere)

Images acquired from the heart tissue of control embryos are shown in Figure 8.7A and B. Normal myocardial architecture can be observed in Figure 8.7A whereas Figure 8.7B shows the ultrastructure of a cardiac myocyte with clear nucleus, sarcomeres, the contractile units of these myocytes, as well as numerous mitochondria.

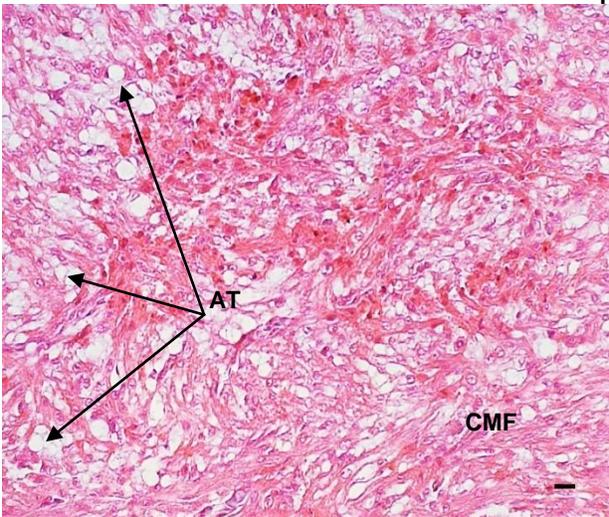


A

Eph5.0

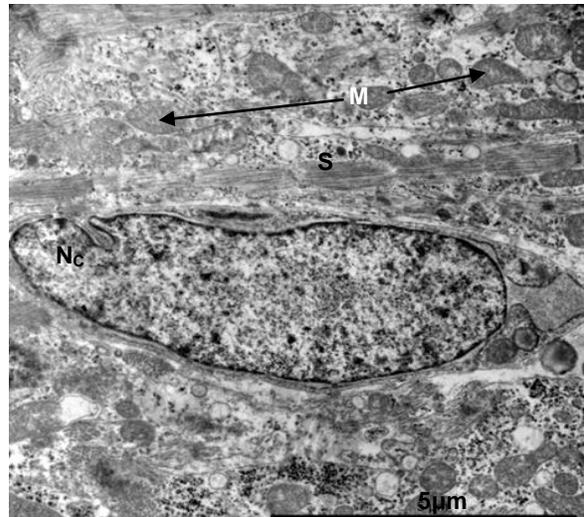


B

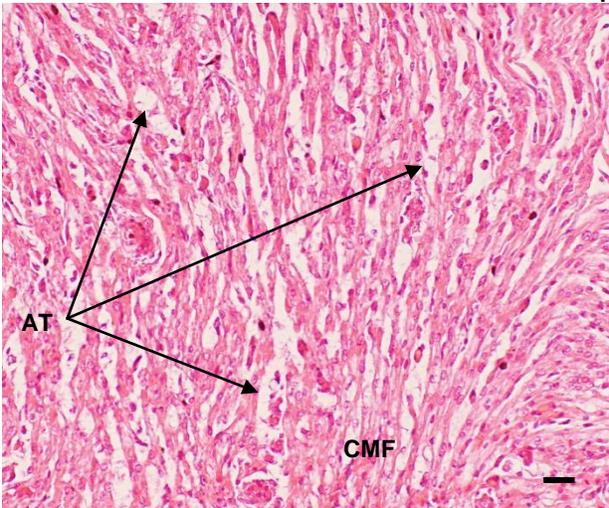


C

Eph2.5

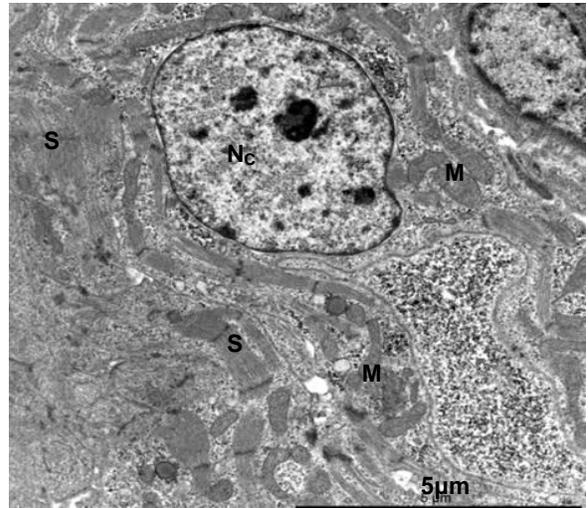


D



E

Eph5.0



F

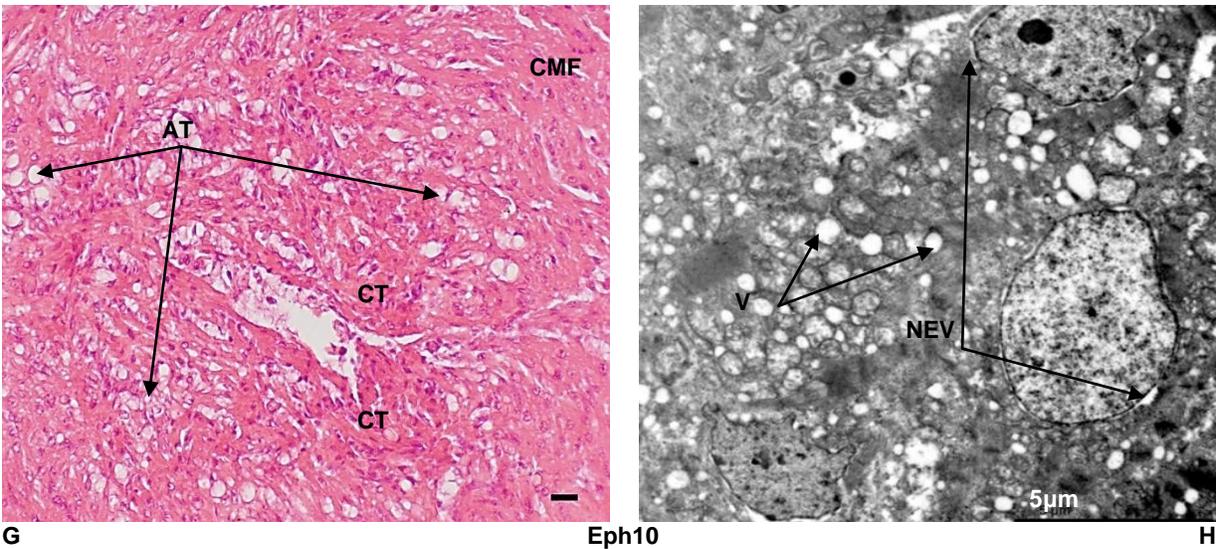
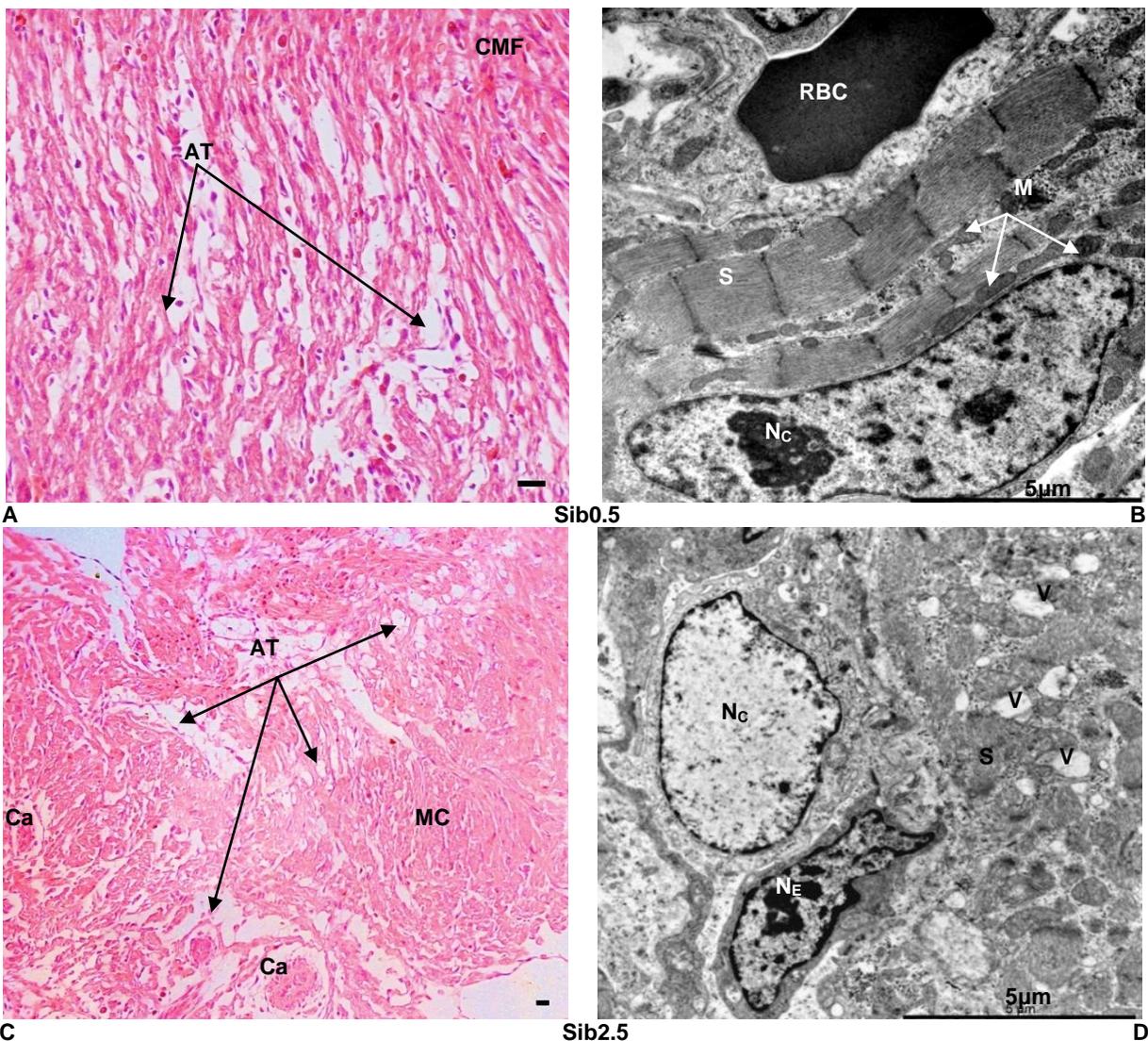


Figure 8.8: Heart samples collected from the ephedrine experimental groups. **A and B:** Eph0.5 LM and TEM respectively; **C and D:** Eph2.5 LM and TEM respectively; **E and F:** Eph5.0 LM and TEM respectively; **G and H:** Eph10 LM and TEM respectively. LM scale bar=20 µm, TEM=5 µm. (AT=adipose tissue; Ca=capillaries; CMF=cardiac muscle fibres; CT=connective tissue; ED=oedema; G=glycogen; N_c=nucleus of cardiac muscle cells; NEV=nuclear envelope vacuolisation; S=sarcomere; V=vacuoles)

Figures 8.8A-H and 8.9A-F are representative of heart samples obtained from the different experimental groups exposed to the two respective compounds. Compared to the control animals, the histology micrographs revealed increased adiposity as well as chord-like arrangement