Riboflavin protective role against mitochondrial toxicity and lipodystrophy due to stavudine and lamivudine

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Declaration

This Thesis is my original work and to the best of my knowledge it has never been presented in any university for an award.

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Dedication

This thesis is dedicated to my wife, Regina Mukomwaa, my children Joy Nkirote and Bernice Makena, and my parents.

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List of abbreviations

3TC:	Lamivudine
ABC:	Abacavir
ADP:	Adenosine diphosphate
APTT	Activated partial thromboplastin time
ARV:	Antiretroviral drugs
ATP:	Adenosine triphosphate
AZT:	Zidovudine
cAMP:	cyclic adenosine monophosphate
CK:	Creatine kinase
ct:	Cycle threshold
d4T:	Stavudine
DAAD:	German Academic Exchange Services
DADP:	Diaminopurine Dioxolone
dCTP:	deoxycytidine triphosphate deaminase
ddc:	Zalcitabine
ddl:	Didanosine
DNA:	Deoxyribonucleic acid
EFV:	Efavirenz
FAD/$FADH_2$:	Flavin Adenine Dinucleotide
FIAU:	Fialuridine
FMN:	Flavin Mononucleotide
g:	gram
HAART:	Highly Active Antiretroviral Therapy
HIV:	Human Immunodeficiency Virus
Kg:	kilogragm
MELAS:	Mitochondrial encephalomyopathy with lactic acidosis and Stroke-like episodes
Mg:	Milligram
Min:	minute

Ml:	millilitre
mtDNA:	Mitochondrial DNA
NAD+/NADH:	Nicotinamide Adenine Dinucleotide
NaOH:	Sodium hydroxide
nDNA:	nucleic DNA
Neg:	negative
NFV:	Nelfinavir
ng:	nanograms
NNRTI:	Non Nucleoside Reverse Transcriptase Inhibitor
NRTI:	Nucleoside Reverse Transcriptase Inhibitor
NVP:	Nevirapine
PCR:	Polymerase Chain Reaction
PI:	Protease Inhibitor
PK:	Prekallikrein
PLWHA:	People Living With HIV/AIDS
PT:	Prothrombin time
PTPC:	Permeability transition pore complex
RNA:	Ribonucleic Acid
RTI:	Reverse transcriptase inhibitor
rt-PCR:	Real time PCR
Temp:	temperature
TK-1:	mitochondrial thymidine kinase
TK-2:	cytosolic thymidine kinase
TNF:	Tumor Necrotic Factor
UNAIDS:	United Nations AIDS
UON:	University of Nairobi
USAMRU-K:	United States of America Military Research Unit-Kenya
WHO:	World Health Organization
ZDV:	Zidovudine

Definition of terms

Analogue: Something that is similar to something else in design, origin or use

Antiretroviral drugs: Drugs used in the treatment of HIV infection

CEM Cells: A cell line derived from human T cells

Deleterious: Anything hhaving harmful effect or injurious

Derangement: Disruption of the order or arrangement of set norm or upsetting the normal condition or functioning

Double human equivalent dose: A dose of stavudine equivalent to 2.28mg/kg or lamivudine equivalen to 8.6mg/kg or riboflavin equivalent or riboflavin equivalent to 11.4mg/kg

Genome: The total genetic content contained in a haploid set of Chromosomes in eukaryotes, in a single chromosome in bacteria, or in the DNA or RNA of viruses

Half human equivalent dose: A dose of stavudine equivalent to 0.57mg/kg or lamivudine equivalen to 2.15mg/kg or riboflavin equivalent or riboflavin equivalent to 2.85mg/kg

Human equivalent dose: A dose of stavudine equivalent to 1.14mg/kg Or lamivudine equivalen to 4.3mg/kg or riboflavin equivalent or riboflavin equivalent to 5.7mg/kg

Hyperglycaemia: Describes blood glucose levels above reference range (7.5mmol/L)

Juvenile: Describes young ones of mice below 6weeks old

Mitigation: Effort to reduce loss of life and property by lessening the impact of disasters. Mitigation is taking after a disaster before the next one happens

Nucleoside: Any of various compounds consisting of a sugar, usually ribose or deoxyribose, and a purine or pyrimidine base,

	especially a compound obtained by hydrolysis of a nucleic acid, such as adenosine or guanine
Oxidation:	Combination of a substance with oxygen
Toxicity:	Defined by the skin texture, wounds, shivering, eating habits, hepatomegally, hyperglycemia, mitochondrial genome derangement and prolonged coagulation time

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Abstract

Background Nucleoside analogues: stavudine and lamivudine represent the cornerstone of antiretroviral regimens. These drugs are associated with a range of toxicities such as peripheral neuropathy, myopathy, pancreatitis and lactic acidosis with hepatic steatosis. Fat lipodystrophy is also seen on long term antiretroviral therapy with nucleoside analogues. Given the low cost of their production compared to other antiretroviral drugs, this study was carried out to establish the role of riboflavin in protecting against mitochondrial toxicity and lipodystrophy due to their use.

Methodology Swiss albino mice models were used for this study. Three dose levels of test drugs were used: half human; human equivalent and double human dose for stavudine, lamivudine and riboflavin. Four drug combinations were experimented consisting of stavudine only; stavudine and lamivudine; stavudine, lamivudine and riboflavin; and stavudine and riboflavin. The mitochondrial DNA damage was established using real time (rt) polymerase chain reaction (rt-PCR). Experimental toxicity was monitored by assessing body weight, general body appearance, glucose, lipid profile, haematology (prothrombin time-PT and activated partial thromboplastin time-APTT) and histology. Liver and adipose tissue were extracted and blood collected. Data were analysed using SPSS version 17.0.

Results The reference ranges for the parameters: live weight, liver, blood glucose, lipid and haematological profile for healthy untreated mice were established. These wre subsequently applied in the interpretation of experimental data.There was statistical significant rise in blood glucose levels in groups treated with stavudine and lamivudine at all dose levels (p= 0.001-0.011). Although there was an increase in blood glucose levels in groups treated with combination of stavudine and lamivudine plus riboflavin, the increase was not statistically significant except for the groups treated with double human equivalent dose (p= 0.001-0.007). However, groups treated with double human equivalent dose of stavudine and riboflavin showed decrease in

blood glucose (p= 0.003). There was no dyslipidaemia noted in the groups treated with human and double human equivalent dose. However, the total cholesterol and triglycerides levels were below the reference range for groups treated with combination containing riboflavin for half human equivalent group. The statistical significance for the total cholesterol and triglycerides in the group treated with combination of half human equivalent dose stavudine and riboflavin were statistically significant at 0.034 and 0.02 respectively. Adipose tissue was depleted in the groups treated with stavudine human equivalent and double human equivalent doses. There was significant increase in PT for groups treated with human equivalent dose of stavudine and stavudine plus lamivudine (p= 0.024-0.03). However, addition of riboflavin to double human equivalent dose of stavudine was associated with a significant decrease in PT (p= 0.000). Similarly, the APTT significantly increased for group treated with human equivalent dose of stavudine and combination of stavudine and lamivudine (p= 0.008-0.016) but significantly decreased on addition of riboflavin to double dose of stavudine (p= 0.000). This is a beneficial effect. Generally, there was liver derangement leading to hepatitis, steatosis and granuloma however, these were found to be less severe in groups treated with riboflavin. Mitochondrial DNA was measured by decrease in ct values compared with untreated group. For the group treated with half human equivalent dose of stavudine, the ct values for the liver (p=0.02) and adipose tissue (0.02) were increasing. The same trend for increasing ct values (liver p=0.03; and adipose tissue p=0.012) was reported in the group treated with half human equivalent dose combination of stavudine and lamivudine. The ct values decreased on addition of riboflavin. The ct values for the liver in group treated with human equivalent dose combination of stavudine and lamivudine was reported to increase compared to untreated group (p=0.003). The ct values for the liver reduced in the groups treated with human equivalent dose combinations of: stavudine, lamivudine and riboflavin (p=0.021); and stavudine and riboflavin (p=0.002). The group treated with double human equivalent dose

combination of stavudine and lamivudine, liver and adipose tissue ct values were increased with statistical correlation of 0.001 and 0.007 respectively.

Conclusion The reference ranges for biochemical and haematological parameters were established. Toxicity due to the drugs stavudine and lamivudine was confirmed for human and double human equivalent doses. Riboflavin was found to have protective effect to mtDNA when in combination with stavudine; and stavudine and lamivudine in animal model. Half human equivalent dose was found to cause minimal toxicity.

Chapter one

1.0 Introduction

Current guidelines for the management of HIV infected individuals recommend the use of combinations of antiretroviral drugs including at least three agents—highly active antiretroviral therapy (HAART). This term was coined because of the magnitude of the effects seen in the early clinical studies of regimens combining the new HIV protease inhibitors (PIs) with the established nucleoside reverse transcriptase inhibitors (NRTIs). In addition to profound effects on viral load and CD4 cell count, these regimens were shown to significantly delay disease progression and death. Analyses of population data have shown reductions in morbidity and mortality associated with increased use of HAART. The initial enthusiasm for PI regimens has waned somewhat with the realisation that these agents may be associated with long term toxicity in the form of a metabolic syndrome that has become known as HIV associated lipodystrophy (Mallal and Nolan, 2000). This, coupled with the development of cross resistance between currently available agents in the PI class, has led to a search for other regimens that may be employed either before or after PI therapy. Other triple regimens that have been studied have replaced the PI by either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or another NRTI.

The NRTI stavudine in combination with other antiretroviral drugs is used in the treatment of HIV/AIDS. It is valuable in repressing HIV replication and is affordable in low and medium income countries. However, common and severe adverse drug effects have led to calls for its withdrawal (Kampira et al., 2014). Many of the adverse drug effects of stavudine are similar to patterns found in inherited mitochondrial diseases (Nicholas et al., 2007) and indeed stavudine can affect mitochondria, either through inhibition of mtDNA transcription, or

through impact on the activity of mitochondrial polymerase-gamma, an enzyme required for mtDNA replication and repair. In both incidences, mitochondria depletion is common and once this reaches a critical limit, inadequate energy generation develops (Kampira et al., 2014), triggering tissue or organ dysfunction. Histologic evidence demonstrates abnormal mitochondrial structure and/or depletion in affected tissues of HIV-infected patients on NRTI containing ART (Morse et al., 2012).

Another complication seen in treated patients is a lipodystrophy syndrome, with peripheral fat wasting, central adiposity, hyperlipidemia, and insulin resistance (Carr et al., 1999). Although the pathogenesis of this syndrome is unknown, several factors including the HIV infection, a putative genetic predisposition and antiretroviral treatments could play a role (Brinkman et al., 1999; Carr et al., 1999). Both HIV protease inhibitors (Carr et al., 1999) and nucleoside analogues (Strobel et al., 1999) could modify lipid metabolism in these patients. Although a possible involvement of mitochondrial dysfunction in nucleoside-induced metabolic disorders has been suggested (Brinkman et al., 1999), definite data are currently lacking.

Stavudine is currently one of the most widely used nucleoside analogues in numerous countries (Carpenter et al., 2000). Like other nucleoside analogues, stavudine has caused neuropathy, pancreatitis, microvesicular steatosis, and lactic acidosis in a few patients (Mokrzycki et al., 2000) and stavudine may also increase the risk of fat wasting and hypertriglyceridemia (Strobel et al., 1999). Whereas, the effects of stavudine on mitochondrial function and lipid metabolism have been assessed in invitro studies, its interventions have not been studied. There have been no studies done in vivo mitigating the effects of nucleoside analogues. This study addressed mitigations to the adverse effects of nucleoside analogues on cellular processes in mice models.

Chapter two

2.0 Literature Review

2.1 Mitochondrial biology

2.1.1 Structure

The identification of filaments (mito) and grains (chondria) under the light microscope by scientists in the 19th century provided early clues for mitochondrion, but it was only the advent of electron microscopy that led to the widespread visualisation of mitochondria as the sausage-shaped organelles as shown in figure 2.1 (Enger et al., 2003). While this classic picture of mitochondria has been accepted, studies have shown it to be an oversimplification. Rather than always being present as discrete organelles, mitochondria have also been shown to form a highly integrated network, and to undergo what appears to be a frequent process of fusion and fission. In addition, depletion in mitochondrial DNA has been shown to cause morphological changes in mitochondria from cultured human cells, and a high energy demand or oxidative stress induces proliferation of the mitochondrial network to satisfy the cell's energy needs.

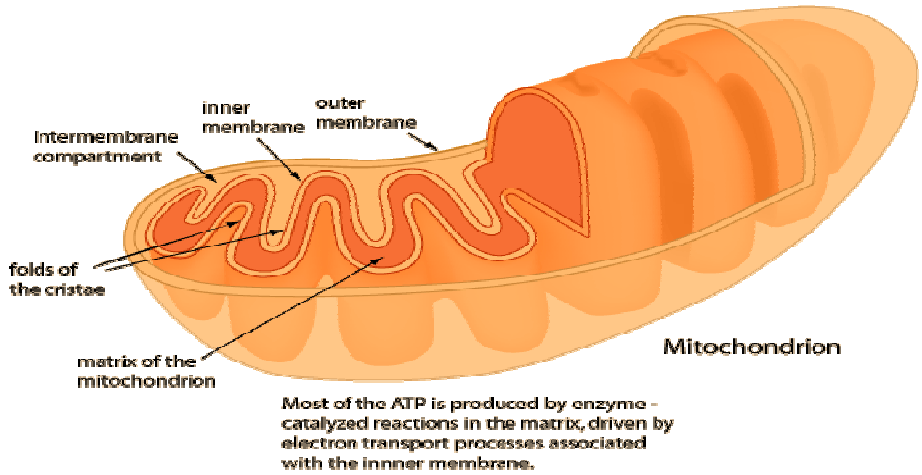


Figure 2.1: Structure of mitochondria (adapted from Enger et al., 2003)

2.1.2 Mitochondrial function

The main function of mitochondria is to produce energy for the cell in form of adenosine triphosphate [ATP], via the process of oxidative phosphorylation. Acetyl-CoA is generated either via glycolysis in the cytosol or β-oxidation of fatty acids in the mitochondria. The passage of acetyl -CoA through the tricarboxylic acid cycle generates NADH and $FADH_2$, which are powerful reducing agents. The mitochondrial electron transport chain takes electrons from these reducing agents and passes them down the electron transport chain, eventually reducing oxygen to water. The transport of electrons down the different components of the electron transport chain also leads to the pumping of protons out of the mitochondrial matrix. This creates an electrochemical gradient between the mitochondrial matrix and the inter-membrane space leading to the return of protons into the mitochondria matrix via electron carriers located in the inner mitochondrial membrane. As protons pass through this channel integral component catalyses the synthesis of ATP. The ATP is then exchanged with ADP from the cytosol by a specific carrier, the ADP/ATP translocator. In addition, while considering mitochondrial function it is important to acknowledge that mitochondria are known to participate in other cellular processes, particularly apoptosis (Kroemer et al., 1998). Thus, mitochondria are not only essential for energy generation within the cell but also function as key regulators of cellular survival (White, 2001).

2.1.3 Fatty acid oxidation

2.1.3.1 Fatty acid oxidation and transport into mitochondria

Fatty acids arise in the cytosol either through biosynthesis or through triacyglycerol or fatty acid transport from fat depots outside the cell. A special transport mechanism carries long-chain acyl CoA molecules across the inner mitochondrial membrane. Activated long-chain fatty acids are transported across the membrane by conjugating them to carnitine. The acyl group is

transferred from sulphur atom of CoA to the hydroxyl group of carnitine to form acyl carnitine. This reaction is catalyzed by carnitine acyl transferase I, which is bound to the outer mitochondrial membrane. Acyl carnitine is then shuttled across the inner mitochondrial membrane by a translocase. The acyl group is transferred back to CoA on the matrix side of the membrane. This reaction is catalyzed by acyl transferase II (Mathews et al., 1999; Berg et al., 2001).

A saturated acyl CoA is degraded by a recurring sequence of four reactions: oxidation by flavin adenine dinucleotide (FAD), hydration, oxidation by nicotinamide adenine dinucleotide (NAD+), and thiolysis by CoA. The fatty acyl CoA is shortened by two carbons as a result of these reactions, and $FADH_2$, NADH and acetyl CoA are generated. Oxidation takes place on the β-carbon (Berg et al., 2001).

2.1.3.2 The oxidative phosphorylation system

The mitochondria play a major role in oxidative phosphorylation for ATP production. The ATP produced is useful in the provision of energy for reactions in the matrix or is shuttled to the cytosol by the adenine nucleotide translocator in exchange for cytosolic ADP (Scholte, 1988). The oxidative phosphorylation system is made up of four multisubunit enzyme complexes of the mitochondrial respiratory chain (complexes I-IV) and the ATP synthetase complex (complex V). These are found in the lipid bilayer of the inner mitochondrial membrane. Apart from the oxidative phosphorylation system in the mitochondria, ATP can also be produced through anaerobic respiration of glucose in the cytoplasm. However, unlike oxidative phosphorylation which produce large amount of energy (ATP), glycolysis produces little and therefore cannot sustain cellular energy requirements in multicellular organisms (Scholte, 1988).

The respiratory chains complexes I, II, IV and V are coded by both nuclear and mitochondrial DNA. The mtDNA is circular double stranded and code for 22 transfer RNA, two ribososmal RNA and 13 subunits of the oxidative phosphorylation system. The total mtDNA is 16 569 base pairs.The mtDNA can be expressed independent of nDNA (Johns, 1996). However, more than 90% of mitochondrial proteins are controlled by nDNA (DiMauro and De Vivo, 1994).

The nDNA and mtDNA differ both in function and structure.Firstly, mtDNA is predominantly maternally inherited whereas nDNA is directly inherited from the cytoplasm (Johns, 1996; Alberts et al., 1994). Secondly, mtDNA is not recombinant but undergoes replicative segregation during cell replication processes: mitosis and meiosis. All human cell contains several mitochondria and each of them contain two to ten mtDNA molecules. During cell division, the mutated and non-mutated forms of mtDNA are arbitrarily segregated into the descendant cells, resulting in mixtures of mutant and wild-type mtDNA in cells and human pedigree (DiMauro and De Vivo, 1994, Moraes et al., 1991). This coexistence of mutant and wild-type mtDNA (heteroplasmy), may lead to persistence of fatal transformations (Johns, 1996). The gravity of a defect due to mtDNA alteration depends on the nature of the mtDNA mutation and on the quantity of mutant mtDNA within the cell; mtDNA mutations will consequently lead to cellular malfunction when a certain threshold is achieved. This, incident is called threshold expression (DiMauro and De Vivo, 1994). This expression depends on the severity of the oxidative phosphorylation defect and the relative reliance of each organ system on mitochondrial energy production. Mitochondria are more dynamic than nuclei through replication and therefore a relative proportion of mutant and wild-type mtDNA may change within a cell cycle (DiMauro and De Vivo, 1994). More replications provide a higher possibilty to develop replication abnormalities. Since mtDNA has no introns, a random mutation will usually attack a coding DNA sequence. Besides, mutations and defects can easily occur to mtDNA since it has neither an

effective repair mechanism nor protective histones, and above all it is exposed to oxygen radicals generated by the respiratory chain (Lewis and Dalakas, 1995; Johns, 1996; Alberts et al., 1994; Lewis and Perrino, 1996).

Generally, mtDNA is extremely vulnerable to mutations acquired genetically and exogeneously. The DNA polymerase γ appears is the only regulating enzyme of mtDNA replication, therefore its inhibition with RTI might easily down regulate this replication leading to decrease in mitochondrial energy generation (Brinkman et al., 1999).

2.2 Genome and Replication

Mitochondria are distinctively different from other organelles in that they have own DNA unique from that of the nucleus (Taanman, 1999). The products expressed by mitochondrial DNA are quite limited, as most of the organelle phenotype is encoded for by nuclear DNA (nDNA). Mitochondrial gene, present certain features that are highly significant (Shoffner, 1992). Firstly, mitochondrial DNA is maternally inherited. Paternal mtDNA copy number in sperm cells is low by comparison with the large number of mtDNA molecules in the oocyte. In addition, it appears that although paternal mtDNA is transferred during fertilisation, it is lost early in embryogenesis. One of the most important observations of mitochondrial genetics is that different mtDNA variants may coexist in a single cell, the state of heteroplasmy. It has also been noted that there is a genetic bottleneck in mitochondrial DNA at some point between oogenesis and development of the embryo. This means that although there may be a large degree of heteroplasmy in the mother, the restriction and amplification that occurs during the bottleneck results in a very small number of mtDNA variants ultimately populating the embryo (Stacpoole, 1997).

2.2.1 Mitochondrial DNA polymerase

There are at least nine polymerases involved in the replication and maintenance of cellular DNA; however, only one, DNA polymerase gamma, is responsible for mitochondrial DNA replication. Human mitochondrial DNA polymerase is a family A DNA polymerase and was cloned and characterised by Ropp and Copeland (Ropp and Copeland, 1996). It has been shown that polymerase gamma is expressed and translated in cells which have been depleted of mitochondrial DNA (Davis et al., 1996). Polymerase gamma has to perform both replication and repair for mtDNA although for some time it was believed that repair activity was absent. However, polymerase gamma has been shown to participate in base excision repair, and other repair proteins have also been shown to be present in mitochondria (Rasmussen and Singh, 1998).

