The Effects of Multivitamin-Multimineral Supplementation on the Spontaneously Hypertensive Rat Model of Hypertension.

By

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DECLARATION

I, Rosemarie Ursula Höfler, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

All of the experiments were conducted by myself, and contributed towards experimental and project design.

3. That the contributions of others to the project are as follows:

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Dr. A. Nadar (University of KwaZulu-Natal), was Co-supervisor for the project, and made conceptual contributions towards experimental and project design.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACE	Angiotensin-converting enzyme
Ag	Silver
AHA	American Heart Association
Ang I/ II	Angiotensin I/II
Apo	Apoptosis
Atro	Atrophy
BHT	Butylated hydroxytoluene
BP	Blood pressure
BRU	Biomedical Resources Unit
BUN	Blood urea nitrogen
C	Carbon
°C	Celsius
Ca	Calcium
Cd	Cadmium
CHD	Coronary heart disease
$ClHO_4$	Perchloric acid
Co	Cobalt
CO	Cardiac output
CO_2	Carbon dioxide
CPD	Critical point dryer
Cr	Chromium
CRP	C-reactive protein
Cu	Copper
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DCT	Distal convoluted tubules
Dil	Dilation
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DPX	Distyrene Plasticizer Xylene
ECF	Extra-cellular fluid
EDX - SEM	Energy Dispersive X-ray analytical system and Scanning Electron Microscopy
ELISA	Enzyme-Linked Immunosorbent Assay
F	Fluoride
FDA	Food and Drug Administration
Fe	Iron
FFA's	Free fatty acids
g	gram
GFR	Glomerular filtration rate
GH	Growth hormone
GPX	Glutathione peroxidase

Hb	Haemoglobin
HCL	hydrochloric acid
HCT	Haematocrit
HDL's	High density lipoproteins
H&E	haematoxylin and eosin
Hg	mercury
H_2O_2	Hydrogen peroxide
Hya	Hyalinization
I	Iodine
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
IGF-1	Insulin-like growth factors
IL-2/IL-6	Interleukin
Inflam	Inflammation
JG	Juxtaglomerular
K	Potassium
LDL's	Low Density Lipoproteins
Li	Lithium
LLD	Low Limit of Detection
MANOVA	Multivariate Analysis of Variance
Mb	Molybdenum
MDA	Malondialdehyde
Mg	Magnesium
mg	Milligram
ml	Milliliter
mm	Millimeters
mm Hg	Millimetres Mercury
m.mol/ℓ	Millimole per Litre
MMPs	Matrix-Metalloproteinases
Mn	Manganese
MVMM	Multivitamin-Multimineral
N	Nitrogen
n	Sample Size
Na	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NASC	National Animal Supplement Council
NF-kB	nuclear factor-kappaB
ng	Nanogram
NHANES	National Health and Nutrition Examination Survey
Ni	Nickel
NIH	National Institute of Health
nm	Nanometre
NO•	Nitric oxide
O	Oxygen
$•O_2^-$	Superoxide

OH•	Hydroxyl
$ONOO^-$	Peroxynitrite
P	Phosphorus
PAI-1	Plasminogen-Activator Inhibitor-1
Pb	Lead
p-casts	Protein Casts
PCT	Proximal Convoluted Tubules
PLT	Platelets
PT	Parathyroid
PTH	Parathyroid Hormone
PUFA's	Polyunsaturated Fatty Acids
RAAS	Renin-Angiotensin-Aldosterone System
RBC's	Red Blood Cells
RDA	Recommended Dietary Allowance
REDOX	Reduction Reactions
Reg	Regeneration
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
S	Sulphur
SBP	Systolic Blood Pressure
Sclero	Glomerulosclerosis
SD	Standard Deviation
Se	Selenium
SEM	Standard Error of the Mean
SHR	spontaneously hypertensive rat
SHR S	spontaneously hypertensive rat supplemented
Si	Silicon
SNS	Sympathetic Nervous System
SOD	Superoxide Dismutase
TA	Tunica Adventitia
TBARS	Thiobarbituric Acid Reacting Substances
TGF-β1	Transforming Growth Factor-beta 1
TI	Thallium
TI	Tunica Intima
TM	Tunica Media
TNF-α	Tumor Necrosis Factor alpha
TPR	Total Peripheral Resistance
μl	Microlitre
USA	United States of America
UTI	Upper Tolerable Intake
UKZN	University of KwaZulu-Natal
V	Vanadium
Vac	Vacuolation
VLDL	Very Low Density Lipoproteins

VVG	Verhoeff-Van Gienson
WBC	White Blood Cells
WHO	World Health Organization
WIS	Wistar
WIS S	Wistar Supplemented
Zn	Zinc

ABSTRACT

The nutraceutical industry has proliferated in recent years with the most popular form of supplementation being the multivitamin-multimineral (MVMM) supplement. In the animal health sector, supplement use has also expanded.

The objective of this study was to determine the effects of MVMM supplementation, beneficial or otherwise, on the general health status of the spontaneously hypertensive rat (SHR) strain, an animal model used in hypertension research.

A commercially prepared MVMM supplement was given tri-weekly via oral dosing for eight weeks to two groups of seven adult female SHR and Wistar rats. Their corresponding control groups were dosed with deionised water only.

Systolic and diastolic blood pressure, fasting blood glucose, growth rate and food and water intake were measured weekly. At the end of eight weeks, the animals were euthanized and a full blood profile, urine sodium potassium ratio, blood urea nitrogen levels and total plasma cholesterol was measured for all groups. Further biochemical tests included determining plasma C-reactive protein, angiotensin-converting enzyme and kidney lipid peroxidation levels. Blood and tissue trace element profiles were determined by ICP and EDX analysis. A histological study was conducted on the kidney and aorta.

MVMM supplementation had significant metabolic, cardiovascular and renal effects in the SHR group. It increased growth and caused a significant reduction in the diastolic blood pressure of both SHR S and WIS S groups over the eight week period. It also lowered total cholesterol and increased RBC count in the SHR S group.

Supplementation also had some renoprotective properties as the SHR S group exhibited lesser hypertension related morphological changes in the kidney due to lowered blood pressure and possible antioxidant effects of the supplement. This was however not translated into any improvement in renal function.

No adverse effects on the general health status of the animals were observed. MVMM supplementation may therefore be useful in aiding growth and delaying the onset of hypertension and the related complications. It may also assist in the longevity of the breeding stock of SHR rats.

1 INTRODUCTION

In recent years there has been a proliferation in the nutrient supplementation industry which, spurred by various marketing claims, in 2007 was worth an approximated 23.7 billion dollars in America alone. The most commonly purchased supplement was the multivitamin-multimineral (MVMM) supplement, showing a 3.9% increase in sales during that year alone (Marra & Boyar 2009). In the United States, multiple surveys known as the National Health and Nutrition Examination Survey (NHANES) were carried out over a period of years by the National Centre for Health Statistics, to monitor dietary supplement use by its citizens. It revealed that between the years 1988 to 2006, there was a steady increase in MVMM supplement use in adults aged 20 and above, with women being the largest consumers (Gahche *et al* 2011).

The primary role of pharmaceutically manufactured MVMM supplements is to target specific vitamin and/or mineral deficiencies that arise from primary or secondary malnutrition, which can be caused by a variety of disease conditions. Through high impact marketing strategies, otherwise healthy individuals are now also being encouraged to take these MVMM supplements in order to, as some claim, promote growth and general health, improve metabolism and enhance cognitive function (Ames, Atamna & Killilea 2005). It is also claimed that the MVMM supplements act as 'nutritional insurance' against the effects of ageing, fatigue, cancers and strokes, as well as chronic conditions such as hypertension, diabetes and cardiovascular disease (National Institute of Health 2015a).

Predictably, the MVMM supplementation industry has now also spread into the veterinary and animal health sectors with a large number of preparations and combinations being made available for livestock, poultry and companion animals, all with similar health promoting purposes as for humans (National Animal Supplement Council 2002). New MVMM formulations using nano emulsion technology allows a multiple variety of supplements to be presented together in one suspension and delivered simultaneously in a single bolus (Srinivas *et al* 2010). In this growing industry, there is a concomitant need to include more research with a specific focus on both the beneficial effects and possible health risks associated with both human and animal MVMM supplementation (Comerford 2013). The National Animal Supplement Council (NASC) also raises the concern that there is no proper regulation for animal supplementation and many of the marketed supplements contain unsafe and/or unapproved ingredients, jeopardizing the safety of the animals (NASC 2002).

Hypertension, branded as a 'silent killer' has become a major health issue in recent years, especially among the middle to lower-income groups. The health systems in these particular groups are not as advanced and less action is taken to decrease the risk factors associated with hypertension such as obesity, inactivity, smoking, high alcohol consumption and stress. This in turn results in an increased risk for the development of coronary heart disease (CHD) and strokes (World Health Organization

2013; World Heart Federation, n.d.). Annually, hypertension is responsible for approximately 9.4 million deaths worldwide, with deaths due to hypertension anticipated to increase from 18% in 2008 to 24 % by 2030 (WHO 2013). In South Africa there are approximately 6.2 million people who have a blood pressure of 140/90 mm Hg or higher, and of that 6.2 million, 3.2 million exceed 160/95 mm Hg (Hasumi & Jacobsen 2012; Steyn 2005; Rayner 2014).

Conventional treatment of hypertension includes the use of beta-blockers, Angiotensin converting enzyme inhibitors, Angiotenisin II receptor blockers, diuretics as well as a variety of other multi-drug combinations due to hypertension having a multifactorial and polygenic aetiology (American Heart Association 2015; Calhoun *et al* 2008). However, vitamin and/or mineral supplements are being marketed as either an alternative way to treat hypertension or in conjunction with conventional drugs to aid in relief from the side effects. For example, some diuretics can result in urinary potassium loss, insomnia and depression and angiotensin-converting enzyme (ACE) inhibitors can cause a dry cough and skin rash to name a few (AHA 2015). Vitamin and/or mineral supplements are therefore promoted as being the 'natural' and more healthy alternative for treating hypertension since they are claimed to have little or no side effects. Another important aspect especially for the lower-income groups is that costing isn't as exorbitant as conventional prescription drugs (Afrolayan & Wintola 2014). The marketing claims cannot be dismissed entirely. Various studies have also shown the positive effects of some vitamin and mineral supplements in having antihypertensive effects and reducing side effects. For example vitamins E and C, and minerals such as calcium and manganese have all shown to have a lowering effect on blood pressure in both human and animal studies (Vasdev *et al* 2001, 2002; Makynen *et al* 1995; Burgess *et al* 1999; Cheng *et al* 2001).

1.1 Aims and Objectives of the Study:

The overall aim of the study was to determine the effects of MVMM supplementation on the spontaneously hypertensive rat (SHR) and its effects on hypertension at a biochemical and morphological level.

The objectives of the study were:

1. To investigate the effects of MVMM supplementation on the general health status in two rat strains, namely the spontaneously hypertensive rat (SHR), a well-established animal model of hypertension, and the Wistar strain which served as a control.

2. To evaluate the effects of MVMM supplementation on hypertension and its associated biochemical parameters.

3. To show the effects of MVMM supplementation on renal and vascular histology in the SHR after the establishment of hypertension.

A more practical outcome of this study would be to apply the findings to aid animal breeding units to decide whether MVMM supplementation is necessary for optimizing the health and maintenance of their breeding stock of SHR rats which can be prone to a variety of health problems that accompany hypertension.

2 LITERATURE REVIEW

2.1 Vitamin and Mineral Supplementation: A Brief History

The specific components of food that provide vital nutrition were officially named 'vitamins' by biochemist Casimir Funk in 1912, considered to be the 'father of vitamin therapy'. He, together with Sir Frederick Gowland Hopkins formed the hypothesis that a lack of one of these important vitamins and/or minerals would lead to the development of 'deficiency diseases' such as rickets and pellagra (Bellis 2013; Piro *et al* 2010). Subsequently in 1913 when the first vitamin 'thiamine' was isolated, it was found that its deficiency resulted in the tropical disease beri-beri (Piro *et al* 2010). However the use of vitamins and minerals via food sources to prevent disease goes back much further, to the Ancient Egyptian, Greek and Arabic periods. In 1750 Scottish sailor James Lind conducted the first controlled trial showing the importance of citrus fruit, which was later discovered to contain the vitamin C, in eradicating scurvy (Carpenter 2012).

The fortification of foods only began to occur in the early 20th century on a much larger-scale, starting in the United States of America (USA), in order to prevent nutritional disorders. This included the addition of iodine into regular table salt in 1924 to prevent goitre, followed by the fortification of milk with vitamin D in 1933 to prevent rickets. Then there was the fortification of flour with thiamine, riboflavin, niacin and iron in 1941 (McGinnis *et al* 2006).

In the mid-1930's grocery stores and pharmacy's alike started providing MVMM products containing more than the standard vitamins A and D to the public. This then lead to the development of the first MVMM supplement tablet in the early 1940's which contained a variety of vitamins and minerals given in one single dose for ease and convenience. To date there have been 15 essential minerals and 13 vitamins identified as key to our basic human nutrition and in which majority are found in most commercially available MVMM supplements (McGinnis *et al* 2006).

2.*1.1. Functions of Vitamins.*

Vitamins are organic compounds that cannot be manufactured in the body, and are required only in small quantities on a daily basis through the diet for the maintenance of a normal metabolism. The storage of vitamins in the body is, to a large extent, in the liver but can also be stored in fatty tissue (NHS 2015). Fat-soluble vitamins, such as vitamin A, can be stored in the liver in sufficient quantities to last for up to 5-10 months without any further intake. Vitamin D can also be stored in sufficient quantities in the liver for up to 2-4 months. Water soluble vitamin storage is relatively non-existent in comparison. As seen with most of the vitamin B complexes (all water-soluble vitamins), when a diet is deficient in one or more of the vitamin B compounds, clinical symptoms will begin to develop and show within a few days. A deficiency in vitamin C, also a water soluble vitamin, results in clinical symptoms

within a few weeks and if left untreated can eventually lead to decreased immune function, gingivitis and anaemia within 20-30 days (Evert 2013).

Certain vitamins can act as powerful antioxidants, having either a direct or indirect role in the antioxidant defence system. The vitamins directly involved in the antioxidant system are vitamins C (ascorbic acid) and E (tocopherols and tocotrienols). Vitamin C acts as a very powerful antioxidant and is commonly known as a 'radical scavenger' (McDowell *et al* 2007; Evans & Halliwell 2001; Sies, Stahl & Sundquist 1992). Vitamin E functions mainly within the cell membrane acting as a chain breaking antioxidant, where it neutralises free radicals and prevents the oxidation of the phospholipids in the membrane during oxidative stress. Studies have shown that only after the long term consumption of vitamin E supplements is there any significant decrease in cardiovascular associated diseases (McDowell *et al* 2007; Evans & Halliwell 2001). Both vitamins are also promoters of a greater immune response by increasing synthesis of interlukin-2 (IL-2), lymphocytes and macrophages (Maggini *et al* 2007).

Vitamins A and B6 act as antioxidants as well as play a vital role in the immune system. Vitamin A in the immune system is able to regulate the innate and cell mediated immune systems, as well as the humoral antibody response. Therefore a deficiency would lead to an increase in oxidative stress therefore leading to an increase in inflammation, macrophage activity and increased synthesis and release of IL-12 and tumor necrosis factor alpha (TNF-α). Therefore supplements containing vitamin A may decrease oxidative stress and inflammation in hypertension, as well as lead to an increase in antibody production, namely the lymphocyte cells Th1 and Th2 (Maggini *et al* 2007). Vitamin A in its precursor form as a carotenoid is also able to act as an antioxidant, especially as the precursor beta-carotene, which has shown to destroy free-radicals in lipid phases, as well as increase the effect of other antioxidants (Paiva & Russell 1999; Sies, Stahl & Sundquist 1992).

Vitamin B6 is an essential component for the synthesis of proteins and nucleic acids using amino acids, therefore a deficiency would directly affect antibody and cytokine production (Maggini *et al* 2007). In its capacity as an antioxidant, it is also able to deactivate free radicals, therefore inhibiting oxidative damage (Natera, Massad & Garcia 2012).

Vitamin D also plays a large role in the immune system, where most cells of the immune system contain vitamin D receptors for activation, for example antigen-presenting cells, T-cells and B-cells (Aranow 2011). As previously discussed vitamins C and E are powerful antioxidants, as well as promoters of a greater immune response by increasing synthesis of IL-2, lymphocytes and macrophages (Maggini *et al* 2007).

2.1.2 Functions of Mineral Elements.

Minerals are inorganic compounds found in foods, and are essential for general well-being and growth. The most important minerals required in human nutrition are: sodium (Na), potassium (K), calcium (Ca), phosphorus (P), sulphur (S) and magnesium (Mg). Cobalt (Co), also an essential mineral for human health, is supplied by vitamin B12. Vitamin B12, along with other co-enzymes named cobalamins, contains cobalt-carbon bonds making them the only organometallic complex to occur naturally in the body (Lindsay & Kerr 2011). Therefore a deficiency in Co can only be rectified by an increase in vitamin B12 as the body only accepts the metallovitamin form of Co (Gropper & Smith 2012). Carbon, oxygen, hydrogen and nitrogen are macro elements that are organically bound and occur in large amounts in mammals and are therefore essential minerals that form the basic building blocks for all life (Pani 2007). There is evidence showing that chromium, boron, and other inorganic elements have some part in human nutrition, but their specific role as yet has not been properly determined (Vincent 2010; Nielsen 1998).

Some of these minerals also have antioxidant properties, for example potassium has shown to have antioxidant properties in salt-sensitive hypertension, where it inhibits the cardiac diastolic dysfunction as well as neointima formation in the vasculature, mainly in the arteries. This in turn helps reduce reactive oxygen species (ROS) production, typical for salt-sensitive hypertension (Ando *et al* 2010). Magnesium has also shown to have antioxidant properties, especially in hypertension where it decreases oxidative stress, therefore preventing endothelial dysfunction, platelet aggregation and inflammation (Cunha *et al* 2011).

2.1.3 Functions of Trace Elements.

Trace elements are found in such minute quantities in the body hence they are called 'trace elements' (Underwood 1977). The amount of these elements found in food is typically low; however a deficiency in any one of these will most certainly lead to a specific deficiency syndrome (Nielson 1998). For example an iodine deficiency would lead to impaired thyroid activity and can affect ovarian function in women (Hidrioglou 1979). Examples of trace elements are: selenium (Se), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), iron (Fe), cobalt (Co), molybdenum (Mb), chromium (Cr), iodine (I) and fluoride (F) (Underwood 1977). Some of these trace elements also can exert their effects in the form of metalloproteins (mineral elements bound to a protein) as well as metalloenzymes (mineral elements bound to an enzyme) (Williams 2008). A good example is selenium which forms selenoprotein P (a metalloprotein) and/or glutathione perioxidase (a metalloenzyme), both of which have strong antioxidant properties (Arthur, McKenzie & Beckett 2003). Iron is also incorporated as a metalloprotein and is required for various cellular activities in the form of ferritin for red blood cell production, part of the heme molecule in cytochromes for energy production, haemoglobin and myoglobin for oxygen

transport and storage and catalases and perioxidases to neutralise ROS (Lu *et al* 2009; Laclaustra *et al* 2009; Hill *et al* 1996; Ellis 2006; Abbaspour, Hurrell & Kelishadi 2014; NIH 2015b). Zn has a co-active role in the enzymatic activity of superoxide dismutase (SOD), as well as being involved in metabolism, hormone production and immune function. Cu and Mn are both involved in reduction (REDOX) reactions as components of metalloenzymes for processes such as respiration and lipid metabolism and can also act as antioxidants (Gressley 2009). Trace elements Cu, Zn, Fe, Mn and Se are components of some of the most powerful endogenous antioxidant systems, playing an important role in cellular protection against oxidative stress. (Okuonghae *et al* 2011).

2.2 Therapeutic Uses of MVMM Supplements.

The primary use of a MVMM supplement is to treat a vitamin and/or mineral deficiency brought on usually by either primary or secondary malnutrition. Primary malnutrition is the most common cause of a deficiency, where the subject has limited access to the required essential vitamins and minerals due to a lack of food or variety of food available, resulting in protein-calorie malnutrition and/or a deficiency in macronutrients and micronutrients. Primary malnutrition of vitamins and minerals in the diet may also be caused by the poor quality of food available to the public due to lack of fortification and/or a failure to meet the required food industry standards. Other factors to take into account is the accessibility of good nutritious food, for example the ‘urban poor’ who don’t have the opportunity to grow and produce their own food, nor the time to prepare nutritious meals due to modern-day living (Gagné 2006). The occurrence of secondary malnutrition is linked to any diseases or disorder affecting a person’s appetite, digestion and/or the absorption of the required nutrients irrespective of whether they have access to adequate amounts of vitamins and minerals on a daily basis (Fuchs 1990).

2.3 Marketing of MVMM Supplements.

The marketing of the dietary supplement was not largely recognised until the late 1950’s when it took a huge leap forward after chemist Denham Harman, better known as the ‘father of the free radical theory of aging’, suggested that ageing was due to the damage caused by an increase in the production of free radicals. This in turn led to the possibility that antioxidants could slow down the ageing process, and thereby extend one’s life-span, something which has been difficult to prove consistently (Gutteridge & Halliwell 2010). However, some research has shown that when there is a deficiency in one or more micronutrients (vitamins, minerals, trace elements), mitochondrial oxidative decay as well as a breakdown of cellular membranes increases, thereby contributing towards the ageing process (Ames *et al* 2005). Various vitamins and minerals which have proven to have antioxidant properties, as previously described, have been marketed in the forms of supplements, foods, drinks and creams, with claims of slowing down ageing. The supplement and nutraceutical industries have promoted to the

general public that MVMM supplements and other antioxidants may well be the 'elixirs of youth' (Gutteridge & Halliwell 2010).

Over the years, there has been a steady increase in MVMM supplement use, especially in the United States. Multiple continuous surveys, called NHANES (National Health and Nutrition Examination Survey) are conducted in the USA. Data is collected every 2 years by the National Centre for Health Statistics to monitor the dietary supplement intake used by majority of its citizens. The first survey was conducted between the years 1971 to 1975 and estimated that 28% of men and 38% of women over the age of 20 were using supplements. The second survey (NHANES II) conducted from 1976 to 1980, found there was a noteworthy increase in supplement use amongst the general population, especially among women (43%) when compared to men (32%) over the age of twenty years. NHANES III, conducted between the years 1988 to 1994, showed that in total (combined percentage population of men and women) supplement use was at 42%. Another survey, conducted from 2003 to 2006, showed that 53% of the population were specifically using MVMM supplements (Gahche *et al* 2011). Women are the greatest consumers of MVMM supplements as well as individual component supplements such as folic acid, vitamin D and calcium which have also increased in use over the years (Mann 2011). Figure 1 below shows the progressive yearly increase in supplement use, especially amongst women who are the greatest consumers.

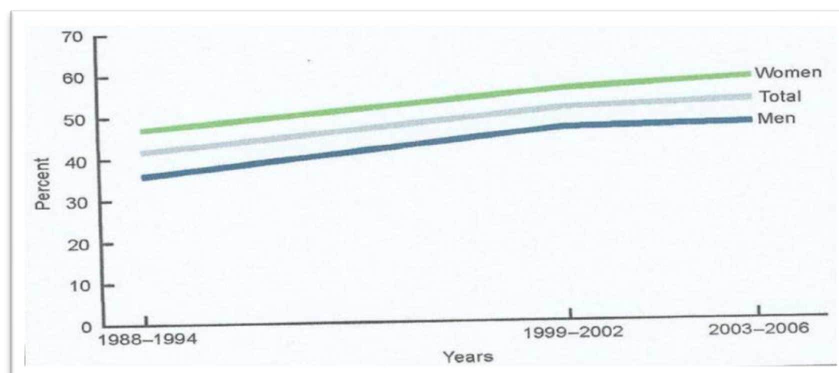


Figure 1: The NHANES surveys showing trends in the percentage of persons using dietary supplements, by gender, for adults aged 20 and over. Women are the greatest consumers, United States, 1988-2006 (Gahche *et al* 2011)

2.3.1 Reasons for Supplement Use

The main role of pharmaceutically manufactured MVMM supplements is to target specific vitamin and/or mineral deficiencies caused by either a primary or secondary malnutrition. However, through various high-impact marketing strategies, otherwise healthy individuals are now being encouraged to take MVMM supplements in order to promote their general body health status. The target market is the health conscious consumer who wishes to prevent and/or treat a disease. The marketing of the MVMM

supplements has also become more gender and age based with MVMM supplements being produced specifically for women, men and teenagers (Gahche *et al* 2011; NHS 2011). Therefore the greatest consumers are women and children, as well as the elderly and those who wish to recover from or prevent a chronic condition from reoccurring (Comerford 2013).

Possible reasons for the MVMM supplementation industry to show such positive growth over the years could be due to the public's desire to remain in good health, slow down aging and prevent diseases. The marketing of supplements, and specifically certain vitamins and minerals, has created an awareness of the possible positive effects in terms of their antioxidant capacity, as well as the increased accessibility of them in the form of supplements, cosmetics, fortified foods and drinks amongst other things (Gahche *et al* 2011; McGinnis *et al* 2006). Some researchers regard the MVMM supplement as a "nutritional insurance", a Miles Laboratories concept (NIH 2015a). This term is now being used mainly for marketing purposes, where the supplements provide an "insurance" against the effects of ageing, fatigue, cancers, strokes, maintain general health, improve antioxidant deficiencies, prevent fatigue, strengthen the immune system, as well as act as a supplementary or alternative treatment for chronic conditions such as hypertension, type-2 diabetes and cardiovascular disease (Ames *et al* 2005). The down turn of the economy in recent years has also played a role in the rise specifically in supplements where people opt for self-treatment using supplements rather than pay for exorbitant medical fees and medicine (Mann 2011).

2.4 Regulation of the MVMM Supplementation Industry

2.4.1 The Effectiveness of MVMM Supplements

Currently there is not enough reliable data to determine the effectiveness of MVMM supplementation in humans or animals. This would include the effect on populations who range from the healthy to the nutrient deficient, and those suffering from chronic conditions such as diabetes and hypertension. There are also methodological difficulties in scientific studies including the problem that there are many unknown factors and confounding variables to take into consideration, such as; individual health status, fortified food intake, cultural variability in diets and food sources, variability in vitamins and mineral content in similar food types, as well as the unclear Food and Drug Administration (FDA) guidelines for MVMM supplements (McGinnis *et al* 2006).

Furthermore there are certain shortcomings in the Recommended Dietary Allowance (RDA) and Upper Tolerable Intake Levels (UTI) concepts that are central to the supplementation industry.

- The RDA's is a guideline used to determine the ideal intake of vitamins and minerals required per day. Supplements whose components meet these RDA requirements should not run the risk of over-supplementation. Nonetheless, these requirements may vary significantly for each

individual according to factors such as body size (height and weight), rate of growth, amount of exercise, gender, pregnancy as well as the nutrient intake from food sources which may be already fortified, therefore leading to the risk of over-supplementation or not meeting the individuals specific nutrient requirement (Murphy & Barr 2006).

- The UTL's are defined as "the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population'' (WHO 1996). Some supplements do contain vitamins and minerals above the UTL are which increases the risk for over-supplementation, therefore having a counter-effect (Zlotkin 2006).

Previous studies that have been conducted to determine the efficacy of MVMM supplements may have been compromised due to the difficulty in controlling multiple variables. Even in self-report studies, test subjects may not have been taking the correct MVMM supplements, as well as not taking note of their dosage frequency, compromising accurate feedback as to the effectiveness of MVMM supplementation (McGinnis *et al* 2006). In animal studies, only individual supplement components have been predominantly studied. They also face similar challenges as human studies but have certain advantages.

These shortcomings can however be rectified to some extent by conducting more controlled tests, improving communication with consumers, providing scientific information to aid when choosing the correct supplement in order to avoid the risks of over-supplementation as well as developing an accessible and up to date data base on the exact composition of the various MVMM supplements available on the market today (McGinnis *et al* 2006).

2.4.2 Role of the Food and Drug Administration (FDA) in supplement regulation

The Definition of a dietary supplement as defined by the U.S. Congress in the Dietary Supplement Health and Education Act in 1994, states that "a dietary supplement is a product (besides tobacco) that is intended to supplement the diet; contains one or more dietary ingredients (including vitamins, minerals, herbs or other botanicals, amino acids, and other substances) or their constituents; is intended to be taken orally, capsule, tablet or liquid, and is labelled on the front panel as being a dietary supplement" (Gahche *et al* 2011).