of muscle fibres, with mild oedema between wavy muscle fibres. Figure 8.8A and B represent embryos from the Eph0.5 experimental group. Figure 8.8A shows an increased oedematous appearance (ED) of myocardium with capillaries (Ca) distributed between muscle fibres (CMF) and the corresponding TEM image (Figure 8.8B) shows loss of structural integrity as tissue appears less organized. Small vacuoles (V) can be seen and the nuclear envelope appears dilated (NEV). Muscle fibres (M) as well as capillaries (C) can be seen. Connective tissue (CT) is present between the muscle fibres. In Figure 8.8C, acquired from the Eph2.5 experimental group, the infiltration of adipose tissue (AT) can also be observed between muscle fibres and the related TEM image (Figure 8.8D) also appeared less organized with less visible contractile components. Samples obtained from the Eph5.0 experimental groups also

showed adipose infiltration (Figure 8.8E) although TEM images (Figure 8.8F) did not reflect these findings. LM (Figure 8.8G) and TEM (Figure 8.8H) images obtained from embryos administered the highest ephedrine dose (i.e. 10µmol) also presented with adipose tissue infiltration, increased connective tissue and ultrastructurally showed signs of severe vacuolisation which could be mitochondrial degeneration, and nuclear envelope vacuolisation. Sarcomeres were not clearly distinguishable.