2.2.2 Mitochondrial disease

Mitochondrial disease can result from mutations or rearrangements in both mitochondrial and nuclear DNA (Leonard and Schapira, 2000), and generally involves post-mitotic tissues. The clinical presentation includes organs such as the central and peripheral nervous system, the bone marrow, skeletal and cardiac muscle, the gastrointestinal tract, the kidneys, the pancreas, and the liver. An important feature that relates to the heteroplasmy and threshold effects discussed above is the heterogeneity of the clinical presentation of many of these inherited defects (Chinnery and Turnbull, 1999). This is thought to relate to the mutation load in particular tissues of different individuals. For example, Kearns-Sayre syndrome, an encephalomyopathy, and Pearson syndrome, a disease of the pancreas and bone marrow, both result from the same deletion of nearly 5000 base pairs in mitochondrial DNA. A another example is the mutation in a tRNA gene at position 3243, which when present at high levels is associated with the MELAS syndrome (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), but with maternally inherited diabetes and deafness when present at low levels. The

critical significance of the level of mutant mitochondrial DNA in determining the clinical presentation has naturally led to a focus on the patterns and maintenance of heteroplasmy. There has been some controversy over whether mutant or normal mitochondria have a selection advantage, since results using hibrid cells have not been consistent. A recent model suggests that although damaged mitochondria may replicate as quickly as normal mitochondria, they are degraded more slowly and thus persist in post-mitotic cells, but mitotically active tissues may be able to rejuvenate the population (White, 2001).

2.2.3 Oxidative phosphorylation disorders

Defects of mtDNA inherited genetically or from nuclear genes encoding the oxidative phosphorylation system lead to its dysfunction, giving rise to a variety of clinical diseases due to failure in ATP synthesis (Johns, 1996; DiMauro and De Vivo, 1994). This malfunction affects all organ systems, with tissues having the highest energy demand most susceptible (Lewis et al., 1997). Decline in mitochondrial energy-generating capcity below the threshold of an organ could manifest disese symptoms (Johns, 1996; Wallace, 1992; Moraes et al., 1991; Chinnery and Turnbull, 1997). Many organ systems have been described to be possibly affected include: liver, pancreas, heart, skeletal muscle, nervous system, haematopoietic system, inner ear, kidney (renal failure is rare), and the eyes (Table 2.1). Liver cells and pancreatic β cells rely highly on oxidative metabolism and are therefore easily vulnerable to energy reduction, leading to liver disease and diabetes. Other (genetically inherited) clinical manifestations encountered in mitochondrial cytopathies are blindness, deafness, dementia, movement disorders, weakness, cardiac failure, and renal dysfunction (Johns, 1996; Chinnery and Turnbull, 1997).

Table 2.1: Clinical manifestations of oxidative phosphorylation disorders

Disorder	Manifestations
Neurological	Peripheral neuropathy, encephalopathy, dementia, seizures, stroke
Myopathy	Hypotonia, muscle weakness, exercise intolerance
Cardiac	Cardiomyopathy, conduction disorders
Endocrine	Diabetes mellitus
Gstroinestinal	Colonic pseudo-obstruction, exocrine pancreas dysfunction, pancreatitis, hepatomegaly, steatosis, liver failure, lactic acidosis
Nephrological	Non-selective proximal tubular dysfunction with acidaemia, phosphaturia and glucosuria, glomerulopathy
Haematological	Anaemia, thrombocytopenia, pancytopenia
Psychiatric	Depression
General	Multiple systemic lipomas, fatigue

(Brinkman et al, 1999)

The amount of mtDNA defects is one of the principal factors that determine whether a defect is expressed clinically. Usually the highest levels of mtDNA defects are in post-mitotic tissues such as skeletal muscle. Lower levels are seen in rapidly dividing tissues such as blood. Tissues with a slow turnover of mtDNA accumulate the largest number of mtDNA defects (Johns, 1996; Chinnery and Turnbull, 1997). When a certain threshold is reached after accumulation of mtDNA defects, a deficient production of ATP with its consequences for a specific tissue will emerge. As deficient oxidative phosphorylation increases with mitochondrial damage, mitochondrial ATP production declines until it falls below the minimum energy levels (threshold

expression) necessary for oxidative tissues and organs to function (Lewis and Dalakas, 1995).

A disturbed function of the oxidative phosphorylation system will give rise to an altered oxidoreduction status: a disturbed redox state (increased $NADH/NAD^+$ ratio) shifts the pyruvate/lactate equilibrium in the direction of lactate and leads to a functional impairment of the Krebs cycle. Consequently, lactate, leading to lactic acidaemia or even lactic acidosis, as well as the lactate/pyruvate ratio increase. This is particularly true in the post-absorptive period, when more NAD^+ is required for the adequate metabolism of glycolytic substrates (Munnich et al., 1992). Similarly, a postprandial increase of ketone bodies synthesis can be observed, related to the channelling of acetylcoenzymeA towards ketogenesis (Rotig et al., 1990). Fat (triglycerides and free fatty acids) will accumulate intracellularly, which can be demonstrated histologically (macrovesicular hepatic steatosis).

In electron microscopy, histological damage can be demonstrated as enlarged mitochondria, with or without loss of cristae, matrix dissolution, lipid droplets, paracristalline and scattered vesicular inclusions (Lewis and Dalakas, 1995; Olano et al., 1995).

2.2.4 Nucleoside analogues and mitochondrial toxicity

Other than inheritable route, mtDNA defects can also be acquired exogeneously through toxic agents such as alcohol, tobacco and drugs (Lewis and Dalakas, 1995; Johns, 1996). Nucleoside analogues drugs: cytarabine, vidarabine, aciclovir and ribavirin, used in chemotherapy and antiretroviral therapy have been shown to induce mitochondrial toxicity. These nucleoside analogues elicit complete mtDNA replication shortage; clinical features can be regarded as a compilation of those seen in the genetic mitochondrio-cytopathies. These features include myopathy, cardiomyopathy, neuropathy, lactic acidosis,

exocrine pancreas failure, liver failure and bone-marrow failure (Lewis and Dalakas, 1995; Chinnery and Turnbull, 1997; Aggarwal et al., 1996).

2.2.4.1 NRTIs and mitochondrial DNA

After HIV has entered the cell, it is required to integrate its genome with the host cell genome. To do this, it needs to convert single stranded viral RNA into double stranded DNA and this task is performed by the viral enzyme reverse transcriptase. This process can be inhibited by NRTI and is the target for therapy. The NRTIs resemble the natural nucleosides but do not have a free 3` hydroxyl group, and thus once they are added to the growing DNA chain, termination occurs. Many NRTIs have been investigated for anti-HIV activity, and some knowledge of the structure-activity relation of these agents has been established. Since these drugs resemble natural nucleosides, the potential for inhibition of DNA polymerases exists, although there are sufficient differences between the enzyme kinetics to enable selective inhibition to occur (White, 2001).

2.2.4.2 In-Vitro Studies with NRTIs

Early studies of NRTIs demonstrated minimal effect on DNA polymerase alpha, the main enzyme responsible for nuclear DNA replication, but polymerase beta and gamma were affected to some degree (Wright and Brown, 1990). The clinical significance of the inhibition of DNA polymerase beta is unknown this enzyme synthesizes short sections of DNA as part of a group of enzymes involved in repair. It has been shown that Tat, a gene product of HIV, induces the expression of DNA polymerase beta (Srivastava et al., 2001). The effects of NRTIs on polymerase gamma have been studied and it appears that inhibition of this enzyme and chain termination would lead to mitochondrial DNA depletion, which upon falling below the critical threshold would lead to insufficient energy generation and subsequent cellular dysfunction (Cheng et

al., 1990). Initial in vitro studies with NRTIs examined the toxicity of these agents in murine bone marrow progenitor cells, since the first NRTI licensed for the treatment of HIV, zidovudine, was associated with anaemia and neutropenia in the clinic. These studies showed that zidovudine exhibited toxicity in these models, appearing to confirm what was observed in patients (Luster et al., 1989). Later studies in neuronal cell models showed that the ddC, ddI, and d4T caused toxicity, whereas AZT and 3TC did not, again reflecting what was seen in clinical practice (Cui et al., 1997). The recognition that NRTIs may interfere with mitochondrial DNA synthesis led to many studies evaluating these effects in vitro, as was reviewed by Kakuda (Kakuda, 2000). These studies suggested a ranking of ddC >ddI >d4T > 3TC > ZDV > ABC for effects on mitochondrial polymerase gamma. Martin and colleagues examined both the inhibition of polymerase gamma and the inhibition of mitochondrial DNA synthesis, since some correlation was expected (Martin et al., 1994). Although a similar ranking of the NRTIs for effects on mitochondrial DNA synthesis was noted, there was no clear correlation with the potency of mitochondrial DNA inhibition. For example, 935U83 was shown to be a highly potent inhibitor of polymerase gamma and yet showed no effect of mitochondrial DNA synthesis (Martin et al., 1994).

Considering all in vitro experiments, it is important to note that these studies can only provide information on the effect of a drug in a particular cell type under given experimental conditions. Sensitivity to drugs varies between cell types and there are many other factors involved. One that has obvious implications if the mechanism of NRTI toxicity is indeed incorporation into mitochondrial DNA is removal by exonuclease activity. Gray and colleagues demonstrated that lamivudine (3TC) is a substrate for the exonuclease activity of polymerase gamma, thus it can be excised if incorporated (Gray et al., 1995). Studies with other NRTIs have shown that they are substrates for cytosolic

exonucleases, but the exonuclease activity may be inhibited by high levels of the NRTI monophosphate (Skalski et al., 1995).

2.2.5 Activation of the NRTIs

Before their addition to the growing DNA chain by HIV reverse transcriptase or other polymerases, the NRTI agents need to be phosphorylated three times. This process is known to vary with the activation state of the cell, with stavudine (d4T) and zidovudine (ZDV) being more active in activated cells and other NRTIs being more active in resting cells. In addition, intermediary anabolites may be implicated in toxicity of NRTIs, as has been shown for ZDV monophosphate in a study of CEM cells (Tomevik et al., 1995). Considering the mitochondrial effects of NRTIs, it is important to note that many cellular kinases exist in both mitochondrial and cytosolic forms. In addition to their subcellular localisation, these kinases frequently differ in their substrate specificity and regulation through the cell cycle. Studies of zalcitabine (ddC) suggest that the drug was phosphorylated in the cytosol and then transported into the mitochondria. In the neuronal cell model referred to above, it was noted that ddC was only phosphorylated to the monophosphate in mitochondria, compared with the monophosphate, diphosphate, and triphosphate in the cytosol (Cui et al., 1997). Other investigators have reported similar results, and transport activity for both dCTP (Bridges et al., 1999) and 2',3'-dideoxycytidine 5'-diphosphocholine (ddCDP-choline) (Rossi et al., 1999) has recently been described, suggesting that mitochondrial toxicity due to ddC is the result of transport of anabolites into the mitochondria, rather than phosphorylation therein.

Although less relevant for ddC, studies with other NRTIs demonstrate that mitochondrial phosphorylation can be a factor in determining drug toxicity. Fialuridine (FIAU) was investigated for the treatment of hepatitis B but unfortunately led to profound liver toxicity in most patients exposed to the drug

for longer than 10 weeks (Honkoop et al., 1997). The liver toxicity was a consequence of severe mitochondrial dysfunction and in vitro experiments showed that FIAU was a much better substrate for mitochondrial thymidine kinase (TK-2) than the cytosolic form (TK-1), although the fact that FIAU is not a chain terminator like other NRTIs and may be incorporated also played a significant part (Wang and Eriksson, 1996). The NRTIs zidovudine and stavudine are also activated by thymidine kinase, and initial studies suggested that they could inhibit both TK-1 and TK-2, although d4T was not shown to act as substrate for either of these enzymes (Munch-Peterson et al., 1991). Subsequently it has been recognised that d4T is relatively poorly phosphorylated compared with thymidine and ZDV, (Ho and Hitchcock, 1989) and later studies have shown that d4T is indeed a substrate for these enzymes (Wang et al., 2000). In the neuronal cell model referred to above, d4T was shown to be phosphorylated to the triphosphate within the mitochondria (Cui et al., 1997). Considering other nucleosides, while the formation of ddA-monophosphate from didanosine (ddI) occurs through a slightly different enzymatic mechanism than other NRTIs, the second phosphorylation step is catalysed by adenylate kinase. This enzyme exists as cytosolic (AK-1) and mitochondrial forms (AK-2), and thus the potential for differential inhibition exists. The kinases involved in the activation of guanosine analogues, such as abacavir and DAPD, are not as far advanced in terms of a link between activation and toxicity, although studies with nucleosides used in cancer therapy have shown a direct relation between mitochondrial deoxyguanosine kinase activity and cytotoxicity (Zhu et al., 1998).

2.2.6 NRTI Transport

The entry of NRTIs into cells has been observed to occur at different rates, and there are many transport systems available for nucleosides. Since the phosphorylation of NRTIs may differ between subcellular compartments, it follows that movement of the drugs and their anabolites between the cytosolic

and mitochondrial compartments is of considerable interest (Dolce et al., 2001). Early studies with lamivudine (3TC) showed synergistic or additive activity against HIV in vitro, and also protection against the delayed mitochondrial toxicity associated with d4T, ZDV, ddC, and ddI (Bridges et al., 1996). The protection conferred by 3TC in this study was thought to be due to interference with the uptake of the other agents into mitochondria. Subsequent experiments have shown that other "unnatural" NRTIs in the same class, such as L (-) Fd4C, also show similar properties. Further, the activation and transport of the NRTIs within different subcellular compartments may lead to molecules or strategies in which efficacy can be enhanced and toxicity reduced (White, 2001).

2.3 Effect of HIV infection on Mitochondrial function

It is known that many of the toxicities associated with NRTI therapy may also be related to HIV infection itself, but it is not often appreciated that there is also known to be a direct interaction between HIV and mitochondria (White, 2001). Early studies showed that HIV RNA could be found in mitochondria of infected cells, and that there were mitochondrial alterations in patients with the acute HIV syndrome and stable infection. Other studies have shown that the HIV TAT protein may promote mitochondrially induced apoptosis (Macho et al., 1999), consistent with the immune cell destruction caused by the virus. A specific interaction between the HIV viral protein R and the mitochondrial permeability transition pore complex (PTPC) has been demonstrated by Jacotot et al, (2001), and with the recognised involvement of the PTPC in apoptosis it seems likely that HIV affects the immune system at least in part by interacting with mitochondria leading to programmed cell death. Such viral effects are not uncommon; indeed the hepatitis B virus protein X has also been shown to interact with a component of the PTPC (White, 2001).

2.3.1 Effects of NRTIs on mitochondrial function

Nucleoside analogues: zidovudine, stavudine, didanosine and lamivudine are used in combination with non-nucleoside drugs such as nevirapine in the treatment of HIV (Carpenter et al., 2000). However, the outstanding benefit of these antiretroviral drugs on mortality and morbidity is overshadowed by possible occurrence of serious side-effects. Nucleoside analogues can occasionally cause myopathy, cardiomyopathy, pancreatitis, peripheral neuropathy and micro vesicular steatosis of the liver, with lactic acidosis and/or liver failure (table 2.2).

Table 2.2: Adverse effects of ARV drugs

NRTIs	**NNRTIs**	**PIs**
• Peripheral neuropathy • Pancreatitis • Lipodystrophy • Hepatitis • Lactic acidosis • Mitochondrial toxicity	Rash Fever Nausea diarrhea Hepatoxicity CNS	• Lipodystrophy • GI intolerance • Hyperglycemia • Lipid abnormalities

(Ministry of Health, 2005)

These adverse effects have been ascribed to drug-induced impairment of mitochondrial DNA (mtDNA) replication, causing mtDNA depletion, impaired oxidative phosphorylation and ATP deficiency (Gaou et al., 2001). Available data suggest that nucleoside analogues could also impair mitochondrial function and cellular metabolisms independent of mtDNA depletion (Szabados et al., 1999; Pan-Zhou et al., 2000).

Lipodystrophy syndrome with peripheral wasting, central adiposity, hyperlipidemia, and insulin resistance also occurs in patients on first line regimen (Gaou et al., 2001). Several factors including HIV-infection, a putative genetic predisposition, and antiretroviral treatment could cause this syndrome (Brinkman et al, 1999; Carr et al., 1999; Gaou et al., 2001). Nucleoside analogues could modify lipid metabolism in HIV patients on treatment (Gaou et al., 2001). Involvement of mitochondrial dysfunction in nucleoside-induced metabolic disorders has been suggested (Brinkman et al., 1999), but there is no proving data and intervention to this problem.
Stavudine is currently one of the first regimen in HIV treatment. It is suspected of causing neuropathy, pancreatitis, micro vesicular steatosis, and lactic acidosis among patients on antiretroviral therapy (Mokrzycki et al., 2000; Gaou et al., 2001). Stavudine may also increase the risk of fat wasting and hypertriglyceridemia (Saint-Marc et al., 1999; Strobel et al., 1999; Gaou et al., 2001).

Since the tissue involvement and clinical presentation often resembles aspects of inherited mitochondrial disease, and it is known that NRTIs may affect mitochondrial function. Many authors have proposed that mitochondrial toxicity of the NRTIs is the underlying pathophysiology behind most of these toxicities (Brinkman et al, 1999). Lewis and Dalakas put forward the "polymerase gamma hypothesis" in their review (Lewis and Dalakas, 1995), suggesting that the manifestations of NRTI toxicity relate to the combined effects of four principal factors. Firstly, the tissue must have some dependence on oxidative phosphorylation; secondly, the NRTI must pass into the tissue itself; thirdly, the NRTI must be phosphorylated by cellular kinases; and, finally, it must inhibit polymerase gamma activity by competing with the natural substrate or by chain termination (Lewis and Dalakas, 1995). The data reviewed earlier identify some refinements that may be made to this hypothesis such as the role of phosphorylation in different subcellular compartments,

particularly the mitochondrion itself. More fundamental is the lack of correlation between polymerase gamma inhibition and mitochondrial DNA depletion as identified by Martin and colleagues (Martin et al., 1994). In addition, the neuronal cell model showed that the neurotoxic effect of d4T did not correlate with mitochondrial DNA depletion, whereas there was a correlation between the effect of ddC and mitochondrial DNA levels (Cui et al., 1997).

2.3.1.1 Haematological Toxicity

HIV infection and AIDS are known to be associated with significant haematological toxicity, including anaemia, neutropenia, and thrombocytopenia (Moses et al., 1998). This toxicity is generally reversible and may be managed by dose reduction or drug withdrawal. Other interventions also appear to confer varying degrees of benefit, including growth factors for neutropenia (Hermans et al., 1996), and recombinant haemoglobin (Moqattash et al., 1997) or erythropoietin for anaemia (White, 2001). In vitro studies also confirm the haematological toxicity of HIV and zidovudine (Luster et al., 1989), although the mechanism of this toxicity is unclear. One study suggested that permeation of the drugs into canine bone marrow progenitor cells might be an indicator of drug specific toxicity, since although ZDV permeation was itself slow, ddI was even slower and ddC did not permeate at all (White, 2001). Törnevik et al, (1995) suggested that the cytotoxic effect correlates with ZDV monophosphate levels. Faraj et al, (1994) confirmed these findings and did not demonstrate a correlation between toxicity and mitochondrial DNA inhibition, suggesting that this effect may occur through some other mechanism. Although it is known that mitochondrial dysfunction may result in haematological toxicity, it seems possible that zidovudine associated haematological toxicity may result from alternative effects, perhaps on haem metabolism or gene expression. Both zidovudine and stavudine treatment are known to be associated with macrocytosis, although the mechanism and clinical significance is unclear

(Genne et al., 2000). HIV infection is also known to be associated with thrombocytopenia (Cole et al., 1998), possibly through the action of specific viral strains in the bone marrow, and this has been shown to improve with zidovudine treatment (Pottage et al., 1998).

2.3.1.2 Myopathy

HIV infection may be associated with myopathy at all stages of the disease (Chariot and Gherardi, 1995). It was also noted that zidovudine therapy may be associated with myopathy in the early studies with this drug (Dalakas et al., 1990), although distinguishing the disease and drug-related myopathies has proved difficult (Simpson et al., 1993). Simpson and colleagues analysed data from a placebo controlled study of zidovudine (Simpson et al., 1997), and conducted a prospective myopathy substudy in a large trial of combination therapy (Simpson et al., 1998). In the first study, using ACTG 016, five out of 279 (1.8%) zidovudine treated patients had a composite myopathy diagnosis, compared with none in the placebo group. ACTG 016 examined response to a dose of 200 mg/day of zidovudine every 4 hours and in three instances of dose reduction, the five patients with a composite myopathy diagnosis led to improvements in creatine kinase levels, but not in strength. However, CK levels were also observed to improve independent of dose reduction, and in these instances improvement in strength was noted on one occasion (Simpson et al., 1997).

Benbrik and colleagues studied the effects of ddI, ddC, and ZDV on cultured human muscle cells and showed that although ZDV was the most potent inhibitor of cell proliferation, ddC and ddI were the most potent inhibitors of mitochondrial function (Benbrik et al., 1997). Depletion of mitochondrial DNA has been reported in patients with zidovudine related myopathy, and this has been shown to be reversible on drug withdrawal (Masanes et al., 1998). Chariot et al, (1993) showed that the histological assessment of zidovudine myopathy

may be improved by assessing cytochrome c oxidase activity, and studies in rats have reported similar observations. Many inherited mitochondrial diseases are known to be associated with myopathy (Rose, 1998), and the changes observed in mitochondrial DNA levels in clinical and laboratory studies of zidovudine myopathy strongly support a mitochondrial pathophysiology for this effect, although it should be acknowledged that there are data suggesting other mechanisms (Masini et al., 1999).

2.3.1.3 Cardiomyopathy

It is has been observed that mitochondrial dysfunction may frequently be associated with heart disease (DiMauro and Hirano, 1998). In vitro studies have identified that cardiac mitochondrial DNA polymerase may be inhibited by zidovudine (Lewis et al., 1994), and studies in rats have shown that the drug may also induce ultrastructural changes in cardiac myocytes (Lewis et al., 1991). A study by Lewis and colleagues demonstrated that zidovudine and HIV infection led to the independent development of cardiomyopathic changes in a transgenic mouse model (Lewis et al., 2000).