According to the FDA Centre for Food, Safety and Applied Nutrition which monitors the supplement industry, dietary supplements are classed as a subcategory of food, and therefore do not need to be screened for safety or effectiveness. Supplement labels do however have to display the following for FDA approval:

1. The health claims they are making, between the dietary ingredient and the disease/condition it is meant to target.

2. The contents of the supplement (e.g.: vitamin and/or mineral content and their quantities)

3. The structure and function of the individual vitamins and minerals.

No FDA approval is required for supplements, as the main purpose is to describe the overall effect the supplement will have on the targeted structures/functions of the body, and/or the way in which the supplement will maintain these structures/functions of the body and provide general well-being (Marra & Boyar 2009).

The FDA does however maintain that the manufacturer put a disclaimer stating that the FDA has not proven that what they, the companies are claiming, is scientifically accurate. It is illegal for the label to state that the product can 'cure, treat or prevent' any disease (Marra & Boyar 2009).

The FDA is also unable to prevent any new dietary ingredient from being marketed, as long as the manufacturer informs the respective parties on the particular ingredient and provides sufficient information that the new ingredient is safe for consumption. However once a product has been marketed the FDA can remove it if it has shown to be unsafe through customer complaints or any adverse reactions from the consumers (Marra & Boyar 2009).

2.4.3 Risks Associated with Incorrect Supplement Use

Due to the lack of clarity concerning MVMM labelling and health claims, there are many contradictory reports questioning the effectiveness of these supplements. Numerous claims have been made that incorrect usage of MVMM supplements could lead to damage where the supplement's vitamin and mineral concentrations go beyond the RDA's and UTL's (McGinnis *et al* 2006). A study conducted on US adult citizens showed that the majority in the supplemented group were exceeding the UTL's due to the fact that subjects already had a vitamin and mineral intake from their various food sources (Bailey *et al* 2012).

Besides the risk of over-supplementation which can be harmful and/or toxic, there can also be adverse interactions between individual supplement components. There are also some vitamins and minerals in supplements that have been proven to be toxic or have adverse effects.

The following are some examples of vitamins and/or minerals that can be potentially harmful:

- β-Carotene (a precursor for Vitamin A) supplementation when it is above the UTL's, has been shown to increase the incidence of cancer, such as gastric cancer and particularly lung cancer in smokers (Druesne-Pecollo et al 2010).

- Vitamin A, when paired with β-Carotene or Zn causes an increase in lung cancer and cardiovascular disease. (McGinnis *et al* 2006).

- Se is known for its role as an antioxidant during oxidative stress usually associated with hypertension. However in quantities above the RDA's recommendation, it may have more of a pro-oxidant effect, and has been linked to a higher occurrence of hypertension (Laclaustra *et al* 2009).

- Zn and Cu are also important components of the antioxidant system and should both be present in an MVMM supplement in quantities of about 8 - 15 mg Zn for every 1 mg Cu in order to prevent an imbalance occurring. If there is an imbalance by an increase or decrease in either Zn or Cu there may be adverse effects. If for example, there is too much Zn, especially over a long period of time, this will cause a decrease in Cu absorption in the intestines, anaemia as well as a decrease in the body's antioxidant capacity (Osredkar & Sustar 2011; Vivoli *et al* 1995).

- Vitamin E studies have been inconclusive in determining whether vitamin E is beneficial or not, however it has shown to cause haemorrhages, decrease blood coagulation and inhibit platelet aggregation if taken in excessive amounts that are above the UTL's (NIH 2013).

- Fe can act as a pro-oxidant in its ferrous form (Fe II and III) where it is involved in the Fenton reaction which results in the oxidation of organic matter. Therefore when there is an overload, there is an increase in oxidative stress and lipid peroxidation (Kohgo *et al* 2008; Barbusiński 2009) Studies have indicated that when vitamin C and Fe are paired together, especially at higher concentrations, they also have a pro-oxidant effect, causing changes to leucocyte DNA base pairs, therefore affecting the immune system (Evans & Halliwell 2001).

- Some added components of MVMM supplements may possibly increase oxidative stress by being targets themselves for oxidative damage if the ratio of nutrients is not balanced. (Laclaustra *et al* 2009). For example the omega 3 and 6 polyunsaturated fatty acids (PUFA's) are major targets for lipid peroxidation due to their high lipid content and structure containing double carbon bonds (Ayala, Muñoz & Argüelles 2014; Halliwell & Chirico 1993).

2.5 Animal Health Sector

Predictably, the MVMM supplementation industry has now also spread into the veterinary and animal health sectors with a large number of preparations and combinations being made available for livestock,

poultry and companion animals, all with similar health promoting purposes as for humans (NASA 2002). New MVMM formulations using nano emulsion technology allows for multiple supplements to be presented together in one suspension and delivered simultaneously in a single bolus (Srinivas *et al* 2010). However there is a concomitant need to include more research with a specific focus on the effects and possible health risks associated with both human and animal MVMM supplementation (Comerford 2013). The National Animal Supplement Council (NASC) also raises the concern that there is no proper regulation for animal supplementation and many of the marketed supplements contain unsafe and/or unapproved ingredients, jeopardizing the safety of the animals as well as humans, where the supplemented animals are used as a food source (NASA 2002).

Experimentation and research testing the effectiveness of a specifically designed animal MVMM supplement in specially bred or established animal models could be a good indicator as to what might be expected of MVMM supplementation in humans. Especially if that animal, through genetic modification, is bred to reflect a chronic lifestyle disease such as diabetes mellitus or hypertension which are widely prevalent in modern day society. Information from these studies in turn can be extrapolated to humans or alternately provide a basis for future research in human studies.

2.5.1 Nano technology in Animal Supplementation

Nano technology, as defined by Professor Wang, is the construction of functional structures at an atomic and/or molecular level. This allows these molecules to exhibit novel properties at physical, biological and molecular level (Wang n.d.). Nano technology in recent years is also now being utilized by the cosmetics industry, pharmaceutical companies and in food and dietary supplements due to their ability to deliver bioactive material effectively (Kosowski 2014).

Nano capsulation in supplements allows for the protection and controlled release of various micronutrients trapped in the nano particles. This therefore allows for nutrients or drugs to be administered more effectively by a variety of delivery systems, increasing bioactive food or drug components in the circulation and improving bioavailability. With the ability to now combine water soluble as well as fat soluble vitamins and minerals and deliver the combination in one bolus, effective delivery of supplements is increased (Srinivas *et al* 2010; Rajendran 2013). Nano technology allows for more effective absorption of a variety of nutrients but the drawback is that overconsumption and toxicity could be a major risk. The FDA has stated that altering the physical and chemical states of these properties in food could alter their absorption, metabolism and distribution, as well as their biological interactions which can lead to toxic effects, and that more research is required to investigate any adverse effects nano technology cause in these systems (Kosowski 2014).

The application of nanotechnology to the field of nutrition and specifically animal nutrition has however shown positive results so far with animal growth, reproduction and immunity. Not enough research in the animal health and nutrition sectors has been conducted as yet in order to fully understand and explain the efficiency of nano minerals, as well as investigating the possible toxic effects they may cause (Kosowski 2014). Future fields of exploration in the field of nano technology include the creation and production of nano medicine to increase delivery and effectiveness of conventional medication (Srinivas *et al* 2010; Rajendran 2013; Joye, Davidov-Pardo & McClements 2014).

2.5.2 Relationship with the Biomedical Resources Unit (BRU) at UKZN.

The study was also conducted in response to a problem area in our facility, the BRU, which inter alia breeds, houses and maintains a wide variety of rat strains such as the Wistar rats, Sprague Dawley, Dahl salt sensitive and the spontaneously hypertensive (SHR) rat models. The latter two strains serve as models for the study of salt sensitive and genetic hypertension respectively, and are used extensively in research projects both at this and other research institutions. Unfortunately, as these animals age, they develop various sequelae of hypertension like lethargy, cardiovascular complications, decreased memory and learning as well as loss of libido and fertility, therefore requiring increased husbandry to maintain their health status (Linz *et al* 1999; Hernandez, Hoifodt & Terry 2003; Pepeu 2004; Meneses & Hong 1998; Azu 2013).

Therefore, by introducing a MVMM supplement, we would not only be able to observe its effect on the pathogenesis of hypertension, but also whether MVMM supplementation could assist in maintaining the general health status of the breeding stock and to extend and improve the quality of animal life. The outcome of the study could therefore provide recommendations to the BRU as to the use of supplements for their breeding stock

In this particular study, the animals were in a constant and controlled environment, a high quality quantified MVMM supplement supplied by an international manufacturer was used, there was accurate and controlled dosage, a strict scientific protocol and timeline were followed and standard scientific procedures were used in data analysis.

2.6 The Crisis of Hypertension

Hypertension has been branded a 'silent killer" as it can go undetected for long periods of time and it has no early warning signs or symptoms. Over time uncontrolled blood pressure can lead to various complications such as heart attacks, strokes and kidney failure (WHO 2013; CDC 2015) Hypertension or high blood pressure is responsible for approximately 9.4 million deaths worldwide, and is a major risk factor for CHD and strokes (WHO 2013; WHF n.d.). According to WHO, in 2008 40% of the global adult population (25 and over) were diagnosed with hypertension, with the number of people

suffering with this condition having risen from 600 million in 1980 to 1 billion in 2008. This still does not account for the large number of people who are not even diagnosed. It is one of the most common health problems in developed, as well as in developing countries which have weaker health care systems and where majority of patients usually go undiagnosed, causing hypertension to become uncontrollable and eventually untreatable (WHO 2013).

In South Africa there are approximately 6.2 million people with a blood pressure of 140/90 mm Hg or higher, and of that 6.2 million, 3.2 million have a blood pressure exceeding 160/95 mm Hg. There is a greater prevalence of hypertension among women, especially black African women, compared to the general male population. These statistics however are not complete, as there are a high number of people who have not as yet been diagnosed (Hasumi & Jacobsen 2012; Steyn 2005; Rayner 2014).

Hypertension is defined as a pathological condition where the arterial pressure is raised, and the systolic blood pressure (SBP) value is 140 mmHg and above, and diastolic blood pressure (DBP) is 90 mmHg and above (Kashyap *et al* 2005; Sulakova & Feber 2013).

Table 1: Classification of Blood Pressure Levels (Adapted from the Heart Foundation 2010)

Category	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Normal	< 120	< 80
Pre-hypertensive	120 - 139	80 – 89
Stage 1 (mild) hypertension	140 - 159	90 – 99
Stage 2 (moderate) hypertension	160 - 179	100 – 109
Stage 3 (severe) hypertension	≥ 180	≥ 110

Hypertension usually results in end organ damage such as nephropathy (renal failure and proteinuria), vasculopathy (endothelial dysfunction, remodelling and aneurysms), heart disease (myocardial infarction and heart failure) and cerebrovascular damage (strokes and retinopathy) ultimately leading to a reduced lifespan and death. However early detection and control of hypertension can prevent further organ damage, slow down its progression and even allow for regression where the damage is still in its reversible stage (WHO 2013; Schmieder 2010). Conventional control and treatment of hypertension include angiotension-converting enzyme (ACE) inhibitors, vasodilators and diuretic drugs accompanied by changes in an unhealthy lifestyle. Treatment can cause a decrease by 40 % in strokes, and up to 50 % in myocardial infarction. Treatment with beta blockers causes a decrease in associated cardiovascular disease (CVD) by between 15 % to 45 % (Rafey 2013; Law, Wald & Morris 2003; Oparil *et al* 2003).

2.6.1 Essential Hypertension

The term "Essential hypertension" is used where no one cause can be identified, and it is also the most common form of hypertension (Beg, Gupta & Khanna 2010). Research has suggested that essential hypertension may be genetically linked with loci on multiple genes. Apart from being polygenic, hypertension is also multifactorial, where factors such as disruption to the renin-angiotension-aldosterone system (RAAS), increased activity of the sympathetic nervous system (SNS), dysfunction in the vasculature (endothelium, smooth muscle cells) and impaired platelet function among other factors, increase the risk for developing hypertension (Kashyap *et al* 2005; O'Shaughnessy 2001; Wang & Peng 2013).

Other types of hypertension that are not as common include:

- Secondary (non-essential) hypertension, where the cause is known, for example sleeps apnoea, thyroid problems and excess alcohol consumption.
- Resistant hypertension which is rare and usually genetically linked (Lliades 2009).
- Malignant hypertension characterised by acute frequent and severe increases in blood pressure, and is usually associated with a high mortality and morbidity rate (Kitiyakara & Guzman 1998; Lima *et al* 1999).
- Renovascular hypertension arises from renal-artery stenosis which in turn activates the renin-angiotensin-aldosterone system (RAAS), leading to elevation in blood pressure (Higashi *et al* 2002).

2.6.2 Pathophysiological Mechanisms for the Development of Essential Hypertension

The equation for the arterial pressure is the cardiac output (CO) multiplied by the total peripheral resistance (TPR), which is derived from the standard equation for CO which is the arterial pressure divided by the TPR (Mayet & Hughes 2003). TPR is the resistance determined in the pre-capillary vessels, i.e.: arterioles and small arteries (Oparil *et al* 2003). Therefore any factors effecting the CO or TPR will have a direct effect on the arterial blood pressure leading towards the development of hypertension. Figure 2 demonstrates the many possible factors that can affect TPR thereby altering BP.

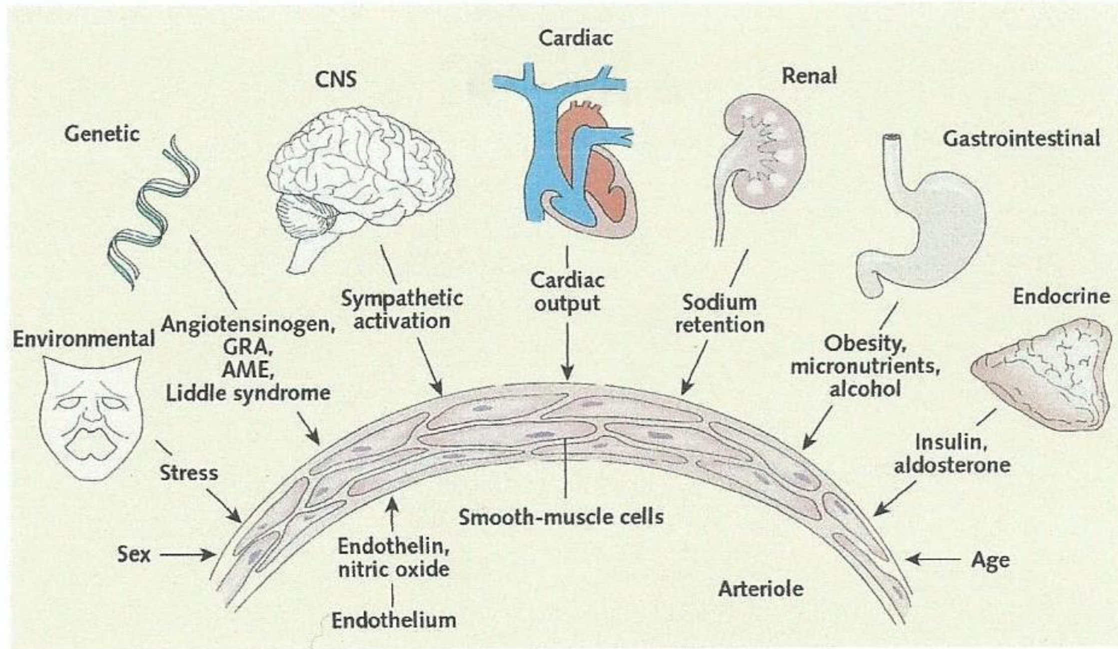


Figure 2: Factors affecting the total peripheral resistance in blood vessels that can lead to the development of hypertension (Adapted from Oparil *et al* 2003)

2.6.2.1 Genetics and Hypertension

Hypertension does not develop solely from a genetic predisposition in humans, but also involves environment factors. Genetic influences have been demonstrated in population studies amongst families who have similar blood pressure profiles (Longini *et al* 1984). Chromosonal studies have shown that there may be numerous genetic loci each exerting an effect on the blood pressure, resulting in essential hypertenison being classed as polygenic (O'Shaughnessy 2001). One study has shown that there are at least 2 loci in the genes, one Y-linked and the other autosomal, which are responsible for the inheritance of hypertension (Ely & Turner 1990). However the possibility of identifying a single-gene responsible for hypertension is highly unlikely as it is more likly a combination of multiple genes and other biological or external factors (Oparil *et al* 2003).

2.6.2.2 Renal Damage and Hypertension

The Na^+/K^+ ratio, plasma creatinine, blood urea nitrogen (BUN), urine output and water intake are all parameters typically indicative of kidney function (Duarte, Zhang & Ellis 2001; Gowda *et al* 2010). Kidney damage due to hypertension leads to renal dysfunction shown by a decrease in the urine output, increase in the BUN, and a decrease in the creatinine clearance (Karam *et al* 1996). Potassium (K^+) has a vasodilator effect in the endothelial cells of blood vessels. Excess K^+ can lead to vasodilation induced increase in blood flow by stimulating the endothelial cells under the smooth muscle cell layer in the blood vessel (Haddy, Vanhoutte & Feletou 2006).

K^+ is a vital component for neural communications via the glial cells, and can be released by endothelial cells (Eckman & Nelson 2001). During oxidative stress and increased lipid peroxidation there is damage to the red blood cells (RBC) membrane resulting in a "leakage" intracellular K^+ ions , leading to further damage in the form of lipid peroxidation in the RBC's (Halliwell & Chirico 1993; National Academies 1980).

BUN measures the amount of urea nitrogen in the blood and whether the kidney is excreting it sufficiently or not. Therefore when BUN levels increase this is a typical marker for renal dysfunction (HIN 2012). Creatinine is a waste product from the breakdown of creatinine in the muscle tissue, and is filtered through the kidneys and excreted in the urine. This is a good indication of kidney function, where the kidneys ability to deal with creatinine, known as the creatinine clearance, helps determine the glomerular filtration rate (GFR) in the kidneys (Perrone, Madias & Levey 1992).

2.6.2.3 Other Factors in the Pathogenesis of Hypertension

- Stress leads to the overstimulation for the sympathetic nervous system, which plays a vital role in blood pressure regulation. This leads to an increased heart rate and increased stimulation of the kidneys and peripheral vasculature causing fluid retention, increased CO and TPR, and ultimately high blood pressure (Esler 2000; Oparil *et al* 2003).

- Obesity is linked to an increase in TPR and has also been shown to cause activation of the SNS as well as the renin-angiotensin system. Adipose tissue acts as an endocrine organ secreting bioactive substances such as TNF-α and IL-6 which are all involved in the development of hypertension (Oparil *et al* 2003; Yanai *et al* 2008; Lavie & Messerli 1986).

- A high salt diet or the over-production of salt-retaining hormones like aldosterone may also elevated sodium levels, leading to an increase in blood pressure (Oparil *et al* 2003).

- Gender has also shown to play a role, where females are less likely to develop hypertension early in their lives compared to their male counterparts (Daugherty *et al* 2011).

- Age plays a major role in the pathogenesis of hypertension where increasing age causes an increase in aortic thickness and stiffness, therefore making hypertension an age-related disease (Pearson *et al* 1994; Afolayan & Wintola 2014).

2.6.2.4 The Renin-Angiotensin-Aldosterone System (RAAS) in Blood Pressure Control

The RAAS is one of the most important control systems for blood pressure regulation as it affects renal function as well as vascular tone (Yanai *et al* 2008). Renin, a proteolytic enzyme in the juxtaglomerular (JG) cells in the walls of the afferent arterioles is released when there is a decrease in systemic blood

pressure (Persson 2003). Renin enters the blood where it converts the plasma protein angiotensinogen into angiotensin I (Ang I), a precursor for angiotensin II (Ang II). The ACE, produced in the lungs, converts the Ang I to Ang II, which then in turn binds to AT_1 and AT_2 receptors. This activates various mechanisms to increase blood pressure including vasoconstriction of the peripheral blood vessels, increased aldosterone secretion and decreased salt and water excretion by the kidneys (Bachmann *et al* 1992).

One of the numerous ways in which to treat hypertension is to inhibit the formation of Ang II using ACE inhibitors. This therefore prevents the constriction of blood vessels and lowers blood pressure (Sweitzer 2003; Freiberg *et al* 1994). Elevated levels of ACE activity in the blood can be an indicator for the development of hypertension (Rakugi *et al* 1994).

Aldosterone, produced in the cortex of the adrenal gland is activated and released in the circulatory system by elevated levels of Ang II, causing sodium re-absorption in the kidney. If elevated it can exert hypertrophic effects on the heart and alter the endothelial function of the blood vessels. Elevation of aldosterone production and secretion would lead to excessive sodium re-absorption and vasoconstriction. It has also been implicated in fibrotic and renal inflammatory processes (Briet & Schiffrin 2010; Wenzel 2008).

2.6.2.5 Oxidative Stress in the Pathogenesis of Hypertension

Essential hypertension has various underlying mechanisms leading to its development as previously discussed, and not all are fully understood. However research has shown that there is a positive correlation between oxidative stress and hypertension, and that oxidative stress plays a central role in its pathophysiology. Through increased knowledge in oxidative stress and its various mechanisms, particularly in hypertension, new strategies are developed in order to combat it mainly in the form of antioxidants, such as vitamins C and E, and minerals Se, Zn and Cu. These antioxidants may exert different effects, but all aim to reduce oxidative stress that can ultimately lead to the development of hypertension (Mylonas &Kouretas 1999).

Oxidative stress is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to tissue damage" (Sies 1997). During aerobic metabolism in cells, there is generally the production of ROS. However the rate and extent of ROS being produced is usually balanced by the rate and magnitude of oxidant removal by the antioxidant systems. When there is an imbalance between these pro-oxidants and antioxidants, with an overproduction of oxidants, oxidative stress occurs, as the antioxidant capacity of the cells have been overwhelmed (Beg *et al* 2010).

ROS, along with antioxidants, are also involved in many regulatory functions at a biochemical and physiological level in various organ systems. They are involved in hormone biosynthesis, fertilization,

host defence, cellular signalling and the regulation of blood vessel endothelial function and vascular tone. However when there is an increase in the production of ROS or decreased levels of antioxidants, the balance between the two is disturbed. This may then lead to one of many chronic pathophysiological conditions including hypertension, arthrosclerosis, cardiac hypertrophy, heart failure, diabetes and chronic kidney disease to name but a few (Paravicini & Touyz 2008).

ROS are also known as “oxygen-derived species” or “oxidants” and comprise of 2 major groups:

a) Free Radicals, which can exist independently but contain 1/more unpaired electrons.

e.g.: superoxide ($\bullet O_2^-$), hydroxyl ($OH\bullet$), nitric oxide ($NO\bullet$)

b) Non-radical derivatives of O_2, which are more stable, less reactive, have a longer life-span, and are therefore more likely to cross cell membranes.

e.g.: Hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Paravicini & Touyz 2008).

There are 2 main sources of ROS:

a) Exogenous ROS - produced from pollution, cigarette smoke, drugs, UV light and radiation.

b) Endogenous ROS - produced intracellularly in all cell types (endothelial cells, smooth muscle cells, adventitial cells) (Frei 1994).

Antioxidants are defined by Gutteridge & Halliwell (1989) as “any substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate” (Sies 1997). The antioxidant defense system is an extensive and layered system that protects at different sites, and caters for the various ROS produced in the body (Beg *et al*, 2010). Antioxidants can be sourced exogenously from the diet where they naturally occur, or even supplied through dietary supplements, for example Vitamin A, C, E and beta-carotene (Frei 1994). They are also produced endogenously in the body in the form of antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase) and metalloproteins (ferritin, metallothionein and ceruloplasmin). They are essential in counteracting the effects of the pro-oxidants by limiting the oxidative damage, and maintaining a balance in order to allow normal metabolic processes to occur by reducing oxidative stress (Sies 1997).

It has been shown that ROS play a vital role in maintaining endothelial function and vascular tone; therefore as a result there is a direct link between oxidative stress, which causes an increase in ROS production, and the development of hypertension (Swei *et al* 1997). The most important ROS associated

with the pathogenesis of hypertension are the superoxide radical, hydrogen peroxide, hydroxyl radical and nitric oxide. Studies have shown that there is a direct relationship between the mean arterial pressure and the hydrogen peroxide levels (Swei *et al* 1997). Some radicals, such as nitric oxide, have a dual role, acting either as either a pro-oxidant or antioxidant depending on its location and the various factors/stresses affecting its production.

2.6.2.6 Mechanisms of Oxidative Stress

Nitric oxide, produced in the mitochondria and cytosol of the endothelial cells, has the ability to act as a vasodilator. Nitric oxide production and release is triggered by chemical or physical stimuli like elevated blood pressure, pulsatile stretch and shear stress. Shear stress occurs when the blood in the vessels drag against the walls, causing the production and release of nitric oxide which in turn leaves the endothelial cell of the arterioles and moves into the surrounding smooth muscle cells. It then causes various physiological responses such as relaxation of the smooth muscle, inhibition of platelet aggregation and suppression of the migration and proliferation of vascular smooth muscle cells (Förstermann 2010; Oparil *et al* 2003). It's this ability of the nitric oxide to act on the surrounding tissue to relax and cause dilation of the blood vessels and therefore lowering the blood pressure, which makes it so vital in blood pressure regulation (Oparil *et al* 2003).

There is now a significant amount of research proving the link between oxidative stress, endothelial dysfunction and the development of hypertension. In endothelial dysfunction, nitric oxide, which acts as a vasodilator and is released by the endothelial cells, is inactivated by increased superoxide levels during oxidative stress. Since nitric oxide's half-life is only a few seconds it can be rapidly degraded by the superoxides (Kashyap *et al* 2005). This is accompanied by endothelin release from the endothelial cells which reduces vasodilation, and increases vasoconstriction/contractility and eventually leads to the development of hypertension. Studies have shown animal models with compromised antioxidant system resulted in the development of hypertension (Beg *et al* 2010). Previous studies have also shown that ROS produced in other organ systems besides the endothelium of the vascular system, such as the heart, kidneys and nervous system; also contribute towards the development of hypertension (Beg *et al* 2010).

Other evidence linking nitric oxide to hypertension is its role in the inhibition of lipid peroxidation. During classic lipid peroxidation there is usually a production of free radicals, mainly hydroxyl radicals and hydroperoxyl radicals, which attack mainly lipids containing carbon double bonds in polyunsaturated fats, glycolipids, cholesterol and phospholipids. The attacked cells respond in one of 2 ways:

a) If the lipid peroxidation is at a low level then cell survival will be promoted through various antioxidant defense systems and/or activation of signaling pathways to produce more antioxidant proteins (Ayala *et al* 2014).

b) If lipid peroxidation is at a medium to high level then the cells' repair capacity will become overwhelmed and cell apoptosis and/or necrosis will occur. This damage at molecular level in turn contributes to structural damage to the cell membrane, including an increase ion permeability, altered fluid exchange and channel dysfunction leading to a disruption in membrane-dependent signaling. There is also increased toxicity from the products of the lipid peroxidation, as well as deoxyribonucleic acid (DNA) damage, mutagenesis and accelerated cell ageing. This may all in turn contribute towards the renal and vascular damage commonly seen in hypertensive patients (Kashyap *et al* 2005; Russo *et al* 1998). Therefore lipid peroxidation inhibition by nitric oxide reduces the occurrence of these effects (Hogg & Kalyanaraman 1999; Ayala *et al* 2014).

Previous studies have shown that essential hypertension is associated with elevated levels of lipid peroxidation, emphasising the role oxidative stress plays in the pathogenesis of hypertension. Therefore testing the extent of tissue lipid peroxidation is a good way to determine oxidative stress (Ayala *et al* 2014; Russo *et al* 1998).

There is a positive correlation between essential hypertension, antioxidants and the ROS. Consequently, hypertension can be linked to either a decreased availability of antioxidants, or an increase in oxidant production resulting in the development of free radicals.

2.6.2.7 Inflammation and Hypertension

Inflammation is commonly known to accompany hypertension due to vascular damage and endothelial dysfunction. Plasma C-Reactive Protein (CRP) is a powerful biomarker for the presence of inflammation. High levels of CRP are usually observed in bacterial infections, Crohn's disease, rheumatoid arthritis, myocardial infarction and atherosclerotic vascular disease. In hypertension where CRP levels increase along with vascular stiffness, atherosclerosis and end-organ damage (Hage 2014; Clifton 2003; Semple 2006; Yanai *et al* 2008; Du Clos 2000).

Research has shown a direct relationship between hypertension, inflammation and CRP levels. It has been accepted that elevated CRP is a biomarker for hypertension and an independent risk factor for CHD and the metabolic syndrome (Bautista *et al* 2001). Testing for plasma CRP levels could be an added diagnostic criterion for these conditions. (Ridker, Wilson & Grundy 2004; Grundy 2003; Semple 2006).