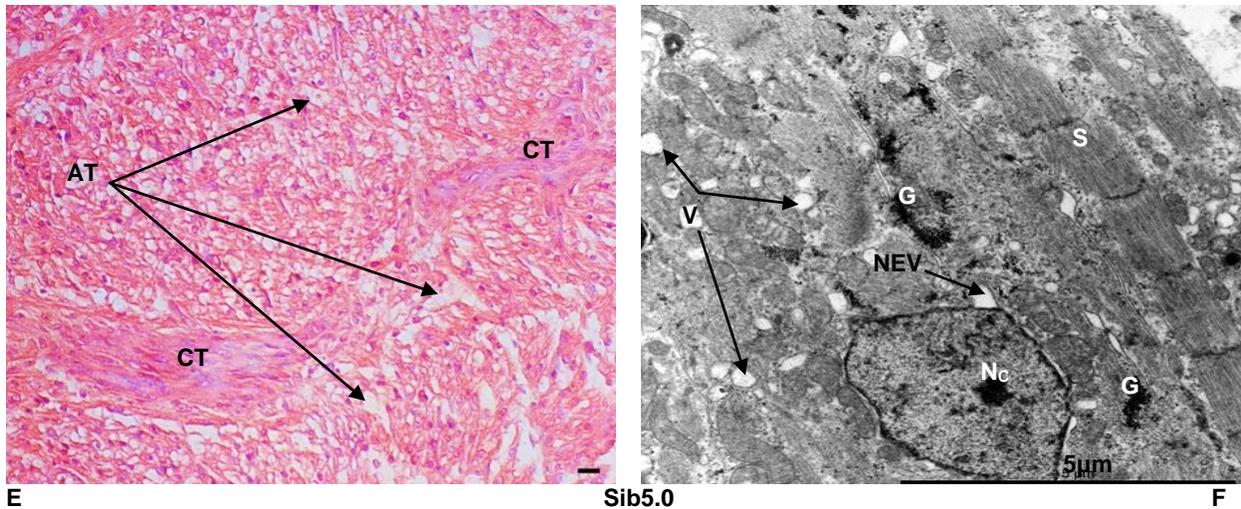


Figure 8.9: Heart samples collected from the sibutramine experimental groups. **A and B:** Sib0.5 LM and TEM respectively; **C and D:** Sib2.5 LM and TEM respectively; **E and F:** Sib5.0 LM and TEM respectively. LM scale bar=20 µm, TEM=5 µm. (AT=adipose tissue; Ca=capillaries; CMF=cardiac muscle fibres; CT=connective tissue; ED=oedema; G=glycogen; MC=myocardium; N_c=nucleus of cardiac muscle cells; NEV=nuclear envelope vacuolisation; S=sarcomere; V=vacuoles;)

Images acquired from the Sib0.5 experimental group are shown in Figures 8.9A and 8.9B. Again, adipose tissue (AT) can be observed distributed between the thin, wavy cardiac muscle fibres (CMF). TEM images (Figure 8.9B) showed normal structural arrangement. In embryos in the Sib2.5 group images show increased adipose tissue between cardiac muscle fibres (Figure 8.9C) and with TEM analysis increased vacuolisation (V), which could be due to dilation of the sarcoplasmic reticulum, was observed. The general arrangement of tissue also appears disrupted and these findings are also evident in the Sib5.0 experimental groups where connective tissue is increased as well as adiposity (Figure 8.9E). Vacuolisation is also evident in the TEM images (Figure 8.9F) with nuclear envelope dilations (NEV).

8.3.4 Brain

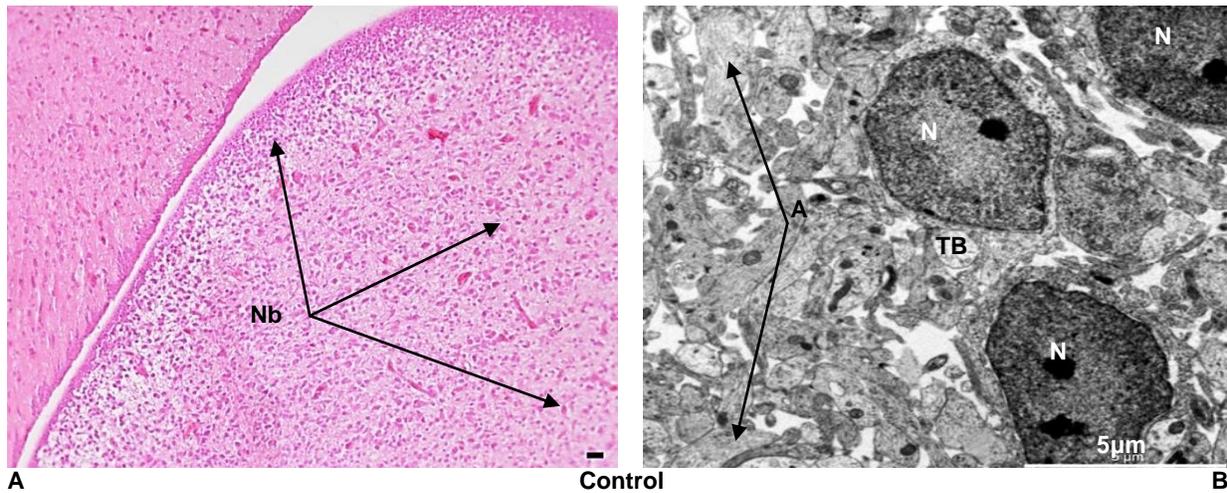
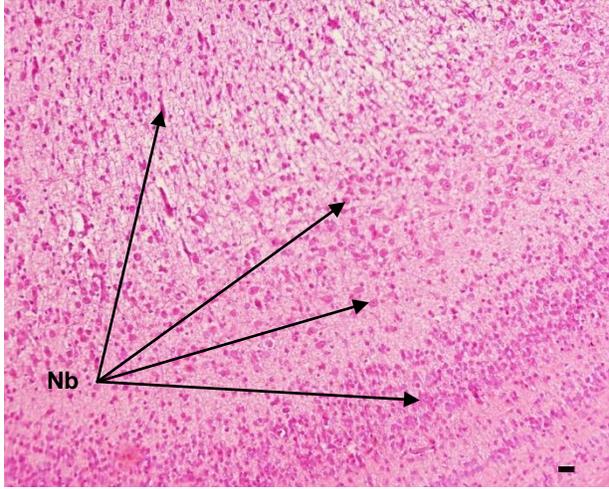


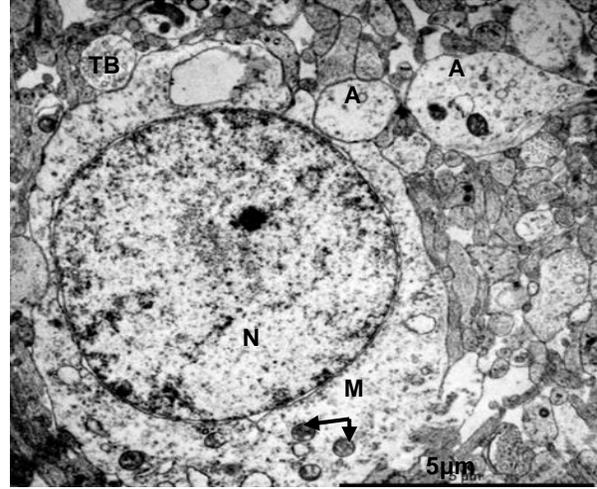
Figure 8.10: Brain samples collected from the control groups. **A and B:** Control LM and TEM respectively. LM scale bar=10 µm, TEM=5 µm. (A=axon; N=neuron; Nb=neuronal cell bodies; TB=terminal bouton)

Figures 8.10A and B show images acquired from the control embryos. Figure 8.10A shows the normal histological arrangement of control embryos where various neuronal cell bodies can be seen within the interstitial tissue. TEM micrographs (Figure 8.10B) show axons (A), dendrites (D) and neuron cell nuclei. A terminal bouton (TB), an axonal end, is also visible.