2.3.1.4 Neuropathy

Peripheral neuropathy has long been recognised as a complication of HIV infection, the incidence of which increases with the degree of immunosuppression (Wuff et al., 2000). Three of the currently licensed NRTIs, ddC, ddI and d4T, have also been associated with the development of distal symmetrical polyneuropathy in clinical studies (Simpson and Tagliati, 1995). Although there is limited information on the risk associated with combinations of neurotoxic NRTIs, Moore et al, (2000) reported an analysis in 1116 patients on the Johns Hopkins HIV database, which showed that the risk of peripheral neuropathy in patients treated with ddI and d4T was 3.5-fold greater than for patients treated with ddI alone in a multivariate model adjusted for other factors. Hydroxyurea was shown to increase the risk of peripheral neuropathy

still further, since patients taking ddI/d4T hydroxyurea were shown to have a 7.8-fold greater risk than ddI alone (Moore et al., 2000), findings that have also been confirmed by other groups (McCarthy et al., 2000). Since studies have shown that the development of neuropathy with ddC, ddI, and d4T is dose related, management by dose reduction or discontinuation is normally recommended (Moyle and Sadler, 1998).

2.3.1.5 Pancreatitis

Individuals with HIV infection are at greater risk for the development of pancreatitis as a result of immunodeficiency and exposure to a variety of pancreatotoxic agents (Dassopoulos and Ehrenpreis, 1999). These include drugs used to treat opportunistic infections, such as pentamidine, and drugs to treat the HIV infection itself, principally didanosine. The reported incidence of pancreatitis with didanosine varies. Maxson and colleagues suggested it was relatively common with clinical pancreatitis present in 23.5% of their patients and asymptomatic elevations of amylase and lipase in an additional 39.2% (Maxson et al., 1992). Moore and colleagues reported a fourfold increased risk for pancreatitis when hydroxyurea was used with ddI or ddI and d4T. They also reported a similar incidence associated with either ddI or d4T alone and an increased risk of pancreatitis if these two agents were used together (Moore et al., 2001).

2.3.1.6 Lactic Acidosis

Severe liver toxicity, manifesting as acute lactic acidosis with evidence of hepatic steatosis is another toxicity of the NRTIs. It can be fatal and progression may be rapid (White, 2001).

When a cell is unable to generate enough energy through oxidative phosphorylation, anaerobic respiration occurs via the conversion of pyruvate to lactate in the cytoplasm. This also results in excess production of hydrogen

ions, which if uncontrolled, may lead to a cellular and subsequently metabolic acidosis. Clearance is normally performed by the liver and kidneys but if the production is excessive or these organs are damaged, accumulation of lactate and hydrogen ions may occur and severe lactic acidosis may result. Lactic acidosis is a known feature of many mitochondrial disorders (Stacpoole, 1997) and steatosis results from inhibition of fatty acid oxidation, leading to the accumulation of lipid droplets. In vitro studies and recent reports showing evidence of mitochondrial dysfunction after detailed clinical and laboratory investigations in patients with lactic acidosis associated with d4T and ddI (Brivet et al., 2000) or ZDV confirm a mitochondrial pathophysiology. Cases of severe lactic acidosis have been reported with all NRTIs (Brinkman and ter Hofstede, 1999).

Some studies primarily focused on lipodystrophy have also noted an association between d4T therapy and hyperlactaemia (Carr et al., 2000). Saint Marc and colleagues noted that patients switched from d4T to either ZDV or ABC had significant reductions in their plasma lactate levels, which were elevated on d4T therapy (Saint-Marc et al., 2000).

2.3.2 Lipodystrophy

Reports of metabolic and body habitus changes in HIV patients led to the description of what has become known as HIV associated lipodystrophy. Initial studies associated this syndrome with the protease inhibitors although subsequently it has become clear that the NRTIs, particularly d4T, also play a part. Lipodystrophy has been associated with NRTI therapy (Brinkman et al, 1999) and also related with mitochondrial toxicity (Kakuda et al., 2000). It can be seen that overall duration of NRTI therapy and d4T therapy in particular are associated with the development of lipodystrophy. Since time on therapy has been identified as a risk factor the association with d4T could simply be because it was the most recently used nucleoside (Schwenk et al., 2000).

Brinkman et al, (1999) and colleagues proposed that NRTI associated lipodystrophy may be related to mitochondrial toxicity partly due to the similarities between Madelung's disease and some of the clinical features of the lipodystrophy syndrome. While Saint-Marc et al, (2000) noted a reduction in plasma lactate levels as lipodystrophy improved and Carr et al, (2000) described a syndrome of lactic acidaemia and peripheral fat wasting, both of these observations also related to the presence or absence of d4T therapy, which is known to be associated with a higher risk for the development of both hyperlactataemia and peripheral fat wasting. Other studies, Walker et al, (2000) and Shikuma et al (2001) have demonstrated that fat biopsies from patients with lipodystrophy show depletion of mtDNA compared with controls, and Mallal and Nolan, (2000) have demonstrated ultra structural abnormalities of mitochondria in similar tissue. Paulik and colleagues have described an association between metabolic complications associated with NRTIs and changes in the expression of genes and markers of oxidative stress in animal models and cell culture. They also reported that antioxidants such as ascorbate and tocopherol ameliorated these adverse effects, suggesting future studies of these agents may be warranted (Paulik et al., 2000).

2.3.3 Role of riboflavin in mitochondrial transport

Riboflavin is essential in the use of oxygen and in the metabolism of carbohydrates, amino acids, and fatty acids. It is also required in the activation and absorption of pyridoxine (vitamin B_6) and iron, and helps to create niacin (vitamin B_3). It assists in the adrenal gland, in red blood cells formation, in cell respiration and growth, in antibody production, and in the maintenance of the mucus membranes in the digestive tract (Roe & Coates, 1995).

Riboflavin acts as an integral component of two coenzymes: FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide). These flavin coenzymes are critical for the metabolism of carbohydrates, fats, and proteins

into energy. Because riboflavin is an important component of these flavin coenzymes, riboflavin supplementation is believed to increase the efficiency of energy metabolism in cells. Impaired energy metabolism is associated with mitochondrial myopathies. Because of the role of riboflavin derivatives in the electron transport chain, riboflavin supplementation is thought to be a way of improving energy metabolism (Metzler et al., 2001).

Bernsen *et al* (1993) evaluated the effects of riboflavin treatment in five patients with mitochondrial myopathies. The participants in the study had a deficiency of Complex I, the largest of the electron transport chain enzymes. Before treatment, the participants suffered from high lactate levels, exercise-induced weakness, muscle atrophy and other motor problems. Treatment with riboflavin resulted in varying degrees of improvement in three of the five patients. Two patients experienced no improvement, and the remaining three patients with improved conditions showed normalized lactate levels and improved muscle strength and motor abilities.

Ogle *et al* (1997) reported the effects of riboflavin in a case involving a female patient with a myopathy caused by Complex I deficiency. The patient had a mutation that caused instability in the assembly of the complex I protein and consequent deficiency in complex I activity. She suffered from frequent falls and could no longer climb the stairs due to muscle weakness. She also showed increased lactate levels. Treatment with riboflavin during a 3-year period showed normalization in blood lactate levels. The participant was also able to walk longer distances and to rise from the floor without difficulty. An obvious worsening of symptoms occurred during one period when the participant failed to take riboflavin. Exercise tolerance deteriorated, muscle tone worsened, and lactate levels rose during the period when riboflavin was not used. The symptoms observed when riboflavin was not used suggest that the previous improvements were associated with riboflavin supplementation.

2.3.4 Mitochondrial treatment using natural cofactors

The best treatment for all forms of mitochondrial toxicity, but especially lactate acidosis is early detection and cessation of the NRTI treatment. If the treatment is stopped in time, the body will have an opportunity to make new mitochondria, which is the only chance for recovery. This can take either days or weeks. There has been much speculation regarding possible supportive treatments, such as therapies with riboflavin (vitamin B_2), which may be able to increase somewhat (and perhaps sufficiently) the reserve capacity of the mitochondria (Stankov et al., 2010). A number of reports justify (because they are not damaging) the use of riboflavin (vitamin B_2) supplement to such a degree that their administration has been included in the Dutch Association of AIDS Physicians' protocol for lactate acidosis (Brinkman et al., 2000).

There are some potential but poorly researched ways to counteract mitochondrial toxicities. The agents involved are natural cofactors for mitochondrial energy production and supplying them might increase the efficiency of that process. For example, there have been three individual cases of treating severe lactic acidosis with large amounts of riboflavin (vitamin B_2) (Boxwell and Styrt, 1999), a micronutrient that is commonly deficient in people with HIV (Fouty et al., 1998).

Garcia de la Asuncion et al, (1998) tried a high dose of the antioxidant vitamins C (1 g per day) and E (0.6 g per day) in HIV infected patients. Chemical markers of muscle damage and oxidative stress decreased in eight out of twelve HIV positive persons on AZT plus the vitamins compared with a control group on AZT alone. Examination of the mitochondria in the mice muscle cells further showed that the mice receiving AZT plus the vitamins retained normally organized mitochondria whereas the mice on AZT alone had swollen, disrupted mitochondria (Dalakas et al., 1990).

It has also been reported that 12 HIV infected patients with peripheral neuropathy on regimens including ddC, ddl, or d4T had acetyl carnitine deficiency compared with controls with no disease or non-drug related neuropathies (Fouty et al., 1998).

A study in rabbits showed mitochondrial alterations in peripheral nerves after ddC treatment (Feldman & Anderson, 1994), but similar studies with ddl and d4T showed no neuro-toxic effect in the same species (Warner et al., 1995), although ddl has been shown to cause neurotoxicity in rats given very high doses (White, 2001).

2.3.5 Fatty acid transport into mitochondria and clinical manifestations due to defects in this process

Mitochondria oxidation of fatty acid is the principal source of energy for the heart. Consequently, inherited defects of fatty acid oxidation or carnitine assisted transport appear as a serious heart disease called inherited cardiomyopathy. This may manifest as heart failure, pulmonary edema or sudden infant death (Metzler et al., 2001; Roe & Coates, 1995). The proteins that may be defective include a plasma membrane carnitine transporter, carnitine palmitoyl-transferases, carnitine/glycarnitine translocase, long chain, medium chain and short chain acyl CoA dehydrogenases, 2-4dienyl-coA reductase and long -chain 3-hydrxyacyl-coA dehydrogenase. Children with genetically transmitted carnitine deficiency have weak muscles and their mitochondria oxidize long-chain fatty acids slowly. If the inner mitochondrial membrane carnitine palmitoyl-transferase II is lacking, long-chain acylcarnitines accumulate in the mitochondria and appear to have damaging effect on the membranes (Metzler et al., 2001).

Acute myocardial ischemia (lack of oxygen, e.g., during a heart attack) is caused by large accumulation of long-chain acylcarnitines (Roe & Coates, 1995). These compounds may induce cardiac arrhythmia and may also

account for sudden death from deficiency of carnitine palmitoyl-transferase II. Treatment of disorders of carnitine metabolism with daily oral ingestion of several grams of carnitine is helpful, especially for deficiency of plasma membrane transporter (Metzler et al., 2001). One of the most frequent defects of fatty acid oxidation is deficiency of a mitochondrial acyl-coA dehydrogenase. If the long-chain specific enzyme is lacking, the rate of β oxidation of such substrate as octanoate is much less than normal and afflicted individuals excrete in the urine hexanedioic (adipic), octanedioic, decanedioic acids, and all products of ώ oxidation (Roe & Coates, 1995). Much more is the lack of mitochondrial medium-chain acyl Co A dehydrogenase (Metzler et al., 2001).

2.3.6 Advantages of triple NRTl regimens

Triple NRTI regimens potentially afford important advantages for long-term therapy. Dosing is simpler than for protease-inhibitor (PI)-based highly active antiretroviral therapy (HAART) (Arribas, 2004). Patients on triple NRTI regimens generally have a reduced pill burden, rendering therapy more attractive to patients who wish to maintain as normal a lifestyle as possible (Arribas, 2004).

Furthermore, a triple NRTI strategy spares other antiretroviral drug classes, i.e. non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) (Arribas, 2004; Pallela et al., 1998). This allows these other drug classes to be kept in reserve for later intensification or rescue regimens, if required, while limiting the adverse events associated with antiretroviral treatment (Arribas, 2004; Mocroft et al., 2003; Patella et al., 1998). The potentially increased risk of cardiovascular disease linked with long-term PI therapy has attracted particular concern (Friis-Moller et al., 2003): PI-containing regimens have been linked with a significant increase in dyslipidaemia and insulin resistance (Fellay et al., 2001; Mulligan et al., 2000). However, HIV infection is itself associated with an increased prevalence of

decreased high-density lipoproteins, hypertriglyceridaemia, hypercoagulability, endothelial dysfunction and abnormal coronary artery pathology, and it is hence difficult to determine the differential effects of disease and therapy (Passalaris et al., 2000; Riddler et al., 2003). Available guidelines stress that viral suppression should remain the prescriber's prime consideration (Dube et al., 2003).

2.4 Burden of HIV/AIDS

HIV has found a wealth of opportunities to thrive among tragic human conditions fueled by poverty, abuse, violence, prejudice and ignorance. Social and economic circumstances contribute to vulnerability to HIV infection and intensify its impact, while HIV/AIDS generates and amplifies the very conditions that enable the epidemic to thrive. Just as the virus depletes the human body of its natural defenses, it can also deplete families and communities of the assets and social structures necessary for successful prevention and provision of care and treatment for persons living with HIV/AIDS. This is demonstrated by the estimated 30 million people living with HIV/AIDS, mostly in developing countries. Over 2 million people die annually from HIV related illnesses adding to nearly 12 million deaths attributed so far to the epidemic. The impact of HIV/AIDS extends beyond those living with the virus, as each infection produces consequences, which affect the lives of the family, friends and communities surrounding an infected person. The overall impact of the epidemic encompasses effects on the lives of multiples of the millions of people living with HIV/AIDS or of those who have died.

Since the first case of Acquired Immunodeficiency Syndrome was reported in Kenya in 1984, HIV/AIDS has continued to have a devastating impact on all sectors of the society. According to the Kenya AIDS Epidemic Report 2012, adult HIV prevalence in Kenya in 2011 was 6.2%. This includes about 1.6 million adults between 15 and 49 years, another 60,000 age 50 and over, and

approximately 100,000 children (The Kenya AIDS epidemic update 2012). The number of people living with HIV worldwide in 2012 is estimated to be 35.3million with 9.7million on antiretroviral drugs. Twenty six million are eligible for antiretroviral therapy (WHO et al., 2013). The decline in HIV prevalence from 14% to current less than 6.2% has been associated with the antiretroviral therapy.

In the early years of the pandemic, the major interventions were aimed at prevention of new infections through creating awareness as well as advocacy for behaviour change. The majority of those who were already infected could not access palliative care as the cost of antiretroviral drug was prohibitive and beyond the reach of many. Over the last few years, there has been introduction of services that can ameliorate the suffering of people living with HIV/AIDS (PLWHA). An example is widespread availability of antiretroviral therapy.
The NRTI backbone consisting of stavudine has been preferred due to their efficacy, cost-effectiveness, availability and ease of adherence. However, there has been an array of effects from these drugs necessitating change of drugs in the course of treatment. Lack of solution to these toxicities has complicated the application of the first line regimen.

Stavudine (d4T)–related toxicities reviewed above have led to various recommendations by the World Health Organization (WHO). In 2007, the WHO recommended dose reduction of d4T from 40 mg twice daily (BD) to 30 mg twice daily for all adults (WHO, 2007) and in the guideline of 2010, the WHO recommended that d4T should be phased out in all countries (WHO, 2010). Kenya has adopted these recommendations, and is subsequently phasing out stavudine in favour of tenofovir (Ogola et al., 2013). Stavudine phase out is ongoing with only 15.4% of pediatrics currently on Stavudine based regimens, while 49.7% are on Zidovudine based and 27.6% on Abacavir based regimens (Kenya Operational plan 2012).

Whereas decision is being made by public health authorities to replace d4T in HIV/AIDS management programs, the question of resource constraints need be articulated in view of cost of available options such as tenofir and abacavir (Ogola et al., 2013; Innes et al., 2011).

The logistics and cost of switching are significant, and the World Health Organization had estimated 1.55 million people to be on stavudine-based antiretroviral therapy by the end of 2012. Stavudine is co-formulated in many countries, is very cheap and effective, and is well tolerated in initial therapy (Innes et al., 2011).

Despite generic tenofovir now being cheaper than zidovudine, tenofovir consumes the majority of adult antiretroviral programme medication budgets in programmes in Africa, where it is used in first-line therapy (Ogola et al,. 2013). Abacavir is far more expensive than zidovudine or tenofovir, and is a major cost driver in paediatric programmes with access to abacavir-based first-line treatment. Low-dose stavudine may offer the only cheaper (and possibly as effective and safe) alternative to programmes grappling with limited financial resources (Innes et al., 2011).

The logistics and cost of switching all antiretroviral-treated individuals to non-stavudine therapy is significant. Generic tenofovir costs 6 times more per month than stavudine, while tenofovir co-formulated with emtricitibine costs 4 times more than a month's supply of stavudine and lamivudine combined. In addition, the use of tenofovir, which requires additional renal function monitoring, substantially increases the programme costs of safety monitoring (Wood, 2006). Taking the costs of toxicity management into account, the cost-effectiveness ratio (measured in cost per year of life saved) of tenofovir is double that of stavudine (when ART is initiated at 350 CD4 cells/µl in a one-line

setting) with similar 5-year survival (89% v. 87%) when using the incidence of stavudine toxicity associated with 40 mg BD (Innes et al., 2011).

2.5 Beneficiaries/Impact

2.5.1 HIV positive patients on antiretroviral therapy including stavudine

The HIV patients on the first line regimen are changed to another regimen when adverse effects develop from the first one. The second regimen includes protease inhibitor for a nucleoside analogue. The second line also has complications. For instance, some patients develop hypersensitivity to abacavir. The NRTI backbone of stavudine (d4T) and lamivudine (3TC) is cost effective and efficacious. Stavudine is also preferable as it does not cause bone marrow suppression. Alternative regimen from the one containing stavudine is zidovudine (AZT) or tenofovir (TDF). The AZT/3TC combination is more costly than d4T/3TC. Zidovudine also causes bone marrow suppression which magnifies the problem of anaemia - common among HIV positive patients in our population.

Therefore, the findings of this study, may give a solution to the problem of toxicity hence minimizing the number of patients changing from the first line regimen. Besides this, there is peace of mind to the patient that comes in maintaining the same treatment pattern. The patients will also save the cost of constant clinic attendance for briefing and follow-up owing to change of treatment regimen.

2.5.2 The Government of Kenya (GoK)

2.5.2.1 Minimal cost of changing regimen

The goals of antiretroviral treatment are to improve the quality of life of the HIV-infected, reduce HIV related morbidity and mortality, and restore or preserve their immune function through maximal suppression of viral replication. This can only be done effectively if the treatment is administered carefully bearing in mind the efficacy of the regimen as well as the tolerability, affordability and availability of the drugs. These qualities are found in the first line regimen containing the stavudine.

The other regimen comes with added cost. The Government which is offering free antiretroviral drugs shoulders the extra burden. With time, it will be a big burden to government as more HIV patients change from the initial first line treatment. Maintaining patients on the first line regimen will save the government from added cost.

2.5.2.2 Improved management of HIV/AIDS patients

Managing the HIV patients requires a lengthy procedure including rigorous follow up. This is particularly during initiation of treatment. Laboratory investigations are of prime importance at this time. More follow-up and investigations are done during clinic visits. This is meant to ensure adherence to treatment and maintaining the gains of antiretroviral treatment. Changing from one regimen to another means repeating the process all over again. More laboratory tests and frequent visits to clinic are inevitable.

The clinician will find it of great convenience if the patients remain in the initial treatment. The government will also save in constant briefs, workshops, follow-up and training on changing the regimen.

2.5.3 The World Health Organization (WHO)

The outcomes of this study are expected to help the HIV/AIDS patients on the first line regimen. This study is in line with WHO mission which is "*the attainment of highest level of health by all people*" through working out an intervention to the current problem of mitochondrial toxicity. The findings of this study are expected to promote general health of HIV positive patients on first line regimen by treating mitochondrial toxicity and lipodystrophy. This is one of the tasks of WHO. The findings of this may trigger the need to revisit and review the position taken by WHO, UNAIDS and other regulatory agencies on antiretroviral therapy particularly with respect to salvaging stavudine. The results will also prompt more human based research on use of stavudine.

2.6 Rationale

Nucleoside analogues represent the cornerstone of antiretroviral regimens. However, these drugs are associated with a range of drug or tissue-specific toxicities such as peripheral neuropathy, myopathy, pancreatic and lactic acidosis with hepatic steatosis. Fat lipodystrophy seen also on long term antiretroviral therapy is related to nucleoside analogues. The remedy tenable in clinics is changing to another regimen which introduces protease inhibitor in the regimen.

Stavudine and lamivudine are nucleoside analogues believed to be the major cause of these effects. There is no established intervention to these toxicities. This has led to recommendation for its withdrawal by the WHO. There has been suggestions to reduce the dose of stavudine in effort manage its effects. Given the low cost of production of NRTIs including stavudine compared to other antiretroviral drugs and its efficacy in combating the HIV replication, this study was carried out to establish the nature and extent of mitochondrial toxicity and interventions during antiretroviral treatment with these drugs. Riboflavin was

used as interventions in this study. Whereas riboflavin has been used before, there have been no systematic studies on its efficacy. This study tested the efficacy of riboflavin and its effect on different dose concentrations and combinations of nucleoside analogues. Riboflavin is a natural cofactor and useful in contribution to FMN and FAD. These molecules are vital in mitochondrial function.

2.7 Hypothesis

Mitochondrial toxicity is minimized by use of natural cofactors: riboflavin.

2.8 Objectives

2.8.1 General objectives

To assess the role of riboflavin in protecting against mitochondrial toxicity and lipodystrophy due to stavudine and lamivudine.