The synthesis and release of CRP mainly occurs in the liver hepatocytes due to stimulation by inflammatory mediators; however it can also be released in the tubular epithelial cells and even the epithelial cells in the respiratory tract (Semple 2006; Hage 2014; Cheung *et al* 2012; Savoia & Schiffrin 2007; Rosalki 2001).

Inflammatory mediators, such as TNF-α and IL-6 are but a few of the inflammatory triggers for CRP elevation seen in the pathophysiology of hypertension. Previous studies have shown that when these inflammatory markers are elevated, there is a direct relationship with elevated blood pressure, heart rate and insulin resistance. All these factors contribute towards the development of metabolic syndrome and its associated hypertension (Yanai *et al* 2008).

As previously mentioned ACE is a vital component of Ang II production which if elevated abnormally can in turn cause vascular damage via various mechanisms, one of which is inflammation. Ang II modulated inflammation induces the release of cytokines (TNF-α & IL's), plasminogen-activator inhibitor-1 (PAI-1) which increases thrombosis, and inflammatory factors such as nuclear factor-kappaB (NF-kB), resulting in vascular wall inflammation, depletion of extra-cellular fluid (ECF) and hypertrophy and/or hyperplasia of the vascular smooth muscle cells. Ang II is also involved in the activation of matrix-metalloproteinases (MMPs) which cause plaque instability and plaque ruptures in the vasculature (Savoia & Schiffrin 2007).

Ang II, via increase in ACE, is also able to modulate and increase activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, one of the main sources of ROS in the vasculature and expressed in the endothelial cells. This results in increased activation of various NADPH oxidase subunits, leading to increased oxidative stress in the vascular wall. The resulting vascular damage leads to a decrease in NO release from the endothelial cells, and an increase in vascular contraction (Savoia & Schiffrin 2007).

2.6.3 Trace element Status in Hypertension

Trace elements such as copper (Cu), manganese (Mn), selenium (Se), magnesium (Mg), iron (Fe), silicon (Si), cobalt (Co), lithium (Li), vanadium (V) and zinc (Zn) are all classed as essential trace elements. It has been demonstrated that a deficiency in one or more of these elements, especially Zn, Cu, Se and Mn, increases the risk of hypertension and cardiovascular disease development. During a deficiency in these trace elements, is has been shown that there is an increased uptake from the diet in these toxic elements such cadmium (Cd), lead (Pb), silver (Ag), mercury (Hg) and thallium (TI) which also have been shown to increase the risk for hypertension and cardiovascular disease. The main trace elements that have the greatest impact on blood pressure regulation and the maintenance of the vascular

system are Zn, Cu, Fe, Se, Mg and Mn especially during dietary insufficiency or metabolic imbalances (Tubek 2006).

2.6.3.1 Role of Trace Elements in Hypertension

Table 2: Elements and their Effect on Blood Pressure.

Increase Blood Pressure	Decrease Blood Pressure	Combined
Sodium	Potassium	Cadmium
Iron	Magnesium	Lithium
Lead	Zinc	Selenium
Arsenic	Calcium	Mercury
Thallium		
Copper		
Barium		

(Adapted from Loyke 2002)

2.6.3.2 Antioxidant Associated Trace Elements in Hypertension

Trace elements, when bound to proteins and enzymes are referred to as metalloproteins and metalloenzymes respectively (Martin, Puerta & Cohen 2014; Hogbom *et al* 2005; Nadar & Lip 2009).

The metalloproteins, apart from their role in metal transport and storage, also function as antioxidants primarily binding to active metal ions such as copper and iron in order to prevent the production of ROS via the Fenton reaction (Kodali *et al* 2012; Maggini *et al* 2007; Lemire, Harrison & Turner 2013).

These metalloproteins include ceruloplasmin (Cu) metallothioneins (Cu, Zn, Hg), albumin, (Cu) transferrin (Fe), ferritin (Fe) and myoglobin (Fe). These proteins are mobilized during oxidative stress and acute-phase responses during trauma (Evans & Abraham 1973; Nordberg & Nordberg 2000; Roche *et al* 2008; Beard 2001). Metal binding enzymes known as metallo enzymes, are also involved in cellular antioxidant defenses and have trace elements as integral components, include superoxide dismutase (Zn, Mn,Se), glutathione peroxidase (Zn, Mn, Se) and catalase (Fe) (Maggini *et al* 2007; Zhan *et al* 2004; Laclaustra *et al* 2009; Ognjanovic *et al* 2008).

Trace elements therefore play a vital role in the normal regulation of blood pressure, as well as the antioxidant defence systems within cells and in the blood, especially in the forms of metalloproteins and metalloenzymes (Loyke 2002; Kodali *et al* 2012; Nadar & Lip 2009; Odusan 2012). Oxidative

stress plays a major role in the pathogenesis of hypertension and a deficiency or imbalance in trace element metabolism could therefore potentially lead to increased free radical production. By determining trace element levels in blood pressure regulatory organs such as the kidney, brain and liver, one can assess firstly to what extent their profile is altered due to hypertension, and secondly by gauging in an indirect manner the antioxidant system status.

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) and Energy Dispersive X-ray analytical system and Scanning Electron Microscopy (EDX – SEM) are two of the most advanced analytical methods for elemental analysis in a variety of materials including plant and animal tissue. Used together, they can provide an elemental content within tissue or a scan of their topographical distribution in specific areas of the tissue surface.

The ICP technique gives a more accurate display of specified element content, including trace elements in the whole tissue i.e.: inside and outside. However, even though ICP is more accurate, it is unable to differentiate between metal ions bound on the outer surface of the cell to those that have accumulated inside the cells, and cannot determine trace element content for specific areas of the tissue. Therefore EDX-SEM is used in a complementary fashion as it allows for the macro, micro and trace elemental composition to be mapped along the surface of the tissue in designated areas and determines the distribution of these metal ions along the surface of the cells. There seem to be more advantages than disadvantages for both techniques and both may be necessary in order to accurately determine elemental distribution of the tissue, using ICP for precise quantification, and EDX for a quantitative and qualitative overview (Michalak *et al* 2011).

2.6.4 Renal Pathology in Hypertension: End Organ Damage in the Kidneys and Blood Vessels

End organ damage in hypertension includes retinopathy, myocardial infarction, cardiac failure, strokes, vascular damage and renal failure (Schmieder 2010). The kidneys are particularly vulnerable to damage as they are greatly vascularised and have a high regional blood flow. High blood pressure damages vasculature in and around the kidneys, causing them to narrow and/or weaken, and therefore impairing delivery of sufficient blood to the kidneys. This affects normal kidney function in multiple ways (AHA 2014).

In the kidney the functional component is the nephron, with approximately 900 000 to 1 000 000 per kidney. They are unable to regenerate therefore any disease or injury as well as ageing causes a decrease in nephron number, leading to impairment in renal function (Weinstein & Anderson 2010; Bertram *et al* 2011). In hypertension the most common sign of end organ damage is nephropathy, which is defined by Schädlich as "disease induced renal glomerular damage", which occurs and can be detected during

the very early stages of hypertension through the mild occurrence of albuminuria and a decreased GFR (Schädlich *et al* 2001; Schmieder 2010).

The nephron, located in the renal cortex, comprises of the renal corpuscle and the renal tubule. The renal corpuscle contains the glomerulus, a network of anastomosing capillaries, which is supplied with blood by the afferent arteriole and drained by the efferent arteriole. The glomerulus filters small molecules and water from the blood plasma into the Bowman's space, thereby forming an 'ultra-filtrate'. The filtration area between the capillaries of the glomerulus and Bowman's space has various layers acting as a barrier preventing larger molecules from crossing including plasma proteins such as albumin and blood clotting factors. The layers of this barrier comprise of the capillary endothelium, podocyte layer and a glomerular basement membrane which are essential for proper filtration of the plasma. They are also the main target for injury, damage and/or scarring in various kidney disorders and during hypertension (Eremina *et al* 2003).

The glomerular capillaries display a high hydrostatic pressure for rapid filtration to occur, whereas the hydrostatic pressure in the proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) is lower to allow for reabsorption. The kidneys, by adjusting to the resistance of the afferent and efferent arterioles, can control the rate of filtration and absorption as a response mechanism to changes in blood pressure (Sherwood 2011). Glomerulus damage occurs due to various factors, including hypertension, which interferes with this internal arterial pressure regulation system.

Nephron damage caused by hypertension can be identified mainly by morphological changes of the glomerulus. Glomerulosclerosis is the most common indicator for renal damage, as well as the presence of tubular atrophy, vacuolation, dilation, protein casts (p-casts) and localised cell regeneration and apoptosis. These features may be accompanied by inflammation of the interstitium and hyalinization in the blood vessels, all which can be used as biomarkers/ indicators for chronic kidney disease and renal damage (Rouse *et al* 2013; Klahr, Schreiner & Ichikawa 1988).

2.6.4.1. Histopathological Indicators of Renal Damage in Hypertension

- Glomerulosclerosis is the scarring of the glomerulus caused by lesions and replaced by fibrous tissues. It occurs mainly in the glomerular capillary wall affecting the podocytes in the filtration barrier. Due to the compromised status of the barrier wall, abnormal ultra-filtrate, containing large amounts of plasma proteins and other larger molecules, filters through into the nephron causing tubulointerstitial damage as well as an inflammatory and apoptotic response from the mesangial cells (Zoja, Abbate & Remuzzi 2014). The mesangial cells are found in between the glomerular capillaries and are centrally located in the glomerulus to provide structural support.

They also have contractile properties, and by contracting or relaxing they can alter the GFR (Schlondorff 1987; Schlondorff & Banas 2009).

- Tubular dilation, seen mainly in the proximal tubule, is characterized by the flattening of the renal tubular epithelial cells, loss of brush border and occasional shedding of cells (Lerma, Agraharkar & Kelly 2014).

- Inflammation of the interstitium, characterized by an accumulation of monocytes and macrophages, may occur due to the production and release of chemokines, cytokines and growth factors from the mesangial cells in the glomerular capillaries and tubular epithelial cells, with the inflammatory cells moving into the interstitium (López-Novoa *et al* 2011).

- Hyalination is characterized by material deposits within the glomerulus and scarring of the glomeruli, and renal blood vessels, and is usually in conjunction with glomerulosclerosis (Maselle 2012).

- Atrophy is observed as cell shrinkage and in tubular atrophy there will be a decrease in the tubular diameter with a thick and wrinkled basement membrane (Vahed, Samadi & Ardalan 2014; Nadasdy *et al* 1994). Apoptosis occurs in all cells and is known as programmed cell death. It can be identified by cell shrinkage and nuclear condensation, and usually follows after or is in conjunction with atrophy (Rouse *et al* 2013; Venkatachalam *et al* 2010). Apoptosis under normal circumstances occurs in abundance to counterbalance cell division and growth, therefore it aids in balancing the number of renal, inflammatory, fibroblast and immune cells being produced after injury. However it may also lead to the loss of glomerular cells therefore may also contribute towards renal damage (Sanz *et al* 2008; Elmore 2007).

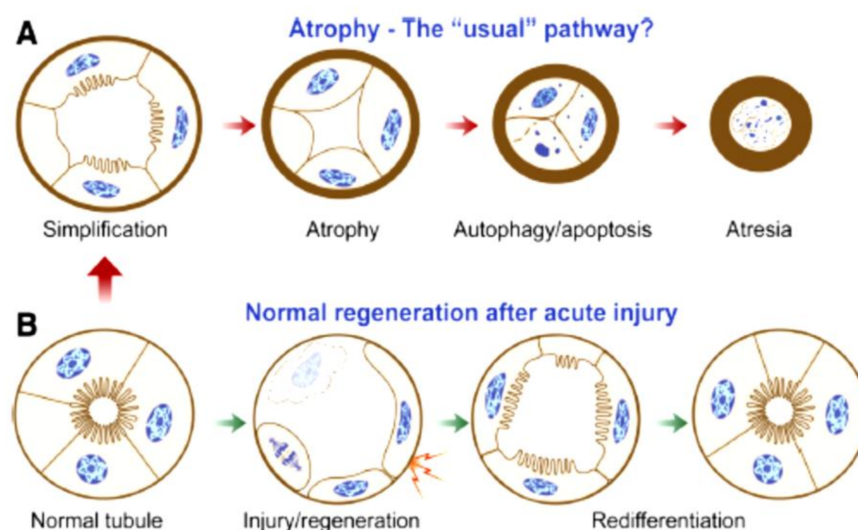


Figure 3: Diagram showing the progression of tubular atrophy, apoptosis and regeneration (Adapted from Venkatachalam *et al* 2010)

- Vacuolation, usually seen in the proximal tubule epithelium when the cytosomes such as the membrane-bound lysosomes are distended and there is dilation of the endoplasmic reticulum and various other cell components by ECF (Jennette 2007).

- Periartritis, also known as polyarteritis nodosa or panarteritis nodosa, is classified as having necrotising arteries, with small nodules along the walls of the arteries and commonly affects the celiac, renal and coronary arteries (Brogan *et al* 2002).

- P-casts in the renal tubules are characterized by flattened atrophic tubular epithelium surrounded by pink protein casts, which look similar to thyroid follicles and is a characteristic feature of vascular damage due to hypertension. When there is renal tubular damage the epithelial cells move into the lumen where they will mix with the material deposits from hyalinization of the tubule, forming first a cellular cast. However as degradation of the tissue occurs, the cast becomes coarsely granular then finely granular, until finally forming a waxy cast which is the final cast formation and an indicator for chronic tubular disease (Didio & Motta 2012).

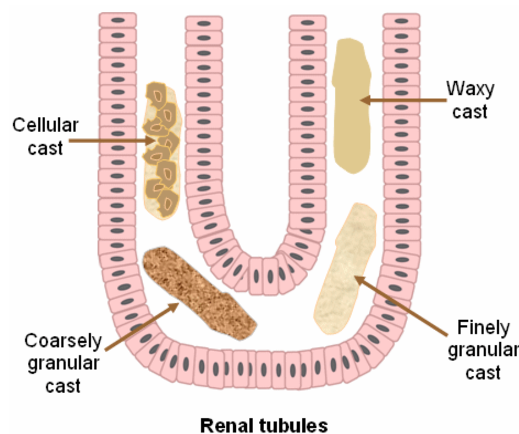


Figure 4: Types of Protein Casts in the Renal Tubules (Adapted from eClinpath 2012)

2.6.4.2. Vascular Remodelling and Hypertension

The basic structure of all arteries comprises of 3 layers, namely:

1. The tunica intima which is the innermost layer and is made up of endothelial and subendothelial layers containing collagen, elastic and smooth muscle cells.

2. The tunica media which is the middle and thickest layer contains smooth muscle cells as well as a large amount of elastic fibres, especially in the elastic arteries, with the elastin arranged on concentric circles allowing for the artery to stretch under pressure from the blood flow.

3. The final layer is the tunica adventitia which is the outermost layer and is usually very thin, comprising mainly of loose connective tissue, nerves, lymphatic's and blood vessels (Juang, Braverman & Eagle 2008; Krstic 2013; Intengan & Schiffrin 2000).

As hypertension progresses, there are numerous alterations in the structure, function and mechanical properties of large and small arteries (Mayet & Hughes 2003; Intengan & Schiffrin 2001). Structural alteration of the blood vessels usually involves either eutrophic or hypertrophic remodelling where there is usually an alteration in the media/lumen ratio (Intengan & Schiffrin 2001). This leads to an elevated TPR which in turn aids in further elevating blood pressure and leading to associated end-organ damage.

a) Eutrophic remodelling – where the outer diameter and the lumen diameter changes but the tunica media/lumen ratio is relatively unchanged. This is the most common remodelling seen in hypertension, in the SHR rat model and patients with mild essential hypertension. A combination of chronic vasoconstriction, along with activation in deposition of collagen and mild inflammation leads to a characteristic smaller lumen for eutrophic remodelling, which can therefore result in an increase in peripheral resistance (Oparil *et al* 2003; Intengan & Schiffrin 2001).

b) Hypertrophic remodelling – where the media thickness increases and lumen diameter decreases, resulting in an increase in media/lumen ratio and cross-section of the tunica media. This usually occurs in the 'one kidney, one clip' Goldblatt rat model of renal stenosis and the deoxycorticosterone acetate (DOCA) salt rat model, and in severe hypertensive states, This therefore can result in loss of elasticity in the arteries and increase blood pressure (Van Varik *et al* 2012; Intengan & Schiffrin 2001).

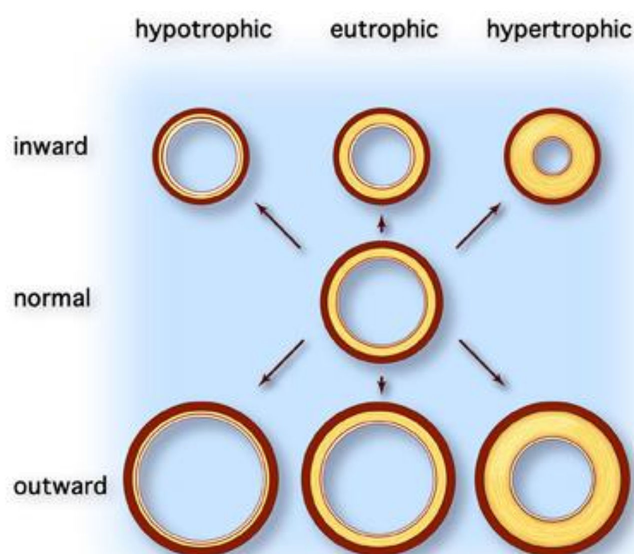


Figure 5: Eutrophic, hypotrophic and hypertrophic remodelling that can occur in the arterial blood vessels during cardiovascular diseases, especially hypertension (Adapted from Van Varik *et al* 2012)

Factors that contribute towards the vascular remodelling of arteries during hypertension are growth, inflammation, apoptosis and fibrosis. Apoptosis increases due to hypertension, where both ROS and Ang II can cause apoptosis in the vascular cells (Intengan & Schiffrin 2001). Oxidative stress and inflammation at low levels act as a trigger for fibrosis (Oparil *et al* 2003). Growth involves the tunica media which encroaches on the lumen area, leading to either eutrophic or hypertrophic remodelling (Intengan & Schiffrin 2001).

2.7 Animal Model Selection.

Various animal models of hypertension, predominantly rats, have been developed in the last five decades. It involves outbreeding an animal that displays spontaneous hereditary hypertension. Hypertensive animal models are widely used to study the pathogenic processes leading to hypertension.

The SHR are a descendant from a hypertensive Wistar male from a colony in Kyoto, Japan. This allows for Wistar rats to serve as control animals for the SHR rat model (Okamoto & Aoki 1963, Okamoto *et al* 1966; Kurtz & Morris 1987).

Hypertension in the SHR is genetically predisposed and multifactorial, with the SHR developing hypertension from five to six weeks of age, and the systolic blood pressure reaching 180 – 200 mm Hg during the adult phase (Yang, Zuo & Fogo 2010). The pathogenic sequence in this model is characterized first by endothelial dysfunction followed by smooth vascular muscle dysfunction, then a decrease in renal function due to proteinuria and renal damage in the form of glomerulosclerosis, tubular

atrophy, p-cast formation and juxtaglomerular damage - much as in a human subject with essential hypertension (Bernatova *et al* 2007; Bernatova 2014; Puzerova, Kopincova & Bernatova 2010; Balakumar *et al* 2007; Feld *et al* 1990). Female SHR rats with female Wistar controls were used in this study as the NHANES I, II, III and IV surveys has shown that MVMM supplement use is more prevalent amongst females than in males (Gahche *et al* 2011).

Previous studies with regards to nutrient supplementation in the SHR model focused on macro and micro nutrients which are known to play vital roles in the regulation of the blood pressure. Apart from their normal nutrient function, they additionally also play a major cellular protective role as antioxidants. In the literature there are various studies on the SHR model that have used mineral and vitamin supplementation either individually or in combination. They are as follows:

- Dietary vitamin B6 has shown to lessen the effects of hypertension in the SHR rat strains, specifically decreasing the SBP (Vasdev *et al* 1999).

- Ca and Mg supplementation in SHR has shown Ca to decrease the rate of hypertension development whereas the Mg seemed to be more dose dependent in order to lower hypertension (Makynen *et al* 1995).

- Dietary vitamin E and C supplement have shown, individually, to lower mainly the SBP in the SHR rat (Vasdev *et al* 2001; Vasdev *et al* 2002).

- Prolonged use of Ca and vitamin D supplements may preserve vertebral mineral content in the ageing Wistar rats (Schapira *et al* 1995).

- Lupeol is a triterpene compound found in fruits, vegetable and most medicinal plants and has displayed both anti-inflammatory and antioxidant properties. Lupeol supplementation in SHR rats, and showed a significant decrease the systolic blood pressure and suppressed the hepatic mRNA gene expression levels for genes concerned with triglyceride and cholesterol synthesis (Ardiansyah *et al* 2012).

As seen in the above studies, targeting hypertension with single vitamin and/or mineral supplements has produced positive results. The present study is unique as it uses a combination dose of vitamin and mineral nutrients that have been formulated using new nano emulsion technology that may also improve nutrient delivery and bioavailability (Houston 2005).

2.8 Reiteration of Aims and Objectives of this Study

The aim of the study was to determine the effects of MVMM supplementation on the spontaneously hypertensive rat (SHR) with reference to its health status and its effect on hypertension at a biochemical and morphological level.

This study constituted of three main parts:

1. The first part involved investigating the effects of MVMM supplementation on the general health status in two rat strains, namely the spontaneously hypertensive rat (SHR), a well-established animal model of hypertension, and the Wistar strain which served as a control.

2. The second part focused more specifically on the effects of MVMM supplementation on hypertension and its associated biochemical parameters. This also included oxidative stress and the antioxidant effects of MVMM supplementation.

3. The third part involved investigating the effects of MVMM supplementation on renal and vascular histology in the SHR after the establishment of hypertension.

A more practical objective of this study would be to apply the findings to aid animal breeding units in deciding whether MVMM supplementation is necessary for optimizing the animals' health and maintenance of their breeding stock of SHR rats which can be prone to a variety of health problems that accompany hypertension.

3 METHODS AND MATERIALS

3.1 Study Design

3.1.1 Ethical Approval

This study was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal, Durban, South Africa.

Ethics No. 026/15/Animal

3.1.2 Animal Grouping

Twenty-eight female rats, 14 SHR and 14 Wistar (WIS), weighing ±150 g and therefore aged between 38-42 days (6 weeks) old, supplied by the University of KwaZulu-Natal Biomedical Resource Unit (BRU) were used for the study. The SHR were randomly divided into supplemented (SHR S) and unsupplemented (SHR) groups of seven animals each. The Wistar animals, which normally serve as controls for the SHR strain, were also similarly divided into supplemented (WIS S) and unsupplemented (WIS) groups of seven animals each.

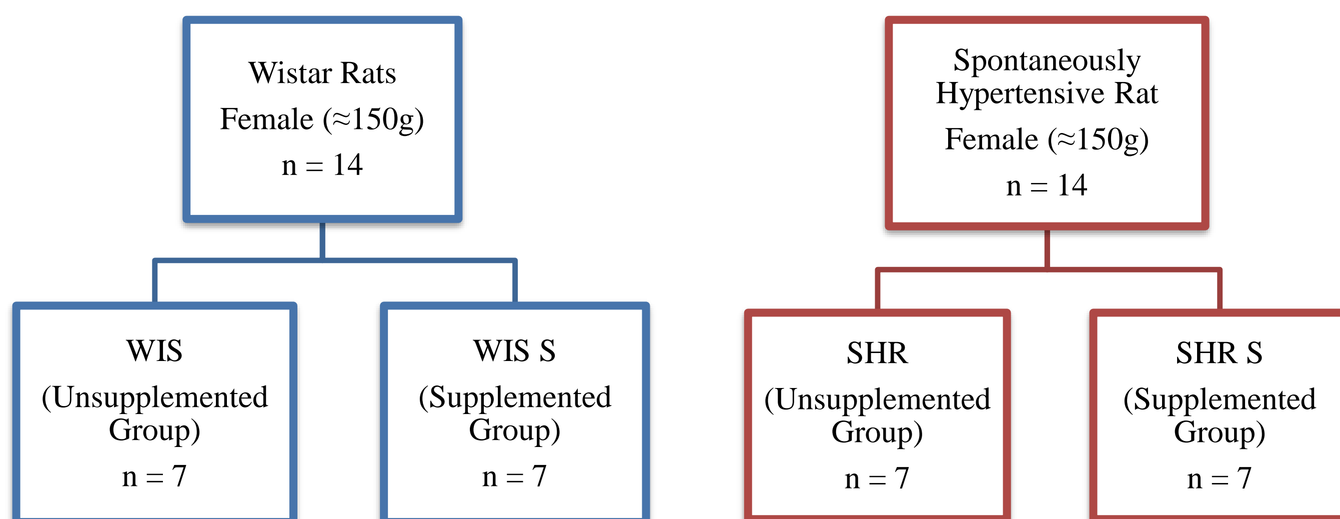


Figure 6: Animal groups used for the study

All animals were maintained at a temperature of 22 ± 1 °C, exposed to a 12 hour light-dark cycle and each group accommodated in a 50×40×18 cm polycarbonate cage. The study was designed around a seven day time line repeated for the duration of eight weeks. All animals had *ad libitum* access to water and commercial rat chow (MeadowFeeds™ standard maintenance Rat Chow). General appearance and behaviour were monitored daily. Body mass was monitored tri-weekly and food and water intake and

urine output were monitored weekly by placing the animals individually in metabolic cages for a 24 hour period. Blood pressure and fasting blood glucose were measured weekly for all rats.

3.2 MVMM Supplement Selection and Dosage

When selecting the MVMM supplement for this study, its composition, whether or not it was water-soluble and the method of administration of the supplement were all taken into consideration. The MVMM supplement selected for this particular study was Byboost™ (for Sheep and Goats) multivitamin-multimineral supplement with added Copper, manufactured and provided by Bayer Health Care (Pty) Ltd, South Africa.

Figure 7: Byboost™ MVMM supplement packaging

Indication/Description	A liquid drench booster for nutritionally aiding growth, fertility and good livestock performance.
Active Ingredients	Vitamin A 1 000 000 iu, vitamin D3 152 000 iu, vitamin E (as a tocopherol acetate) 15 000 mg, vitamin B1 2 700 mg, vitamin B2 1 000 mg, vitamin B3 3 500 mg, vitamin B5 (cal. pantothenate) 1 250 mg, vitamin B6 1 250 mg, folic acid 1 250 mg, vitamin B12 13 000 mcg, vitamin C 1 000 mg, vitamin H (biotin) 3 750 mcg on a high energy base. Vitamin K (MSB) 410 mg, iodine 1,000 mg, selenium 300 mg, cobalt 1 350 mg, copper (MAAC) 3 400 mg, manganese (MAAC) 2 000 mg, zinc (MAAC) 6 000 mg, lysine hydrochloride 2 200 mg, methionine 3 500 mg, amino acids range 2 100 mg, essential fatty acids 1,500 mg. (MAAC= Metal Amino Acid Chelates).
Category	Nutritionals
Target Specificity	Sheep, Goats
Administration	Use in feed, drinking water, milk, drench or top dressed on feed.
Dosage	Ewes/goats/rams pretupping: Rams: 20 ml 6-8 weeks before turnout & again at turnout. Hill ewes and small breeds: 15 ml 3-4 weeks prior to ram turnout. Low ground ewes and larger breeds: 20 ml 3-4 weeks prior to ram turnout. Intensively flushed low ground ewes: 15 ml 3 weeks prior to ram and 15 ml at ram introduction.Pre-lambing: Hill ewes & small breeds: 15 ml 4-6 weeks pre-lambing. Lowland ewes & larger breeds: 20 ml 4-6 weeks pre-lambing. Lambs and hoggs:Very young lambs: 2,5 ml. Young lambs: 5 ml at marking or first worming/vaccinating. Hoggs and ram lambs: 10 ml repeat every 3-4 months or whenever handled No withdrawal period.

Figure 8: Actual labelling on Byboost™ MVMM supplement

3.2.1 Dosage Calculation

The dosage of the supplement was calculated based on the guideline provided by the manufacturer and was extrapolated for the mass of an average 200 g rat. For dosing purposes 0.5 ml of Byboost™ was added to 4.5 ml of distilled water, making up a final volume of 5 ml. Each rat was then orally dosed with 0.5 ml of this composition, which contained 0.05 ml of the supplement suspended in 0.45 ml of distilled water.