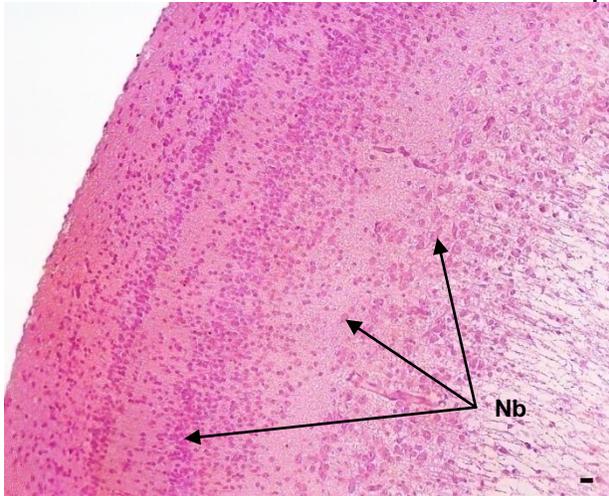


A

Eph0.5

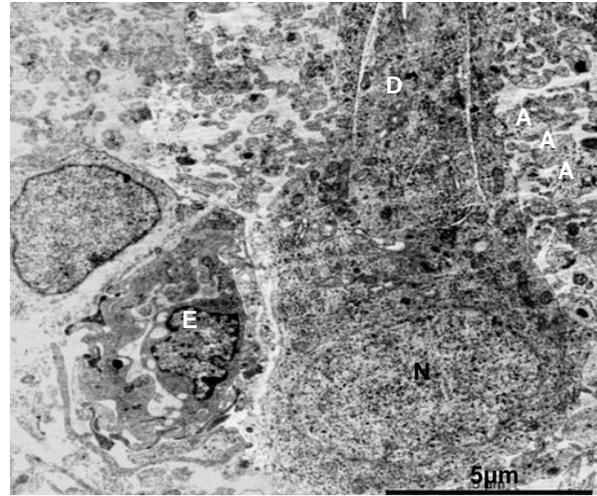


B

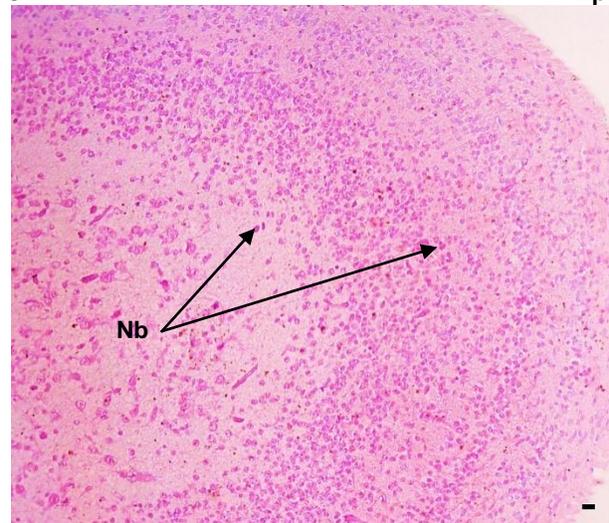


C

Eph2.5

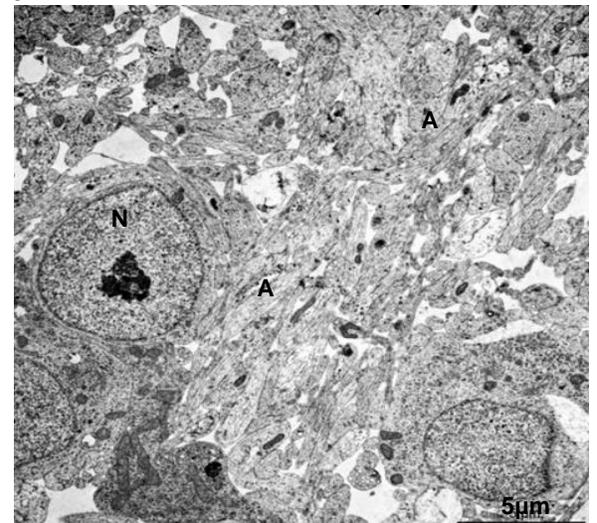


D



E

Eph5.0



F

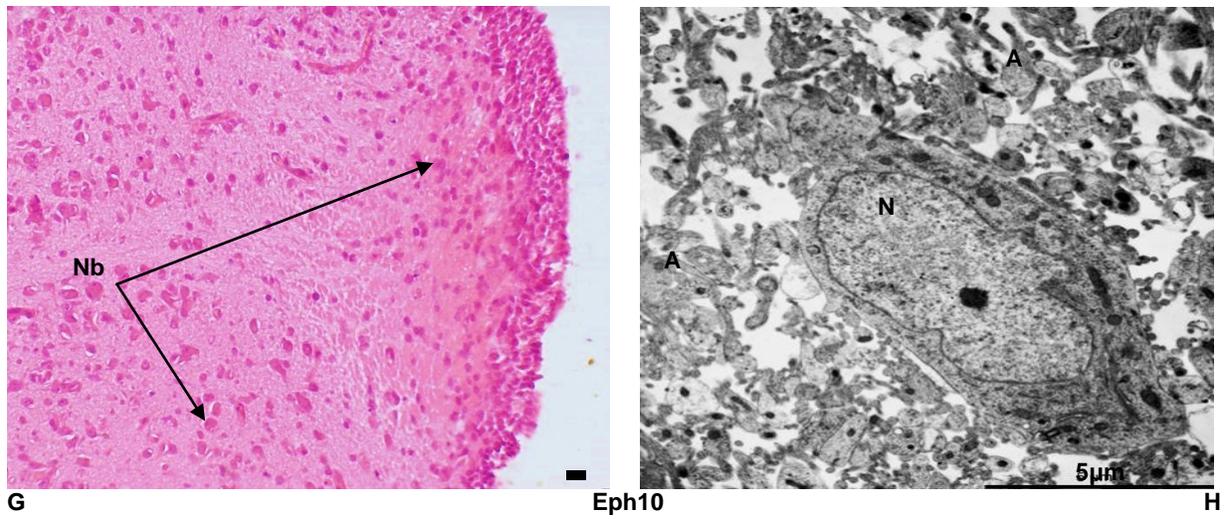
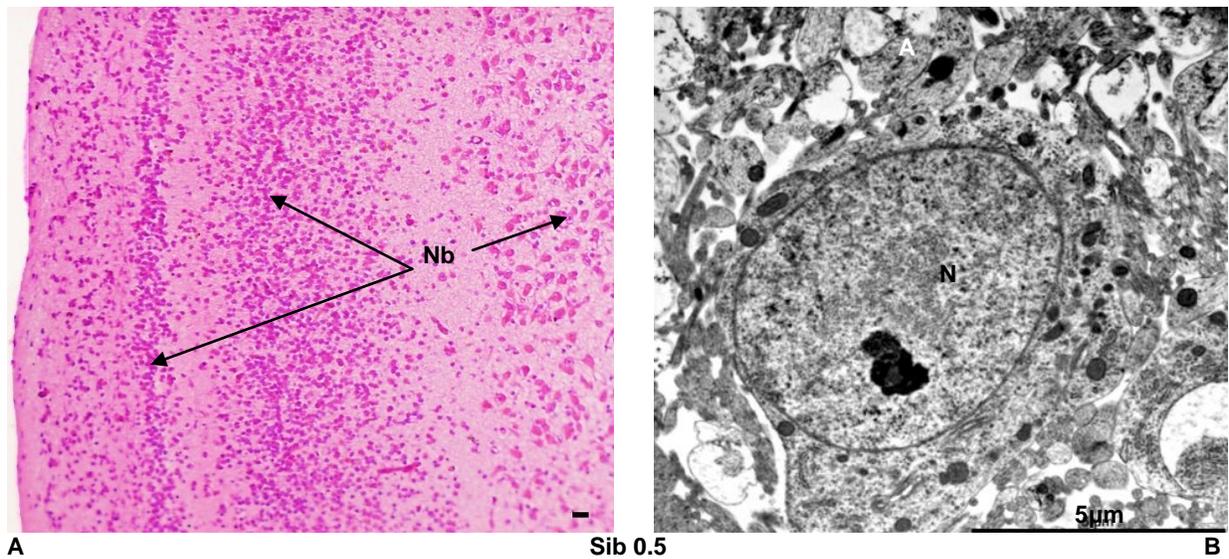