2.8.2 Specific objectives

The specific objectives of this study are to:

1. Establish reference ranges for live weight, liver weight, haematology and biochemical parameters using untreated swiss albino mice for interpretation of study data.

2. Assess toxicity arising from the use of nucleoside analogue drugs on biochemical and haematological parameters.

3. Determine the toxicity caused by the use of nucleoside drug analogues on mitochondrial genome.

4. Determine the hepatoxicity caused by nucleoside analogue drugs.

5. Document the safety and efficacy of riboflavin as an intervention to toxicity caused by use of nucleoside analogue drugs.

Chapter three

3.0 Materials and Methods

3.1 Methodology

The study was carried out in two phases. Phase one focused on determination of acute toxicity for one month and phase two aimed at determination of chronic toxicity for four months. Initial experiments were done to optimise conditions. These experiments included: determination of toxic dosage for lamivudine and riboflavin; testing of results generated in mitochondrial DNA (mtDNA) and genomic DNA and thermocycling conditions for mtDNA. The main experiments included determination of toxic levels for the drugs: stavudine, an equivalence of human dose of stavudine-lamivudine combined, equivalence of human dose for stavudine-lamivudine-riboflavin combined, twice equivalence of human dose for stavudine-lamivudine combined, twice combination of human equivalence of stavudine-lamivudine-riboflavin, half human equivalent dose for stavudine, half human equivalent dose for combination of stavudine-lamivudine and human equivalent dose for riboflavin (table 4.1).

3.2 Mice Acquisition and Breeding

Thirty (twenty female and ten male) Swiss albino mice were purchased from the animal laboratories at the International Livestock Research Institute, Kenya (ILRI). They were assessed for general health before transporting them to animal house at Department of Biochemistry University of Nairobi, Chiromo.

Transportation of animals is stressful and leads to physiologic changes, such as increased cortisol levels, which may potentially alter research results. Duration of acclimatization depends on the distance/time involved in transporting the mice. The mice for this study were transported a distance of ten kilometres on tarmac road. They were placed in cage with wood chips beddings. Male and females were separated and transported in different cages.

They were allowed five days to acclimatize after which they were prepared for breeding.

3.3 Care of Experimental Mouse

3.3.1 Mouse Husbandry

Mice were reared in accordance with good animal husbandry as described in appendix 1. The cleaning was done regularly and food replenished. Water was given using watering bottles. The veterinary doctors from the college of Agriculture and Veterinary services were consulted for regular check-ups on the mice in the course of the study.

All mice were kept in solid bottomed cages throughout the experimental period. The bedding material provided was hardwood chips which catered for thermal insulation, and absorption of faecal and urinary wastes. The bedding materials are appropriate because they are absorbent, not readily eaten, free of infectious agents and injurious substances, and comfortable for the animals. The aromatic wood shavings such as pine and cedar shavings were avoided in the laboratory setting as they induce activation of hepatic microsomal enzymes, and this may interfere with experiment results. Cotton or shredded paper were not used at all in breeding cages for the safety of the pups so that they are not entangled in the fibres and may suffocate or lose appendages.

The mouse room was maintained at temperature range from 18^{o} to 24^{o}C with an average temperature of 21^{0} C and relative humidity of between 40% and 70%. Mice were inspected daily in the morning for ringtail and swollen and reddened feet. This occurs as a result of extreme decrease in humidity (less than 40%) or elevated temperature (greater than 26^{o}C). The humidity was measured daily by the Department of Meteorology in the University of Nairobi.

Mouse room was well regulated by automatic timers to provide cycles with 12-14 hours of light and 10-12 hours of dark. All cages were placed on surface of a uniform bench to ensure equal light distribution. This was done because mice on the lower racks will receive less light than those housed on the top racks. Noise was controlled in the mouse room and the environment.

Mice were fed with pellets obtained from registered animal feed manufacturers-Unga Farm Care EA limited. Maintenance diets contained 4-5% fat and 14% protein. Young animals and those used for breeding were fed with higher nutrient diets containing 7-11% fat and 17-19% protein.

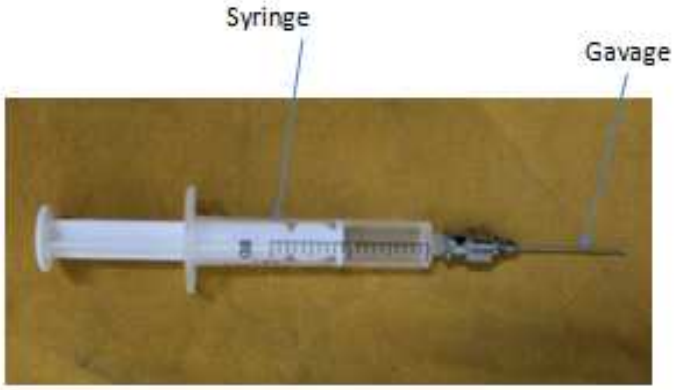
Water was provided using bottles and sipper tubes. Leakage was checked every day per cage to prevent leakage soaking the bedding.

Juvenile and adult mice were caught and picked up by grasping the base or middle third of the tail with the fingers. Once caught, the mouse was restrained by placing it on a wire cage lid, grasping the loose skin behind the neck and ears with the thumb and forefingers, and holding the tail against the palm of the hand using the fourth and fifth fingers (photograph 3.1). Precaution was observed while holding mouse to make sure that the skin around the neck is not pulled so tightly that the mouse cannot breathe. This technique was used when examining a mouse, oral administration of the drug and when giving an injection.

Photograph 3.1: Handling of mouse during oral administration of drugs

3.3.2 Oral drug optimization experiments

Each mouse was identified by marking the tail with an indelible marker.
A special gavage was used to administer experimental drugs orally (photograph 3.2). This gadget was chosen because it can deliver a precise amount of test material. The special gavage needle has a ball at the end and is useful for deliver of materials directly into the stomach. The ball on the needle prevents entry into the trachea. The length of the gavage tube required was determined by measuring the distance from the mouth to the last rib. Each mouse was held with the head and neck extended. In cases when the gavage tube does not easily pass into the esophagus, it was removed and tried again.



Photograph 3.2: Gavage and syringe used to administer drugs to the mice

The drugs lamivudine and riboflavin were used for drug toxicity. Sixty six mice between 8-12 weeks old and with mean weight of 30g were used for preliminary experiments. They were placed in groups of six to constitute a treatment group. The groups were administered staggered doses as shown in the table 3.1 below.

3.4 Experimental procedures

3.4.1 Dose Calculation and Definition

The doses selected for use in the experiments were cascaded from human therapeutic dose. The conventional therapeutic dose administered to a human being of approximately 70kg for stavudine is 40mg twice a day totalling to 80mg a day, lamivudine daily dose administered to human similar weight is 150mg twice a day equivalent of 300mg an equivalent dose of stavudine for 30g mouse was 0.0354mg and 0.13mg for lamivudine. The riboflavin dose was set at 0.17mg which is equivalent to 400mg a day for 70kg human being. The treatment groups were set at half human equivalent dose, human equivalent dose and double human equivalent dose of stavudine, lamivudine and their combinations. The dose concentrations per kilogram body weight are shown in table 3.1.

Table 3.1: The experimental drug dose regimen

Drug	Human equivalent		
	Half (mg/kg)	Human (mg/kg)	Double (mg/kg)
Stavudine (d4T)	0.57	1.14	2.28
Lamivudine (3TC)	2.15	4.3	8.6
Riboflavin (R)	2.85	5.7	11.4

The range weight of mice used in this experiment was 29.5-31.3g. The weight was averaged at 30g for the purpose of calculating drugs concentrations.

The drug concentration in table 3.1 will be used to define experimental drug doses as half human dose, single human dose and double human dose. Each dose level will consist of stavudine alone, combination of stavudine and lamivudine, combination of stavudine, lamivudine and riboflavin, and combination of stavudine and riboflavin.

3.4.2 Dosage Administration

The dosage for riboflavin and lamivudine were serialized as per table 3.2. These serialized drug concentrations were used in optimization experiments. The mice were administered drugs in different concentrations as follows:

Table 3.2: Dosage concentration

Drug	Dosage concentration (mg/kg)				
	Cage I	Cage II	Cage III	Cage IV	Cage V
3TC	2.15	3.2	4.3	6.5	8.6
Riboflavin	2.85	4.3	5.7	8.55	11.4

N=6.

Each cage had six mice. Another group of six mice was placed in a group which was not in any treatment. This was the control group. The mice were on treatment for four months.

At the end of four months, the mice were sacrificed (appendix 2) and the following specimens were collected: blood, liver, skeletal muscles, heart, brain and adipose tissue. The blood was used for lipid profile and blood glucose determination. Mitochondrial genome was assessed for damage.

3.4.3 Monitoring for Toxicity

The mice were monitored throughout the experiment period. The weight, skin texture, room temperature, water and food consumption were monitored. Toxicity was defined by the derangement of mitochondrial DNA (mtDNA), change in feeding habits, rough skin and shivering.

3.5 Optimising PCR Conditions

3.5.1 Optimization of Conditions for Amplifying Mitochondrial DNA (mtDNA) using Polymerase Chain Reaction (PCR)

The PCR conditions mainly the concentration of the reaction mixture and thermocycling profile was optimized. Nine samples were used for this experiment (table 3.3). The DNA concentration was determined using UV camera (Nano Drop 2000C; USA). The PCR was done using four specific primers for mtDNA as described in section B below.

A. DNA quantification

DNA had earlier been extracted using QIAamp DNA Mini Kit for extracting DNA from tissues (QIAGEN, USA) appendix 4.

The following specimens were selected and DNA concentration determined as per table 3.3 below.

Table 3.3: Specimen and DNA concentration

Tissue	Treatment	DNA conc (ng/µl)
Liver	Riboflavin	100
	Control	91
Skeletal muscles	Lamivudine	78.8
Heart	Lamivudine	141.9
Adipose tissue	Control	60.6
	Control	74.7
Liver	Lamivudine	65.4
	Control	89.0
	Riboflavin	157.4

B. Primer Design

Long and short DNA fragments from the mouse mitochondrial DNA was amplified using four specific primers as follows:

Forward primer (I) 5'-GACAGCTAAGACCCAAACTGGG-3' (nt 470-492 of the mouse mtDNA sequence) and reverse primer (II) 5'-CCCATTTCTTCCCATTTCATTGGC-3' (nt 785-762) were used to amplify 316 bp mtDNA fragment, while forward primer (III) 5'-TACTAGTCCGCGAGCCTTCAAAGC-3'(nt 4964-4987) and reverse primer (IV) 5t-GGGTGATCTTTGTTTGCGGGT-3l (nt 13599-13579) to amplify an 8636 bp mtDNA fragment. The PCR was performed with 50ul primers, mtDNA (50-200ng), each dNTP (200uM), magnesium chloride (Mg^{2}+) (1.5mM), and DNA polymerase in reaction tubes.

C. Thermocycling Profile

The thermocycler profile included initial denaturation at 95°C for 2 minutes, 26 cycles of 95°C for 45 seconds, 57°C for 45 seconds and 68°C for 3.0 minutes, and final extension at 68°C for 7 minutes for short PCR. The thermocycling profile long PCR included initial denaturation at 95°C for 2 min, 26 cycles of 95°C for 45 s, 58°C for 45 s and 68°C for 3 min, with final extension at 68°C for 7 min. The PCR products were electrophoresed on agarose gels and were stained with ethidium bromide. Photographs were taken and scanned.

3.5.2 Titrating Conditions

It was essential to determine optimum conditions for PCR. These conditions include the concentration and volume of reagents and thermocycling profile. Various titration experiments for different parameters were done. During titration, the temperature for cycling and annealing was determined. Annealing time, magnesium ion concentrations and final extension time were determined.

3.5.3 Comparison of Results for DNA from Isolated Mitochondria and Homogenized Tissue

An experiment was done to determine if DNA extracted from isolated mitochondria is comparable to genomic one. The purpose of this experiment was to determine if it was necessary to isolate mitochondria before extracting DNA.

3.5.4 Isolation of Mitochondrial DNA

Mitochondria were isolated from the liver, hind limb skeletal muscles and adipose tissue (appendix 3). The mtDNA was thereafter extracted from each these tissues and quantified as described in section 3.5.1 above. The PCR conditions were applied as described above (section 3.5.1 A, B and C).

3.5.5 Testing Optimized Conditions for Long and Short MtDNA Fragments

The purpose of this experiment was to test the optimized conditions for PCR. The long and short fragment primers were used to run twenty one samples after DNA quantification.

Conventional PCR conditions: The reaction mixture components and the thermocyling profile were optimised. The reaction mixture was established as consisting of:1 µM primer (F), 1µMprimer (R), 1.25 U *Taq* polymerase, 200 µM of each dNTP and 1µgDNA template in a *Taq* polymerase buffer containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40 and 1.5 mM MgCl2. The total reaction volume was 50µl. The thermocycling profile was established as an initial denaturation at 95^{o}C for 2 min, followed by 26 cycles consisting of denaturation at 95 ^{o}C for 45 sec, primer annealing at 57 ^{o}C for 45 sec and strand extention at 68 ^{o}C for 3min. These were followed by a final strand extension to fill-in incomplete recessed ends of the amplicons at 68^{o}C for 7 min.

These conditions were applied in assessing the integrity of mtDNA from total DNA (a mixture of nuclear and mtDNA) and from mtDNA obtained from isolated mitochondria. Primers used in both cases were specific to mtDNA with a second control set specific to a housekeeping nuclear gene (β-actin). The results of amplicon concentrations obtained when total DNA and DNA extracted from isolated mitochondria were used as templates with mtDNA-specific primers, was comparable. The housekeeping gene was only amplified when the template was total DNA and not when pure mtDNA was used as template.

The conditions established above for the conventional PCR were only effective in amplifying short fragment of 300bp. It was not possible to amplify the 8.6kb long fragment using these parameters or a series of others that were tried. Therefore, it was also not possible to establish the definite derangement per

treatment group due to the use of a short 300bp amplicons and not the larger 8.6 kb region. Thus, real time PCR (rt-PCR) was used in the determination of the mtDNA damage.

3.5.6 Real Time PCR (rt-PCR) to Determine Damage on MtDNA after Treatment with Nucleoside Analogue Drugs

An experiment was set to determine the mtDNA damage in tissues: liver, heart, brain, skeletal muscles and adipose tissue. The mice consisted of groups treated with lamivudine and riboflavin for a period of four months. Before the amplification was done, DNA concentration was determined as described in section 3.5.1. The DNA concentration in each of the specimen is shown in table 3.4 below.

A. Specimen and DNA quantification

The following specimens were used for this experiment.

Table 3.4: Specimens label and DNA concentration

Tissue	Treatment	DNA conc (ng/ul)
Liver	Lamivudine	123.3
		78.4
	Riboflavin	35.7
		84.7
	Control	37.8
		32.6
Heart	Lamivudine	76.5
		7.0
	Riboflavin	7.4
		4.5
	Control	167.8
		3.6
Brain	Lamivudine	7.8
		2.9
	Riboflavin	4.8
		2.0
	Control	6.3
		3.2
Skeletal muscle	Lamivudine	2.0
		6.5
	Riboflavin	3.9
	Control	18.1
		3.4
Adipose tissue	Lamivudine	3.1
		3.0
	Riboflavin	15.8
		13.9
	Control	1.5
		6.7

The primers and probes are as shown in table 3.5 and the plate arrangement in table 3.6. These specimens were selected dependent on DNA concentration. The representation of the tssues and treatment was also considered. Each

treatment level was represented by two mice. For all the six tissues under experiment, there was lamivudine, riboflavin and untreated group.

B. Probes and primers

The probes and primers were acquired from Applied Biosystems (UK). Four primers and two probes were used (table 3.5).

Table 3.5: Primers and probes used for rt-PCR

Forward fragment primer	
Sequence Detection:	5'-AGA GAA CTA CTA GCC ACA-3'
Reverse fragment primer	
Sequence Detection:	3'-GAA GAT GGC GGT ATA TAG-5'
Probe	
Sequence Detection:	AGA TCG TGA GGT AGA GCG G
Probe	
Sequence Detection:	ACC ATC TCT TGC TAA TTC

C. Plate arrangement

The samples were set on the plate as shown in the table 3.6 below.

Table 3.6: Plate arrangement of the samples for the determination of mtDNA damage as a result of lamivudine and riboflavin

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	9	17	26		1	9	17	26			NEG C
B	2	10	18	27		2	10	18	27			NEG C
C	3	11	19	28		3	11	19	28			
D	4	12	20	29		4	12	20	29			
E	5	13	22			5	13	22				
F	6	14	23			6	14	23				
G	7	15	24			7	15	24				
H	8	16	25			8	16	25				
	Specimen					Duplicate						

Legend: NEG C means Negative Control

The table 3.6 shows the arrangement of the specimen according to the numerical values. This table is a schematic arrangement of the specimen (table 3.4) on the plate for PCR analysis.The rt-PCR thermocycling conditions used in this experiment included storage for 10 minutes at 45^0C, denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and extension at 55°C for 1 minute.

3.5.7 Determination of Reference Ranges for Swiss Mice

It was essential to determine reference ranges of biochemical and haematological profiles of experimental mice so that the data can be applied in the interpretation of results obtained after drug treatment.

The parameters of interest include: blood glucose concentration; lipids profile; haematology and biometric data.

Thirty healthy mice were used. They were fed with water and mice pellets from Unga Farm Care Limited, Kenya for one month. During this period, the mice were monitored on health, reactions to weather fluctuations, weight and water consumed. The blood glucose was also measured and blood smear taken three times during the period they were under observation. After one month, the mice were sacrificed and blood was obtained through cardiac puncture. Liver was obtained and weighed. Adipose tissue was also obtained. The specimen were collected in ice and transported in a cool box to Clinical Chemistry Laboratories for analysis of blood glucose levels, haematology and lipid profile and United States of America Medical Research Unit-Kenya (USAMRU-K) laboratories where mtDNA from the liver and adipose tissue was analyzed.

The histology and cell morphology of liver tissue were measured in the Anatomic Pathology laboratories. The array data was analysed and outliers were removed. This data was used to determine reference ranges for parameters as shown in section 4.1.

3.6 Biochemical parameters

3.6.1 Blood glucose and Lipids Profile

Random blood glucose and plasma triglycerides, HDL-Cholesterol and total cholesterol concentrations were measured. The specimen for blood glucose was whole blood collected through tail nick. The specimen for lipid profile was serum.

The lipid profile: total cholesterol, triglycerides and HDL-Cholesterol was analysed as per appendix 8.

3.6.2 Liver Histology

Hematoxylin and Eosin (H&E) Staining Protocol

The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The in situ oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate. Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the overstaining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed "differentiation". Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei (Mayer, 1896). The procedure is outlined in appendix 6.

3.6.3 Haematology Profile

Leishman's stain

Leishman's stain was used in microscopy for staining blood smears because it provides excellent stain quality. It is generally used to differentiate and identify leucocytes, malaria parasites, and trypanosomae. It is based on a methanolic mixture of "polychromed" methylene blue (i.e. demethylated into various azures) and eosin. The methanolic stock solution is stable and also serves the purpose of directly fixing the smear eliminating a prefixing step. It is similar to

and partially replaceable with Giemsa stain, Jenner's stain, and Wright's stain. Procedure is detailed in appendix 7.

3.6.4 Coagulation Test

The prothrombin time test was introduced originally as a test for prothrombin activity hence the name. The test is now known to measure in addition, and more importantly, factors V, VII and X. It is also relatively sensitive to the presence of heparin in the blood and hypofibronogenaemia. The test in its various modifications can be used to screen for deficiencies of the above mentioned factors occurring in disease or produced by the administration of drugs. The test measures the clotting time of plasma in the presence of optimal concentration of thromboplastin (tissue extract) indicating the efficiency of extrinsic clotting system. Incubation of plasma with optimal quantities of phosphplipids and a surface activator leads to activation of the intrinsic coagulation system. The addition of calcium chloride ($CaCl_2$) triggers the coagulation process. The activated partial thromboplastin time (APTT) is a non-specific test of the intrinsic system. It is the most useful screening test for deficiencies of factors VIII, IX, XI and XII when taken together with a normal prothrombin time. The test is done to demonstrate the competency of intrinsic factor pathway (Dacie and Lewis, 2000).

The detailed procedure is on appendix 9.

3.7 Quality Assurance

Random Blood glucose

The random blood glucose was measured out using glucometer (Softstyle®, Hungary). The glucose strip was inserted in the glucometer and a drop of blood placed on it. A reading was obtained from the glucometer. Internal quality control was used. The glucometer was calibrated before analysis was done.

Lipid profile

The lipid profile was determined using kit (Human®, Germany). The quality control was purchased with the kit and values were provided as follows:

Total cholesterol: 3.57-4.74mmol/L

Triglcerides: 1.53-2.21 mmol/L

HDL-C: 1.18-1.77 mmol/L.

The quality control was run together with the test. If the control value obtained was not within the reference values indicated above, the test was considered invalid and was repeated.

Mitochondrial DNA

Three quality controls were used: two positive controls and a negative control. Positive controls used were RNAP housekeeping nuclear gene (β-actin) and mtDNA extracted from the isolated mitochondria of mouse not on treatment. The negative control was water. If there was reading on negative control and/or RNAP, the test was invalidated and repeated.

All the results reported in this book were valid.

3.8 Ethical Approval

Ethical approval to conduct this study was given by Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH-UON ERC) (appendix 10).

3.9 Data Management

Data entry tools

The primary data was collected in two laboratory books: one for the animal house and another in the laboratory. The laboratory book for the animal house contained information on animal care, feeding, random blood glucose, drug combinations and drug concentrations. The data collected was entered in spreadsheets (MS Excel software) and data editor (SPSS software). Data on weight: food, liver, mice; concentration of drugs; results of biochemistry, haematology and mtDNA were entered. This data was entered against the mouse number, cage number and treatment concentration and dose combinations. Histology data was entered in narrative form in spreadsheets (MS Excel software) and MS Word. Photographs were taken to capture the state of health of the mice during treatment. Further photographs were taken for the tissues that were considered pathological or abnormal.