The calculated dosage fell within the range of the recommended daily allowance for the rats according to the Nutrient Requirements of Laboratory Animals (1995) for rats and mice (National Academic Press 1995). The unsupplemented groups were orally dosed with 0.5 ml of deionised water.

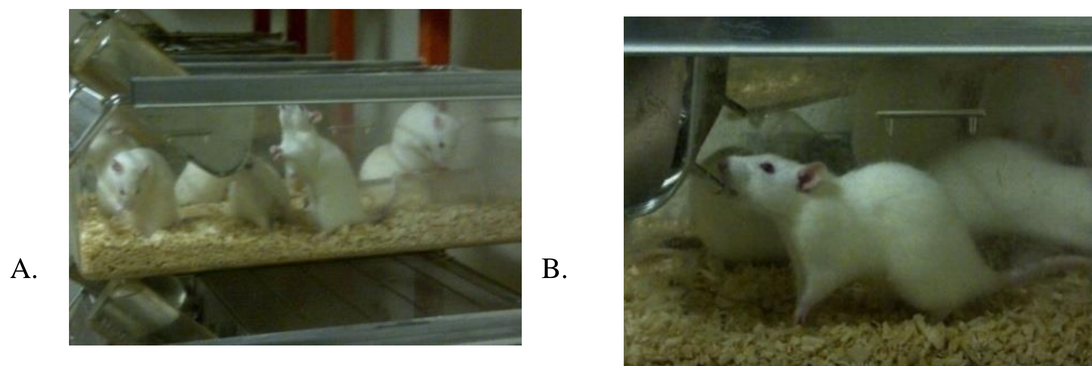


Figure 9: A & B: Animal group housing

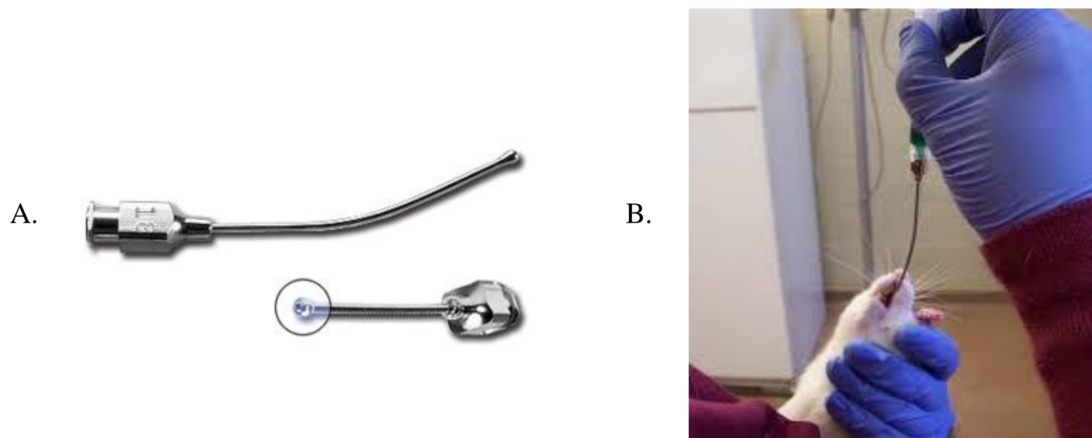
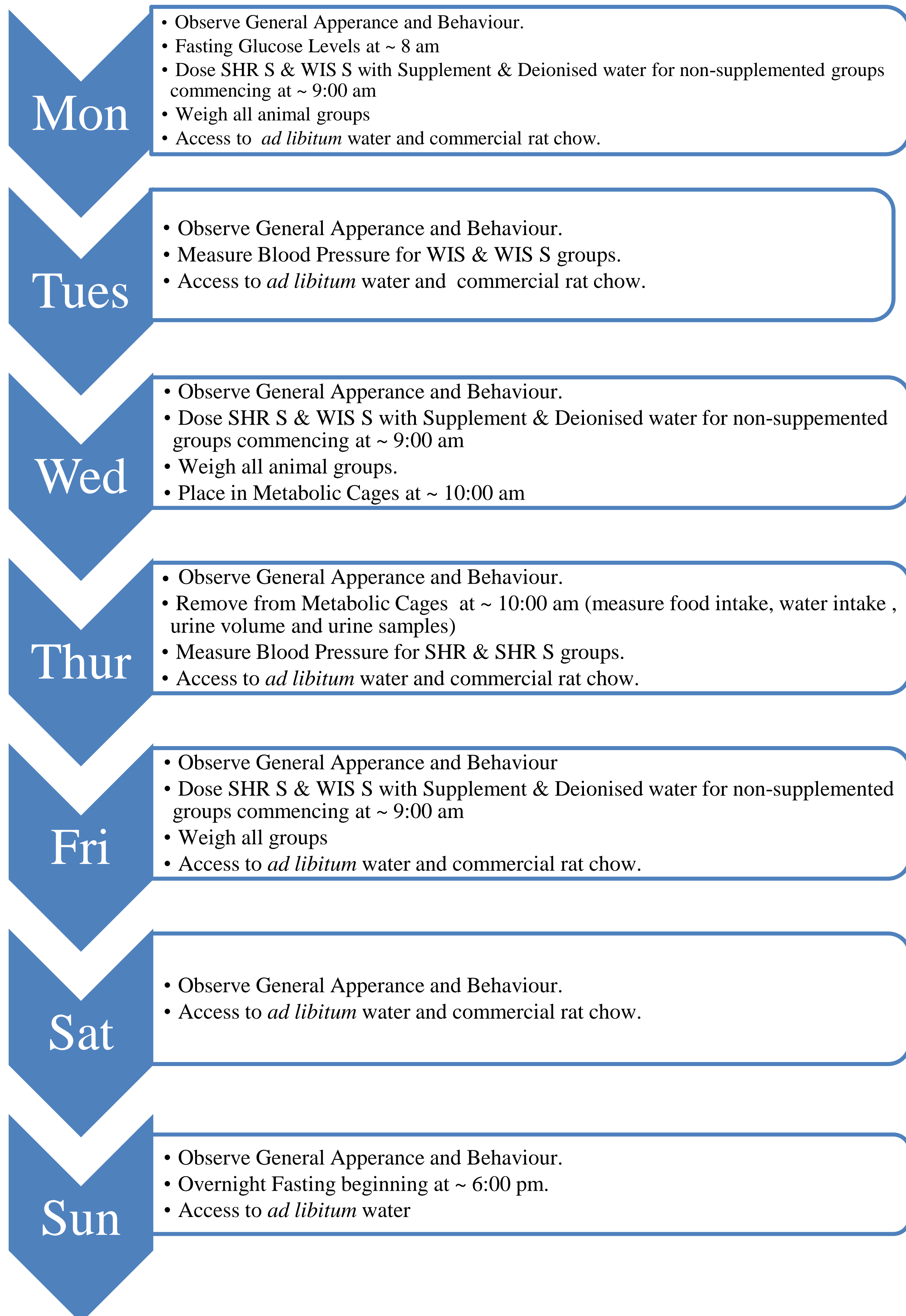


Figure 10: A & B: The dosing needle and the oral dosing method

3.3. Study Protocol

Timeline of daily activities over eight weeks of supplementation, designed around a seven day cycle



3.4 Growth and Health Status Parameters Measured over Eight Weeks of Supplementation

3.4.1 Growth Rate

Although animals were weighed tri-weekly (a requirement by the animal ethics committee), only the weights taken on Friday for each week were used to plot animal growth curves. Each rat was individually weighed each week for eight weeks and the increase in body mass was expressed as a percentage increase based on the weight of the rat from day zero of the study.

3.4.2 Systolic and Diastolic Blood Pressure Measurements

Systolic and diastolic blood pressures as well as heart rate were recorded using the standard non-invasive tail cuff method (IITC Instruments, USA). Equipment required for this comprised of restrainers and tail occlusion cuffs in various sizes to accommodate the growing animals, a warming platform and a computer system with the program software for cuff inflation and measuring the blood pressure. Blood pressure measurement was conducted in an area with minimal noise and at a temperature of 20 °C or more.

The software protocol was first set up and then the warming platform turned on and warmed to a preset temperature of 25 °C. Animals were placed in their respective restrainers according to size so as to minimise movement which affects the accuracy of blood pressure recordings. The front of the animal faced the rear of the holder where the nose cone is located for breathing, and to prevent it from turning around, a screw cap was then secured carefully behind at the opening. The exposed tail was then placed through the occlusion cuff close to the base and animals were then placed on the warming platform where they were allowed to thermo regulate for a minimum of 5 minutes to approximately 32 °C. This allows maximum vasodilation of the tail artery so that the pulsations due to the systolic blood pressure can be easily detected by the photoelectric tail cuff sensor. Once the required temperature was reached, the tail cuff was inflated; occluding blood flow in the tail artery. The cuff was then slowly deflated and tail pulsations set up when the systolic pressure was registered and recorded by the software which computes the systolic and diastolic blood pressures and heart rate (Daugherty *et al* 2009; Somova, Channa & Khan 1999).

All rats were acclimatised and conditioned five days prior to taking measurements by being placed in a restrainer, warmed and exposed to inflation of the tail cuff to decrease the stress effect on blood pressure, thereby increasing accuracy. Baseline blood pressure was recorded for all groups one week before supplementation began. The non-invasive tail-cuff blood pressure measuring technique is a validated and standardised method used in most laboratories, including ours, to measure long term rodent blood pressure in our laboratory (Somova *et al* 1999).

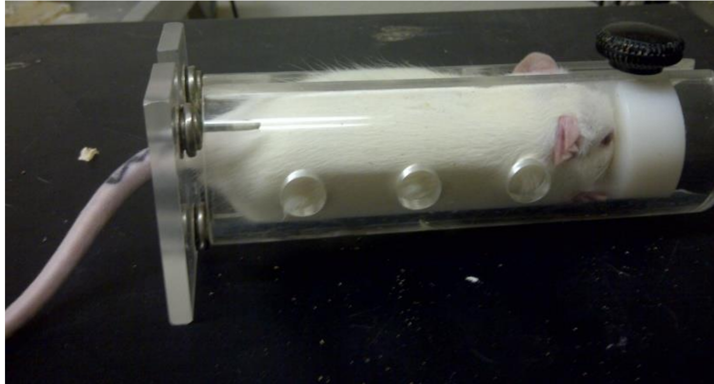


Figure 11: A rat in a restrainer

Figure 12: The non-invasive rodent blood pressure recording system (IITC, USA)

3.4.3 Food and Water Intake and Urine Volume

Food intake, water intake and urine volume for all groups were measured weekly over a 24 hour period by placing animals individually in metabolic cages for 24 hours (Techniplast™, Italy). At end of week eight, urine samples were collected for electrolyte analysis.

Figure 13: Polycarbonate metabolic cage for food & water intake and urine volume measurement

3.4.4 Weekly Fasting Blood Glucose Levels

Each week animals were fasted overnight, and blood glucose levels were measured using the tail-prick method, using the Accu-Chek Active® Blood Glucose Monitor (Roche, Germany) with accompanying test strips. The monitor measured the blood glucose within a defined range of 10-600 mg/dl or 0.6-33.3 mmol/L. Values outside this range were displayed as "Lo" or "Hi".

3.5 Health Status Parameters Measured after Eight Weeks of Supplementation

3.5.1 Urinary Electrolyte Analysis

The urinary Na^+, K^+ and creatinine were measured in urine samples collected after eight weeks of the study by a CX 3 Electrolyte Analyser (Beckman Coulter, USA).

3.5.2 Blood Sample Collection

After week eight, all animals were anaesthetised by halothane inhalation and exsanguinated by cardiac puncture. Approximately 7 ml of blood was collected from each animal in precooled heparinised tubes and kept on ice until analysis.

3.5.3 Full Blood Count

A Full Blood count using 1 ml of blood was done on a COULTER AC•T haematology analyzer (Beckman Coulter, USA) immediately after blood was removed from the animals.

3.5.4. Total Cholesterol

A total cholesterol measurement was also done immediately after sacrifice using a single large drop of blood applied to the reagent test strip. (Accutrend Plus® meter, Cobas USA). It took ± 180 seconds for a total cholesterol reading. Values that fell outside the measurement range were displayed as either 'Lo' or 'Hi'.

3.6 Effect of Supplementation on Hypertension and Oxidative Stress: A Biochemical Analysis.

A further 1 ml of blood was collected and centrifuged (Hermle Z300K, Lasec SA) at 3000 rpm for 10 minutes. The plasma was collected and snap-frozen in liquid nitrogen and stored at -80 ± 2 °C in a bio freezer for further analysis. The remaining blood samples were frozen at -60 ± 2 °C for trace element analysis.

Further tissue samples such as the heart, kidneys, liver, thoracic aorta, femoral artery and brain were collected and stored in either buffered neutral formalin for future histological analysis, or snap-frozen in liquid nitrogen and stored at -80 ± 2 °C in a bio freezer for further trace element and biochemical analysis.

3.6.1 Plasma Angiotensin-Converting Enzyme (ACE) and Blood Urea Nitrogen (BUN)

The ACE and BUN levels were analysed as indicators of kidney function once off in plasma samples collected after the end of the eighth week. Standard Enzyme-Linked Immunosorbent Assay (ELISA) kit protocol was followed by a commercial pathology laboratory (Global Clinical & Viral Labs, Durban).

3.6.2 Thiobarbituric Acid Reactive Substance Assay (TBARS)

Kidney samples were collected and used for TBARS analysis to measure the amount of lipid peroxidation occurring by determining malondialdehyde (MDA) concentrations using a modified protocol from Halliwell & Chirico 1993 of the TBARS ELISA.

This test determines the amount of lipid peroxidation occurring in the tissue due to free radicals being produced during oxidative stress. It is therefore an indicator of tissue oxidative damage. Oxidation of lipids is a common step in the pathogenesis of various diseases, especially hypertension. In normal circumstances, lipid peroxidation occurs due to the production of ROS during oxidative stress, as well as via some of the actions of phagocytosis during an immune response. The naturally occurring antioxidant system would then employ various mechanisms to keep ROS production in check, yet it is sometimes overwhelmed during various pathologies leading to lipid peroxidation and tissue damage.

MDA is one of a number of these free radicals produced due to the peroxidation of polyunsaturated fatty acid and is measured using the TBARS test and is one of the most common indicators for oxidative stress (Gaweł et al 2004).

Determining lipid peroxidation in the kidney is important since it is a primary site of end organ damage in hypertension (Mylonas & Kouretas 1999; Halliwell & Chirico 1993).

3.6.2.1 Tissue Preparation and TBARS Analysis

Kidney tissue including the medulla and cortex were sectioned and weighed (± 50 mg). Samples were placed into clean test tubes and homogenised after adding 450 µl of 0.2% phosphoric acid using an ultrasonic sonicator (Misonix Sonicator XL2000-010 Newtown CT, USA). The solution was centrifuged (Hermle Z300K, Lasec SA) at 10 000 rmp for 10 minutes and the supernatant was then collected. Thereafter 500 µl of 2% phosphoric acid and 200 µl of 7% phosphoric acid were added to the supernatant between vortexing (Heidolph REAX 2000). Finally, 400 µl butylated hydroxytoluene (BHT/TBA) solution and 100 µl 1M HCl was added to the supernatant which was heated in a water bath (Labotec, 132) for 15 minutes at 100 ± 5 °C. Test tubes were then left to cool down to room temperature and then 1.5 ml of butanol was added to all samples before vortexing. The top phase of the solution was then transferred to a 96 well microplate and absorbance was read at 532 nm and 600 nm using the Spectrostar-Nano plate reader (BMG LABTECH, Ortenberg, Germany). Preparation of reagents has been reported (Appendix C).

3.6.3 Determination of Plasma C-Reactive Protein (CRP)

CRP is an inflammatory marker and is also known as an "independent predictor" for cardiovascular disease (Clifton 2003). The purpose of the test is to measure the degree of inflammation accompanying hypertension since it is known that CRP is produced and released by the liver in response to an increase in inflammatory cytokines (Du Clos 2000).

Abcam® CRP (PTX1) rat in vitro ELISA kit was used for the quantitative measurement of CRP in the blood plasma. The assay was performed according to the manufacturer's instructions. It is important to note that CRP levels are very in the circulating plasma, therefore a high-sensitivity assay kit was used (MedlinePlus 2015).

3.6.3.1. Reagent Preparation

All reagents were incubated at room temperature (18-25 °C) prior to use. 1 x Diluent M concentrate was diluted (1:10) with reagent grade water to make up 30 ml of 1 x Diluent M. Wash buffer concentrate was diluted (1:20) with distilled water to prepare 60 ml of 1 x Wash buffer. Stock Biotinylated CRP antibody was diluted with 1 x Diluent M according to the label concentration in order to prepare 4400

µl of 1 x Biotinylated CRP Detector Antibody for use in the assay. 1 x SP Conjugate was spun down and the desired amount diluted (1:100) with 1 x Diluent M (Appendix D).

3.6.3.2 Standard Preparation

Standards were prepared immediately prior to use. Six standards were prepared by reconstituting the CRP Standard vial using a calculated amount of 1 x Diluent M. Standard 6 containing only 1 x Diluent M served as the zero standard (0 ng/mL). The standard results were calculated as per manufacturers protocol (Abcam, UK) (Appendix E).

3.6.3.3 Sample Preparation

Plasma was thawed and centrifuged (Hermle Z300K, Lasec SA) at 3000 rpm for 10 minutes. Plasma was then diluted 1:60 000 into 1 x Diluent M.

3.6.3.4 Assay Protocol

All materials and prepared reagents were equilibrated to room temperature (18-25 °C) prior to use. The 96 well plate strips were supplied ready to use and kept in a sealed pouch to prevent exposure to water vapour and other contaminants. To each well, 50 µl of CRP standard or sample was added and covered with sealing tape provided and incubated for 2 hours. Thereafter the microplate was washed manually five times with 200 µl of 1 x wash buffer. Next 50 µl of biotinylated CRP antibody was added to each well and incubated for 1 hour. The microplate was washed again as previously described. Then 50 µl of 1 x SP conjugate was added to each well and incubated for 30 minutes. After washing the microplate again, 50 µl of Chromogen substrate was added to each well and incubated for about 20 minutes or until an optimal blue density developed. Finally 50 µl of Stop Solution was added to each well and colour changed from blue to yellow. The colour intensity was measured on the Spectrostar-Nano plate reader (BMG LABTECH, Ortenberg, Germany) at 450 nm and results were calculated as per manufacturer’s protocol (Abcam, UK).

3.7 Effect of MVMM supplementation on Antioxidant Associated Trace Elements.

3.7.1 Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)

ICP -OES analysis was conducted in order to determine the antioxidant status of the MVMM supplement in various tissue samples, specifically testing for the antioxidant associated trace elements Zn, Cu, Fe, Se and Mn levels which were measured per gram net weight in the brain, kidney and blood of all animal groups.

3.7.1.1 Preparation of Kidney, Brain and Blood Samples

The capsule of each kidney was removed, and the whole kidney was rinsed with deionised water and placed into a clean test tube, to which 7 ml of 2N hydrochloric acid (HCL) was added. The tissue was then homogenized using an ultrasonic sonicator (Misonix Sonicator XL2000-010 Newtown CT, USA). Whole brain tissue was treated in the same manner. After homogenizing, 1 ml of perchloric acid ($ClHO_4$) was added to both kidney and brain samples for bleaching. All test tubes were then capped and incubated with occasional agitation in a water bath (Labotec 132) at 50 ± 2 °C for digestion for 24 hours. The tubes were then removed and centrifuged (Hermle Z300K, Lasec SA) at 3000 rpm for 1 hour. The supernatant was filtered three times using 0.45 µm cellulose acetate syringe membrane filters (Lasec SA) and put into new plastic vials for analysis.

For the blood samples 1 ml of blood was placed into to a clean plastic test tube and 7 ml of 2N HCL was added directly to this and homogenised. Then 1 ml of perchloric acid was added to the homogenate, and thereafter the same procedure was followed as above for kidney and brain.

Standards solutions for Zn, Cu, Fe, Mn, Se analysis were prepared from stock standard solutions. (Merck, SA). (Appendix F).

The ICP-OES analysis was carried out at the Chemistry department at the University of KwaZulu-Natal, Westville Campus.

3.7.1.2 Principles of ICP-OES Analysis

This analytical technique is used to determine the trace element levels in the various tissues. The digested tissue samples are introduced into the core of inductively coupled argon plasma (ICP), which generates a temperature of approximately 8000 ^{0}C. At this temperature all elements emit light at their own characteristic wavelengths which is collected by the spectrometer and passes through a diffraction grating that resolves the light into a spectrum of its constituent wavelengths. The spectrometer measures the light absorbance and a standard curve of the absorbance verses concentration of each element is constructed to determine its concentration in the sample.

3.7.2 Regional Kidney and Brain Analysis using Energy Dispersive X-ray Analytical System and Scanning Electron Microscopy (EDX – SEM)

EDX is used to determine surface composition and identification of various elements present in a particular area of tissue. Unlike ICP-OES which measure element concentration in a whole tissue sample of a given mass, EDX measures at specified points, and only on the cell surface, allowing for mapping of elements in one specific topographic area. This can be done on a particular area in an organ like the brain cortex or hippocampus. It is also able to show whether there are any aggregated elements

on the surface of the cell which can precede morphological changes (Michalak *et al* 2014). The SEM component is used in order to study any morphological changes that may occur to the surface tissue cells, as it is able to show the ultra-structure of the tissue (Michalak *et al* 2011). The SEM feature was not used in the present study.

Randomly selected single whole kidney and brain sample were collected from each group and used for elemental analysis using the EDX - SEM method as described below. The kidney cortex was specifically chosen for the EDX analysis as this is the area that is most prone to and affected by renal damage due to hypertension (Lubas *et al* 2013). For the brain the hippocampus was chosen due to its easy accessibility. It is part of the limbic system and is involved in functions such as memory, learning, emotions and associated with stress and anxiety responses (Leuner & Gould 2010). The large prefrontal cortex was also chosen for analysis. It functions mainly in cognitive behaviour, personality, decision making, planning, and social behaviour (Buchsbaum 2004).

3.7.2.1 Preparation of Kidney and Brain Tissue for EDX Analysis.

Upon harvesting, kidneys were longitudinally sectioned along the frontal plane and the brain was dissected to remove the hippocampus and both hemispheres of the frontal lobe. The tissue was then fixed in 2.5% glutaraldehyde in phosphate buffer and stored overnight at 4 °C.

For EDX analysis, samples were rinsed in phosphate buffer (3 × 5 minutes) and post fixed in 0.5% osmium tetroxide for 1 hour at room temperature. Tissue was then dehydrated in a graded ethanol series, 2 × 5 minutes in 30% alcohol, 2 × 5 minutes in 50% alcohol, 2 × 5 minutes in 75% alcohol, 2 × 10 minutes in 100% alcohol. Samples were then dried in a critical point dryer (CPD) (Quorom, K850, Quorum Technology Ltd, UK).

The CPD replaced all the alcohol with liquid carbon dioxide (CO_2) over a period of ½ to 3 hours. Liquid CO_2 was fed into the system and the alcohol was simultaneously drained out. Once all the alcohol was removed the dryer was sealed and the temperature increased to 45 °C. The temperature was increased above the critical temperature to ensure that all the CO_2 was converted into vapour. The system was then gradually vented and the dried specimens were removed. The specimens were then orientated and placed on stubs and gold sputter coated.

3.7.2.2. EDX Analysis

The EDX-SEM analysis was done using the Carl Zeiss Evo LS15 ESEM (Oxford detector, UK) and INCA software at the Electron Microscopy Unit at the University of KwaZulu-Natal, Pietermaritzburg Campus. It has an 80 mm^2 detection window and was used in variable pressure (VP) mode. The mounted kidney and brain specimens sections of the kidney, cortex and hippocampus from each animal group

were imaged and recorded using the Scanning Electron Microscope (SEM) as seen in figure 14. The energy dispersive X-ray (EDX) system was used to determine a profile of the elemental composition on the cell membrane surface for each area on each specimen. An X-ray spectrum was obtained for ten areas on each tissue sample which displayed the detected micro/trace elements, as seen in figure 14.

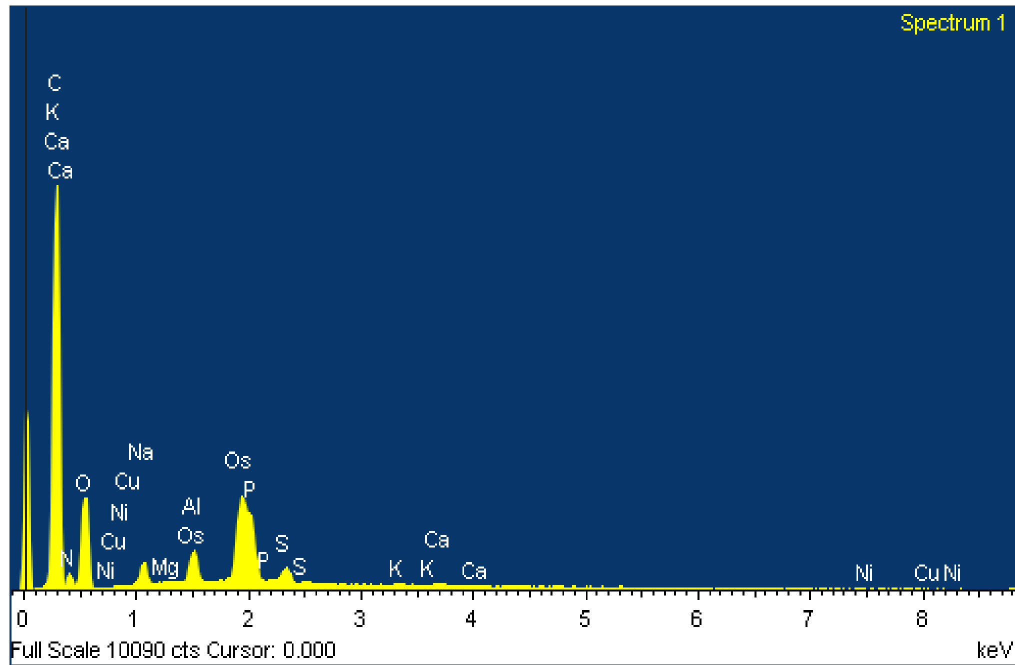


Figure 14: The X-ray spectrum of the surface composition of the trace elements on a frontal area of the kidney cortex of a SHR S rat

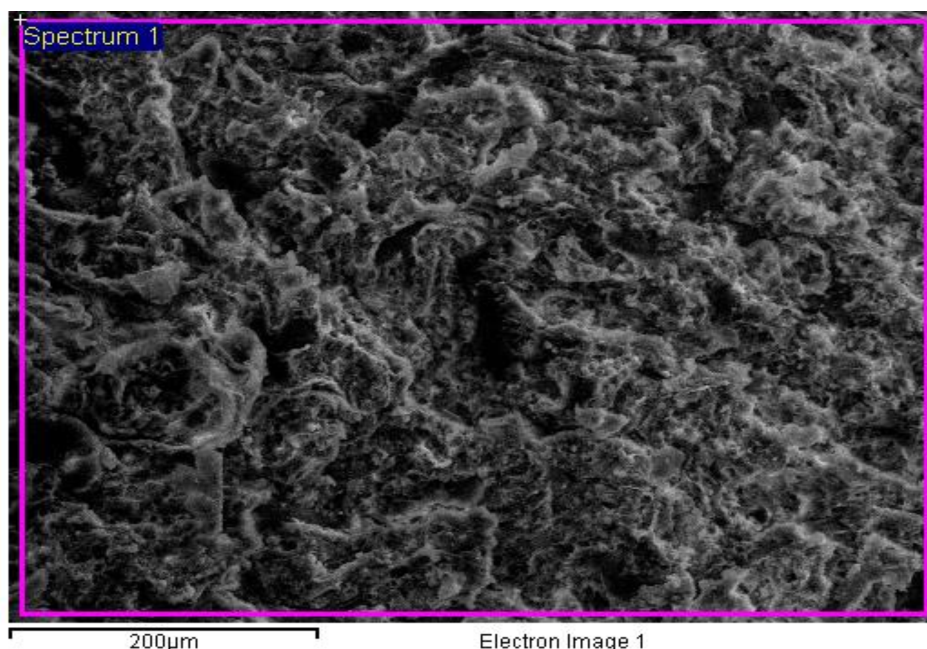


Figure 15: Surface SEM image of an area of the frontal section of a kidney of a SHR S rat

3.8. Effects of MVMM Supplementation on Hypertension: A Histopathological Analysis.

3.8.1. Histological Investigation on the Kidney and Thoracic Aorta

3.8.1.1 Tissue Preparation

Kidneys and aortas from all animal groups were dissected out, with the kidneys sectioned longitudinally and the aortas cross sectioned into segments so that each was approximately 1 cm in length. All kidney and aorta tissue segments were then fixed in 10% buffered formalin for a minimum of 24 hours, followed by the dehydration, clearing and infiltration procedure.