Figure 8.11: Brain samples collected from the ephedrine experimental groups. **A and B:** Eph0.5 LM and TEM respectively; **C and D:** Eph2.5 LM and TEM respectively; **E and F:** Eph5.0 LM and TEM respectively; **G and H:** Eph10 LM and TEM respectively. LM scale bar = 20 µm, TEM=5 µm. (A=axon; E=Endothelial cell; M=mitochondria; N=neuron; Nb=neuronal cell bodies; TB=terminal bouton)



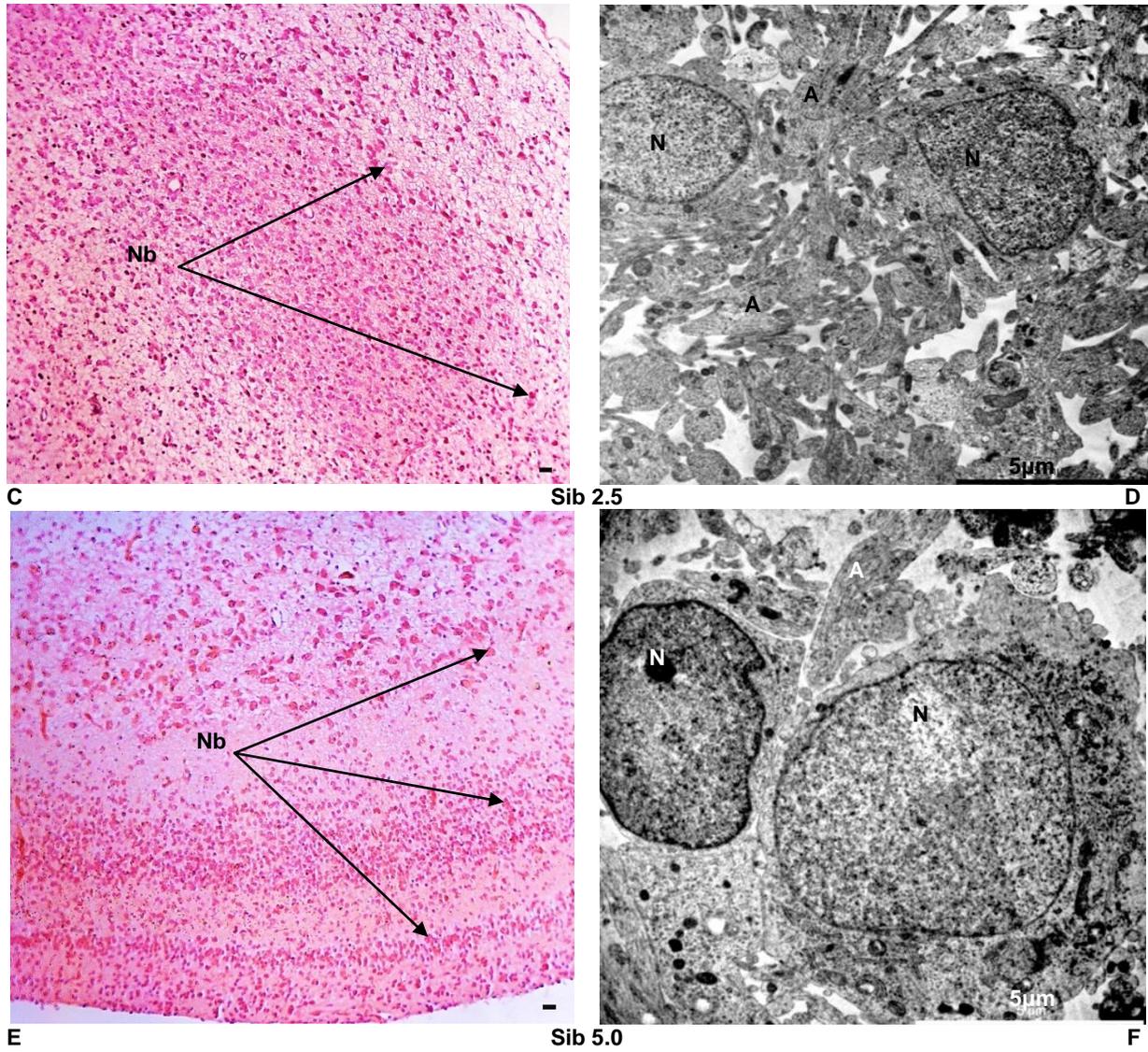


Figure 8.12: Brain samples collected from the sibutramine experimental groups. **A and B:** Sib0.5 LM and TEM respectively; **C and D:** Sib2.5 LM and TEM respectively; **E and F:** Sib5.0 LM and TEM respectively. LM scale bar=20 μm, TEM=5 μm. (A=axon; N=neuron; Nb=neuronal cell bodies)

Figures 8.11A-H and 8.12A-F are images acquired from the collected brain samples of the different experimental groups exposed to either ephedrine or sibutramine. No morphological differences could be observed between the different experimental groups or the control samples. Figures 8.11A, C, E and G and Figure 8.12A, C, and E show histological sections in which various neuronal cell bodies can be seen and present the general arrangement of brain tissue.