Data cleaning procedures

The data entered in computer software was revised to check missing information. Captured information was counterchecked with the raw one to fill any omission for each animal or experiment. The entries were further verified using denominator of each treatment group and dose level. Frequency runs were done to confirm the entries.

Data analysis

The data on animal weight, food intake and biochemistry were analysed using means to describe continuous parameter. Proportions were used to describe categorical values. The data on mtDNA, blood glucose, lipid profile and coagulation were expressed in terms of means, ranges and standard deviation. The biochemistry, coagulation time and mtDNA results were compared using chi-squares, t-test for continuous variables and ANOVA for comparison across groups. The data was presented in bar graphs, boxplots, histograms and tables.

Chapter Four

Results

4.1 Reference Range Determination

4.1.1 Reference Ranges for Blood Count

Table 4.1: The reference ranges of the blood count of the swiss mice

Blood parameter	Mean (%)	SD	2SD	Reference range (%)
Neutrophils	15.8	3.7	7.4	8-23
Lymphocytes	76.7	4.8	9.6	67-86
Monocytes	5.15	2.3	4.6	1.0-9.0
Eosinophils	1.8	1.5	3.0	0- 5

N=21

Table 4.1 shows the blood count parameters include neutrophils, lymphocytes, monocytes and eosinophils.

4.1.2 Reference Ranges for Biochemical Parameters

A) Lipid Profile

Table 4.2: The lipid profiles results for healthy mice

Sample number	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)	High Density Lipoprotein-Choleterol (mmol/)	Low Density Lipoprotein-Choleterol (mmol/)
1	2.8	1.3	0.6	1.6
2	2.9	1.7	0.7	1.4
3	3.02	2.73	1.08	0.74
4	3.09	1.55	0.38	2.01
5	3.1	1.95	0.7	1.5
6	3.1	2.5	1.6	0.38
7	3.11	2.18	0.75	1.4
8	3.3	2.1	0.9	1.45
9	3.4	2.3	1.1	1.3
10	3.4	1.82	0.69	1.9
11	3.5	2.4	1.1	1.3
12	3.6	2.15	1.4	1.2
13	3.68	2.42	0.3	2.3
14	3.7	2.57	1.4	2.3
15	3.9	2.7	1.6	1.1
16	3.92	3.1	2.3	0.22
17	3.94	1.89	0.4	2.7
18	4.1	3.5	1.9	0.6
19	4.2	3.4	2.1	0.6
20	4.26	3.8	1.92	0.64
21	4.4	2.34	0.7	2.6
SUM	74.42	50.4	23.6	29.2
MEAN	3.5	2.4	1.1	1.4
SD	0.48	0.6	0.6	0.7
2SD	0.96	1.2	1.2	1.4

N=21

The results shown in the table 4.2 were obtained from mice not in treatment. The values represent array data which was used to determine the reference ranges for the parameters of lipid profile. Mean and twice the standard deviation was calculated.

B) Reference range for glucose

The reference range of glucose was determined from the 30 untreated mice. The mean was 7.06 mmol/L.

The blood glucose concentration in blood is in figure 5.1.

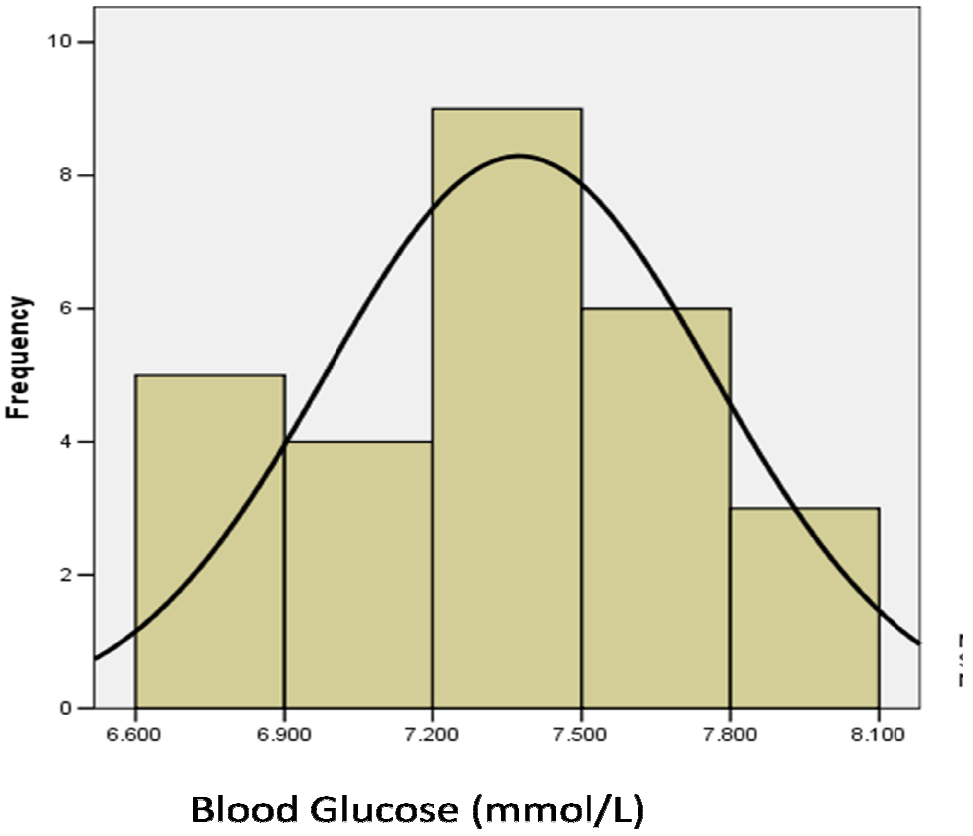


Figure 4.1: The blood glucose concentration

After the removal of outliers, the blood glucose values were 6.7 to 7.6mmol/L. The mean was 7.1mmol/L with standard deviation of 0.21. Therefore, the reference range for glucose was 6.7-7.5mmol/L.

4.1.3 Results for body weight and liver weight

A) Reference range for body weight

The weight of individual mouse was obtained. They were sorted in ascending order and frequency of values that occurred more than once was obtained. The mean value was found to be 29.8g with standard deviation 1.02 (figure 4.2 below). The reference range for body weight for live swiss mouse was 27.8-31.8grams.

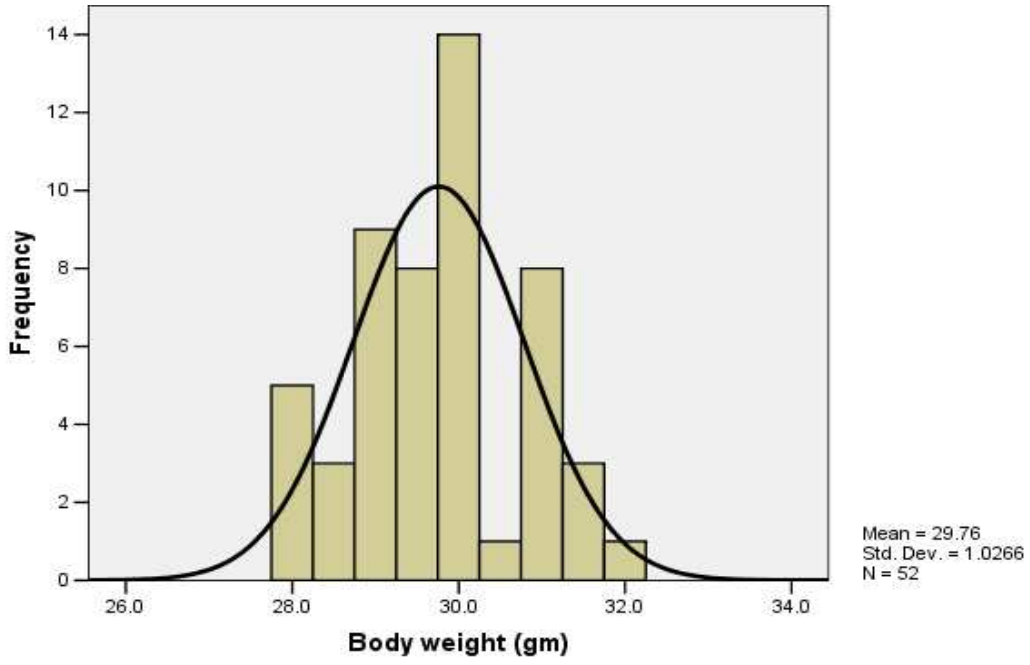


Figure 4.2: Body weight for untreated mice

B) Reference range for liver weight

Reference range for the liver weight was also determined. Mice were sacrificed and liver was obtained and weighed. The results are in figures 4.3.

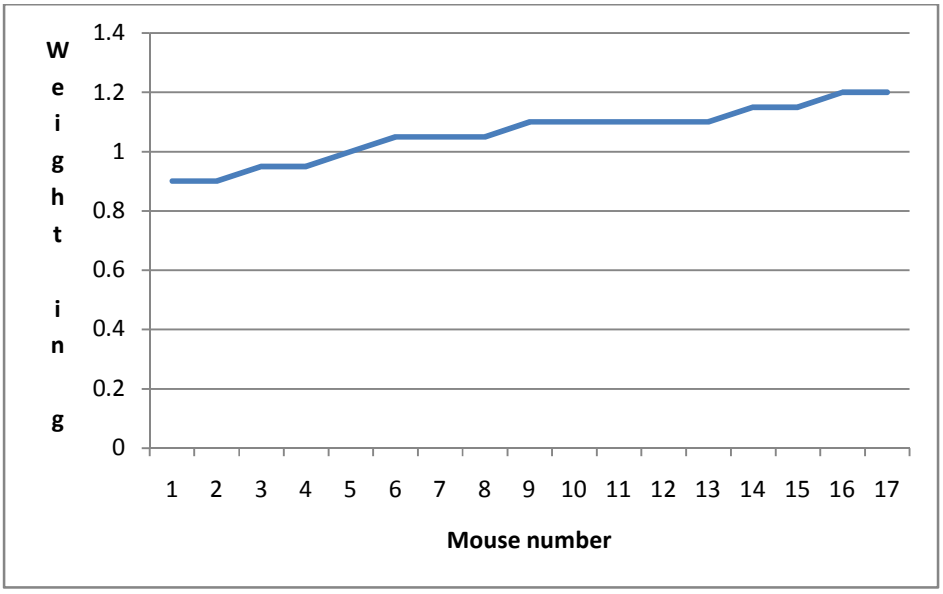


Figure 4.3: The weight for liver in untreated mice

The mean liver weight was 1.05grams with standard deviation of 0.09. Therefore, the reference range for liver weight was 0.87-1.23g.

4.2 Results on the assessment of toxicity

4.2.1 General observation

It was found that the skin texture of the mice on treatment with lamivudine in groups III, IV and V were rough, had sunken eyes and they appeared lethargic. There was no significant change observed on mice on lamivudine treatment in groups I and II. The mice on riboflavin treatment in all the cages had smooth skin, bright eyes and were active. Mice in each cage were weighed individually. The average weight was calculated at the start of the experiment and after one and two months respectively (table 4.3a). The correlation of body weight of mice treated with lamivudine and that treated with riboflavin was not significant (p value was 0.1)

Table 4.3a: Weight of mice treated with lamivudine and riboflavin for up to two months

Group no	Drug name	Drug conc. (Mg/Kg)	Average wt. (g)		
			Start	1month	2months
I	3TC	2.1	31.0	33.4	35.6
	Riboflavin	2.8	30.5	37.0	36.6
II	3TC	3.2	32.3	33.8	36.1
	Riboflavin	4.3	31	28.7	29.6
III	3TC	4.3	29.5	34.5	36.4
	Riboflavin	5.7	31	33.1	34.6
IV	3TC	6.4	30.0	30.4	35.4
	Riboflavin	8.6	29	32.6	32.6
V	3TC	8.6	30.5	34.4	35.9
	Riboflavin	11.4	30.5	30.7	31.0
CTRL	Control	Nil	30.3	29.8	31.0

N=6 mice per cage

3TC= lamivudine

Food consumption declined across the treatment group for the group treated with lamivudine. This was observed across all the dose levels. The food consumption on the populations treated with riboflavin was comparable across different dose levels. There was no difference on food consumption for the untreated group. The findings were reported after one and two months (table 4.3b) below.

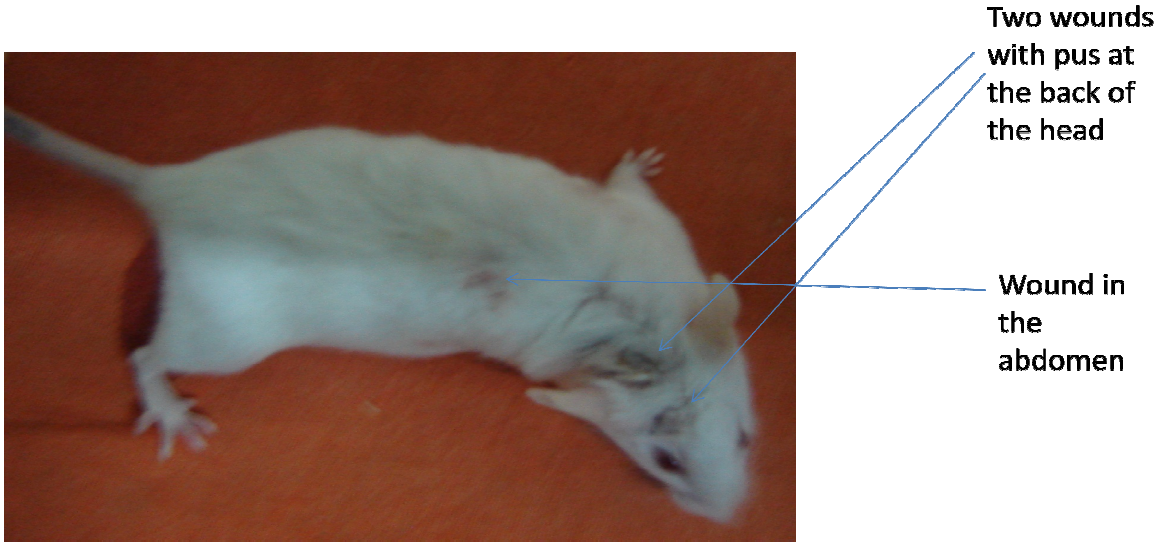
Table 4.3b: Daily food consumption of mice treated in lamivudine and riboflavin treatment for two months

Cage no	Drug name	Drug conc. (Mg/Kg)	Food consumption (g)	
			1month	2months
I	3TC	2.1	183	150
II	3TC	3.2	166	145
III	3TC	4.3	146	110
IV	3TC	6.4	110	97
V	3TC	8.6	110	78
I	Riboflavin	2.8	196	176
II	Riboflavin	4.3	160	157
III	Riboflavin	5.7	168	162
IV	Riboflavin	8.6	153	154
V	Riboflavin	11.4	132	128
CTRL	Control	Nil	172	178

N=6 mice per cage; 3TC=Lamivudine

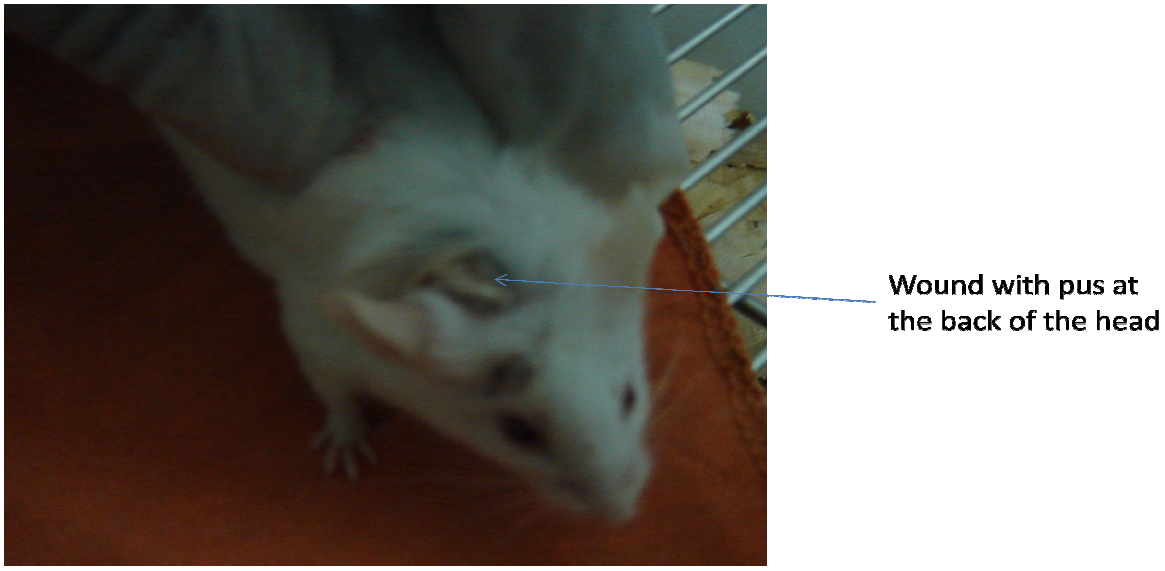
4.2.2 General Observation

After two weeks (14days) of treatment, the mice mainly treated with human equivalent dose of stavudine only and combination of stavudine and lamivudine developed complications. These included: skin rashes, red eyes, skin wounds, swelling at the joints, belly and back. Some of the mice developed wounds on the back, joints and limbs. These complications were more pronounced in the groups treated with double human equivalent dose for stavudine only and combination of stavudine and lamivudine. The mouse on photograph 4.1 below was treated with human equivalent dose of stavudine. It developed wounds on the back and abdomen. The wounds became septic but started healing when pus was drained. Internal complications in the liver were also documented.

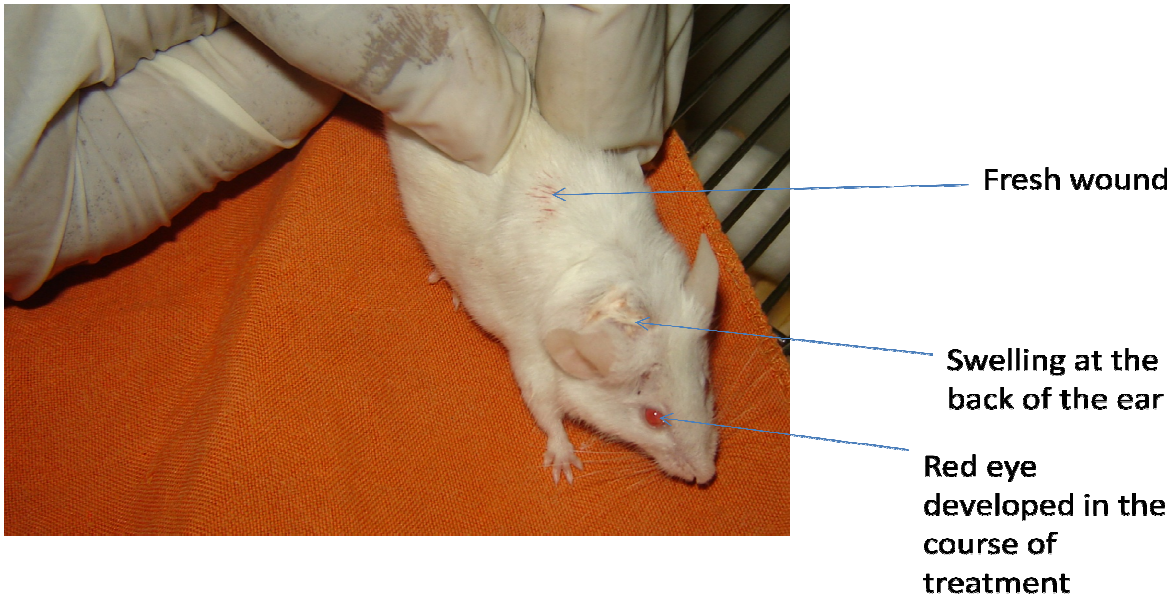


Photograph: 4.1: Photo showing wounds in a mouse treated with daily human equivalent dose of stavudine for two weeks

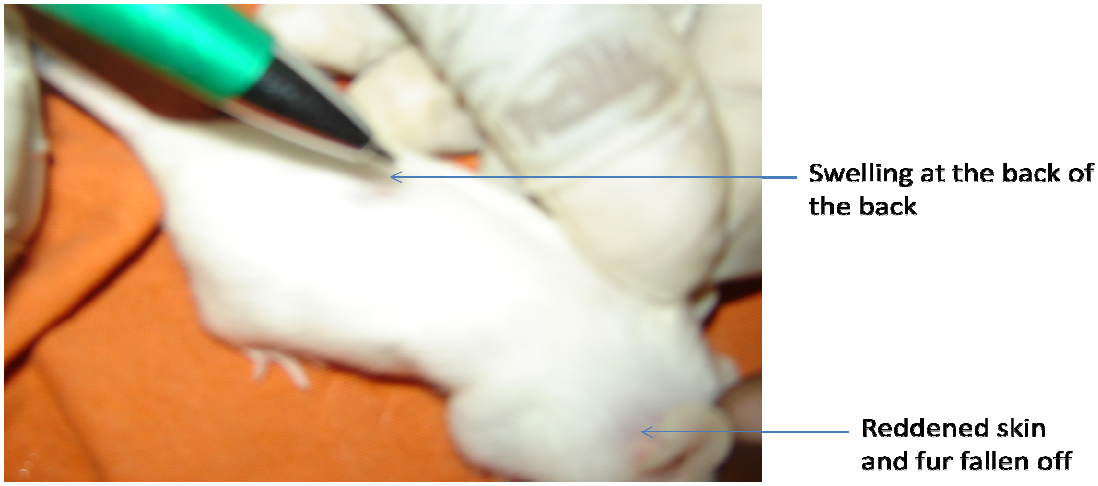
The mouse below photograph 4.2 developed wound with pus at the back of the ear on treatment with human equivalent dose of stavudine.