Tissue was removed from the fixative and first dehydrated in 70% alcohol for 1 hour, followed by 90% alcohol for 1 hour and finally 100% alcohol for 2 hours. The tissues were then cleared in xylene for 2 hours, followed by infiltration using paraffin wax and incubated overnight in a tissue oven to prevent the hardening of the wax. The tissues were then embedded in the wax using the Histocentre (LEICA EG 1160, Wetzlar, Germany) and tissue moulds. Once the wax had hardened, the moulds were removed and the cassettes were then ready for sectioning. After the tissues were sectioned using a microtome (Microtome HM 340E, Walldorf, Germany), 4 µm sections were taken and unfolded in a warm water bath (40 ± 2 °C) and then lifted up out of the water bath using a clean microscope slide and placed on a plate warmer to dry. Once fully dried they were ready for staining.

3.8.1.2 Staining Procedure

Before staining could proceed, slides had to first be deparaffinised by submersing them in xylene for 4 minutes, after which they were rehydrated in 100% alcohol for 2 minutes, 90% alcohol for 2 minutes, 70% alcohol for 2 minutes, 50% alcohol for 2 minutes and finally deionised water until they were ready for staining. The deparaffinising step preceded both staining techniques.

a) For the kidney, a haematoxylin and eosin (H&E) stain was used. This was selected as it is one of the most commonly used staining techniques and allows for easy recognition and differentiation of various types of tissue and their morphological structures. The eosin stains the basic components of the cell in shades of pink, for example the cytoplasm, collagen and red blood cells stain a pale pink or red. The haematoxylin stains the acidic components darker shades of blue or purple, for example, the nuclei of the cells and other structures containing the acidic DNA and RNA structures (Fischer *et al* 2008) (Appendix G).

b) For the aorta a Verhoeff-Van Gienson (VVG) elastic stain was used. This was selected as it stains specifically for elastin, a connective tissue occurring as either branching fibres or sheets in the arteries and aorta. Sections are first over stained using hematoxylin-iodine-ferric chloride solution and then counterstained with the VVG which stains the elastic fibres and cell nuclei a dark brown or black, the collagen fibres red and other tissue components, including the cytoplasm, are stained yellow. Therefore the elastic fibres in the tunica media of the aorta will appear black, the tunica adventitia red and the content of the lumen a pale yellow (Appendix H).

Immediately after staining, the sections were mounted on a slide. A single drop of Distyrene Plasticizer Xylene (DPX™) was placed on the section and a coverslip was then carefully placed on top of the slide, with a slight pressure added to help spread the DPX™ evenly across the tissue sample as well as prevent any formation of air bubbles. The mounted sections were left to dry for ± 24 hours.

3.8.1.3 Kidney and Aorta Image Analysis

Slides were scanned using the Leica Slide Scanner, and analysed using the Leica Slide Path Gateway LAN software (Leica Microsystems CMS, Wetzlar, Germany). Magnification of ×200 was carried out for the kidney, and ×400 for the aorta. Analysis of three areas for two kidney specimens per group was conducted by a blinded observer (i.e.: kidney sections were coded and the analysis of each section was done without any knowledge of which were the treated/control groups). Kidney histopathological evaluation included glomerular structure, presence of glomerulosclerosis, appearance of interstitial and tubular cells and the presence of any protein-casts or inflammation of the interstitium. These are all typical renal markers of hypertension (Rouse *et al* 2013, Venkatachalam *et al* 2010, Feld *et al* 1990). A scale of 0 to 4 was used to describe the level of damage, with 0 = normal, 1= mild, 2 = moderate, 3

=severe, 4 = very severe (Duarte *et al* 1997, Rouse *et al* 2013). For the aorta the medial thickness was obtained from five different areas for three specimens. Measurements were done using the same software as previously described.

3.9 Statistical Analysis of Data.

Statistical analysis was done using GraphPad Instat Version 5.00 (GraphPad Software Inc., San Diego, CA, USA) and StatView Version 5.0. (SAS Institute Inc., CA, USA). Normality testing was done using the Kolmogorov-Smirnov test for normality. A multivariate analysis of variance (MANOVA) was applied to continuous data in the growth rate and blood pressure parameters and was followed by Fisher's post-hoc test. Independent sample *t*-tests were administered for CRP and TBARS, trace element analysis (ICP & EDX), BUN, ACE, creatinine, all haematological parameters, cholesterol, glucose, urine volume and food and water intake. A nonparametric *t* test with a Mann-Whitney post-hoc test was administered to all nonparametric data. All values were expressed as a mean ± SEM, where $p < 0.05$ was considered significant. Graphical representation of the data was plotted using GraphPad (V 5.00) software.

4 RESULTS

4.1 MVMM Supplementation and Growth

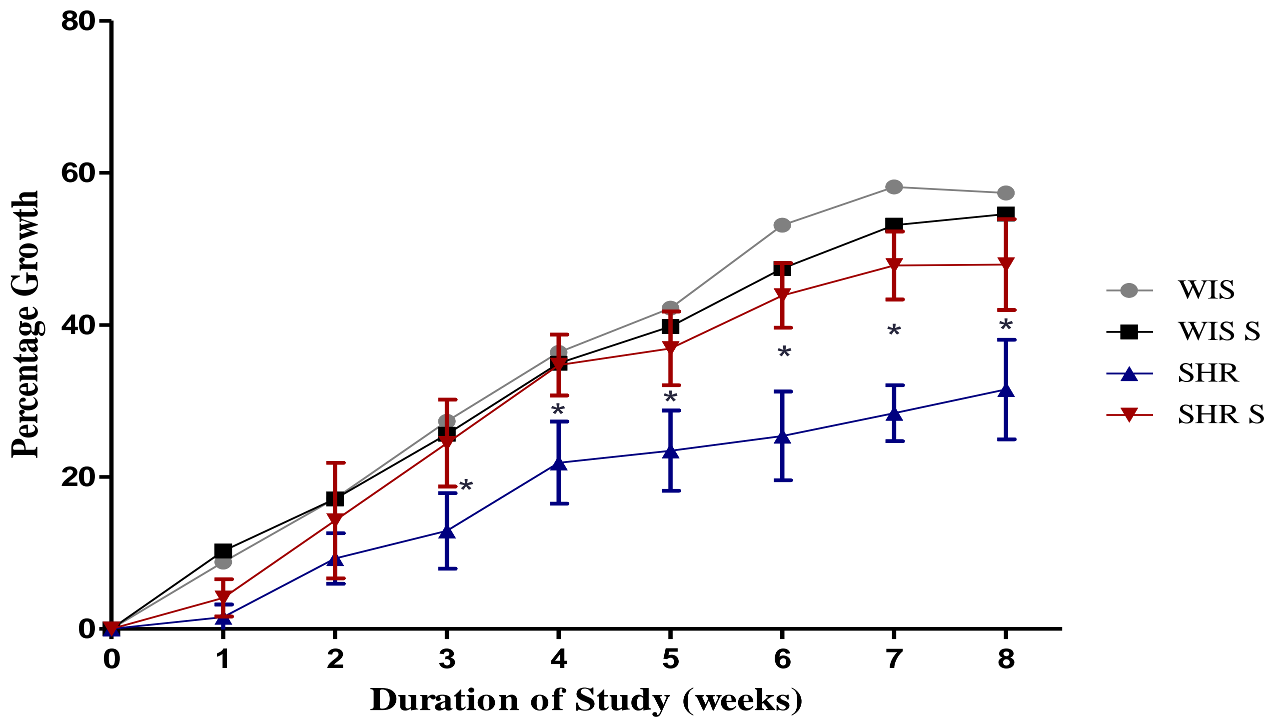


Figure 16: Growth rate in the SHR and WIS rats

Values are mean ± SEM, n=7, * $p < 0.05$ is significant for SHR and SHR S.

The growth rate for all the WIS and SHR groups were initially similar at the start of the study. However, there was a significant increase in growth rate from the third week onwards till the eighth week in the SHR S group when compared to the SHR.

4.2 MVMM Supplementation and Urinary Output, Food and Water Intake.

Table 3: Food, Water intake, and Urinary output in the SHR and WIS rats for week eight

Groups	Food Intake (g)	Water Intake (ml)	Urinary Output (ml)
WIS	17.1 ± 1.73	19.4 ± 2.02	10.3 ± 0.95
WIS S	17.1 ± 1.73	24.4 ± 0.71	10.5 ± 0.81
SHR	16.6 ± 0.87	29.4 ± 2.10 †	10.3 ± 0.92
SHR S	16.9 ± 0.83	30.0 ± 4.08	13.4 ± 2.53

Note: Values are mean ±SEM, n=7, † $p < 0.05$ is significant for WIS and SHR

There was no significant difference observed in the food intake and urine output in all four groups by the end of week eight. Water intake was significantly higher in the SHR than in the WIS group, with supplementation having no significant effect on water intake in both groups.

4.3 MVMM Supplementation and Systolic Blood Pressure (SBP).

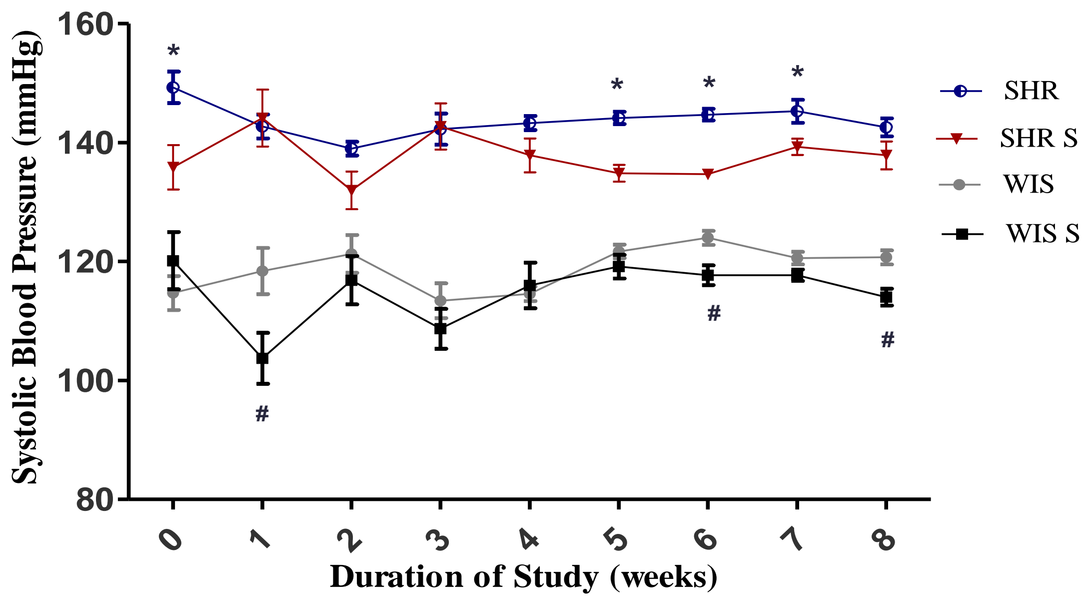


Figure 17: Systolic Blood Pressure in the SHR and WIS rats.

Values are mean ± SEM, n=7, * $p < 0.05$ is significant for SHR and SHR S, # $p < 0.05$ is significant for WIS and WIS S

The SBP was significantly higher in the SHR group when compared to the WIS group. At week eight there was an average significant SBP difference of 22 mm Hg between both strains. Supplementation had no effect on the SBP in both groups except during week one, six and eight in the WIS S group and week five, six and seven in the SHR S group, both showing a transient decrease.

4.4 MVMM Supplementation and Diastolic Blood Pressure (DBP).

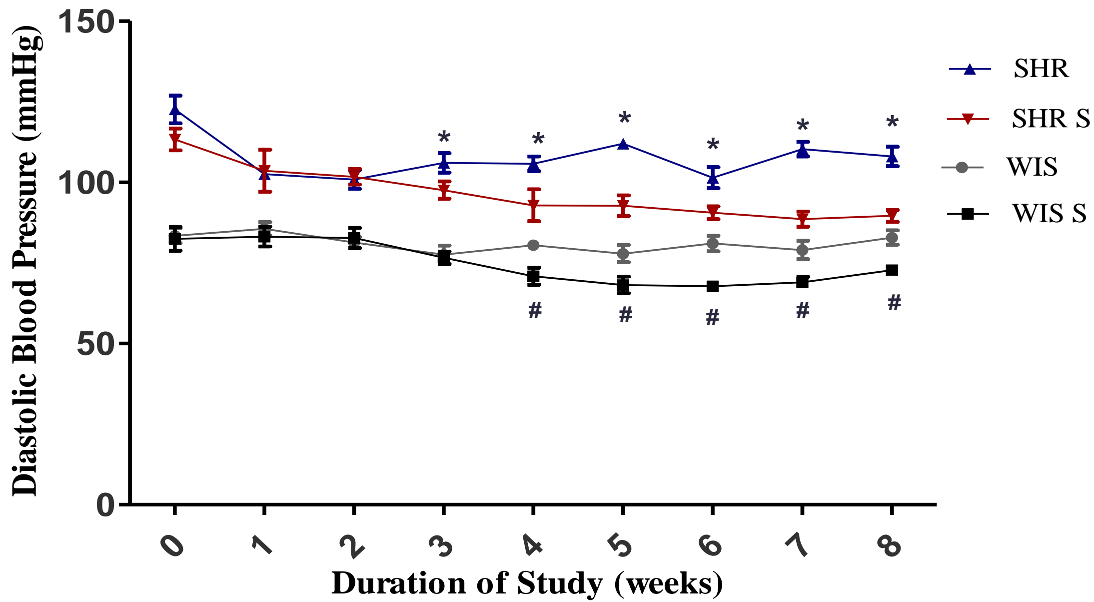


Figure 18: Diastolic Blood Pressure in the SHR and WIS rats.

Values are mean ± SEM, n=7, * $p < 0.05$ is significant for SHR and SHR S, # $p < 0.05$ is significant for WIS and WIS S.

The DBP was also significantly higher in the SHR group than in the WIS group. The SHR can be classed as Stage 1 hypertensive while the WIS were normotensive (WHO 2013). At week eight, there was an average SBP difference of 25 mm Hg between both strains. Supplementation had a significant DBP lowering effect in both the SHR S and WIS S groups in weeks four, five, seven and eight. At week eight, there was a difference of 7 mm Hg between the WIS S and WIS groups and a difference of 16 mm Hg between the SHR S and SHR groups.

4.5. MVMM Supplementation and Fasting Blood Glucose and Total Cholesterol.

Table 4: Total Cholesterol and Fasting Blood Glucose in SHR and WIS rats for week eight

Groups	Total Cholesterol (m.mol/ℓ)	Fasting Glucose (m.mol/ℓ)
WIS	4.29 ± 0.06	5.1 ± 0.11
WIS S	4.55 ± 0.26	4.9 ± 0.18
SHR	4.51 ± 0.03 †	4.4 ± 0.23
SHR S	4.30 ± 0.04 *	4.9 ± 0.19

Note: Values are mean ±SEM, n=7 for Fasting Glucose, n=4 for Total Cholesterol, † $p < 0.05$ is significant for WIS and SHR, * $p < 0.05$ is significant for SHR and SHR S.

No significant difference in fasting blood glucose levels were observed in all four groups at week eight. There was a significant strain difference with the SHR group having higher total cholesterol than the WIS group. Supplementation led to significantly lowered total cholesterol in the SHR S group.

4.6 MVMM Supplementation and Haematological Parameters

Table 5: Red Blood Cells (RBC), White Blood Cells (WBC), Platelets (PLT), Haematocrit (HCT) and Haemoglobin (Hb) in SHR and WIS rats for week eight

Groups	RBC (10^6/µL)	WBC (10^3/µL)	PLT (10^3/µL)	HCT (%)	Hb (g/L)
WIS	7.48 ± 0.28	4.2 ± 0.5	519.7 ± 62.52	41.5 ± 1.10	148 ± 3.89
WIS S	7.67 ± 0.14	3.6 ± 0.3	497.9 ± 64.64	42.2 ± 1.07	152 ± 3.52
SHR	7.79 ± 0.12	4.2 ± 0.3	477.5 ± 49.93	39.2 ± 0.68	138 ± 2.43 †
SHR S	8.26 ± 0.16 *	3.8 ± 0.4	358.4 ± 71.42	41.1 ± 0.86	145 ± 3.12

Note: Values are mean ±SEM, n=7, † $p < 0.05$ is significant for WIS and SHR, * $p < 0.05$ is significant for SHR and SHR S.

No significant differences in the WBC count, PLT's and HCT were observed between all four groups. Haemoglobin (Hb) showed a significant strain difference between the SHR and WIS groups with supplementation having no significant effects in either strain. The SHR S group showed a significantly higher RBC count compared to the SHR group, an effect that was not observed in both the WIS groups. However, this was not accompanied by a concomitant significant increase in the Hb levels in these groups.

4.7 MVMM Supplementation and Urinary Sodium and Potassium Ratio and Blood Urea Nitrogen (BUN)

Table 6: Urinary Creatinine, Na^+/K^+ Ratio and Blood Urea Nitrogen in SHR and WIS rats for week eight

Groups	Creatinine (m.mol/ℓ)	Na^+/K^+ Ratio (m.mol/ℓ)	BUN (m.mol/ℓ)
WIS	3.82 ± 0.95	0.52	6.5 ± 0.51
WIS S	5.34 ± 0.79	0.51	7.6 ± 0.51
SHR	2.24 ± 0.43	0.75 †	8.3 ± 0.30 †
SHR S	3.35 ± 0.71	0.80	8.2 ± 0.44

Note: Values are mean ±SEM, n=7 for Creatinine and Na^+/K^+ ratio, n=6 for BUN, † $p < 0.05$ is significant for WIS and SHR.

There was no significant difference observed in the urinary creatinine in four all groups at the end of week eight. BUN and Na^+/K^+ ratio showed a significant strain difference, with the SHR having higher Na^+/K^+ ratio and BUN levels than the WIS. Supplementation had no effect on both these parameters in both groups.

4.8 MVMM Supplementation and Angiotensin-Converting Enzyme (ACE)

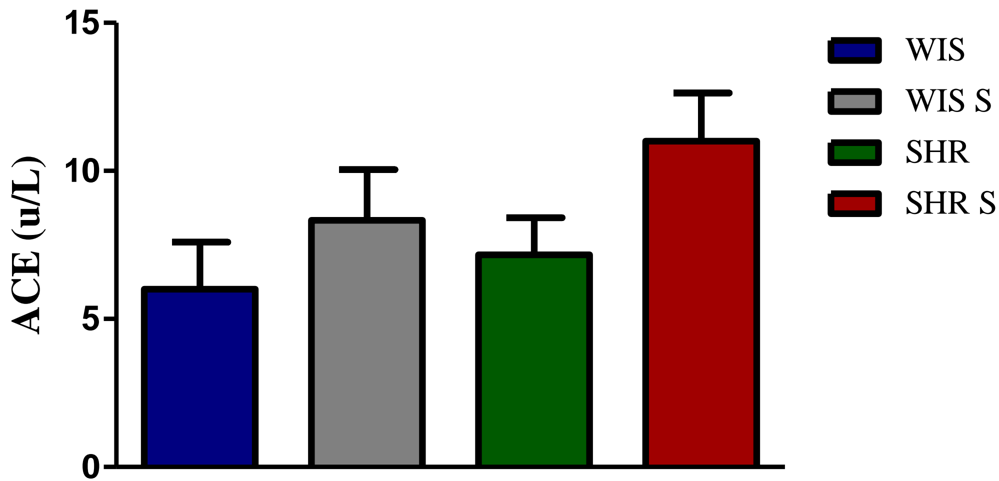


Figure 19: : Plasma ACE in the SHR and WIS rats

Values are mean ± SEM, n=6, $p < 0.05$ is significant.

The Angiotensin-converting enzyme showed no significant difference in either the WIS or SHR groups.

4.9 MVMM Supplementation and Thiobarbituric Acid Reactive Substance (TBARS) in kidneys.

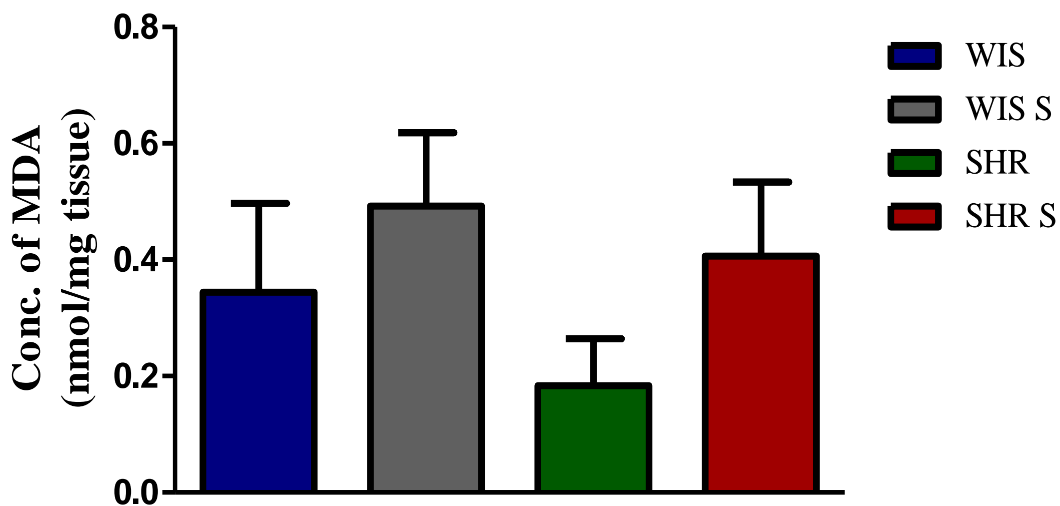


Figure 20: TBARS in the kidney of the SHR and WIS rats.

Values are mean ± SEM, n=6, $p < 0.05$ is significant.

TBARS levels were measured as a preliminary test for lipid peroxidation. No significant differences were seen in either the WIS or SHR group

4.10 MVMM Supplementation and Plasma C Reactive Protein (CRP) levels.

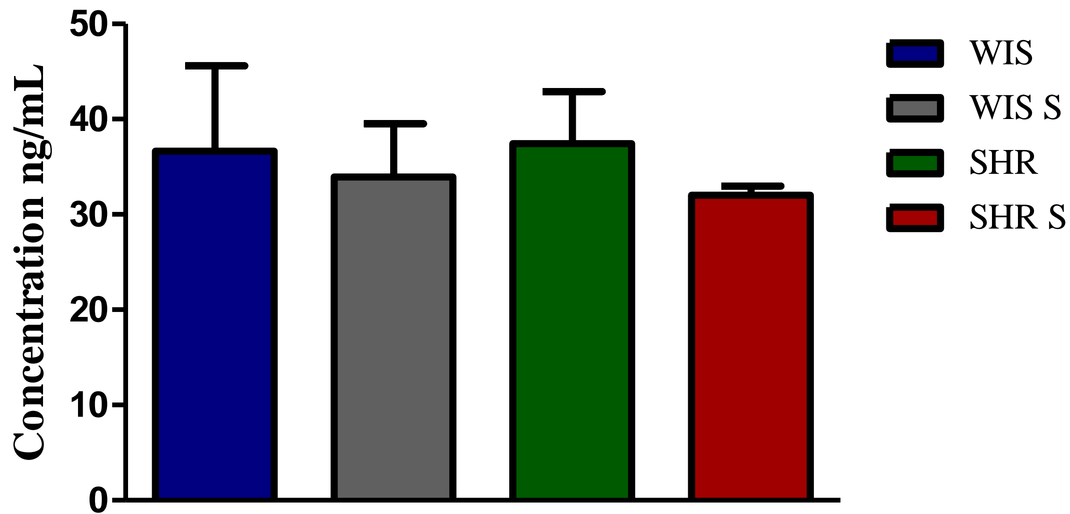


Figure 21: CRP in the plasma of the SHR and WIS rats.

Values are mean ± SEM, n=6, $p < 0.05$ is significant.

CRP levels were measured as a preliminary test for general inflammation. No significant differences were seen in either the WIS or SHR groups.

4.11 MVMM Supplementation and Trace element profile in the Brain, Kidney and Blood

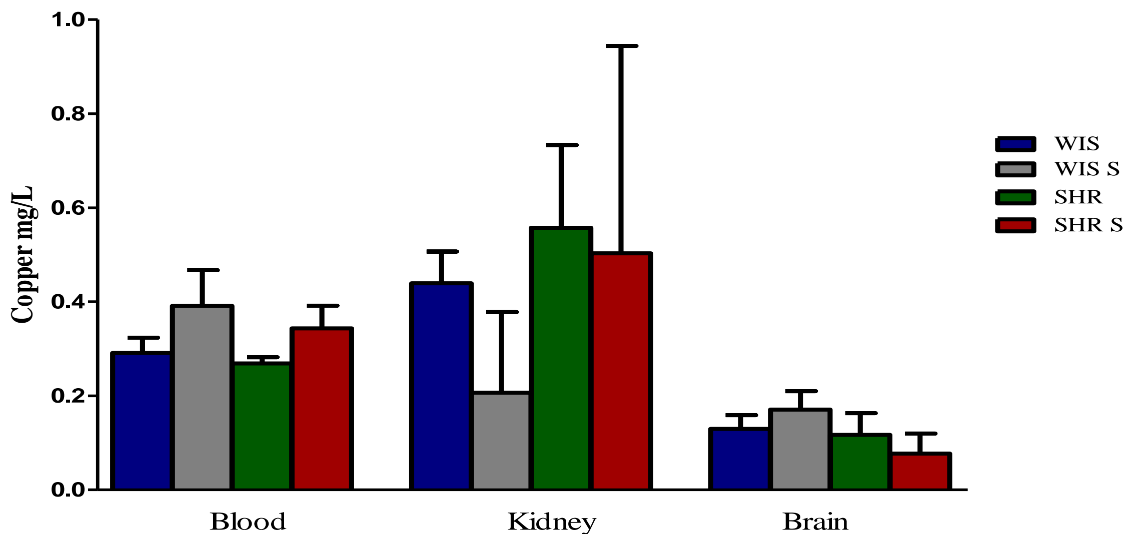


Figure 22: Copper in the blood, kidney and brain in the SHR and WIS rats.

Values are mean ± SEM, n= 7 blood, n=4 kidney, n=4 brain, $p < 0.05$ is significant.

There were no significant differences in blood or organ copper levels between all groups. Supplementation also had no significant effect on the copper levels.

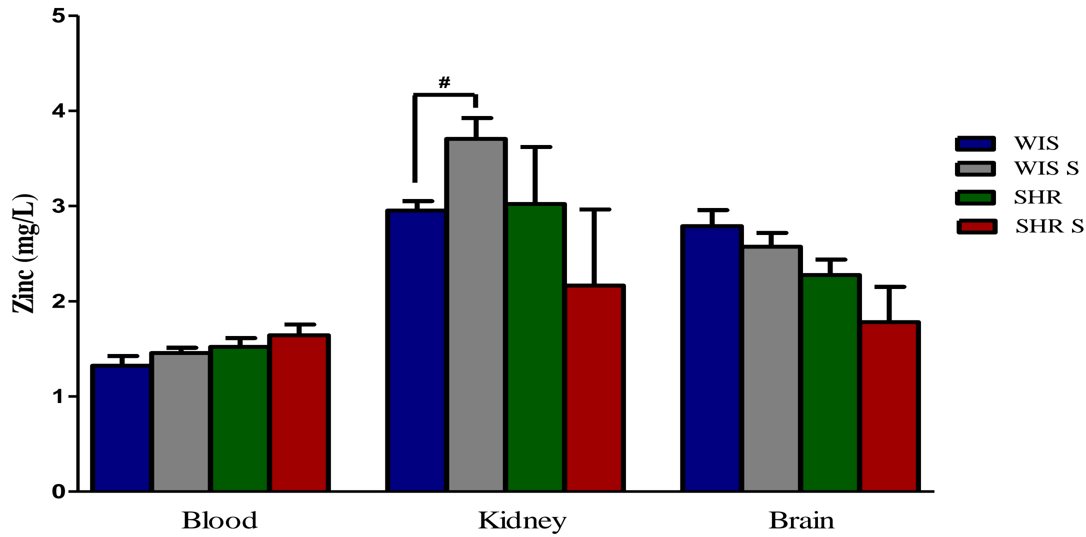


Figure 23: Iron in the blood, kidney and brain in the SHR and WIS rats.