8.3.5 Sib5.0 experimental group

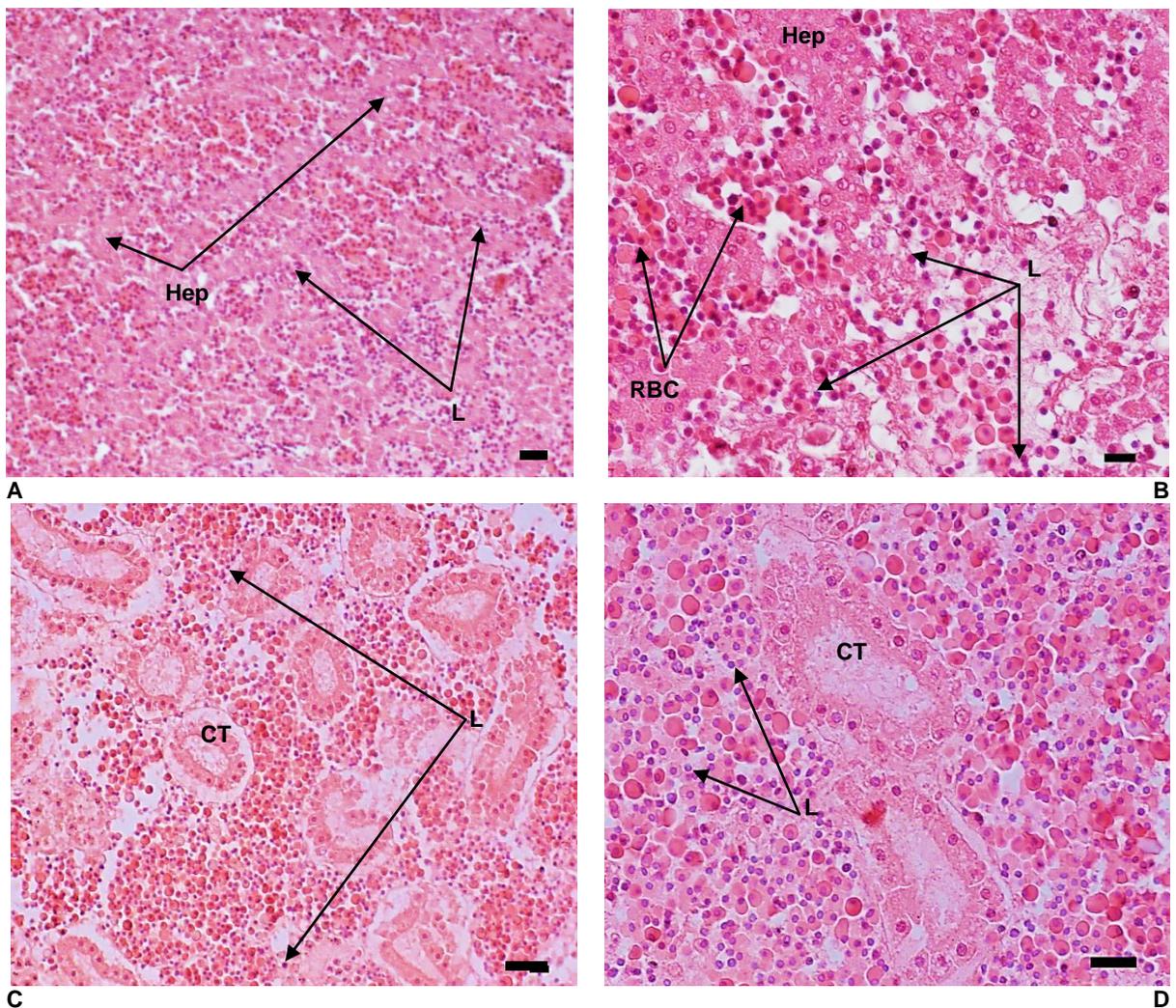


Figure 8.13: Liver and kidney samples obtained from one embryo of the Sib5.0 experimental group. **A and C:** scale bar=20 μ m; **B and D:** scale bar=20 μ m. (CT=collecting tubules; Hep=hepatocytes; L=lymphocytes, RBC=red blood cells)

Figure 8.13A-D shows histological images obtained from liver and kidney samples collected from one of the embryos that developed in the Sib5.0 group. These images show irregular arrangement of liver and renal tissue with congestion, haemorrhage and lymphocyte infiltration. Extravasated erythrocytes (RBC) and round lymphocytes (L) with dark basophilic nuclei are visible. Similar morphology has been observed during post-mortem investigations of fish exposed to heavy metal polluted water (Suxena and Suxena, 2007). Reasons for the observed morphology remain unclear although it suggests severe organ failure due to possible acute toxicity.

Table 8.1 provides a summary of all the histological lesions that were observed.

Table 8.1: Summary of morphological changes observed

Exp. Group	% Viable	Liver	Heart	Kidney and Brain
Control	75.00	Normal histological arrangement	Normal histological arrangement	Normal histological arrangement
Eph0.5	68.75	Normal histological arrangement	Oedema; Dilated nuclear envelope; Vacuolisation	
Eph2.5	75.00	Slight degeneration in tissue arrangement	Adipose tissue infiltration; Loss of structural integrity and contractile components	
Eph5.0	75.00	Slight degeneration in tissue arrangement	Adipose tissue infiltration; Oedema	
Eph10	62.50	Loss of tissue integrity; Dilated Sinusoidal spaces; Lipid deposition; vacuolisation	Adipose tissue infiltration; Increased connective tissue; Severe vacuolisation (possibly due to mitochondrial degeneration); Nuclear envelope vacuolisation	
Sib0.5	75.00	Normal histological arrangement	Adipose tissue infiltration	
Sib2.5	25.00	Lipid accumulation; Decreased structural integrity of space of Disse	Adipose tissue infiltration; Loss of structural integrity; Vacuolisation (possible dilation of sarcoplasmic reticulum)	
Sib5.0	25.00	Lipid accumulation; Decreased structural integrity of space of Disse; Increased connective tissue; Vacuolisation	Adipose tissue infiltration; Loss of structural integrity; Vacuolisation; Nuclear envelope dilations	
Sib10	06.25	Not viable		

8.4 Discussion

This study involved a comprehensive morphological investigation of the possible toxic effects of sibutramine on the developing chick embryo. The results obtained were compared to that of ephedrine, a compound which has been described as a teratogen in a similar experimental model (Nishikawa *et al.*, 1985a; 1985b). Results indicated noticeable differences between the two compounds, and also between different concentrations of the individual compounds.

Apart from the gross morphological abnormalities that were noted which would render the chicks unviable, histological investigation of viable embryos also showed alterations within various organs. Liver sections revealed signs of severe steatosis, with characteristic lipid depositions throughout the liver tissue.

The incidence of hepatic steatosis has been well defined in neonates and is considered a fatal condition in human newborns as it leads to liver failure, coma, and eventually death (Boison *et al.*, 2002). This condition is characterised by a rapid infiltration of hepatic parenchyme by microvesicular fat and the subsequent enlargement of the liver (Satran *et al.*, 1969).