Photograph: 4.2: Photo showing wound at the back of the ear in a mouse treated with single dose of stavudine daily for two weeks



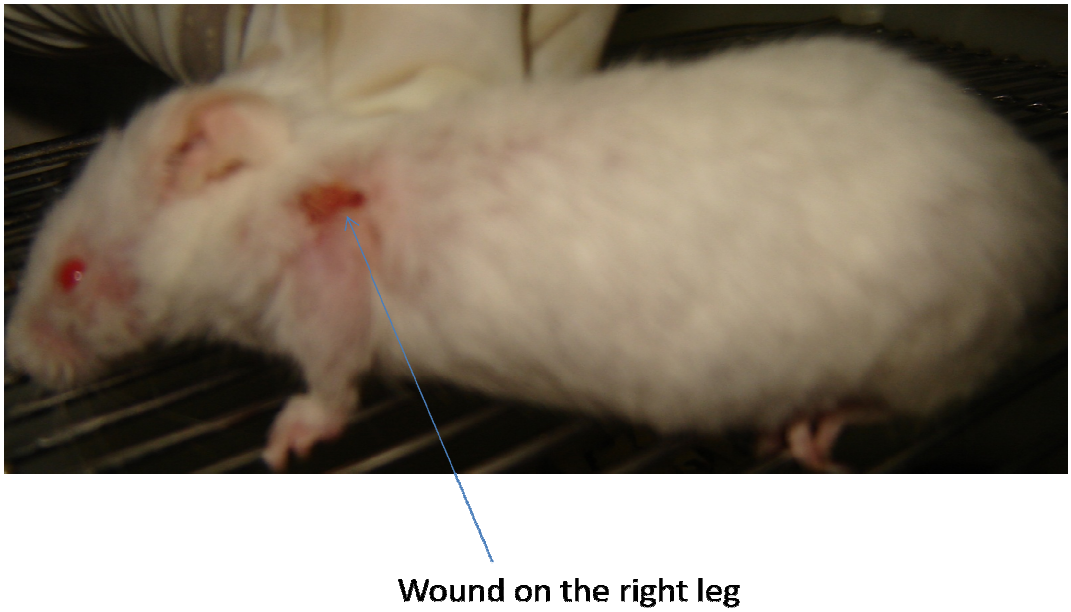
Photograph: 4.3: Photo showing wound and swelling at the back and reddened eyes in a mouse treated with daily human equivalent dose of stavudine and lamivudine for two weeks



Photograph 4.4: Photo showing swelling at the back and reddened skin after fur fell off in a mouse treated with daily human equivalent dose of stavudine for two weeks

Photograph 4.5: Photo showing a wound on the belly and reddened eyes in a mouse treated with daily human equivalent dose of stavudine for two weeks

The mouse (photograph 4.6) developed wound on the right leg.

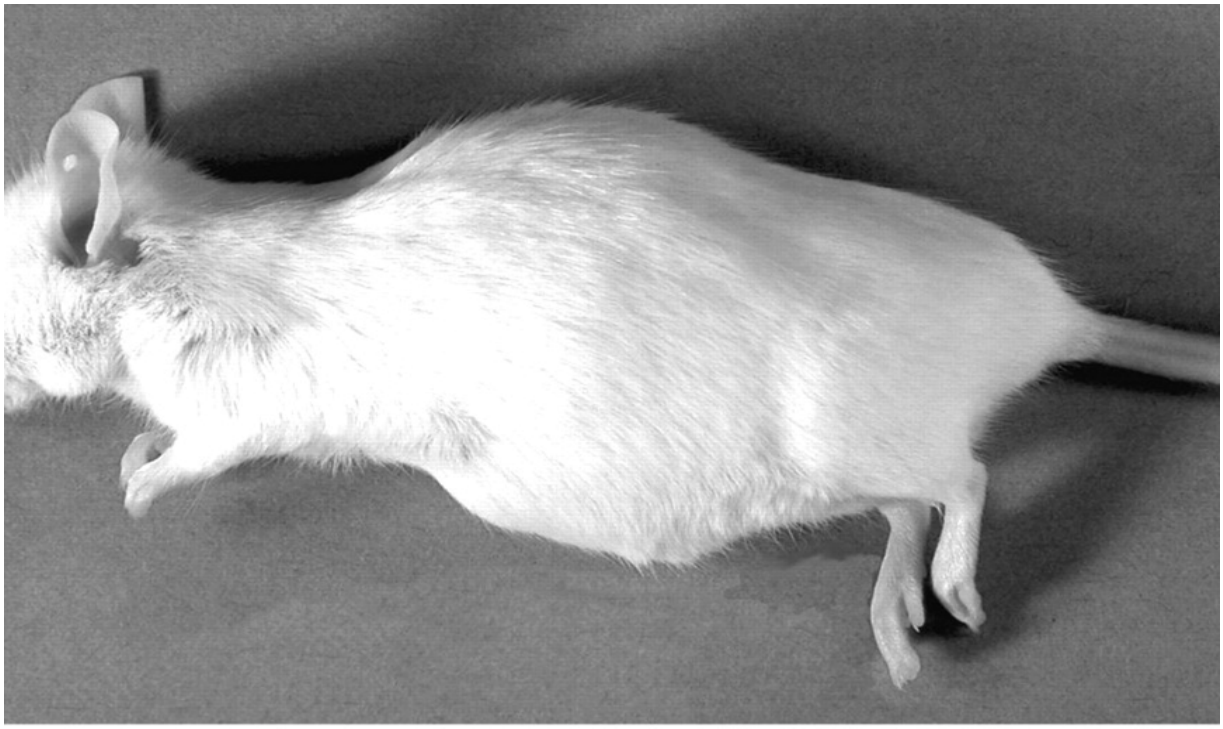


Photograph 4.6: Photo showing a wound on the right leg in a mouse treated with a daily human equivalent dose of Stavudine for two weeks

The affected were mice generally weak. The complications shifted to the internal organs as the mice in this group had swollen abdomen. There were incidences where the tails were nicked as shown in photograph 4.7 below.

Photograph 4.7: Tail nicked. This observation was made in the mice group treated with a daily human equivalent dose of stavudine for two weeks

Comparatively, all mice treated with human equivalent dose of stavudine, human equivalent dose combination of stavudine and lamivudine had swollen abdomen. The same observation was made on the mice treated with double human equivalent dose of stavudine and double human equivalent dose combination of stavudine and lamivudine. This is possibly because of enlargement of the liver as was found on post mortem. Photograph 4.8 illustrates the swollen abdomen. Photograph 4.8 is a mouse treated with human equivalent dose combination of stavudine and lamivudine daily for two weeks.



Photograph 4.8: Mouse with distended abdomen after treatment with a daily human equivalent dose combination of Stavudine and lamivudine for two weeks

There were differences noted in the liver and adipose tissue during sacrifice. All liver tissue in the treatment groups with single and double human equivalent dose of stavudine; the groups treated with combination of human equivalent dose stavudine and lamivudine; and the group treated with combination of double human equivalent dose of stavudine and lamivudine had pale and enlarged liver. The weight of liver ranged between 1.3g to 2.5g for the group treated with human equivalent dose of stavudine. The mice on double human equivalent dose combination of stavudine and lamivudine had liver weight of between 1.42g to 1.67g. The adipose tissue was very little. The weight of liver in the group treated with double human equivalent dose of stavudine was

between 1.6g and 2.5g. There was complete depletion of adipose tissue. The maximum survival period the mice in this treatment group were four weeks.

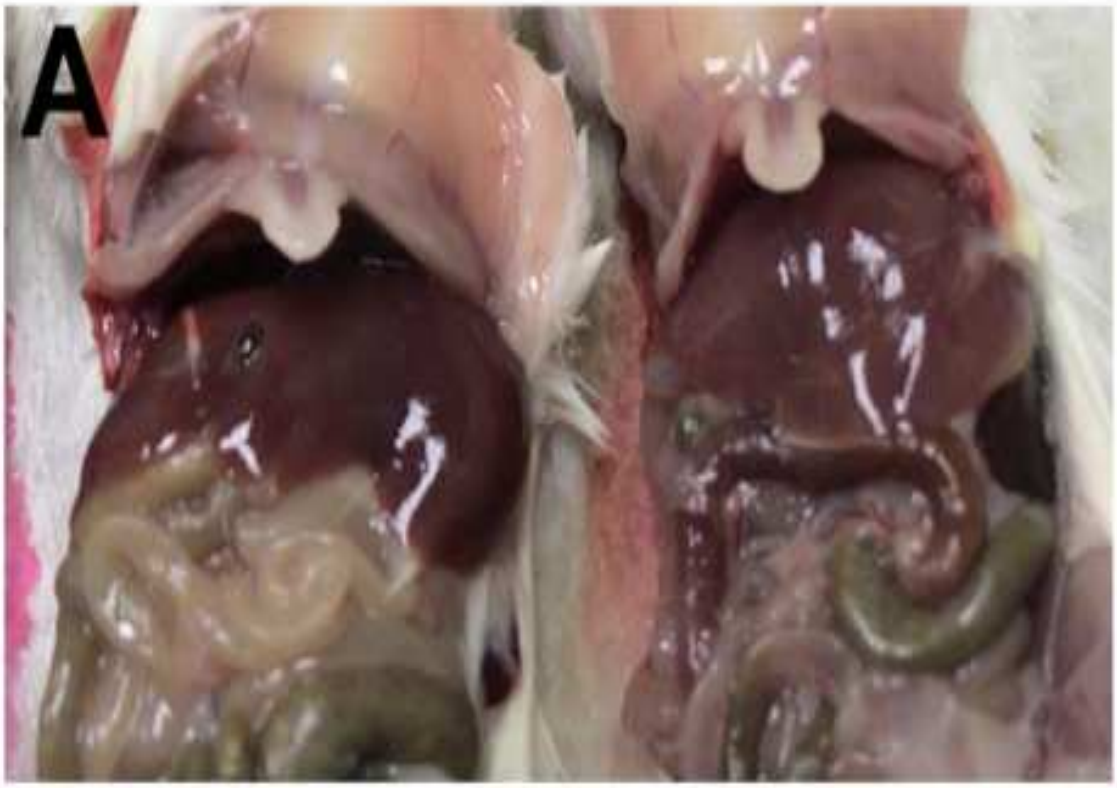
The mice population on half human equivalent dose of stavudine had pale liver. The weight of the liver was between 1.4g and 1.7g. This group did not manifest conditions of general toxicity described above. The adipose tissue was present. The group that was treated with combination of half human equivalent dose stavudine and lamivudine had liver weight between 1.1g to 1.4g. The group that was given half human equivalent dose combination of stavudine and riboflavin had plenty of adipose tissue and liver weight was between 1.0g and 1.2g. Similar observations were made in the group that was treated with combined half human equivalent dose of stavudine, lamivudine and riboflavin. The survival period for this group was four months. The results on liver weight are summarised in table 4.4.

Table 4.4: The mean weight of liver across all treatment groups

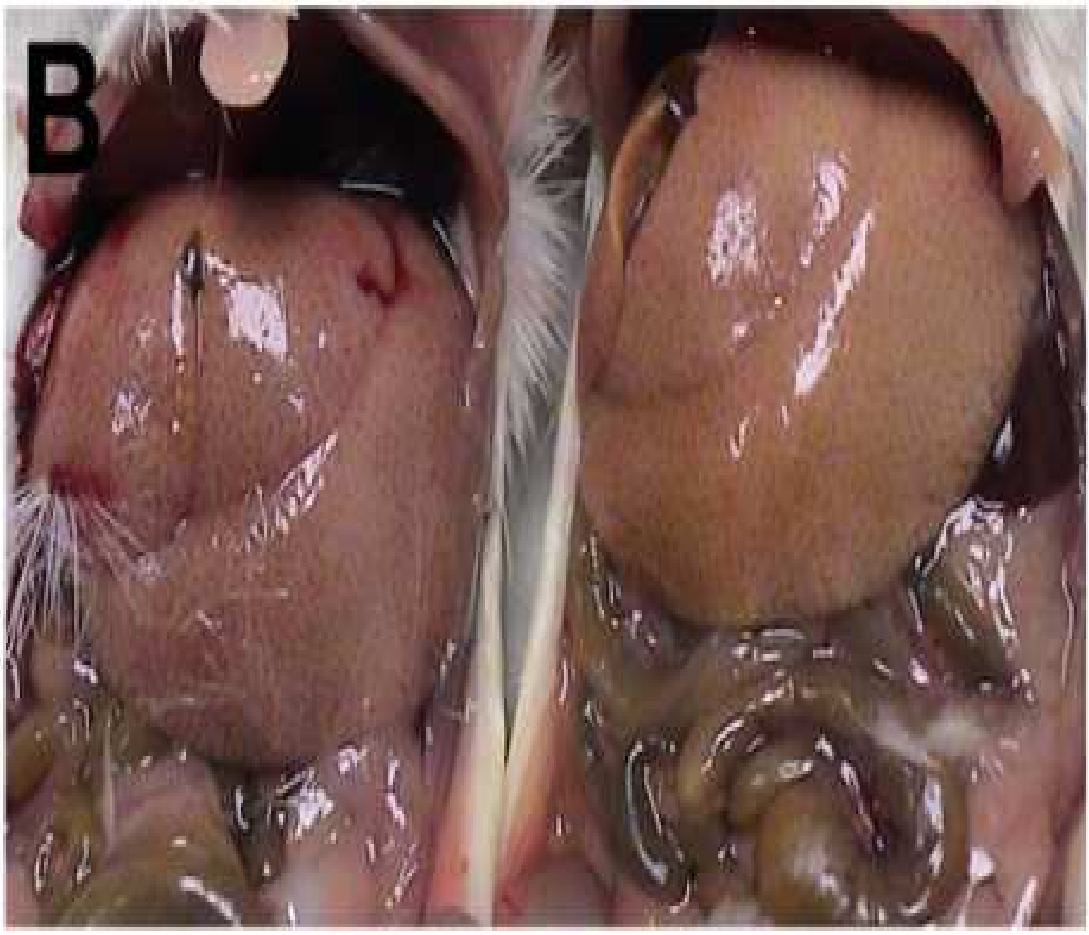
	Dose (mg/kg)	Mean	Std deviation	Range
Control				
No treatment	None	1.05	0.09	0.96-1.14
Riboflavin	5.7	1.35	0.06	1.23-1.47
Half human equivalent dose				
d4T	0.57	1.54	0.18	1.18-1.9
d4T+3TC	0.57 + 2.15	1.2	0.12	0.96-1.44
d4T+3TC+R	0.57 + 2.15 + 2.85	1.25	0.1	1.05-1.45
d4T+R	0.57 + 2.85	1.1	0.13	0.8-1.4
Human equivalent dose				
d4T	1.14	1.6	0.11	1.38-1.88
d4T+3TC	1.14 + 4.3	1.6	0.14	1.32-1.88
d4T+3TC+R	1.14 + 4.3 + 5.7	1.3	0.1	1.1-1.5
d4T+R	1.14 + 5.7	1.4	0.3	1.1-1.7
Double human equivalent dose				
d4T	2.28	2.0	1.3	0-4.6
d4T+3TC	2.28 + 8.6	1.7	0.1	1.5-1.9
d4T+3TC+R	2.28 + 8.6 + 11.4	1.5	0.08	1.3-1.3-1.7
d4T+R	2.28 + 11.4	1.5	0.02	1.46-1.54

The liver weight was recorded during sacrifice. It was found that there was increased liver weight for the mice treated with human equivalent dose of stavudine and combination of stavudine and lamivudine was raised (table 4.4).

Photographs of pale and enlarged liver were taken. The figures below represent the observations made during dissection.



Photograph 4:9A: Liver from control mice, not in any treatment. They appear normal in size and colour.



Photograph 4.9B: Enlarged pale liver from mice treated with daily a combination of human equivalent dose of stavudine and lamivudine for two weeks

Photograph 4.9A shows liver of control mice. These mice were not on any drug treatment. Photograph 4.9B shows liver abnormalities of the mice treated with combination of human equivalent dose of stavudine and lamivudine for a period of two weeks. The liver from these were diffusely enlarged with rounded edges filling up to one-third of the abdominal cavity and mildly displacing other abdominal organs. Additionally, the liver had become distinctively pale with an accentuated reticulated pattern.

4.2.3 Liver Cell Morphology

The slices of liver were prepared to assess for fat deposits. There were no fat deposits demonstrated on the liver. The cell morphology was assessed. The results are summarised in the table below.

Table 4.5: Results for liver cell morphology

Treatment	Dose (mg/kg)	Result
Control		
Negative Control (Untreated)	0.0	Normal
Positive control (riboflavin)	5.7	Normal
Half human equivalent dose		
Stavudine	0.57	Chronic hepatitis
Stavudine+lamivudine	0.57+2.15	Mild chronic hepatitis
Stavudine+lamivudine+Riboflavin	0.57+2.15+2.85	Normal
Stavudine+ riboflavin	0.57+2.15+2.85	Normal
Human equivalent dose		
Stavudine	1.14	Steatosis
Stavudine+lamivudine	1.14+4.3	Steatosis
Stavudine+lamivudine+riboflavin	1.14+4.3+5.7	Mild chronic hepatitis
Stavudine+ riboflavin	1.14+5.7	Normal
Double human equivalent dose		
Stavudine	2.28	Granuloma
Stavudine+lamivudine	2.28+8.6	Granuloma
Stavudine+lamivudine+riboflavin	2.28+8.6+11.4	Hepatitis
Stavudine+riboflavin	2.28+11.4	Mild chronic hepatitis

The positive (riboflavin) and the negative (untreated) groups were found to have normal liver morphology. The group treated with half human equivalent dose stavudine was found to have chronic hepatitis and that treated with half human equivalent dose combination of stavudine and lamivudine had mild chronic hepatitis. The liver morphology was found to be normal for the groups treated with riboflavin. The groups treated with half human equivalent dose combinations of stavudine, lamivudine and riboflavin had normal liver. The group treated with human equivalent dose stavudine only and the one treated with half human equivalent dose combination of stavudine and lamivudine, their liver morphology showed steatosis. The group treated with human equivalent dose combination of stavudine, lamivudine and riboflavin liver morphology showed mild chronic hepatitis while it was normal for the group treated with human equivalent dose combination of stavudine and riboflavin.

The group treated with double human equivalent dose stavudine only and that treated with double human equivalent dose combination of stavudine and lamivudine had liver morphology showing granuloma. Hepatitis was reported in the group treated with double human equivalent dose combination of stavudine, lamivudine and riboflavin and mild chronic hepatitis to the one treated with double human equivalent dose combination of stavudine and riboflavin (table 4.5).

4.2.4 Effects on biochemical parameters

The random blood glucose levels were determined at three intervals: before the oral drug administration and two more times. The two other times were: two weeks after the commencement of experiment and two days before sacrifice for all groups treated with human equivalent dose of stavudine or combination of human equivalent dose of stavudine and lamivudine. The same period was observed for the assessment of blood glucose for the mice treated with double human equivalent dose of stavudine and double human equivalent dose combination of stavudine and lamivudine. However, the groups that were

treated with stavudine half human equivalent dose; half human equivalent dose combination of stavudine and lamivudine; half human equivalent dose combination of stavudine, lamivudine and riboflavin; and half human equivalent dose combination of stavudine and riboflavin, the blood glucose was assessed at intervals of one and four months.

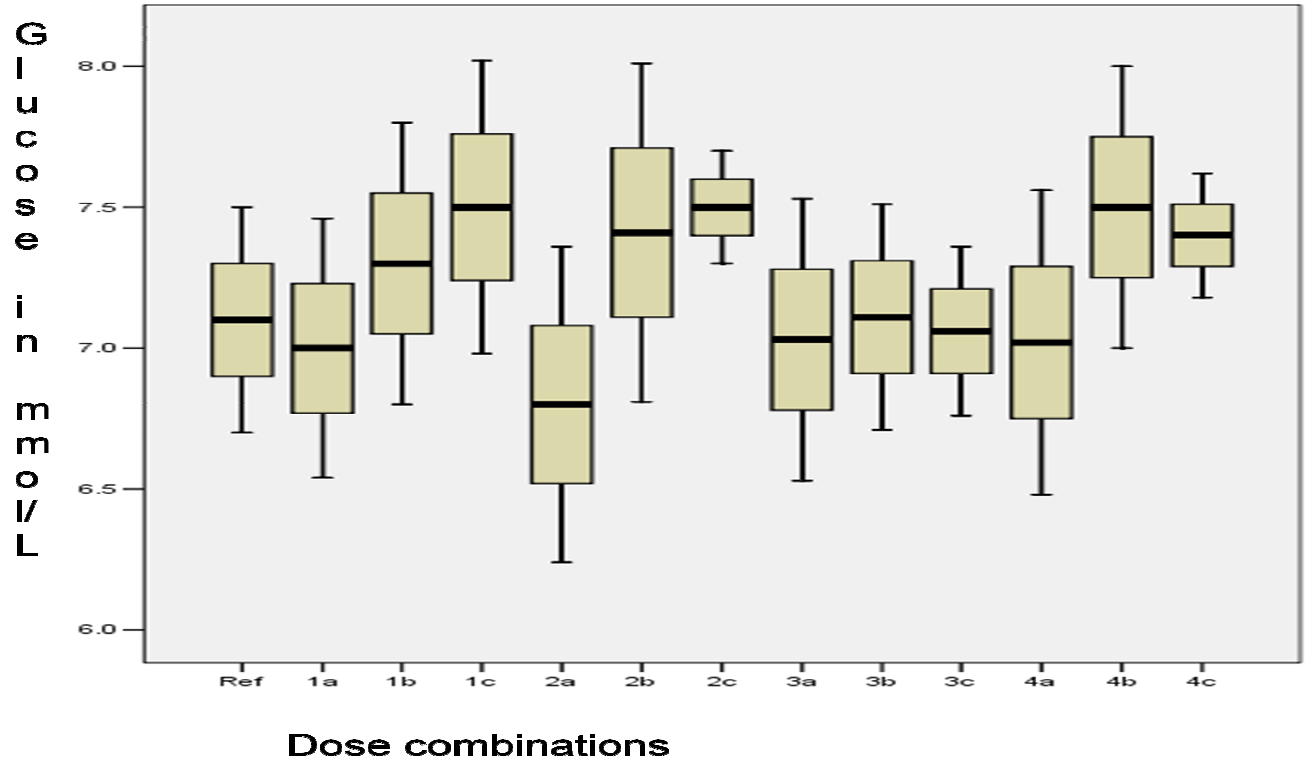


Figure 4.4A: The mean and 2SD of blood glucose concentration for Micetreated with half human equivalent dose of stavudine only, and half human equivalent dose Combinations consisting of stavudine, lamivudine and riboflavin.