Values are mean ± SEM, n= 7 blood, n=4 kidney, n=4 brain, $p < 0.05$ is significant.

There were no significant differences in blood or organ iron levels between all groups. Supplementation also had no significant effect on the iron levels.

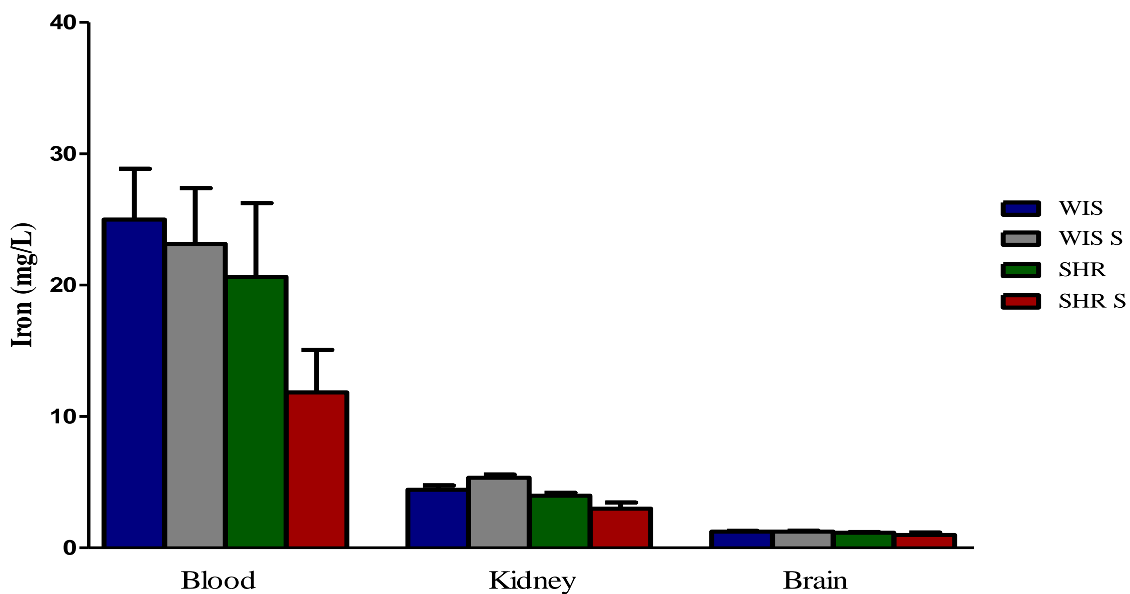


Figure 24: Zinc in the blood, kidney and brain in the SHR and WIS rats.

Values are mean ± SEM, n= 7 blood, n=4 kidney, n=4 brain, # $p < 0.05$ is significant.

There were no significant differences in blood and brain zinc levels between all groups. In the kidney the WIS S had significantly higher levels of zinc compared to the WIS group.

4.12 MVMM Supplementation and Element analysis in the Kidney, Brain and Blood using EDX - SEM.

Table 7: Atomic concentration (%) of the elements in the Kidney for the SHR and WIS rats

Element		Atomic concentration of elements (% of all detected ions)			
		WIS	WIS S	SHR	SHR S
Macro elements	C	68.28 ± 2.82	67.66 ± 1.93	73.74 ± 9.768	68.75 ± 4.68
	N	10.06 ± 2.49	9.96 ± 2.27	6.96 ± 4.580	10.35 ± 4.23
	O	19.36 ± 1.21	20.58 ± 1.19 #	17.17 ± 5.165	18.76 ± 1.63
	P	0.56 ± 0.06	0.45 ± 0.14 #	0.40 ± 0.123	0.49 ± 0.09
	Ca	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.014	0.02 ± 0.01
Micro elements	K	0.03 ± 0.01	0.04 ± 0.02 #	0.02 ± 0.010	0.04 ± 0.02 *
	Na	0.55 ± 0.12	0.54 ± 0.12	0.44 ± 0.19	0.40 ± 0.06
	S	0.32 ± 0.02	0.25 ± 0.02 #	0.21 ± 0.06	0.40 ± 0.10 *
	Mg	<LLD	<LLD	0.03 ± 0.023	0.03 ± 0.01

Note: < *LLD*; below low limit of detection, n= average of 10 areas, values are mean ±SEM, # $p < 0.05$ is significant for WIS and WIS S, * $p < 0.05$ is significant for SHR and SHR S.

For elements in the kidney the WIS S showed a significant increase in O and K, and a significant decrease in P and S compared to the WIS group. The SHR S showed a significant increase only in the K and S levels in the kidney compared to the SHR group.

Table 8: Atomic concentration (%) of the elements in the prefrontal cortex of the brain for the SHR and WIS rats

Element		Atomic concentration of elements (% of all detected ions)			
		WIS	WIS S	SHR	SHR S
Macro elements	C	61.98 ± 4.05	65.60 ± 6.95	71.25 ± 3.79	66.35 ± 3.02
	N	13.63 ± 2.50	10.58 ± 2.06	7.90 ± 2.38	9.19 ± 2.60
	O	23.16 ± 2.97	23.29 ± 3.66	20.23 ± 1.46	22.28 ± 1.33
	P	0.29 ± 0.07	0.37 ± 0.17	0.61 ± 0.01	0.50 ± 0.08 *
	Ca	0.03 ± 0.01	<LLD	0.03 ± 0.01	<LLD
Micro elements	K	<LLD	<LLD	0.04 ± 0.02	0.03 ± 0.02
	Na	0.39 ± 0.10	0.49 ± 0.14	0.61 ± 0.07 †	0.67 ± 0.07
	S	0.14 ± 0.04	0.14 ± 0.06	0.22 ± 0.06	0.14 ± 0.03 *
	Mg	<LLD	<LLD	<LLD	<LLD

Note: < *LLD*; below low limit of detection, n= average of 10 areas, values are mean ±SEM, † $p < 0.05$ is significant for WIS and SHR, * $p < 0.05$ is significant for SHR and SHR S.

There is a significant strain difference with an increase in Na in the SHR group compared to the WIS group. For elements in the prefrontal cortex the WIS group showed no significant increase. The SHR S showed a significant decreased in P and S levels in the prefrontal cortex compared to the SHR group.

Table 9: Atomic concentration (%) of the elements in the hippocampus of the brain for the SHR and WIS rats

Element		Atomic concentration of elements (% of all detected ions)			
		WIS	WIS S	SHR	SHR S
Macro elements	C	67.20 ± 3.52	64.27 ± 4.00	66.70 ± 4.18	71.31 ± 30.91 *
	N	10.79 ± 3.40	13.33 ± 3.48	10.63 ± 3.89	8.27 ± 4.15
	O	20.14 ± 2.05	20.24 ± 2.14	20.59 ± 0.83	18.92 ± 10.05 *
	P	0.42 ± 0.07	0.46 ± 0.12	0.45 ± 0.05	0.56 ± 0.42 *
	Ca	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.00	<LLD
Micro elements	K	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02
	Na	0.39 ± 0.06	0.43 ± 0.05	0.41 ± 0.04	0.40 ± 0.26
	S	0.18 ± 0.04	0.21 ± 0.03	0.19 ± 0.03	0.20 ± 0.16
	Mg	<LLD	<LLD	<LLD	<LLD

Note: < *LLD*; below low limit of detection, n= average of 10 areas, values are mean ±SEM, * $p < 0.05$ is significant for SHR and SHR S.

For elements in the hippocampus, there were no significant differences for any of the elements in the WIS S group compared to the WIS group. The SHR S showed a significant decrease in the O level, and a significant increase in the C and P level compared to the SHR group.

4.13 MVMM Supplementation and Histopathology of the Kidney.

The kidney was examined microscopically for various prominent histopathological markers characteristic of hypertension as well as general tissue damage caused over time. This included

- Glomerulosclerosis
- Apoptosis
- Interstitial inflammation
- Tubular vacuolization, dilation, regeneration and presence of proteinaceous casts (p-casts).
- Blood vessel hyalinization and periarteritis

A histopathological score for each marker of kidney damage was rated using a scale of 0-4 where 0 = normal; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe (Rouse *et al* 2013).

Table 10: Histopathological findings on the effect of MVMM supplementation in the kidney in the WIS and SHRs

ID	Glomeruli		Tubules						Interstitium	Blood Vessels	
	Sclero	Apo	Atro	Vac	Dil	P-cast	Reg	Apo	Inflam	Hya	Periarteritis
WIS 1 – Area 1	0	0	0	0	0	0	0	0	0	0	0
WIS 1 – Area 2	1	0	2	0	2	1	0	0	0	1	0
WIS 1 – Area 3	0	0	0	0	0	0	0	0	0	0	0
WIS 2 – Area 1	1	0	0	0	0	0	0	0	0	0	0
WIS 2 – Area 2	0	0	0	0	0	0	0	0	0	0	0
WIS 2 – Area 3	1	0	1	1	1	1	0	0	0	1	0
Average	0.6	0	0.6	0.2	0.6	0.4	0	0	0	0.4	0
WIS S 1 – Area 1	1	0	0	0	0	0	0	0	0	0	0
WIS S 1 – Area 2	0	0	0	0	0	0	0	0	0	0	0
WIS S 1 – Area 3	0	0	0	0	0	0	0	0	0	0	0
WIS S 2 – Area 1	0	0	0	0	0	0	0	0	0	0	0
WIS S 2 – Area 2	1	0	0	0	0	0	0	0	0	0	0
WIS S 2 – Area 3	0	0	0	0	0	0	0	0	0	0	0
Average	0.4	0	0	0	0	0	0	0	0	0	0
SHR 1 – Area 1	3	0	0	0	2	0	0	0	0	2	0
SHR 1 – Area 2	3	1	0	2	3	1	0	0	0	2	0
SHR 1 – Area 3	4	0	0	3	3	3	0	0	1	3	0
SHR 2 – Area 1	3	0	0	2	3	1	0	0	0	2	0
SHR 2 – Area 2	3	1	0	2	2	0	0	0	0	1	0
SHR 2 – Area 3	3	0	0	3	2	1	0	0	0	2	0
Average	3.8	0.4	0	2.4	3	1.2	0	0	0.2	2.4	0
SHR S 1 – Area 1	2	1	1	2	1	2	2	2	0	1	0
SHR S 1 – Area 2	1	2	1	1	1	1	3	3	0	1	0
SHR S 1 – Area 3	1	1	1	1	1	1	2	2	0	0	0
SHR S 2 – Area 1	2	2	2	1	1	0	2	2	0	1	0
SHR S 2 – Area 2	2	1	1	1	0	1	3	1	0	1	0
SHR S 2 – Area 3	2	1	2	2	1	2	2	2	0	1	0
Average	2	1.6	1.6	1.6	1	1.4	2.8	2.4	0	1	0

generation of tubules; inflam,
l; 1 = mild; 2 = moderate; 3 =

4.13.1 MVMM Supplementation and Histopathological Observations in the Kidney

Table 11: Qualitative Rating for Histopathological markers in the SHR and WIS groups

Markers	**Scores**			
	WIS	WIS S	SHR	SHR S
Glomerulosclerosis	0.6	0.4	3.8	2
Glomerular Apoptosis	0	0	0.4	1.6
Tubular Atrophy	0.6	0	0	1.6
Tubular Vacuolation	0.2	0	2.4	1.6
Tubular Dilation	0.6	0	3	1
Tubules: Presence of P-casts	0.4	0	1.2	1.4
Tubular Regeneration	0	0	0	2.8
Tubular Apoptosis	0	0	0	2.4
Interstitium: Inflammation	0	0	0.2	0
Blood Vessel Hyalinization	0.4	0	2.4	1
Blood vessel Periarteritis	0	0	0	0

Note: Histopathological evaluation on a scale of 0 to 4: 0 = normal; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe (Rouse *et al*, 2013).

In the WIS and WIS S, the histopathological features were at a minimal level ranging between 0 and 0.6, consistent with a normal kidney with an absence of pathology (Rouse *et al* 2013; Freberg *et al* 1994; Dworkin *et al* 1989).The SHR group demonstrated considerable histopathological changes with scores for the various features ranging from mild to severe. There was the presence of severe glomerulosclerosis and tubular dilation, moderate vacuolation and blood vessel hyalinization and mild presence of p-casts. There was mild inflammation of the interstitium. In the SHR S the histopathological profile improved with the presence of moderate glomerulosclerosis, mild tubular dilation, vacuolation, presence of p-casts and blood vessel hyalinization and an absence of inflammation. There was also presence of tubular atrophy, glomerular and tubular apoptosis and also tubular regeneration, features that were less prevalent or absent in all other groups.

The following images show some of the important histopathological markers found in the kidney in the WIS and SHR groups. Further images can be found in Appendix I.

4.13.2. Photomicrographs of the Histopathological Markers used to Rate Kidney Damage.

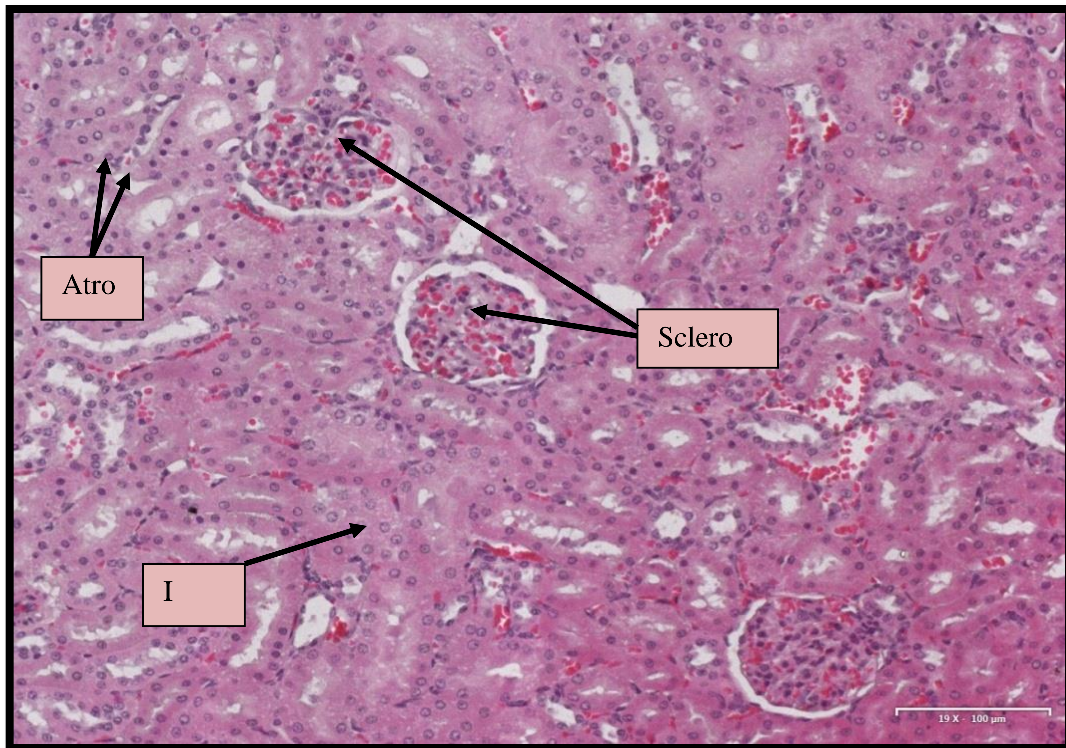


Figure 25: Micrograph (19x100 µm) in the WIS showing slight signs of glomerulosclerosis (Sclero), atrophy (Atro) and minor inflammation (I) of the tubules.

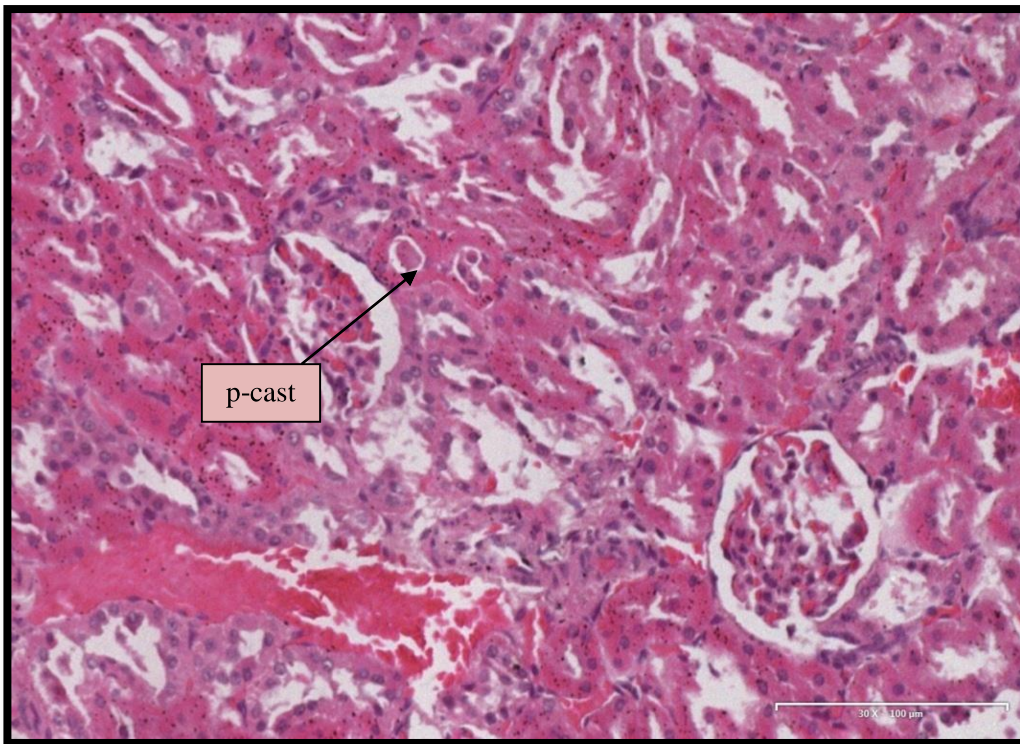


Figure 26: Micrograph (30x100 µm) in the SHR showing a protein cast (p-cast).

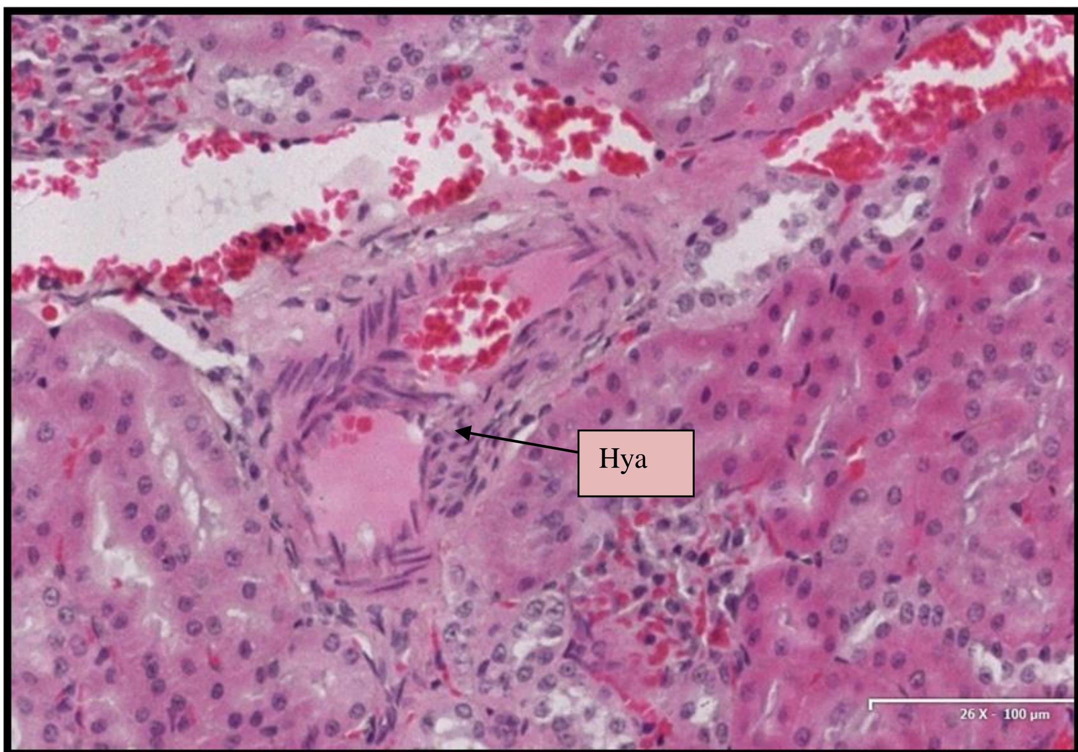


Figure 27: Micrograph (26x100 µm) of the SHR showing signs of hyalinization (Hya)

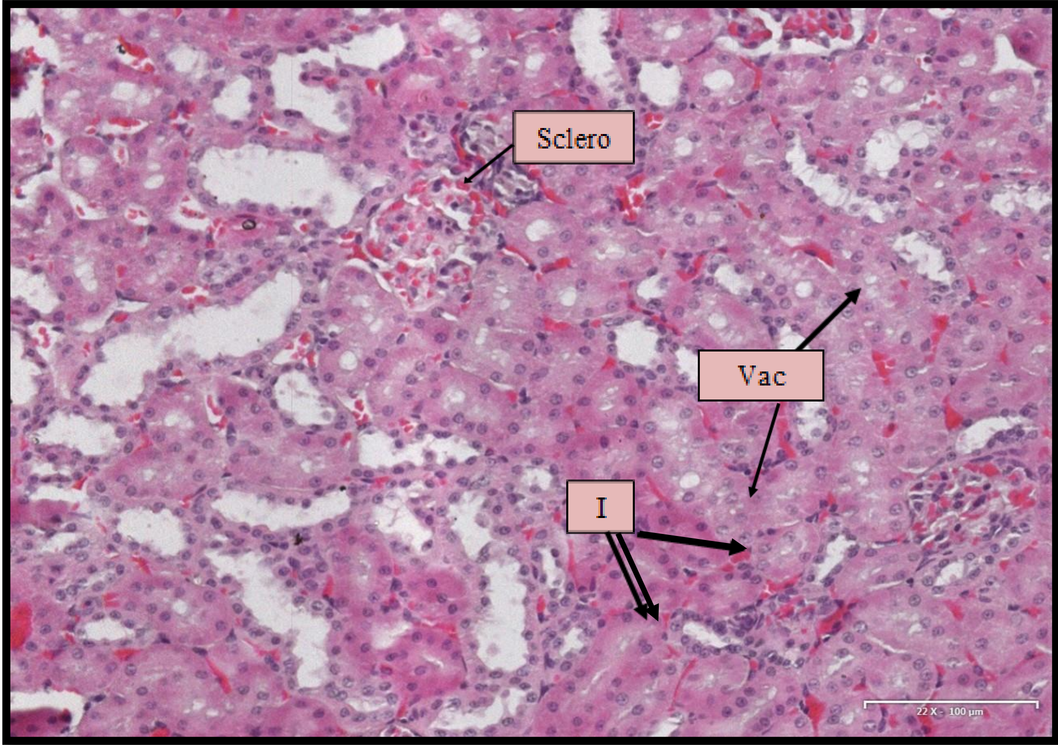


Figure 28: Micrograph (22x100 µm) of SHR showing distal DCT vacuolation (Vac), glomerulosclerosis (Sclero), inflammation (I)

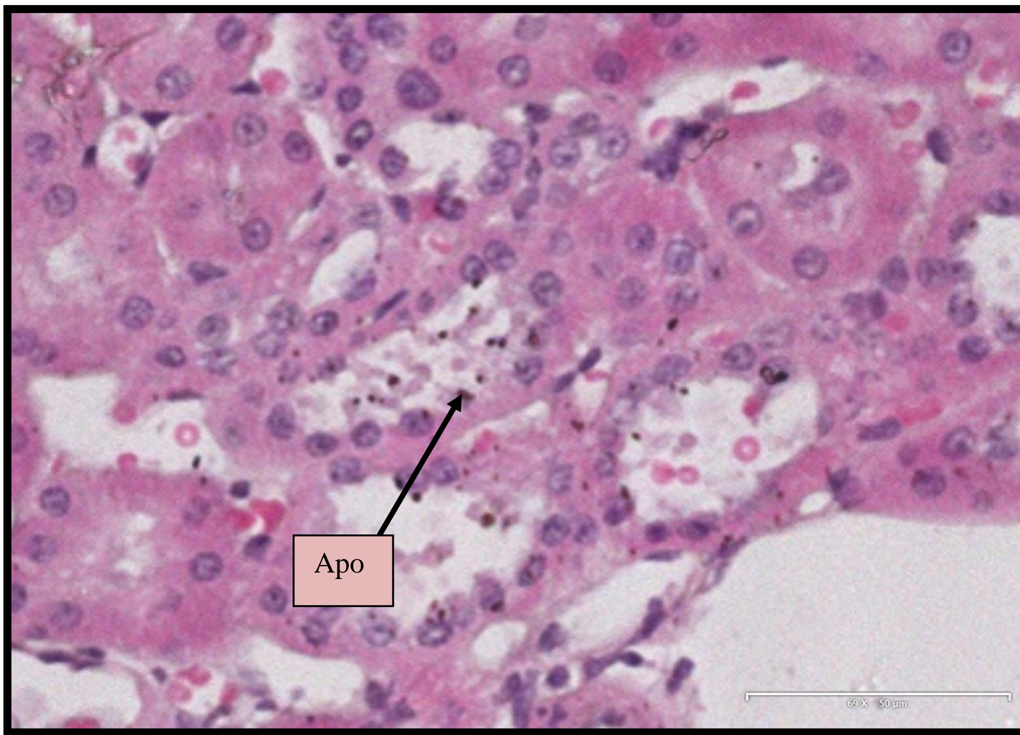


Figure 29: Micrograph (69x50 µm) of SHR S kidney showing signs of apoptosis (Apo)

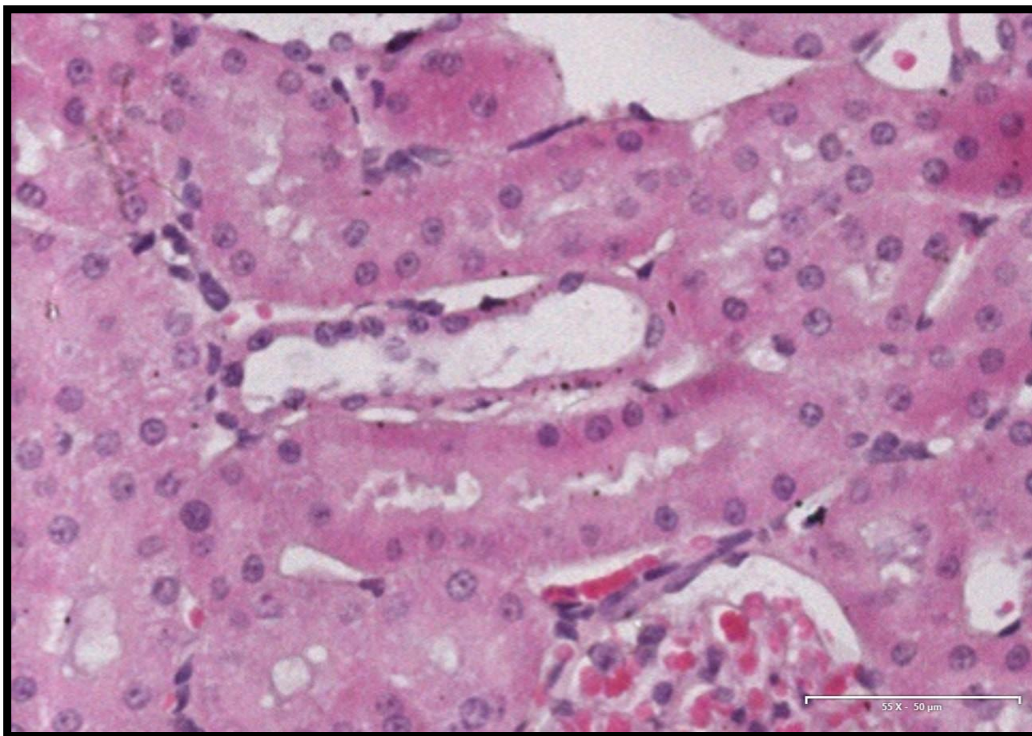
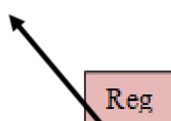


Figure 30: Micrograph (55x100 µm) of SHR S kidney showing signs of attempted regeneration (Reg)



4.14 MVMM Supplementation and Histopathology of the Aorta.

Table 12: Medial wall thickness (µm) of the thoracic aorta in the WIS and SHR rat groups

Group	WIS	WIS S	SHR	SHR S
Medial Wall Thickness (µm)	135.20 ± 3.08	132.25 ± 8.05	133.69 ± 38.05	135.11 ± 21.81

Note: $p < 0.05$ is significant for WIS and SHR

There was no significant difference in the medial wall thickness in the WIS S and SHR S groups when compared to their respective control groups.