The characteristic fat infiltration as observed in this condition is often attributed to severe impairment of mitochondrial function (Burt *et al.*, 1998; Fromenty *et al.*, 1997; Fromenty and Pessayre, 1997) which has been associated with drug-induced inhibition of beta (β)-oxidation (Fromenty *et al.*, 1997; Fromenty and Pessayre, 1997). N is known to play a role in regulating the extracellular uptake and release of carbohydrates and fatty acids and this could provide a mechanism in which sibutramine influences these processes, leading to the development of this condition. Both ephedrine and sibutramine's

mechanism of action involve key interactions within various noradrenergic pathways and preliminary biochemical data suggest that sibutramine could affect the metabolic processes directly by inhibiting various vital enzymes involved in fatty acid oxidation. This topic of research however requires more investigation before possible conclusions can be made. It is clear though that the observed steatosis in the current study would result in severe liver failure and possibly death.

Upon the histological investigation of the cardiac tissue, adipocyte infiltration within the myocardium was seen in the groups exposed to the different concentrations with an increase in severity related to the increasing dosage concentrations. As of yet such histological appearance has not been well defined or previously described in chick embryos although the observed morphology, which shows substantial adipose tissue infiltration of the myocardium with an evident increase in connective tissue surrounding remaining cardiomyocytes (especially evident in the Sib5.0 heart sections), strongly relates to that described in cases of arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) (Fornes *et al.*, 1998). ARVC/D is a lethal condition of unknown cause which affects the heart muscle and is distinguished histologically by the fatty, fibro-collagenous atrophy of the right ventricular myocardium resulting in electrical cardiac irregularities (Marcus *et al.*, 1982; Basso *et al.*, 1996). This process of myocardial loss is also non-inflammatory and occurs via programmed cell death or apoptosis (Mallat *et al.*, 1996). Various genetic mutations within numerous components of the cardiac desmosome have been acknowledged as key mediators in the pathogenesis of ARVC/D as they exhibit variations in penetrance and expression, which further implicates environmental and other genetic factors in the onset and progression of this disease. This is however a late-onset disease. Another condition characterised by fatty infiltration of cardiac tissue is known as Uhl's anomaly. Although this is a congenital

defect, it is defined by the almost complete replacement of ventricular myocardium with adipose tissue or the complete absence thereof, as well as epicardial and endocardial apposition (Gerlis, 2003) and our findings do not coincide with those mentioned. Although very little data is available on the observed histological alterations and possible causes thereof, it is presumably attributable to some form of myocardial dysplasia which is characterised, histopathologically, by fiber size variability, fibre necrosis, regeneration, inflammation and connective tissues deposition, some of which were seen in the current results.

8.5 Conclusion

From the results obtained in the current chapter it can be concluded that both compounds exhibited embryotoxicity when compared to the control groups. Livers and hearts of embryos exposed were severely affected by both compounds in a dose related manner with possible fatal consequences. Heart morphology appears similar to muscle dystrophy, more specifically ARVC/D, in that muscle tissue is replaced by adipose and connective tissue. Severe liver steatosis was also noted. Results are based purely on morphological findings and in-depth investigations into the molecular pathways involved, might shed more light on the exact mechanisms of the observed toxicity of these substances.

CHAPTER 9: Concluding Discussion

Various adverse events related to the cardiovascular system, associated with the use of sibutramine, a serotonin-norepinephrine reuptake inhibitor (SNRI) prescribed for the treatment of obesity, have been reported. This has led to the eventual retraction of this product from most markets but, despite this, the unregulated use of sibutramine continues. Data on the toxic potential of sibutramine within various organ systems is very limited and studies aimed at evaluating the effect of this compound on organ morphology and subsequent function are essential to further ensure safe use thereof. Due to the ban placed on sibutramine, it is often an undisclosed component in herbal remedies indicated for weight loss, and therefore could result in unintentional consumption and subsequent exposure to this compound. Data on the teratogenic potential of sibutramine is also scarce despite its necessity, as exposure of the unborn foetus could possibly occur unknowingly. The aim of this study was therefore to investigate the toxic and teratogenic potential of sibutramine.

To attain these aims, two different animal models were implemented, best suited to this purpose. To assess the effects of sibutramine administration on organ morphology, biochemical markers of organ function and possible hormone alterations, as well as weight changes the Sprague Dawley rat model was implemented. Results obtained from monitoring the weights of animals exposed to sibutramine indicated that female animals within the high dose (HD) group differed significantly from values obtained in both the control (C) and low dose groups (LD), indicating a decrease in the rate of weight gain of animals in this group. Data obtained from the weights of male rats showed no significant differences between any of the experimental groups. These findings however do not

coincide with numerous preceding investigations in which significant weight changes were reported at much lower dosages than those employed in the current study (Weintraub *et al.*, 1991; Bray *et al.*, 1999) using the same methodology.

Upon investigations of alterations in levels of thyroid hormones thyroxine (T₄) and triiodothyronine (T₃) and cortisol, no noteworthy findings were obtained apart from a significant increase in cortisol concentrations of female rats in the HD experimental group. This could be attributed to the reported significant weight loss which was observed within only this experimental group. Despite previous reports that sibutramine administration leads to increased cortisol and thyroid hormone activity, which could point to thyroid or adrenal gland dysfunction, our current findings showed that sibutramine did not alter these hormone concentrations significantly despite the use of concentrations ten times higher than the prescribed doses.

Liver function tests following sibutramine administration also did not show any clinically important changes although long term sibutramine administration could provide different data and such investigations together with periodical analysis could prove advantageous in gaining more information on the safety and efficacy of sibutramine. Hepatic biochemical patterns of injury are frequently misrepresentative. Standard liver function tests are diagnostically correct in approximately 75% of cases (Festi *et al.*, 1983).

Cardiovascular complications such as hypertension, arrhythmias and tachycardia are all adverse events reported with sibutramine use. Therefore, components of blood coagulation were investigated, namely platelets and fibrin networks, using SEM. Blood samples were collected from the rats exposed to sibutramine within the given experimental groups. Given the findings of this particular part of the current research, we

propose that sibutramine administration could lead to a procoagulant state increasing the likelihood of stroke and myocardial infarction especially in already overweight and obese individuals. Investigations of the morphological appearance of the platelets and fibrin networks of animals treated with sibutramine versus controls, revealed differences that are consistent with changes described in previous studies which have been associated with increased coagulability and disease (Pretorius *et al.* 2007). These changes indicate that sibutramine affects either directly or indirectly the coagulation pathway and this is an important area for further research. The observed hypercoagulable state described in the current research can be associated with the previously reported complications such as hypertension, arrhythmias and tachycardia. The sibutramine target population would generally consist of obese and overweight individuals of higher BMI's which attributes to a predisposing risk of cardiovascular complications such as hypertension, coronary artery disease and type II diabetes.