Figure 4.4A Legend

The letters represent random blood glucose as follows: a) at baseline; b) after 1 month of treatment; c) after 4 month of treatment. The figures represent half human equivalent dose of: 1) stavudine; 2) stavudine and lamivudine; 3) stavudine, lamivudine and riboflavin; 4) stavudine and riboflavin.

The mean values per group were calculated. Statistical correlation was done at 0.05 significance level. One way ANOVA was used to analyse blood glucose levels across treatment groups. The correlation between groups treated with half human equivalent dose of stavudine and riboflavin was statistically significant at 0.001. The statistical relationship for the groups treated with half human equivalent dose combination of stavudine and lamivudine to group treated with riboflavin was 0.000 while that of group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin to group treated with riboflavin was 0.548. The statistical relationship for the group treated with half human equivalent dose combination of stavudine and riboflavin to group treated with riboflavin was 0.11. The results on the figure 4.4A represent the results for random blood glucose collected at the onset of treatment, one and four months respectively. The results are shown in figures 4.4A above and summarised on table 4.6.

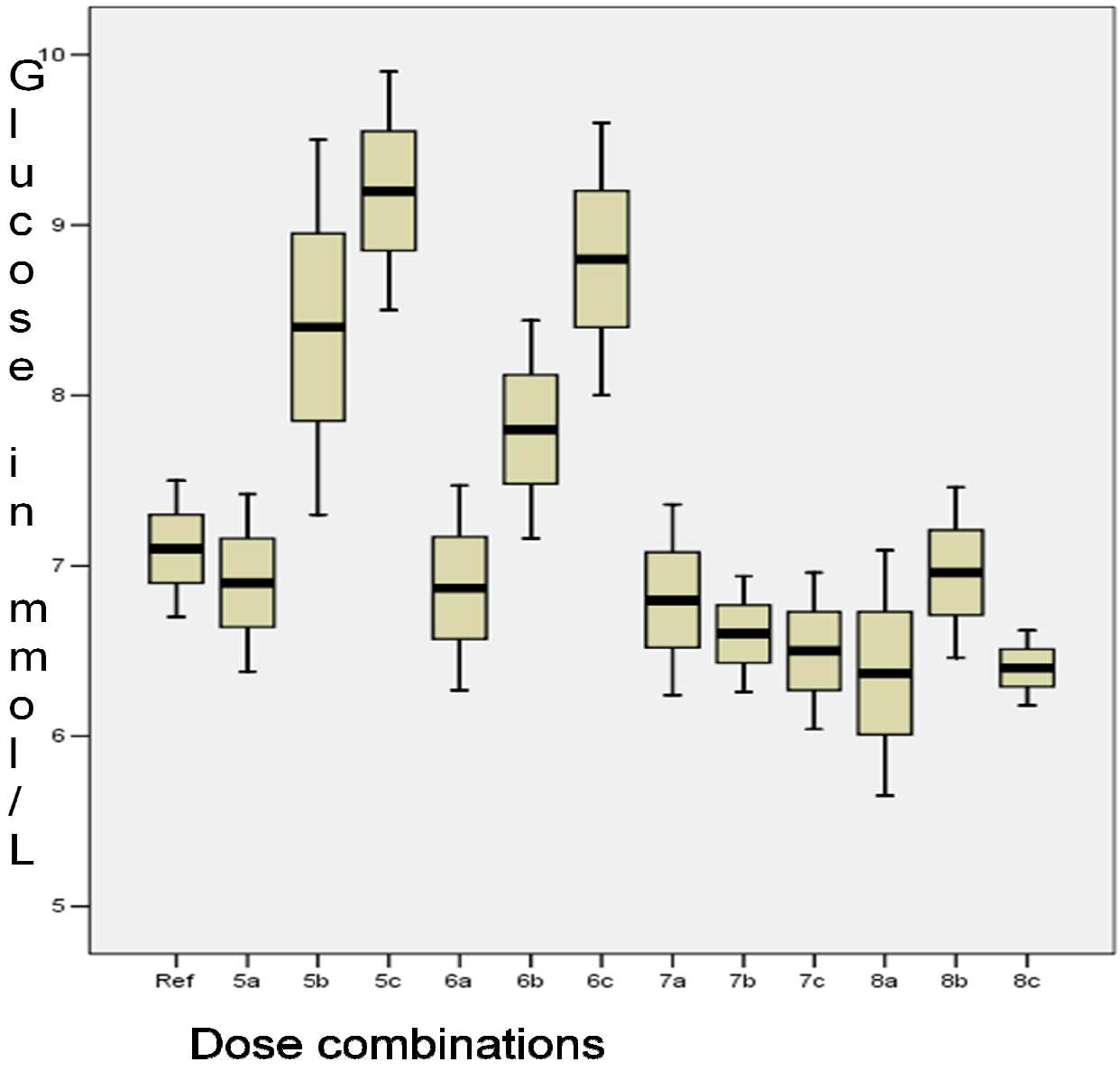


Figure 4.4B: The mean and 2SD of blood glucose concentration for Mice treated with human equivalent dose of stavudine only, and human equivalent dose Combinations consisting of stavudine, lamivudine and riboflavin

Figure 4.4B Legend

The letters represent random blood glucose as follows: a) at baseline; b) after 2 weeks of treatment; c) after 4 weeks of treatment. The figures

represent human equivalent dose of: 5) stavudine; 6) stavudine and lamivudine; 7) stavudine, lamivudine and riboflavin; 8) stavudine and riboflavin.

The blood glucose concentration results for groups that were treated with human equivalent dose of stavudine, human equivalent dose combination of stavudine and lamivudine; and human equivalent dose combination of stavudine, lamivudine and riboflavin were correlated with the group treated with riboflavin. The group treated with human equivalent dose stavudine had statistical significance of 0.011 and the one for the group treated with human equivalent dose combination of stavudine and lamivudine was 0.004. The statistical significance for the group treated with human equivalent dose combinations of stavudine, lamivudine and riboflavin; and the group treated with human equivalent dose combination of stavudine and riboflavin were 0.60 and 0.239 respectively. These results are summarised on table 4.6. The plots are shown for the results of random blood glucose collected at the onset of the treatment, after fourteen and twenty eight days respectively. The mean and twice standard deviation was calculated and presented in the figure 4.4B above.

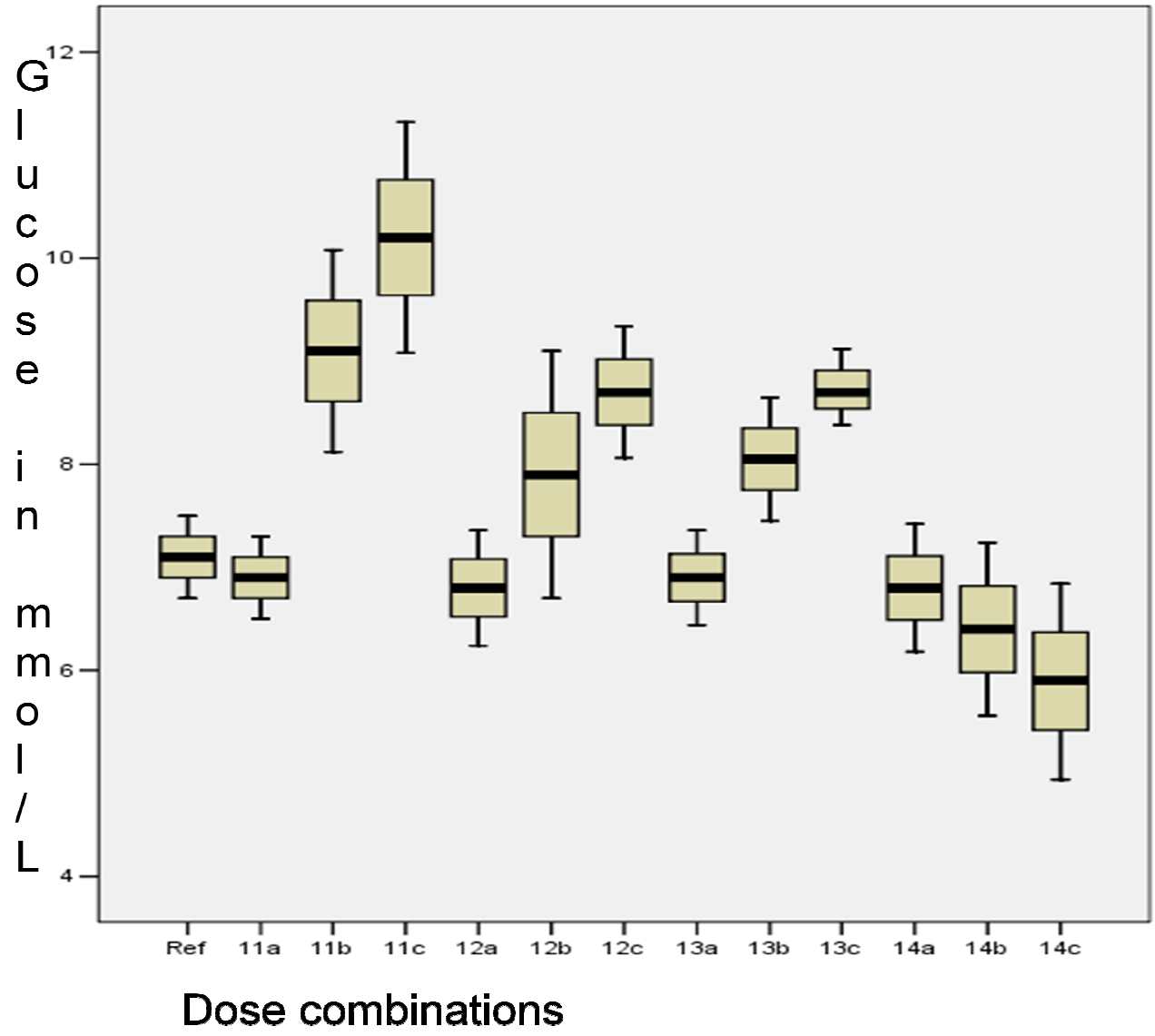


Figure 4.4C: The mean and 2SD of blood glucose concentration for mice treated with double human equivalent dose of stavudine only, and double human equivalent dose combinations consisting of stavudine, lamivudine and riboflavin

Figure 4.4C Legend

The letters represent random blood glucose as follows: a) at baseline; b) after 2 weeks of treatment; c) after 4 weeks of treatment.

The figures represent double human equivalent dose of: 11) stavudine;

12) stavudine and lamivudine: 13) stavudine, lamivudine and riboflavin; and 14) stavudine and riboflavin.

The blood glucose concentration results for groups that were treated with double human equivalent dose of stavudine, double human equivalent dose combination of stavudine and lamivudine and double human equivalent dose combination of stavudine, lamivudine and riboflavin were correlated with the group treated with riboflavin. The group treated with double human equivalent dose of stavudine only showed statistical significance of 0.001 and the one for the group treated with double human equivalent dose combination of stavudine and lamivudine was 0.004. The statistical significance for the group treated with double human equivalent dose combinations of stavudine, lamivudine and riboflavin, and the group treated with double human equivalent dose combination of stavudine and riboflavin were 0.007 and 0.003 respectively. The plots on figure 4.4C above represent the results of random blood glucose collected at the onset of the treatment, after fourteen and twenty eight days respectively. The mean and twice standard deviation was calculated and presented in the figure 4.4C above. The results for the statistical correlations are shown on table 4.6.

There was statistical significance between glucose levels for groups treated with riboflavin and those that were not treated with any drugs. The statistical significance for the group treated with riboflavin at the initiation of treatment was 0.47, after one month treatment it was found to be 0.501 and remained the same after four months of oral feeding with riboflavin 5.7mg/kg.

The reference ranges for blood glucose concentration for swiss mice was established as 6.7 – 7.5mmol/L as established in earlier experiments. It was observed that at the onset of the experiments, blood glucose levels were within the reference ranges. It was observed that there was a general trend in all treatment groups that blood glucose concentration was increasing with continued treatment. However, this rise in blood glucose concentration was not

observed in groups treated with combinations containing stavudine and riboflavin. The blood glucose levels were reducing upon treatment with riboflavin.

The table 4.6 below shows the summary of p values of blood glucose of staggered dose concentrations and combinations correlated with riboflavin.

Table 4.6: The summary of p values of blood glucose at staggered dose concentration and combinations

Drug/drug combination	Dose (mg/kg)	P value
Half human equivalent dose		
d4T	0.57	0.001
d4T+3TC	0.57+2.15	0.000
d4T+3TC+R	0.57+2.15+2.85	0.125
d4T+R	0.57+2.85	0.11
Human equivalent dose		
d4T	1.14	0.011
d4T+3TC	1.14+4.3	0.004
d4T+3TC+R	1.14+4.3+5.7	0.000
d4T+R	1.14+5.7	0.039
Double human equivalent dose		
d4T	2.28	0.001
d4T+3TC	2.28+8.6	0.004
d4T+3TC+R	2.28+8.6+11.4	0.007
d4T+R	2.28+11.4	0.003

All the correlations for the treatment groups were statistically siginificant except the groups treated with half human equivalent dose combinations of

stavudine and riboflavin, and that of the group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin. This means the addition of riboflavin, the blood glucose levels changed. There was general trend for rise in blood glucose among the groups treated with half human equivalent dose of stavudine and half human equivalent dose of combination of stavudine and riboflavin. There was decrease of blood glucose in the group treated with riboflavin. When compared with the group treated with riboflavin, there was no change in blood glucose level during the treatment in the case of groups treated with half human equivalent dose combination combination of stavudine and riboflavin and half human equivalent dose combination of stavudine, lamivudine and riboflavin.

4.4.1 Lipid Profile

The lipid profile determined consisted of total cholesterol, triglycerides and HDL-cholesterol. The results are summarised in the table 4.7 below.

Table 4.7: Lipid Profile

Table 4.7a: Lipid profile for mice treated with half human equivalent dose

Experimental group	Parameter	Test results (mean±SD)	Ref. range (mmol/L)	Remark
d4T	TC	2.6±0.33	2.5-4.5	Within ref range
	TG	1.3±0.08	1.2-3.6	Within ref range
	HDL-C	1.9±0.27	0-2.3	Within ref range
d4T+3TC	TC	2.6±0.5	2.5-4.5	Within ref range
	TG	0.9±0.5	1.2-3.6	Within ref range
	HDL-C	2.4±0.45	0-2.3	Borderline high
d4T+3TC+R	TC	1.9±0.9	2.5-4.5	Low TC levels
	TG	1.0±0.8	1.2-3.6	Low TG levels
	HDL-C	1.1±0.9	0-2.3	Within ref range
d4T+R	TC	2.2±0.8	2.5-4.5	Low TC levels
	TG	0.97±0.05	1.2-3.6	Low TG levels
	HDL-C	1.8±0.6	0-2.3	Within ref. range

The lipid profile for the groups treated with half human equivalent dose combinations of stavudine, lamivudine and riboflavin; and the group treated with half human equivalent dose combination of stavudine and riboflavin had

decreased total cholesterol and triglycerides. The groups treated with half human equivalent dose of stavudine only and one treated with half human equivalent dose combination of stavudine and lamivudine were found to have all lipid profile parameters within reference range. The difference between total cholesterol and triglycerides in the group treated with half human equivalent dose combination of stavudine and riboflavin were significant at 0.034 and 0.02 respectively. The statistical significance of the total cholesterol and triglycerides for the group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin were 0.38 and 0.228 respectively (table 4.7a).

Table 4.7b: Lipid profile for mice treated with human equivalent dose

Experimental group	Parameter	Test results (mean±SD)	Ref. range (mmol/L)	Remark
d4T	TC	2.5±0.56	2.5-4.5	Within ref range
	TG	2.4±1.0	1.2-3.6	Within ref range
	HDL-C	1.7±1.2	0-2.3	Within ref range
d4T+3TC	TC	3.7±1.7	2.5-4.5	Within ref range
	TG	1.5±0.4	1.2-3.6	Within ref range
	HDL-C	1.2±0.37	0-2.3	Within ref range
d4T+3TC+R	TC	3.0±0.9	2.5-4.5	Within ref range
	TG	2.13±1.0	1.2-3.6	Within ref range
	HDL-C	1.14±0.7	0-2.3	Within ref range
d4T+R	TC	2.6±0.9	2.5-4.5	Within ref range
	TG	0.7±0.1	1.2-3.6	Low TG levels
	HDL-C	2.5±1.7	0-2.3	Elevated
Riboflavin	TC	2.7± 0.8	2.5-4.5	Within ref range
	TG	1.0± 0.4	1.2-3.6	Within ref range
	HDL-C	1.3± 0.3	0-2.3	Within ref range

Lipid profile in all treatment groups under human equivalent dose (table 4.7b) was not found to have any significant abnormalities except within the groups treated with human equivalent dose stavudine only and the one treated with human equivalent dose combination of stavudine and riboflavin. The latter group was found to have decreased triglycerides with raised HDL-cholesterol. The statistical significance was 0.125 for triglycerides and 0.038 for HDL-cholesterol. The group treated with human equivalent dose of stavudine only

had total cholesterol borderline low. The statistical significance was 0.142 (table 4.7b).

Table 4.7c: Lipid profile for mice treated with double human equivalent dose

Experimental group	Parameter	Test results (mean±SD)	Ref. range (mmol/L)	Remark
d4T	TC	4.8±1.2	2.5-4.5	Elevated
	TG	2.3±0.9	1.2-3.6	Within ref range
	HDL-C	2.0±0.9	0-2.3	Within ref range
d4T+3TC	TC	2.3±0.8	2.5-4.5	Within ref range
	TG	1.4±0.5	1.2-3.6	Within ref range
	HDL-C	1.45±0.4	0-2.3	Within ref range
d4T+3TC+R	TC	2.8±0.37	2.5-4.5	Within ref range
	TG	1.9±0.45	1.2-3.6	Within ref range
	HDL-C	1.42±0.4	0-2.3	Within ref range
d4T+R	TC	3.6±1.2	2.5-4.5	Within ref range
	TG	2.0±0.2	1.2-3.6	Within ref range
	HDL-C	1.1±0.27	0-2.3	Within ref range

All the lipid profile parameters in the double human equivalent dose level were found to be within the reference ranges except for the group treated with stavudine only. This group had elevated total cholesterol. The statistical significance was found to be 0.02 (table 4.7c).

4.5 Haematological Profile

The full blood count parameters: neutrophils, lymphocytes, monocytes and eosinophils were assessed (table 4.1). Preliminary experiments showed that there were no derangements on these parameters across all treatment groups. However, it was observed that the mice on treatment with stavudine and combination of stavudine and lamivudine had enlarged liver, produced little blood (less than 0.5ml) when cardiac puncture was done and liver was pale. Therefore, the prothrombin time and activated partial thromboplastin time (APTT) were assessed to determine coagulation time.

Table 4.8: Index for interpretation of clotting time

Description	PT (sec)	APTT (sec)
Significantly shortened	<10.6	<34.1
Shortened	10.6 – 13.6	34.1 – 40.7
Normal clotting	13.6 – 16.4	40.7 – 47.5
Prolonged	16.4 – 19.4	47.5 – 54.1
Significantly prolonged	>19.4	>54.1

The reference ranges for the prothrombin time (PT) and activated partial thromblastin time (APTT) were established so that they can be used to interpret data. The reference ranges were 13.6-16.4 second PT and 40.7-47.5 second for APTT. The results were classified as shortened coagulation for prothrombin time if coagulation was reported between 10.6 and 13.6 seconds and significantly shortened for values below 10.6 seconds. The results were classified as prolonged if more than 16.4 seconds and significantly prolonged if coagulation was greater than 19.4 seconds (table 4.8 above).

The results for activated partial thromboplastin time were classified as significantly prolonged if coagulation was more than 54.1 seconds and

prolonged if it happened between 47.5 and 54.1 seconds. Shortened coagulation for the activated partial thromboplastin time was reported between 34.1 and 40.7 seconds. The coagulation time results below 34.1 seconds were classified as significantly shortened.

The general observation was significant prolonged coagulation time for both PT and APTT in all groups treated with stavudine only. The groups treated with containing riboflavin or combination containing riboflavin with a combination of stavudine or stavudine and lamivudine were found to have coagulation time for PT and APTT within reference range.

The statistical correlations of the PT and APTT of all the treatment groups were compared with riboflavin. Riboflavin was used as a positive control. The group treated with riboflavin showed prolonged coagulation time for PT and APTT. The statistical correlation between positive control and negative control for PT and APTT was 0.799 and 0.483 respectively.