The thoracic aorta in both WIS and SHR groups showed normal histological features in the tunica layers. The tunica intima (TI) showed a continuous layer of endothelial cells, and the tunica media (TM) displayed its characteristic feature of distinct elastic laminae, which are arranged concentrically in a wavy pattern with smooth muscle cells in between the concentric lamellae. The tunica adventitia (TA), comprised of fibrous elements, presented normally in both groups

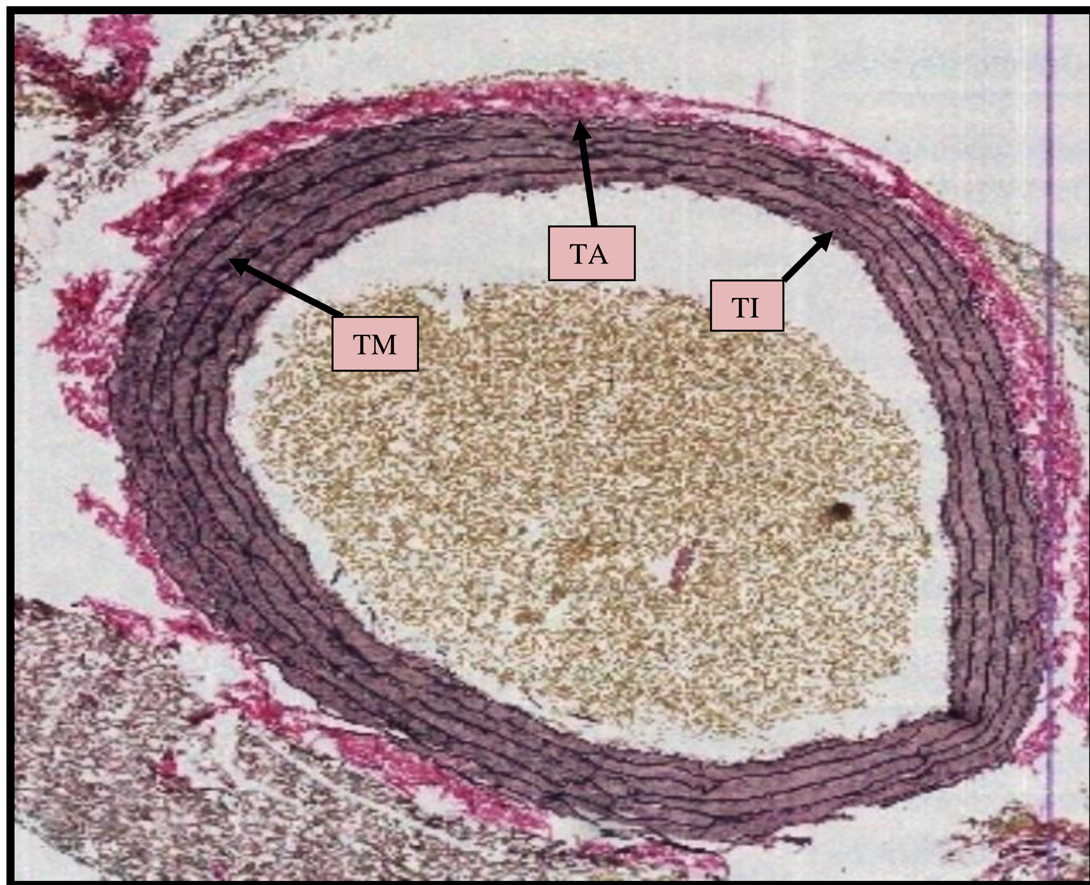


Figure 31: Micrograph showing a cross section of the thoracic aorta from the WIS group. Tunica adventitia (TA), tunica media (TM) and tunica intima (TI) are indicated.

5 DISCUSSION

There has been a proliferation in the availability and use of both human and animal nutritional supplements in recent years, specifically the MVMM supplement. Nano technology, inter-alia, has allowed for multiple combinations of supplements to be presented together in one suspension and hence delivered simultaneously in a single bolus. A typical example would be the Byboost™ supplement which was used for this study. This MVMM supplement was chosen due to its formulation which made use of nanotechnology, a fairly recent application in the field of nutritional sciences, which allows for a single bolus containing a variety of water and fat soluble supplements to be administered (Srinivas *et al* 2010). Due to nano encapsulation, suspended nutrient particles could be administered either in the drinking water, by oral dosing or by drenching the animal feed, as specified in the Byboost™ product information label, where it also claims to aid in growth, fertility and livestock performance.

Extensive research is still being conducted to determine the efficacy of these encapsulated nano particles, particularly nano minerals, in the livestock industry. The prospect of these particles increasing bioavailability may possibly enhance reproduction, growth and immune processes (Rajendran 2013). Research conducted so far shows promising results with new therapeutic formulations more readily entering cells and becoming accessible to intracellular pathways and cellular components thereby providing more effective preventative or palliative treatment (Srinivas *et al* 2010). The composition of the Byboost MVMM supplement stays within the range of the recommended daily allowance for the rats according to the Nutrient Requirements of Laboratory Animals (1995) for rats and mice (National Academic Press 1995).

There are various conflicting claims have been made pertaining to the effectiveness of these MVMM supplements in improving the health of the general population as well as alleviating and preventing the development of diseases and disorders such as hypertension.

The present study was conducted in three parts:

1. The first part involved investigating the effects of MVMM supplementation on the general health status in the spontaneously hypertensive rat (SHR), a well-established animal model of hypertension, and the Wistar rat strain which served as a control. The effects of MVMM supplementation on growth, blood pressure and various general health status parameters were studied for the duration of 8 weeks.

2. The second part focused more specifically on the effects of MVMM supplementation on hypertension and its associated biochemical parameters after 8 weeks. This also included the antioxidant effects of MVMM supplementation and oxidative stress.

3. The third part involved investigating the effects of MVMM supplementation on renal and vascular histology in the SHR after 8 weeks when hypertension was fully established.

5.1 The Effects of MVMM Supplementation on the General Health Status in the spontaneously hypertensive rat (SHR)

5.1.1 Growth Rate

The MVMM supplementation along with a regular diet increased growth by 16.4% in the SHR S group by the end of week eight. A significant difference in the percentage growth rate between the SHR and the SHR S first became apparent at week three and continued over the entire period of supplementation, yet no such effect was observed in the Wistar groups. Initially it was thought that the difference seen in the SHR S group may be related to food consumption, leading to a higher body mass. However individual animal food intake, monitored in a metabolic cage once a week, showed no difference in either the SHR or WIS groups. As observed by week 8, both the SHR and WIS supplemented and control groups had reached a plateau of roughly 16 grams food intake each. However there may be variations in food intake on the other days during communal feeding. This together with various anabolic responses triggered by supplementation such as an increase in growth hormone (GH) release, insulin-like growth factors (IGF-1), and oestrogen could have contributed towards this increase in growth rate in the SHR S group.

GH secretion can be stimulated by alpha 2-adrenoceptors activation which are located in the hypothalamus and have shown to be more sensitive in the SHR compared to the Wistar strain (Eriksson *et al* 1989). These receptors can also be located in the vasculature and have been implicated in the increase in vasoconstriction, therefore may also contribute towards the development of hypertension (Villalobos-Molina & Ibarra 1999). The hormone oestrogen is more relevant for discussion due to the gender of the rats used for this study, as well as their young age. Oestrogen has shown to influence growth rate especially during puberty where there are large amounts of this steroid present, causing an increase in secretion of GH via the hypothalamus and pituitary (Veldhius *et al* 1997). A decrease in oestrogen has also shown a direct link to the increased risk of developing hypertension, as it has a role in regulating vascular function, CNS functioning and the RAAS system (Ashraf & Vongpatanasin 2006).

Vitamins D is used as a treatment for GH deficiency as it has shown to increase circulating IGF-1 (Ameri *et al* 2013). Both IGF-1 and GH affect insulin levels in order to control carbohydrate metabolism (Clemmons 2004). In previous rodent studies vitamins D as well as B6 have shown to increase growth and growth hormone release (Huber & Gershoff 1965; Steenbox & Herting 1955).

Calcium, along with vitamin D supplementation has shown to decrease visceral adipose tissue and reduce weight gain by preventing fat accumulation in adipocytes (Rosenblum *et al* 2012). Another study shows the role of increased levels of conjugated linoleic acid, an essential omega-6 fatty acid, in decreasing the body fat index as well as abdominal adipose tissue (Hernandez-Diaz *et al* 2010). Free fatty acids (FFA's) are known to suppress growth hormone (GH) release, even directly blocking secretion from the pituitary gland (Quabbe *et al* 1972; Casanueva *et al* 1987). This may account for the slight but not significant decrease in body mass in the WIS S group as the combination of calcium, vitamin D and essential fatty acids may have lead to a decrease in abdominal fat being stored and therefore a decrease in body mass.

The above mentioned vitamins, minerals and fatty acids are all present in the Byboost™ supplement. They may be either individually or synergistically responsible for the positive growth effects. This could also be coupled with their increased bioavailability of the nutrients due to modern nano technology used in this supplement (Joye & Davidov-Pardo 2014).

An important question that needs addressing is why did supplementation have little effect on the growth rate of the WIS S group?

The MVMM supplementation did not make any significant difference in the growth rate of the WIS group which does normally exhibit a slightly higher growth rate than the SHR group, as seen in figure 16 (Okamoto & Aoki 1963, Okamoto *et al* 1966). The pathogenesis of hypertension is both polygenic and multi-factorial in the SHR model. It may be accompanied by various metabolic aberrations including alterations in trace element and vitamin metabolism. A marginal MVMM deficiency therefore cannot be excluded in the SHR strain and this could have possibly accounted for the positive response to the supplementation. Alternatively, the WIS groups were essentially healthy and all measured parameters, including growth, fell within the normal range as prescribed by the International Species Information System (ISIS) for a *Rattus Norvegicus* (ISIS 2002; Kurtz & Morris *1987;* Okamoto & Aoki 1963; Okamoto *et al* 1966). Hence they did not exhibit any significant response to MVMM supplementation

Percentage growth rate was used to express the data for body mass where the increase in body mass of each rat was expressed as a percentage increase based on the weight of the rat from day zero of the study. This method was used rather than actual increase in weekly body mass since it takes into account the initial variation in body mass between individual animals and groups at the commencement of the study (Ferry 2005; Creutzberg *et al* 2000; Robbins & Ballew 1984). Furthermore, the SHR rats normally have a slightly lower body mass when compared to the age matched Wistar rats (Dickhout & Lee 1998; Okamoto & Aoki 1963).

5.1.2 Blood Pressure

The significant DBP lowering effect of MVMM supplementation was observed after four weeks in both the WIS S and SHR S groups (Figure 17). This change actually brought the DBP in the SHR S group from Stage 1 hypertensive to almost borderline hypertensive levels (WHO 2013). The lowering of the DBP in particular indicates that supplementation may have more of an effect on the vasculature and peripheral resistance rather than on cardiac output. Blood vessel compliance may also have been improved by supplementation (McVeigh 1996).

Supplementation had a greater DBP lowering effect in the SHR group than the WIS group. The lowering of the DBP by 7 to 16 mm Hg is equivalent to the effects of some of the antihypertensive drugs currently available (Thomopoulos *et al* 2015; Limas & Freis 1972). Previous studies showed the individual antihypertensive effects of vitamins B6, E and C in SHRs, mainly in decreasing the SBP by attenuating adverse changes in the renal vasculature (Vasdev *et al.* 1991; 2001; 2002; Rodriguez-Iturbe *et al* 2003; Ames *et al* 2005). All three of these vitamins are found in the Byboost supplement given to the supplemented groups.

There are also numerous minerals within the Byboost™ supplement that have the ability to alter blood pressure. Selenium and copper are known to strengthen cellular antioxidant enzymes such as glutathione peroxidase (GPX), SOD and catalase, as well as positively influence the nitric oxide vasodilatory mechanism which plays a major role in blood pressure regulation (Arthur e*t al* 2003; Maggini *et al* 2007; Ognjanovic *et al* 2008). Zinc, copper and manganese may also have had direct blood pressure lowering effects (Loyke 1997; 2002; Klahr 2001; Rodrigo *et al* 2007).

The overall effect of the MVMM supplement was the lowering of the DBP in the SHR which therefore may play a role in delaying the onset of severe hypertension. Continued supplementation could possibly also have the potential to delay or mitigate end organ damage in hypertension.

5.1.3 Effects of MVMM Supplementation on other Health Status Parameters

5.1.3.1 Carbohydrate and Lipid Metabolism

Fasting blood glucose and total cholesterol are parameters indicative of carbohydrate and lipid metabolism. There was no significant difference in any of the groups, WIS and SHR, for the fasting glucose levels. For total cholesterol there was a significant strain difference with the SHR having a significantly higher total cholesterol level compared to the WIS group.

In the SHR S the supplement had a significant cholesterol lowering effect. However the total cholesterol level is unable to indicate exactly which of the lipid fractions have increased or decreased, therefore a detailed lipoprotein analysis is required. High density lipoproteins (HDL's) and the low density

lipoproteins (LDL's) as well as the triglycerides and very low density lipoproteins (VLDL) need to be quantified and their interrelationships via ratios further investigated. Certain vitamins with antioxidant properties can also influence lipid fractions, for example vitamins C and E have shown to cause an overall decrease in cholesterol by decreasing LDL's and increasing HDL levels (Jeon *et al* 2005; Carr, Zhu & Frei 2000). The oxidised fraction of the LDL fractions is also strongly associated with atherosclerotic disease and antioxidants, such as vitamin E, which have shown to decrease this particular fraction, therefore can offer a degree of cardio-protection in hypertension (Siess *et al* 1999; Steinberg 1995; Ricciarelli, Zingg & Azzie 2000)

5.1.3.2 Kidney Function

The Na^+/K^+ ratio, plasma creatinine, BUN and water intake are parameters typically indicative of kidney function (Duarte *et al* 2001; Gowda *et al* 2010). Creatinine levels showed no significant differences between all groups whereas the Na^+/K^+ ratio, water intake and BUN were significantly higher in the SHR which, as observed in previous studies, is typical of the strain (Rouse *et al* 2013). This indicates higher sodium retention, expansion of the plasma volume and an impaired renal function in the SHR. It may also in part indicate the renal contribution to the pathogenesis of hypertension in the SHR group (Purkerson *et al* 1976; Fled *et al* 1990; Karam *et al* 1996). Omega-3 fatty acid supplementation has shown to preserve renal function and morphology by decreasing plasma urea, K^+ and creatinine levels (Ahmed & Samad 2013).

Supplementation had no significant effect in this study on any of the renal parameters. The longer term effects of supplementation may show a significant effect. Therefore MVMM supplementation with its DBP lowering effects appears to have had greater effect on the cardiovascular system at this stage in the pathogenesis of hypertension rather than in the renal system.

5.1.3.3 Haematological Parameters

All haematological parameters showed no significant differences between the WIS and SHR strains except for Hb which was significantly lower in the SHR strain. The parameters were not altered by supplementation except for the RBC count which was significantly increased in the SHR S group. The Byboost™ supplement contains essential fatty acids and previous research has shown that an increased intake of fatty acids, specifically n-3 poly-unsaturated fatty acids, results in an increase in RBC's in the SHR rats (Bacova *et al* 2013). Vitamin B6 has also shown to play a role in heme biosynthesis, and its presence in the supplement may therefore have played a role in the increase in RBC count in the SHR S group (Ames *et al* 2005).

5.2 Effect of MVMM Supplementation on Biochemical Parameters Associated with Hypertension and Oxidative Stress

5.2.1 Plasma ACE and the Renin-Angiotensin Aldosterone System (RAAS)

There were no significant differences in plasma ACE levels between the WIS and SHR or in their respective supplemented groups. ACE levels are usually pretty constant during the first 6 months in the SHR development, and usually similar to those of normotensive rat models (Campbell *et al* 1995; Shisheva, Dimitrov & Ikonomov 1991). Due to ACE levels not being elevated at this stage in the SHR, it may indicate that the observed hypertension in the SHR groups would be due to vascular endothelial and possibly cardiac effects rather than via the humoral effects of RAAS (Dickhout & Lee 1998; Bernatova *et al* 2007; Bernatova 2014; Puzerova *et al* 2010; Balakumar *et al* 2007; Feld *et al* 1990).

5.2.2 Lipid Peroxidation in Oxidative Stress

The TBARS test was done to determine the degree of membrane lipid peroxidation in the kidneys. It is a prominent marker for end organ damage in essential hypertension due to oxidative stress (Ayala *et al* 2014; Russo *et al* 1998). No significant difference in MDA levels were observed between the WIS and SHR groups indicating the absence of significant oxidative damage. It may also be possible that due to the age of the animals, lipid peroxidation could be minimised or may not have commenced at this stage. Previous studies have shown that compensatory antioxidant mechanisms also operate during oxidative stress and these may have come into play, for example antioxidant enzymes which are produced endogenously such as GPX, SOD's and catalase. They are essential in counteracting the activity of ROS, reducing oxidative stress and limiting oxidative damage, thereby maintaining a balance in order to allow normal metabolic processes to occur (Sies 1997).

Vitamins E and C have shown to have antioxidant effects in decreasing oxidative stress associated with hypertension development. Vitamin C, in combination with vitamin E or acting individually increases the synthesis of NO, NADPH and superoxide dismutase activity and aids in reducing blood pressure (Rodriguez-Iturbe *et al* 2003; Sies, Stahl & Sundquist 1992; Halliwell & Chirico 1993).

The antioxidant effects of MVMM supplementation were also not apparent at this stage in the supplemented groups which in fact demonstrated a slight non-significant increase in MDA levels when compared to the unsupplemented groups.

5.2.3 Inflammation and Hypertension

Inflammation has been shown in numerous studies to accompany the development of hypertension, and CPR is a good indicator for the presence of this inflammation (Hage 2014; Clifton 2003; Semple 2006;

Yanai *et al* 2008). A direct relationship has been shown between the CRP levels and the degree of hypertension (Bautista *et al* 2001).

CRP levels showed no significant strain differences, and were not altered due to supplementation. Therefore at this stage it can be concluded that hypertension was not accompanied by an increased inflammatory response nor did supplementation have any CRP altering effects.

Human studies conducted in females have demonstrated that CRP levels increase directly with an increase in blood pressure but this was not observed in this study where both normotensive and hypertensive animals had similar levels of CRP (Horiuchi & Mogi 2011; Blake *et al* 2003; Oparil *et al* 2003; Semple 2006).

Consistent with CRP levels, the WBC count, which typically increases during infection and inflammation, was not altered significantly in all animal groups (Pooler 2009). For both the supplemented groups, there was a slight but not significant decrease in WBC count.

The platelet levels were also not altered significantly in all groups. They respond to inflammatory mediators and are involved in the blood clotting process during tissue damage and injury. Previous studies have suggested that hypertension can lead to an increase in thrombosis and a hypercoagulative state (Lip 2003).

To conclude, the ACE, TBARS and CRP parameters showed no changes between the strains or due to supplementation. This may indicative of an early stage in the pathogenesis of hypertension without the onset of the biochemical changes associated with established hypertension. Further, biochemical and/or morphological changes at this stage of the hypertension may be reversible, and may also be responsive to the effect of antioxidants. In the biochemical analysis, although not statistically significant, some interesting “trends” are noted. MVMM supplementation showed a downward trend in plasma CRP levels, WBC and platelet counts pointing to possible antioxidant effects. On the other hand, MVMM supplementation showed an upward trend in plasma ACE and membrane lipid peroxidation pointing to possible pro-oxidant effects.

5.3 Effects of MVMM Supplementation on the Antioxidant Associated Trace Element Status

5.3.1 Trace Element Status

Trace elements play a vital role in the normal regulation of blood pressure and in the antioxidant defence systems within cells and in the blood. They are also integral components in the various metalloproteins and metalloenzymes which also function as antioxidants (Russo1998; Evans & Halliwell 2001). Oxidative stress plays a major role in the pathogenesis of hypertension and there is a direct relationship

between the antioxidants status and the development of hypertension (Mylonas & Kouretas 1999; Oparil *et al* 2003).

By determining trace element levels in the blood and blood pressure regulatory organs like the kidney and brain, one can assess firstly to what extent their profile is altered by hypertension, and secondly, by measuring specific antioxidant associated trace element levels so that the status of the antioxidant system can be gauged in an indirect manner. The main trace elements involved in both blood pressure regulation and the antioxidant system are Zn, Cu, Fe, Mn, & Se.

ICP-OES and EDX – SEM are two of the most advanced a methods for conducting element analysis. They were chosen in order to give a complete and accurate picture of the total trace element content per unit mass of tissue as well as the topographical distribution of elements in specific surface areas of the kidney and brain tissue, namely the kidney cortex and the hippocampus and prefrontal cortex in the brain.

5.3.1.1 Trace Element profile using ICP

The trace element profile was analysed in whole blood, kidney and brain using ICP. As stated in the methods, entire kidneys, brain and whole blood were analysed for all trace elements per gram of tissue or per millilitre blood. There were no significant changes in Fe or Cu levels between the WIS and SHR groups in all tissue samples, and MVMM supplementation also had no effect on their distribution. Zn levels were elevated only in the kidney of the WIS S group otherwise there were no changes observed in the rest of the tissue samples. Se and Mn tissue levels were not in the detectable range using ICP.

The observed beneficial effect of trace element supplementation occurred only in the WIS S group and included only zinc. Zinc is an important component in the extracellular and cytoplasmic superoxide dismutase (Cu-Zn-SOD), an important antioxidant enzyme. Therefore a Zn deficiency can also lead oxidative stress through damage to lipids, DNA and proteins (Rodriguez-Iturbe *et al* 2002; Evans & Halliwell 2001).

The overall tissue trace element profile was stable in all organs and blood, with no major alterations either due to hypertension or supplementation.

5.3.1.2 Elemental profile using EDX

Unlike ICP analysis, EDX analysis showed significant changes in the surface macro and microelement concentrations. High values for macromolecules such as C, N, O, P and Ca were found consistently throughout all tissue samples mainly due to their quantity as they are essential elements for basic cellular structure and function in all living tissues. The K, O, S, Al, P and Na concentrations were either increased or decreased in the kidney, cortex or hippocampus of WIS S and SHR S groups only. No

significant changes were observed between the WIS or SHR groups. This therefore may indicate that these effects were mainly due to supplementation. However the extent of these elemental changes and how they may affect structural or physiological functions needs further investigation as there is little in the literature pertaining to animal studies.

Unlike macro and micro elements, antioxidant associated trace elements were not detected consistently in all areas scanned in both the brain and kidney. Therefore in conclusion, with the exception of Zn, there were no major shifts in the tissue trace element profile during hypertension or supplementation as seen in both the ICP and EDX results. The EDX analysis methodology needs to be further reviewed for our needs and results need to be approached with caution as this technique used in the analysis of animal tissues is still in its early stages and more specific data for rats is required for a comparison.

5.4 The Effects of MVMM Supplementation on Renal and Aortic Morphology

5.4.1 Renal Pathology in the Development of Hypertension

Histological slides of the kidneys of all animal groups were prepared and a qualitative histopathological evaluation (Table 11) was carried out for typical renal markers of hypertension (Rouse *et al* 2013; Venkatachalam *et al* 2010; Feld *et al* 1990). Pathological features were scored on a rating scale from 0 – 4, with 0 = normal, 1= mild, 2 = moderate, 3 =severe, 4 = very severe (Duarte *et al* 1997, Rouse *et al* 2013).

In the SHR group, significant renal pathology was observed. This was characterised by the presence of glomerulosclerosis and glomerular apoptosis, tubular dilation, vacuolation and p-casts, as well as mild localised inflammation of the interstitium and hyalinization in some of the renal blood vessels. The WIS group did show very mild histopathological changes. It is unlikely that the WIS kidneys would have shown zero marker activity, as previous research has indicated that Wistar rats aged three months and older already begin to show signs of renal pathology which are typically associated with normal age-related changes. (Hirokawa 1975; Ofstad & Iversen 2005) The above observations also agree with pre-existing data for the SHR and WIS models (Rouse *et al* 2013; Freiberg *et al* 1994; Dworkin *et al* 1989).

In the SHR S the histopathological profile improved considerably with moderate glomerulosclerosis, mild tubular dilation, vacuolation, presence of p-cast, blood vessel hyalinization and interstitial inflammation. There was also presence of tubular atrophy, glomerular and tubular apoptosis and also tubular regeneration, features that were less prevalent or absent in all other groups. This group therefore exhibited the benefits of both lowered blood pressure and MVMM supplementation. The Wistar S group also showed some benefits of supplementation by exhibiting only mild signs of glomerulosclerosis, and a normal presence for the rest of the renal markers.

The morphological changes in the kidney of the SHR were accompanied by increased BUN and Na^+/K^+ ratio and are indicative that the kidney seems to be the organ most affected during the early stages of hypertension development. These biochemical parameters were not decreased in the SHR S group indicating that although supplementation and the consequent lowered blood pressure improved renal morphology, there were no changes in renal function (Ahmed & Samad 2013; Rouse *et al* 2013).

These observed signs of early renal damage in the SHR would also have worsened as the animals aged, resulting in a decreased GFR, renal excretion and plasma flow (Karam *et al* 1996). Previous studies have monitored the progression of decreased renal function in the SHR as they age, where young SHR at 10 weeks of age and older already show signs of renal damage such as increased protein excretion and decreased GFR when compared to their respective WIS control group. They also display signs of tubular damage and glomerulosclerosis (**Ofstad & Iversen 2005;** Yang, Zuo & Fogo 2010).

5.4.1.1 Further Observations for Biomarkers of Renal Damage

- The SHR group showed mild localized inflammation while the SHR S group showed no inflammation in the interstitium. This corresponded with plasma CRP levels, WBC and PLT counts which were not elevated. This observation indicates that MVMM supplementation may have had some renoprotective effect, either in its antioxidant activity or by its blood pressure lowering effects in the SHR S group.

- There was a large decrease in observed tubular dilation in the SHR S group. Toxins present in the ultra-filtrate, as well as released from the PCT cells may have contributed towards the dilation of the tubules. Dilated tubules have shown to induce apoptosis leading to the disappearance of the epithelial cells to be replaced by a fibrous tissue, leading to tubulointerstitial fibrosis (Kida & Sato 2007). This was therefore a positive effect of both lowered blood pressure and supplementation.

- Hyalinization appeared less frequently in the SHR S compared to the SHR. It occurs near the end stages of renal disease or in full-blown hypertension, and can be located mainly within the renal arterioles and arteries as well as in the glomeruli (Carstens & Allen 1970; Sangle 2013).

- Tubular atrophy and p-casts formation showed an increased in the SHR S group. It is caused mainly by proteins breaking past the capillary barrier and entering into the PCT (Burton & Walls 1996). Apoptosis then usually follows in conjunction with atrophy as a response in acute renal injury (Venkatachalam *et al* 2010). Apoptosis and atrophy, was accompanied by regeneration, only in the SHR S group. This may indicate a possible response mechanism to

ROS production causing oxidative damage. Regeneration could also be due to the significantly decreased blood pressure and antioxidant effects of the supplementation (Elmore 2007).

- Tubular regeneration in injured tubular cell occurs after tubular apoptosis/atrophy mainly in the intrinsic renal cells, where there is a pre-existing stem cell population or surviving tubular cells still intact that can undergo mitosis (Smeets *et al* 2013). Signs of regeneration were only seen in the SHR S group, where supplementation seemed to promote the beginning stages of regeneration in the tubular cells. Erythropoietin, produced primarily in the kidneys in the renal cortical fibroblasts, is a cytokine which can stimulate the proliferation of endothelial cells and angiogenesis, resulting in regeneration. Erythropoietin has shown to have anti-inflammatory and anti-apoptosis properties specifically in the kidney, therefore can play a role in decreasing inflammation associated with renal damage in hypertension (Rafieian-Kopaei, Baradaran & Rafieian 2012; Harvard edu. 2000). It is also used for the production of RBC's and together with the significant increase in the RBC count seen in the SHR S, may demonstrate the role of erythropoietin in early tubular regeneration (Rafieian-Kopaei *et al* 2012).