Light microscope (LM) and transmission electron microscope (TEM) investigations of lung tissues collected from the different experimental groups (i.e. C, LD, HD) showed increases in alveolar wall thickness, and drastic increases in extracellular matrix deposition. This could be attributed to pathological fibrotic processes within lung tissue due to sibutramine interactions. Pulmonary fibrosis is characterised by variable degrees of inflammation and fibrosis within lung tissue (Crystal *et al.*, 1976; Turner-Warwick, *et al.*, 1980; Raghu, 1987). Although direct methods of how sibutramine could induce pulmonary fibrosis were beyond the scope of the current research, these findings provide various aspects that point to an association. Increased thrombin concentrations as well as disturbances in the direct epithelial environment could lead to the development of pulmonary fibrosis. Data from the blood coagulation aspect of this study showed that coagulation factors, such as thrombin, could be increased due to

sibutamine administration, which could lead to shear stress and alterations in the immediate environment of alveolar epithelium which would have to be counteracted in some way. This could lead to the eventual development of increased extracellular matrix deposition and eventual fibrosis.

Although, based on morphological analysis from the results obtained, no significant differences between the liver, kidney and brain samples of animals from the different experimental groups were found, functional and biochemical assays may provide more information on the effects of this compound *in vivo*. In this study the biochemical findings associated with liver and kidney functions were further substantiated by the unaltered histology seen in both these organs upon morphological examination. Heart tissue showed slight increases in collagen deposition between muscle fibres. This also provides motivation for further quantitative research.

Investigations regarding the possible embryo-toxicity of sibutramine, compared to ephedrine, were achieved by implementing the chick *in ovo* model. Upon investigation it was evident that the effects of sibutramine administration were more severe than that of ephedrine with regards to embryo-toxicity. Normal development was significantly decreased in embryos exposed to sibutramine in a dose related manner although abnormalities were noted for both compounds. Retarded growth, limb abnormalities and gastroschisis were amongst the most prevalent macroscopic defects noted whereas histological examination showed signs of liver failure and cardiomyopathy. These defects could be attributed to haemodynamic processes as previously described for compounds with a similar mechanism of action (Gillis *et al.*, 1995; Viscarello *et al.*, 1992). Also, a link might exist between the observed ventral limb-body wall complex and observed histology by means of interactions with the canonical Wnt/ β -catenin signalling

pathway which regulates proliferation, fate specification and differentiation in numerous developmental stages and adult tissue homeostasis (MacDonald *et al.*, 2009). This cell-signalling pathway has been implicated in more than one anomaly seen in the current study. It has been associated with cadmium-induced ventral wall defects within an experimental chick embryo model (Thompson *et al.*, 2008) as well as the process of adipose tissue differentiation within myocardium which has been associated with arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) (Awad *et al.*, 2008).

In general, both animal models proved to be highly effective within their respective applications and provided significant results which should be easily reproducible, further contributing to the reliability of these models.

With regards to cortisone levels, it is well known that both norepinephrine (NE) and epinephrine is capable of stimulating pituitary adrenocorticotrophic hormone (ACTH) release *in vitro* (Calogero *et al.*, 1988; Murakami *et al.*, 1984) and that this effect is additional to the effect of corticotropin-releasing hormone (CRH) (Murakami *et al.*, 1984, Vale *et al.*, 1983). Serotonin (5-HT) has also been shown to induce ACTH secretion *in vitro* (Spinedi and Negro-Vilar, 1983). As sibutramine action involves direct interactions within both serotonergic and adrenergic pathways, and circulating 5-HT elevations could activate the Hypothalamic-Pituitary-Adrenal (HPA) axis (Calogero *et al.*, 1989), future studies investigating the biochemical principle of possible sibutramine administration and HPA axis interaction as a mechanism of cortisol increases might prove highly beneficial due to the important functions of this hormone. Future studies on long term exposure to sibutramine with regular, interval screening of blood for biomarkers of disease or organ function could provide a more detailed, reliable sketch of possible biochemical interactions.

Although platelet and fibrin network morphology was altered due to the administration of sibutramine, the presence or absence of specific molecules cannot be determined by using this scanning electron microscopic analyses despite it providing detailed images of the resultant morphology of the cell and components, given their interactions within these different environments.

Preliminary biochemical analysis of sibutramine and its active metabolites does however propose that a possible direct interaction could exist between these molecules and both co-factor IX and X which are involved in the coagulation cascade. Whether these interactions up- or down-regulate the activity of these serine proteases could be the topic of further research as these biochemical findings could form the basis of future studies aimed towards investigating the exact mechanism in which sibutramine will interfere with this physiological process.

When taking all results into consideration it is clear that sibutramine administration could lead to a number of complications unrelated to the reported cardiovascular difficulties. These results provide a basis for the development of various pathological conditions based on morphological analysis alone. One major limitation of this study is the lack of quantitative data which could, together with these morphological findings, further define the nature of the molecular and cellular mechanisms and interactions associated with sibutramine use as the exact mechanism involved in these processes remain unclear.

Sibutramine research is currently aimed at methods to detect this compound in dietary supplements (Mathonab *et al.*, 2014; Youssef *et al.*, 2014) and recent literature includes clinical reports of severe adverse events associated with its use (Heo, and Kang, 2013; Kim *et al.*, 2013). Functional assays combined with biochemical analysis would provide essential information on the nature of the interactions of sibutramine and its metabolites within the body and the pathological significance thereof. We hope that the current research will motivate more investigation into these aspects of sibutramine interactions within the body so that the risks associated with the administration of this drug can be clearly highlighted and motivate more regulated use thereof.

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APPENDIX

Certificate of Ethical Clearance

H003-13 and H005-13



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	An <i>in vivo</i> and <i>in ovo</i> evaluation of the toxicity of Sibutramine: The effect of Sibutramine Hydrochloride Monohydrate on organ ultrastructure and blood coagulation
PROJECT NUMBER	H003-13
RESEARCHER/PRINCIPAL INVESTIGATOR	Ms. C van der Schoor

STUDENT NUMBER (where applicable)	S28156464
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	Rats	
NUMBER OF ANIMALS	38	
Approval period to use animals for research/testing purposes		May - June 2013
SUPERVISOR	Dr. HM Oberholzer	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	29 April 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	An <i>in vivo</i> and <i>in ovo</i> evaluation of the toxicity of Sibutramine: Teratogenic effects of weight-loss products (Sibutramine and Ephedrine) on chick embryo's
PROJECT NUMBER	H005-13
RESEARCHER/PRINCIPAL INVESTIGATOR	Ms C van der Schoor

STUDENT NUMBER (where applicable)	S28156464
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	Embryonated eggs	
NUMBER OF ANIMALS	180	
Approval period to use animals for research/testing purposes		May - November 2013
SUPERVISOR	Dr HM Oberholzer	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	29 April 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	