5.4.1.2 Proposed Role of Antioxidants in Renal function

The observed markers for renal damage and dysfunction encompasses glomerular injury, nephron destruction and kidney scarring which all ultimately leads to loss of kidney function, and are common pathophysiological features of hypertension (Rouse *et al* 2013; Duarte *et al* 1997; Zoja *et al* 2015; Benhagen 2005). Therefore the decreased occurrence of some of the markers in the supplemented groups could indicate that both decreased blood pressure and MVMM supplementation may have prevented or reversed further renal damage from occurring.

The production of ROS during oxidative stress disrupts endothelial function and increases inflammation, migration and hypertrophy, as well as apoptosis which is traditionally seen as a contributor, along with the previously mentioned markers, to the vascular remodelling and renal damage seen in hypertension (Paravicini & Touyz 2008). Nitric oxide, produced by endothelial cells, can also regulate glomerular vascular tone, inhibit platelet aggregation as well as prevent leukocytes from adhering to the glomerular capillary walls. Therefore damage to the glomerular endothelium results in a decrease these anti-inflammatory and anticoagulant properties, allowing for infiltration of inflammatory factors into the glomerular capillaries and resulting in renal damage, which in turn may contributed towards the development of hypertension (Nahas 2003).

Vitamins C and E acting as antioxidants have shown to decrease renal damage caused by oxidative stress by specifically decreasing the renal cortical and cortical ROS as well as decreasing arterial pressure and increasing renal plasma flow. They have also shown to improve endothelial function which

plays a vital role in blood pressure regulation (Tian *et al* 2005; Yano *et al* 1998). Supplementation with vitamin B6 have shown to improve renal vascular hyperplasia, with minimal wall thickening and narrowing of the lumen in the renal arterioles, therefore aiding in blood pressure management (Vasdev *et al* 1999).

Omega-3 fatty acid has shown to improve renal function and blood pressure by improving glomerular hypercellularity in the form of typically polymorphonuclear neutrophils and leucocytes, vascular lesions, PCT and DCT injury and interstitial cell infiltration. It also has shown to decrease synthesis and release of transforming growth factor-beta 1 (TGF-β1), a profibrotic factor involved in kidney function impairment (Holm *et al* 2001; Ahmed & Samad 2013). Amino acids, such as L-Arginine, have shown to improve renal function, specifically the GFR, decrease interstitial and tubular inflammation and aid in improving blood pressure control (Klahr 2001).

Vitamin D deficiency is a common cause of renal damage. The enzyme 1-alpha-hydroxylase is typically produced in the kidney, and used to convert vitamin D into calcitrol. However due to the kidney damage and dysfunction there is a decrease in 1-alpha-hydroxylase synthesis, and therefore a decrease in calcitrol, leading to hyperplasia of the parathyroid (PT) gland, and an increase in parathyroid hormone (PTH) synthesis. This increase in serum PTH results in a disturbance of the calcium/phosphorous balance in the vasculature (Parry 2004; Levin *et al* 2007; McCarron 1982). Therefore vitamin D supplementation would increase calcitrol levels resulting in a decrease in the PTH levels. It has also shown in separate studies to prevent decrease in the GFR during the early stages of renal damage (De Boer *et al* 2011).

5.4.2 Vascular Remodelling in Hypertension

Vascular remodelling in hypertension is due to an increased load on the vascular walls, resulting in wall tension and eventually increased tensile stress. Therefore to compensate for this, vascular wall thickness increases and/or the lumen diameter decreases. Hypertension is most commonly associated with eutrophic remodelling, where there is an inward growth in the vasculature resulting in a smaller lumen, and an increase in the wall/lumen ratio. Other mechanisms commonly associated with remodelling are apoptosis, vascular fibrosis and inflammation caused by oxidative stress, all resulting in an increased peripheral resistance (Intengan & Schiffrin 2001).

Previous studies conducted in SHR noted an increase in the medial wall thickness of the thoracic aorta as well as in coronary arteries and arterioles (Bachmann *et al* 1992). There were no significant changes to the medial wall thickness in all animal groups, indicating that remodelling in the SHR had not yet begun. Significant changes in the vasculature can be observed in the later and more severe stages

hypertension and remodelling of the blood vessels and/or major arteries like the aorta and femoral artery, can be clearly seen (Lakatta & Levy 2003).

There were significant changes observed in the blood pressure in the SHR group but no significant changes occurring in the vasculature. This may therefore indicate that vasoactive substances, such as TNF and IL-6, were responsible for the development of hypertension rather than vascular morphological changes which are only usually seen in later development of hypertension in the aged rats (Limas, Westrum & Limas 1980). Vasodilatory mechanisms are related to the antioxidant system, such as when there is a deficiency in Zn, this decreases vasodilator receptor functioning in the vasculature, leading to an increase in blood pressure (Browning, Reeves & O'Dell 1987).

Minor vascular structural changes may start occurring already in the pre-hypertensive phase in the SHR but in elastic arteries such as the aorta, changes are not as prominent during the early phase of hypertension (Lee 1985; Van Gorp 1995).

6 CONCLUSION

The pathogenic sequence in the SHR is characterized by endothelial dysfunction followed by smooth vascular muscle dysfunction, then a decrease in renal function due to proteinuria and renal damage in the form of glomerulosclerosis, tubular atrophy, p-cast formation and juxtaglomerular damage, much as in a human subject with essential hypertension (Bernatova *et al* 2009; Bernatova 2014; Puzerova, Kopincova & Bernatova 2010; Balakumar *et al* 2007; Feld *et al* 1990).

MVMM supplementation had significant metabolic, cardiovascular and renal effects in the SHR group. It increased growth and caused a significant reduction in the diastolic blood pressure of both SHR S and WIS S groups over the eight week period. The reduction in diastolic blood pressure was possibly achieved through changes in vasoactive factors in the vascular endothelium as there were no significant morphological changes in the large blood vessels as seen in the aorta. It also to a lesser extent, lowered total cholesterol and increased RBC count in the SHR S group.

Supplementation also had some renal protective properties as the SHR S group exhibited lesser hypertension related morphological changes. These were however not translated into any improvement in renal function. The renal effects could have been mediated both via a decreased blood pressure and may also be due to the possible antioxidant effects of the supplement. Further quantitative analysis and biochemical tests are however needed to determine whether supplementation may have the potential to reverse existing pathological changes.

Overall, the current dosage of supplementation showed no deleterious effects in any of the animal groups. The pathogenesis of hypertension is both polygenic and multifactorial in the SHR model. It may be accompanied by various metabolic aberrations including alterations in trace element and vitamin metabolism which may in turn contribute towards the pathogenesis of hypertension or, alternatively, may arise from it. A marginal MVMM deficiency therefore cannot be excluded in the SHR strain and this could possibly account for its positive response to the supplementation. In a review by Dr. Ioannis A. Delimaris, it is stated that “low dose mixtures, as in multivitamin/multimineral tablets, can sometimes do good, but may be beneficial only for those members of populations whose diet and lifestyle are so bad that they are deficient in certain micronutrients” (Delimaris 2012). This may also be applicable to the present study.

New age MVMM supplementation, using nano technology, incorporated in the diets, especially in the growth phase of young animals, may be effective in maintaining general growth and health status. It may also be important in delaying the onset of the effects of hypertension in the breeding stock of SHR rats. Supplementation has been clearly effective in younger animals in the SHR strain and therefore is recommended for the SHR breeding stock in BRU. It is not labour intensive as it can be used as a drench

on the animal feed. Duration and timing of supplementation in older animals need to be resolved. Breeding pairs are quite expensive to purchase and supplementation would ensure improved breeding and longevity in this group.

6.1 Recommendations to Improve/Complement the Present Study

- Increasing the duration of the study and introducing varied dosages of supplement may assist in getting an accurate display of the long-term effects of MVMM supplementation, even though 8 weeks is a long enough time considering the life span of a rat.
- Extend the study to include the use of male rats in order to determine gender responses to supplementation
- Further research is required at a biochemical and molecular level to study the mechanisms of the effects caused by MVMM supplementation.
- Morphological studies in kidney need to be followed up by further quantitative and biochemical tests.
- Design further studies to investigate the possibility of antioxidants as therapeutic, disease-preventing and anti-aging agents.

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8. APPENDICES

Appendix A: Data for Growth curve – WIS and SHR Groups

Table 13: Data for % Growth curve for Wistar groups

Weeks	Wistar		Wistar Supplemented	
	(g)	%	(g)	%
Baseline	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	12.86 ± 7.17	8.80 ± 4.63	14.71 ± 3.50	10.27 ± 2.65
2	24.86 ± 6.82	17.16 ± 4.10	24.57 ± 5.71	17.13 ± 4.06
3	39.29 ± 9.03	27.31 ± 6.06	36.86 ± 7.03	25.63 ± 4.93
4	52.57 ± 11.47	36.37 ± 6.77	50.14 ± 9.23	34.96 ± 6.84
5	60.86 ± 13.81	42.19 ± 8.80	57.29 ± 10.13	39.79 ± 6.58
6	76.86 ± 17.54	53.12 ± 10.32	68.14 ± 13.33	47.40 ± 9.12
7	84.14 ± 20.54	58.15 ± 12.12	76.14 ± 13.41	53.10 ± 10.26
8	85.86 ± 20.36	57.34 ± 15.45	78.43 ± 14.75	54.57 ± 10.20

Table 14: Data for % Growth curve for the SHR groups

Weeks	SHR		SHR Supplemented	
	(g)	%	(g)	%
Baseline	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	2.14 ± 2.34	1.55 ± 1.67	5 ± 2.94	4.09 ± 2.46
2	13.00 ± 4.80	9.28 ± 3.31	17.71 ± 9.21	14.25 ± 7.59
3	18.00 ± 6.98	12.91 ± 4.96	30.29 ± 6.40	24.44 ± 5.71
4	30.43 ± 7.25	21.87 ± 5.40	43.14 ± 4.14	34.73 ± 3.99
5	32.71 ± 7.09	23.46 ± 5.27	45.86 ± 5.40	36.91 ± 4.85
6	35.43 ± 7.93	25.4 ± 5.84	54.57 ± 5.00	43.87 ± 4.26
7	39.57 ± 4.54	28.37 ± 3.68	59.43 ± 4.50	47.82 ± 4.46
8	43.86 ± 8.45	31.50 ± 6.55	59.57 ± 6.97	47.92 ± 5.98

Appendix B: Systolic and Diastolic Blood Pressure – WIS and SHR Groups

Table 15: Data for Systolic and Diastolic Blood pressure for WIS group

Week	Wistar		Wistar Supplemented	
	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
0	114.71 ± 2.85	83.43 ± 2.34	120.14 ± 4.81	82.43 ± 3.68
1	118.43 ± 3.88	85.57 ± 2.09	103.71 ± 4.29	83.14 ± 3.10
2	121.29 ± 3.16	81.29 ± 1.77	116.86 ± 4.06	82.71 ± 3.11
3	113.43 ± 2.93	77.57 ± 2.77	108.71 ± 3.35	76.57 ± 1.96
4	114.57 ± 1.19	80.43 ± 1.11	116.00 ± 3.83	70.86 ± 2.60
5	121.71 ± 1.15	77.86 ± 2.70	119.14 ± 1.97	68.14 ± 2.60
6	124.00 ± 1.20	81.00 ± 2.42	117.71 ± 1.66	67.71 ± 1.27
7	120.57 ± 1.07	79.00 ± 2.86	117.71 ± 0.94	69.00 ± 1.6
8	120.71 ± 1.19	82.86 ± 2.22	114.00 ± 1.41	72.71 ± 1.15

Table 16: Data for Systolic and Diastolic Blood pressure for SHR group

Week	SHR		SHR Supplemented	
	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
0	149.29 ± 2.65	122.57 ± 4.31	135.86 ± 3.73	113.29 ± 3.40
1	142.71 ± 2.02	102.57 ± 1.13	144.14 ± 4.80	103.57 ± 6.52
2	139.00 ± 1.15	100.86 ± 2.89	132.00 ± 3.18	101.71 ± 2.38
3	142.29 ± 2.62	106.00 ± 3.02	142.71 ± 3.87	97.57 ± 2.69
4	143.29 ± 1.19	105.71 ± 2.24	137.86 ± 2.86	92.86 ± 4.93
5	144.14 ± 1.03	112.00 ± 1.59	134.86 ± 1.40	92.71 ± 3.20
6	144.71 ± 0.97	101.43 ± 3.26	134.71 ± 0.81	90.57 ± 2.00
7	145.29 ± 1.95	110.29 ± 2.25	139.29 ± 1.36	88.57 ± 2.33
8	142.57 ± 1.49	108.00 ± 2.98	137.86 ± 2.33	89.57 ± 1.85

Appendix C: TBARS Reagents

Preparation of Reagents

1. BHT: 20mM → 0.449g in 100µl of ethanol

2. NaOH: 50mM → 0.2g into 100ml of H_2O

Took 5ml of 20mM BHT, and added it 100ml of NaOH solution and mixed, then added 1g of TBA, producing the BHT/TBA solution.

3. 0.2% phosphoric acid → 170µl of 85% phosphoric acid into ± 99.83ml of H_2O.

4. 2% phosphoric acid → 1.7ml of 85% phosphoric acid into ±98.3ml H_2O.

5. 7% phosphoric acid → 5.95ml of 85% phosphoric acid into ±94.05ml H_2O.

6. 1M HCl → 9.5ml HCl into 90.5ml H_2O.

Appendix D: CRP ELISA Reagent Preparation

1. The stock Biotinylated C Reactive Protein Antibody was diluted with 1x Diluent M to prepare 1x Biotinylated C Reactive Protein Antibody.

2. The following calculation was used to calculated the volume of (X) stock Biotinylated Antibody was required for a selected number of wells:

$$(C_F / C_S) \text{ x } V_T = V_A$$

C_S = Starting concentration (X) of stock Biotinylated CRP Antibody (variable)

C_F = The final concentration (always = 1X) of Biotinylated CRP Antibody solution for the assay procedure.

V_T = Total required volume of 1x Biotinylated CRP Antibody solution for the assay procedure (Values were given in a table and depend on number of wells chosen. For this ELISA 80 wells were required, therefore the total volume of 1x Biotinylated CRP Antibody was 4400 µl)

V_A = Total volume of (X) stock Biotinylated CRP Antibody.

3. A final volume of 1x Diluent M was calculated to prepare the 1x Biotinylated CRP Antibody. The calculation is as follows:

$$V_T - V_A = V_D$$

V_D = Total volume of 1x Diluent M required to dilute (X) stock Biotinylated CRP Antibody to prepare 1x Biotinylated CRP solution for assay procedure.

Appendix E: CRP ELIZA Standard Preparation

Reconstituted the C Reactive Protein Standard vial to prepare the 50 ng/mL C Reactive Protein Standard number 1.

1. First consulted the CRP standard vial to determine the mass of the protein.

2. Then calculated the appropriate amount volume of 1x Diluent M to add to resuspend the CRP Standard vial to produce the 50 ng/mL by using the following calculation:

$$(C_S / C_F) \times 1000 = V_D$$

C_S = Starting mass of CRP Standard found on vial

C_F = The 50 ng/mL CRP Standard 1 final required concentration

V_D = Required volume 1x Diluent M for the reconstitution (µL)

3. The reconstituted 50 ng/mL CRP Standard 1was left for 10 minutes.

4. A further 7 tubes were labelled and 120 µl of 1x Diluent M was added to each one.

5. Standard 2 was prepared by adding 120 µl of Standard 1 to it and gently mixed.

6. Standard 3 was prepared by adding 120 µl of Standard 2 to it and gently mixed.

7. Standard 4 was prepared by adding 120 µl of Standard 3 to it and gently mixed.

8. Standard 5 was prepared by adding 120 µl of Standard 4 to it and gently mixed.

9. Standard 6 was prepared by adding 120 µl of Standard 5 to it and gently mixed.

10. Standard 7 was prepared by adding 120 µl of Standard 6 to it and gently mixed.

11. Standard 8 contained only 1x Diluent M and served as a zero standard.

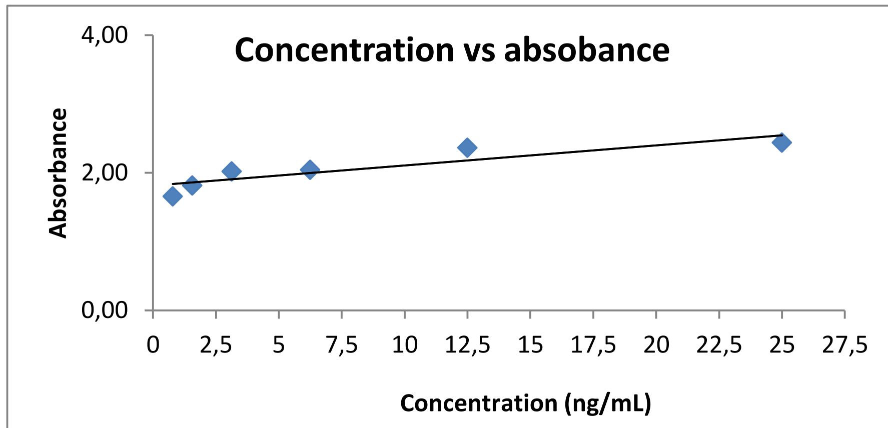

Figure 32: Standard curve for CRP ELISA

Appendix F: Standard Preparations for ICP-OES Analysis

Table 17: ICP-OES Multi-element Standard Preparation

	STD 1	STD 2	STD 3	STD 4	STD 5
Concentration (µg/ml)	0.1	0.5	1	2	5
Stock Volume (µl) in 50 ml standard	5	25	50	100	250

Table 18: ICP-OES Single-element Standard Preparation

	STD 1	STD 2	STD 3	STD 4	STD 5
Concentration (µg/ml)	0.5	1	5	10	50
Stock Volume (µl) in 50 ml standard	25	50	250	500	2500

12N HCL (37%) in distilled water was prepared for the blank.

Appendix G: Histological Investigation - H&E Stain

The Haematoxylin and Eosin stains, Xylene, Absolute alcohol and DPX were provided for.

The staining procedure is as follows:

1. Filled 250 ml xylene into two separate staining jars.

2. Filled the following 4 staining jars with 250 ml each of 100% alcohol, 90% alcohol, 70% alcohol and 50% alcohol.

3. Filled the last staining jar with 250 ml distilled water.

4. Put slides into a staning rack, ensuring the slides did not touch.

5. The racks went through clearing (deparaffinising) and rehydration according to the following steps:

a) Xylene – 3 minutes

b) Xylene – 3 minutes

c) 100% alcohol – 2 minutes

d) 90% alcohol – 2 minutes

e) 70% alcohol – 2 minutes

f) 50% alcohol – 2 minutes

g) Left in distilled water until ready to stain.

6. Two glass rods were placed over a sink with enough space to fit the slides on. Slides were then removed from rack and placed on the glass rods with tissue section oriented up.

7. Flooded slide with haematoxylin using Pasteur pipettes and left for 5 minutes.

8. Removed excess haematoxylin by dropping off into the sink.

9. Flooded slides with tap wate with a Pasteur pipettes, then dropped excess off into sink.

10. Repeated step 9 three times or until the purple stained sections turned blue.

11. Dropped eosin over the tissue sections with a Pasteur pipette and left for 3-5 minutes.

12. Rinsed off the residual eosin with tap water and then placed the slides back into the staining rack.

13. Dipped the rack into 90% alcohol and then into 100% alcohol and gently tapped off the rack on a roller towel.

14. Placed the rack into Xyelne until ready for coverslip.

15. Removed 1 slide at a time from xylene and placed onto filter paper to remove any risual xylene.

16. With an applicator, dropped DPX mounting glue directly over the tissue section

17. Gently lowered an appropriate sized coverslip over the DPX to ensure no air bubbles were trapped.

18. Left overnight to dry.

Appendix H: Histological Investigation - Elastic Stain

The working elastic stain solution was prepared by adding the following reagents in order indicated, into a Coplin jar:

1. Heamatoxylin solution, Alcoholic (20ml)
2. Ferric chloride solution (3ml)
3. Weigert's Iodine solution (8ml)
4. Deionised water (5ml)

The working ferric chloride solution, for differentiation, was prepared by adding 3ml of ferric chloride solution and 37ml deionised water to a Coplin jar and mixed well.

The Van Gieson solution was provided for and ready to use.

The staining procedure is as follows:

1. Placed deparaffinised slides into the working elastic stain for 10 minutes
2. Rinsed in deionised water
3. Differentiated in working ferric chloride solution for 1 minute
4. Rinsed with tap water
5. Checked microscopically. If was over differentiated, returned to working elastic stain solution
6. Rinsed in 95% alcohol to remove iodine.
7. Rinsed in deionised water
8. Stained with the Van Gieson solution for 1.5 minutes
9. Rinsed in 95% alcohol
10. Dehydrated with xylene and the mounted with GPX and coverslip.

Appendix I: Additional micrographs of histological analysis of the kidneys using H&E staining.

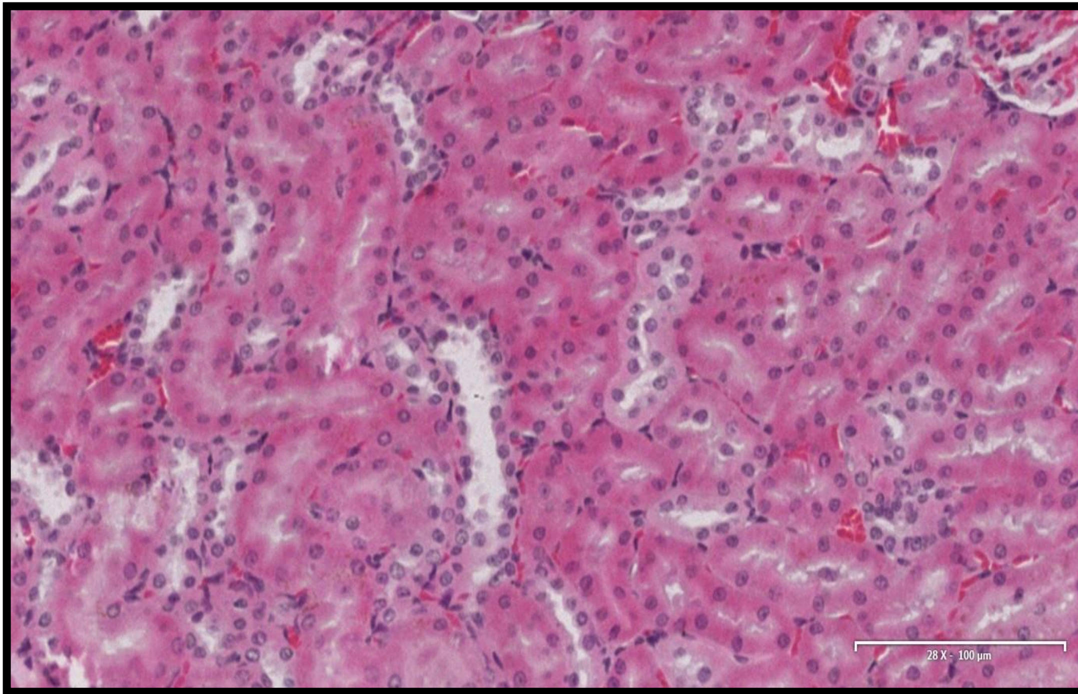

Figure 33: Micrograph (28x100 µm) of WIS S showing normal distal and proximal convoluted tubules (PCT/DCT) with very little inflammation.

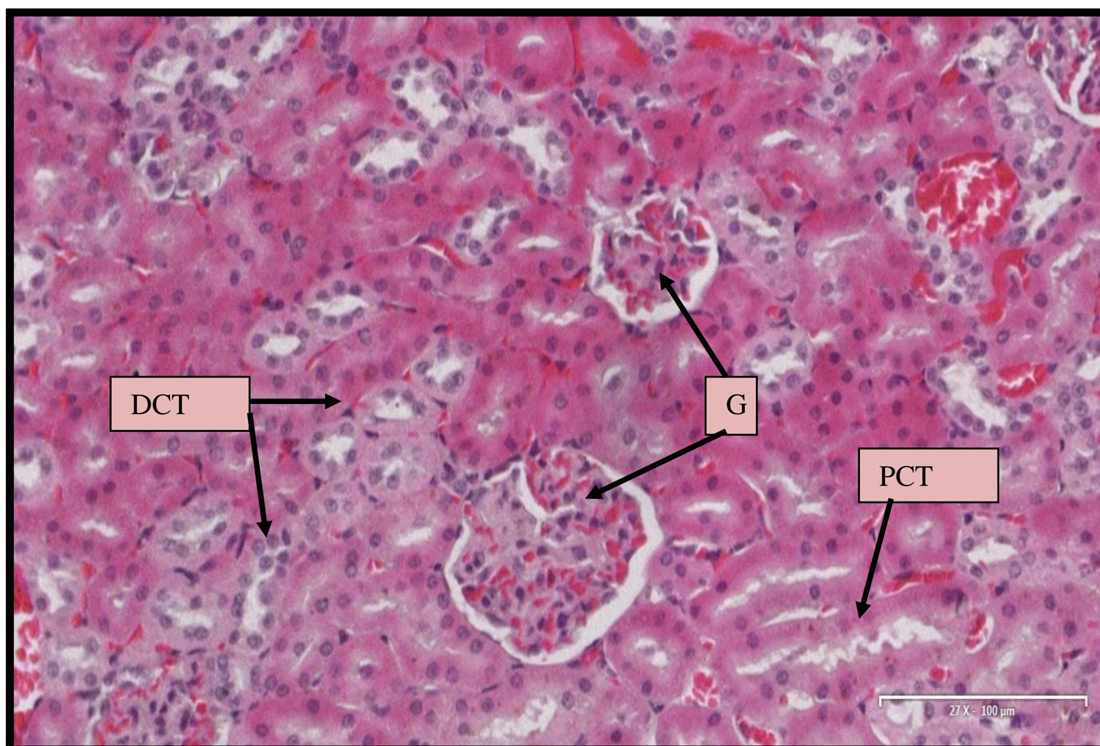

Figure 34: Micrograph (27x100 µm) of WIS S showing normal glomeruli (G) and DCT and PCT.

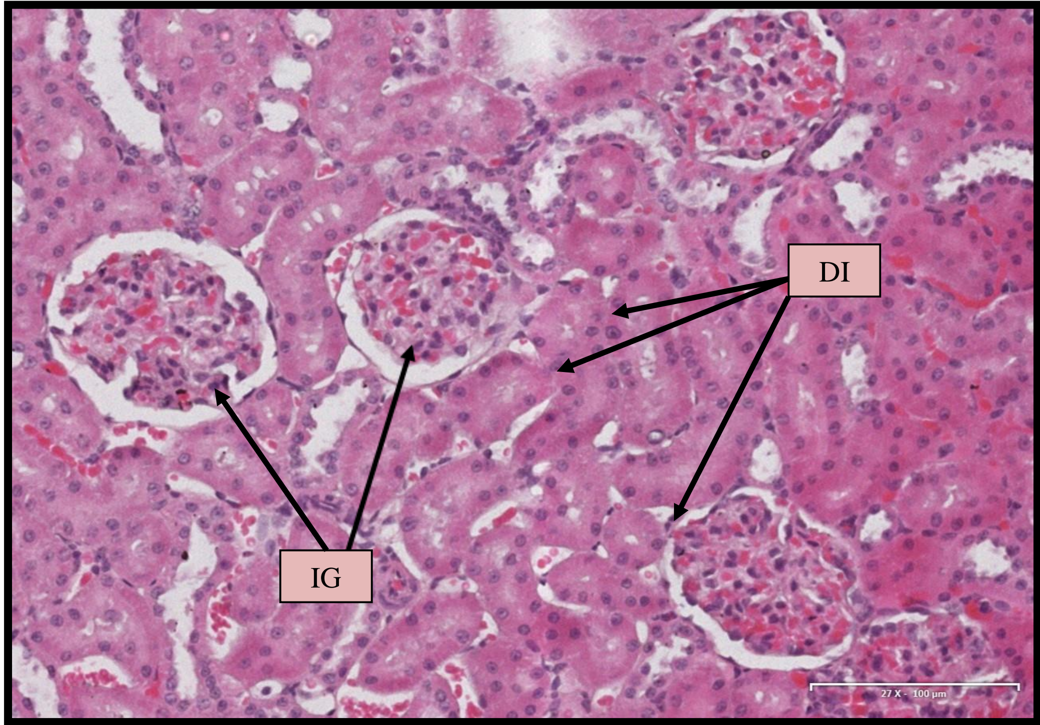

Figure 35: Micrograph (27x100 µm) of SHR S showing signs of improved glomeruli (IG) and decreased inflammation (DI) in the interstitial space.

Appendix J: Plagiarism Report

Please see attached report.

Appendix K: Ethical clearance letter

Please see attached letter.