Table 4.9 A: Coagulation time for the half human equivalent dose

Treatment group	PT (sec) (mean±SD)	APTT (sec) (mean±SD)	Remarks
	13.6-16.4	40.7-47.5	
d4T (0.57mg/kg)	21.3±2.2	54.2±7.2	PT and APTT significantly prolonged
d4T+3TC (0.57+2.15)mg/kg	14.3±2.7	32.3±3.7	APTT significantly shortened
d4T+3TC+R (0.57+2.15+2.85)mg/kg	16.3±1.5	51.3±7.6	APTT prolonged
d4T+R (0.57+2.85)mg/kg	15.4±1.9	60.7±3.4	APTT significantly prolonged
Riboflavin	17.3±1.7	49.4±1.4	PT and APTT prolonged

The group treated with stavudine half human equivalent dose was found to have significantly prolonged coagulation time for the tests PT and APTT. The PT and APTT in this group had a statistical correlation of 0.164 and 0.228 respectively. The group on half human equivalent dose combination of stavudine and lamivudine had similar results as the group on half human equivalent dose of stavudine only. The statistical correlation for PT was 0.139 and that of APTT was 0.093. The group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin reported prolonged PT and APTT. The statistical correlation was for PT and APTT was 0.221 and 0.74, respectively. The APTT was significantly prolonged for the group treated with half human equivalent dose combination of stavudine and riboflavin. The statistical correlation for PT was 0.016 and that of APTT was 0.424 (table 4.9A).

Table 4.9 B: Coagulation time for the Human dose

Treatment group	PT (sec) (mean±SD)	APTT (sec) (mean±SD)	Remarks
Reference range	13.6-16.4	40.7-47.5	
d4T (1.14mg/kg)	25.7±2.2	56.9±2.61	PT and APTT significantly prolonged
d4T+3TC (1.14+4.3)mg/kg	18.3±1.15	54.4±7.6	PT significantly prolonged and APTT prolonged
d4T+3TC+R (1.14+4.3+5.7)mg/kg	17.0±0.87	49.3±1.4	PT and APTT prolonged
d4T+R (1.14+5.7)mg/kg	15.0±1.4	39.8±3.4	PT and APTT shortened

The group treated with stavudine human equivalent dose was found to have significantly prolonged coagulation time for PT and APTT. The statistical correlation for PT and APTT in this group was 0.03 and 0.016 respectively. The

group treated with human equivalent dose combination containing stavudine and lamivudine dose was reported to have prolonged PT and significantly prolonged APTT. The statistical correlation in this group for PT and APTT was 0.0248 and 0.008 respectively. The PT and APTT were prolonged for the treatment group treated with human equivalent dose combination of stavudine, lamivudine and riboflavin. The PT and APTT in this group had statistical correlation of 0.603 and 0.375 respectively. Shortened coagulation time for PT and APTT was reported in group treated with the human equivalent dose combination of stavudine and riboflavin (table 4.9 B). The statistical correlation for PT and APTT in this group was 0.116 and 0.123 respectively.

Table 4.9 C: Coagulation time results for the double human equivalent dose

Treatment group	PT (sec) (mean±SD)	APTT (sec) (mean±SD)	Remarks
Reference range	13.6-16.4	40.7-47.5	
d4T (2.28mg/kg)	38.3±0.6	63.2±3.4	PT and APTT significantly prolonged
d4T+3TC (2.28+8.6)mg/kg	31.4±3.7	60.1±2.3	PT and APTT significantly prolonged
d4T+3TC+R (2.28+8.6+11.4)mg/kg	17.2±0.6	49.4±2.7	PT and APTT prolonged
d4T+R (2.28+11.4)mg/kg	16.5±0.42	40.1±3.2	Within ref range

The group treated with stavudine double human equivalent dose, was found to have significantly prolonged coagulation time both PT andAPTT. The statistical correlation for PT and APTT in this group was similar at 0.00. Similar results were found in the group treated with the double human equivalent dose

combination of stavudine and lamivudine. The PT and APTT statistical correlation was 0.128 and 0.615 respectively. The coagulation time changed from significantly prolonged to prolonged for both PT and APTT for group treated with double human equivalent dose combination of stavudine, lamivudine and riboflavin (table 4.9 C). In this group, the statistical values for PT and APTT were 0.158 and 0.221 respectively. The group treated with a double human equivalent dose combination containing stavudine and riboflavin the coagulation time for PT and APTT was within the reference range. The statistical correlation for PT and APTT in this group was similar at 0.000

4.7 Mitochondrial DNA Analysis

4.7.1 Real Time PCR (RT-PCR) to Determine Damage

On MtDNA after treatment with Nucleoside AnalogueDrugs

The results of rt-PCR were presented in cycle threshold (ct) values. The ct values are inversely proportional to the integrity and concentration of template DNA in the PCR reaction. Therefore, the higher the ct value, the low the DNA concentration and the converse is true. As applied to this analysis, whereas the ct value of the amplification is dependent on the concentration of the template DNA, efficient amplification is actually dependent on the integrity of the DNA in the region of the mtDNA being amplified and can thus be used as a direct measure of the integrity of the mtDNA.

Table 4.10: Results on the assessment of mtDNA damage using rt-PCR

Tissue	Treatment	Mean ct values short fragment (s)
Liver	Lamivudine	23.2
	Riboflavin	20.4
	Control	20.4
Heart	Lamivudine	18.1
	Riboflavin	18.4
	Control	16.9
Brain	Lamivudine	20.2
	Riboflavin	20.6
	Control	17.4
Skeletal Muscles	Lamivudine	20.7
	Riboflavin	20.7
	Control	18.6
Adipose Tissue	Lamivudine	29.8
	Riboflavin	24.8
	Control	20.2

N=6

The ct values for liver from mice treated with lamivudine were higher compared to control and riboflavin treated group. The ct values for the heart were comparable for mice treated with lamivudine, riboflavin and also control. The results for the skeletal muscles and brain did not show rise in ct values. The ct values for the adipose tissue were higher than for the control and riboflavin, respectively (table 4.10). The adipose tissue and the liver of mice treated with

lamivudine had the highest ct values depicting low amount of mtDNA present. Further experiments were done on the two tissues: liver and adipose tissue.

4.7.2 Influence of Stavudine, Lamivudine and Riboflavin Combination Treatments on Mice mtDNA Integrity

Analysis of damage of mitochondrial DNA (mtDNA) was was carried out using realtime PCR (rt-PCR). Amplification results were validated within cycle thresholds (ct) values between 12 and 30.

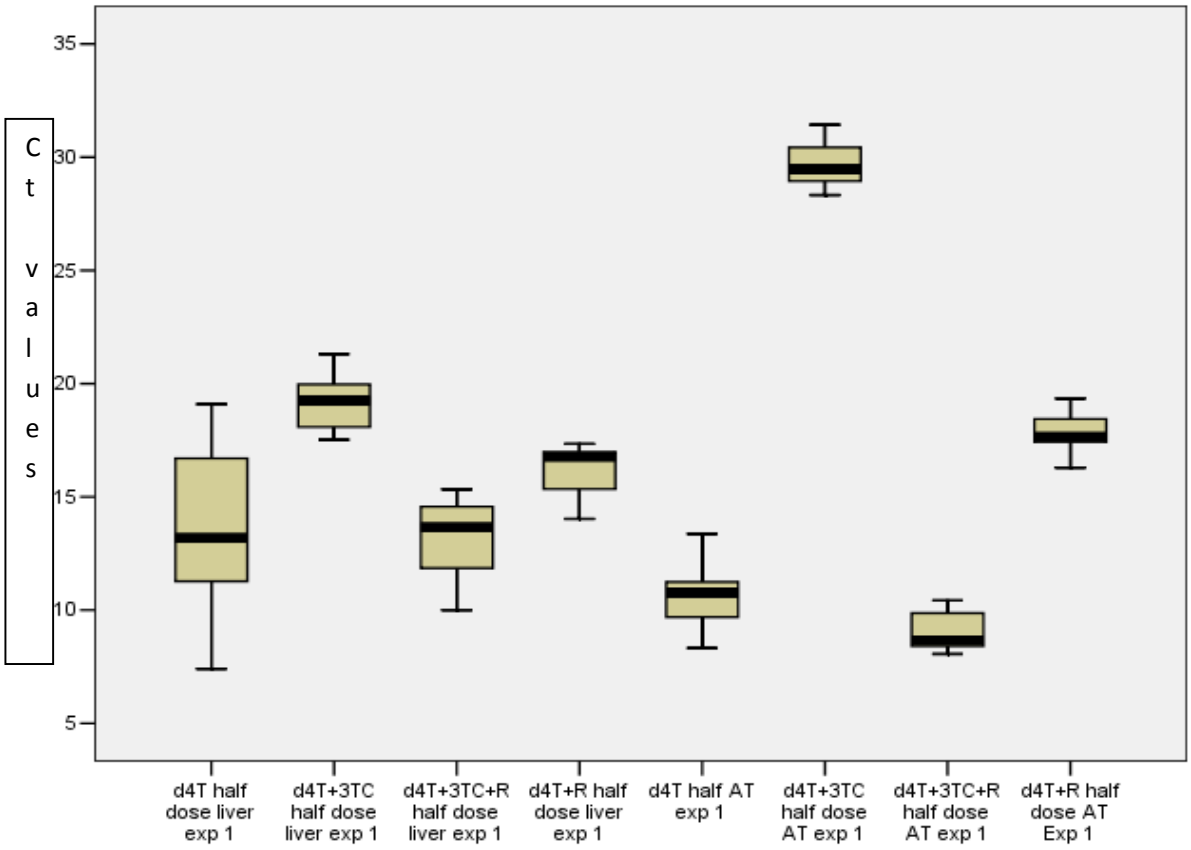


Figure 4:5. Liver and adipose tissue ct values across treatment groups' half human equivalent dose stavudine only and half human equivalent dose combination consisiting of lamivudine and riboflavin experiments 1

The ct values for groups treated half human equivalent dose treatment were compared. Experiment 1 consists of mice treated for one month and experiment 2 consists of the mice treated up to four months. Continued treatment with half human equivalent dose of stavudine caused increase in ct values. The same trend was observed in the group treated with half human equivalent dose combination of stavudine and lamivudine; and group treated with half human equivalent dose combination of stavudine and riboflavin. However, the ct values decreased when riboflavin when used in half human equivalent dose combination consisting stavudine and lamivudine (figure 4.5). Correlation studies between the group in experiment 1 and 2 gave statistical significance for group on half human equivalent dose stavudine only as 0.02 and that of half human equivalent dose combination of stavudine and lamivudine as 0.03. The statistical significance for the group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin was 0.45 and that of group treated with half human equivalent dose combination of stavudine and riboflavin was 0.012.

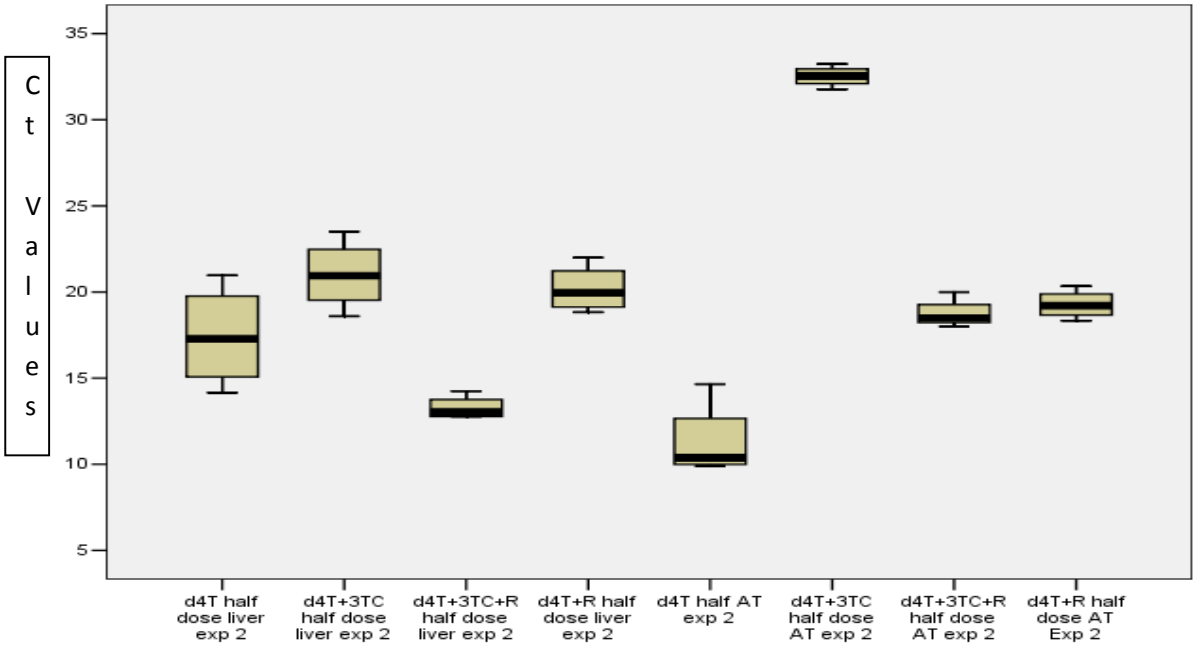


Figure 4.6: Liver and adipose tissue ct values across treatment groups' half human equivalent dose stavudine only and half human equivalent dose combination consisiting of lamivudine and riboflavin experiments 2

The ct values for group treated with half human equivalent dose stavudine only did not change for experiment 1 and 2. Similar observation was made on the groups treated with half human equivalent dose combination of stavudine and lamivudine; and that treated with half human equivalent dose combination of stavudine and riboflavin. However, the ct values increased on treatment with riboflavin when used in half human equivalent dose combination of stavudine and lamivudine (figure 4.6). The correlation was done within the groups (experiments 1 and 2). The statistical significance for half human equivalent dose stavudine was 0.485 and that of half human equivalent dose combination containing stavudine and lamivudine was 0.05. The statistical significance for the group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin was 0.00 and that of group treated with half human equivalent dose combination of stavudine and riboflavin was 0.032.

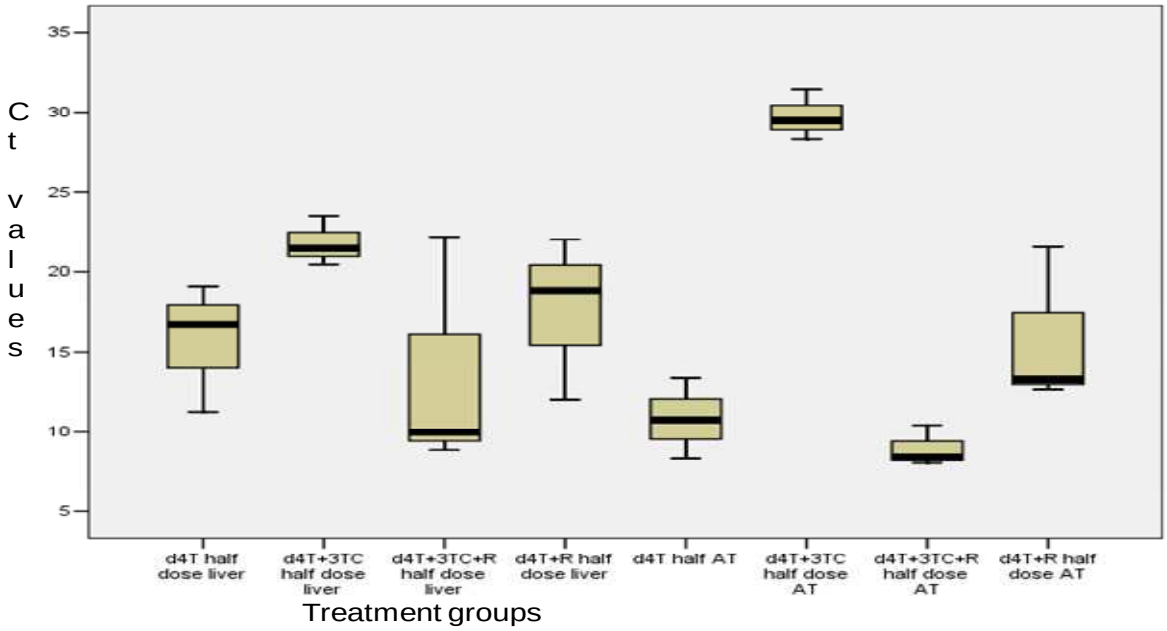


Figure 4.7: The mean ct values for groups treated with half human equivalent dose stavudine only and combination of stavudine, lamivudine and riboflavin

For the groups that were treated with half human equivalent dose stavudine; half human equivalent dose combination of stavudine and lamivudine; half human equivalent dose combination of stavudine, lamivudine and riboflavin and half human equivalent dose combination of stavudine and riboflavin, the mtDNA was assessed at intervals of one and four months. Statistical correlation was done at 0.05 significance level. Paired sample T test was used for analysis of ct values across treatment groups for the tissues: liver and adipose tissue. The correlation between groups treated with half human equivalent dose combination of stavudine and riboflavin for liver was statistically significant at 0.012 and 0.02 for adipose tissue. The statistical relationship for the groups treated with half human equivalent dose combination of stavudine and lamivudine, liver was 0.379 and 0.001 for the adipose tissue. The statistical correlation for the group treated with half human

equivalent dose combination of stavudine, lamivudine and riboflavin for the liver was 0.063 and 0.019 for adipose tissue while the group treated with half human equivalent dose combination of stavudine and riboflavin, liver had p value of 0.83 and adipose tissue was 0.002. The results on the figure 4.7 represent the results for ct values from rt-PCR for mtDNA on one and four months of treatment.

The mice on half human equivalent dose treatment consisting stavudine, lamivudine and riboflavin had higher life span for they lived through the experiment period of four months. It was found that liver mtDNA decreased as the treatment progressed in this dose level. The ct value for liver in the group treated with half human equivalent dose stavudine and the one treated with half human equivalent dose combination of stavudine and lamivudine were elevated. On addition of riboflavin, the ct values decreased (figure 4.7). Comparatively, the ct values for groups treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin and the one treated with half human equivalent dose combination stavudine and riboflavin showed lower ct values than the groups treated with stavudine only or stavudine and lamivudine combination. The higher the ct values, the lower the mtDNA concentration. It was therefore observed that the groups treated with any combination containing riboflavin, had low ct value. An indication that riboflavin preserves the mtDNA.

The ct values for the adipose tissue mtDNA for groups treated with half human equivalent dose of stavudine and half human equivalent dose combination of stavudine and lamivudine were high but decreased on treatment with riboflavin. Comparatively, the groups treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin and the group treated with half human equivalent dose combination of stavudine and riboflavin had lower ct values.

The mtDNA for the groups treated with human equivalent dose stavudine; human equivalent dose combination of stavudine and lamivudine and riboflavin was assessed. The findings are shown in the figure 4.8 below

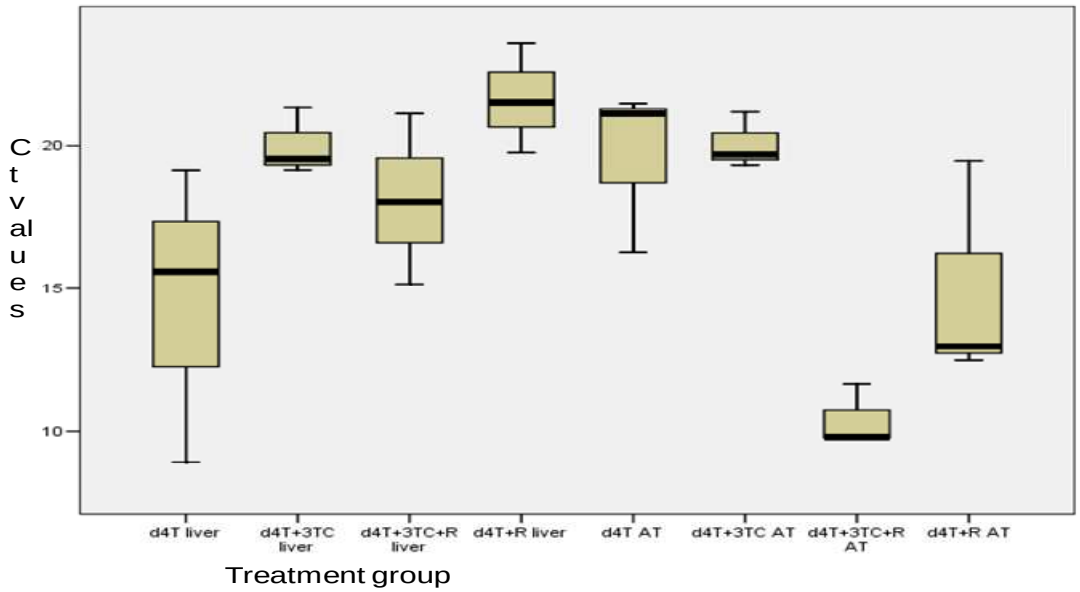


Figure 4.8: The mean ct values for groups treated with single Dose Stavudine, and human equivalent dose combination consisting of stavudine, lamivudine and riboflavin

The ct values for liver in the group treated with human equivalent dose stavudine was lower compared to that of the group treated with human equivalent dose combination of stavudine and lamivudine. The statistical correlation for the liver in the group treated with human equivalent dose stavudine was 0.105 and that of the human equivalent dose combination consisting of stavudine and lamivudine was statistically significant at 0.003. On addition of riboflavin the ct values decreased as seen in groups treated with human equivalent dose combination of stavudine, lamivudine and riboflavin, and human equivalent dose combination of stavudine and riboflavin. The statistical correlation for the ct values for liver in the groups treated with

human equivalent dose combination of stavudine, lamivudine and riboflavin was significant at 0.021, and that of human equivalent dose combination of stavudine and riboflavin was 0.002.

The adipose tissue for the group treated with human equivalent dose stavudine was immensely depleted. The statistical correlation was 0.407. However, the treatment groups with human equivalent dose combination of stavudine and lamivudine; human equivalent dose combination of stavudine, lamivudine and riboflavin; and human equivalent dose combination of stavudine and riboflavin had adipose tissue whose statistical correlation values were: 0.247, 0.056 and 0.174 respectively. It was found that all the treatment combinations containing riboflavin had low ct values compared to the groups without riboflavin.

The mtDNA for the groups treated with double human equivalent dose stavudine only; double human equivalent dose combinations of stavudine and lamivudine and riboflavin was assessed. The findings are shown in the figure 4.9 below.

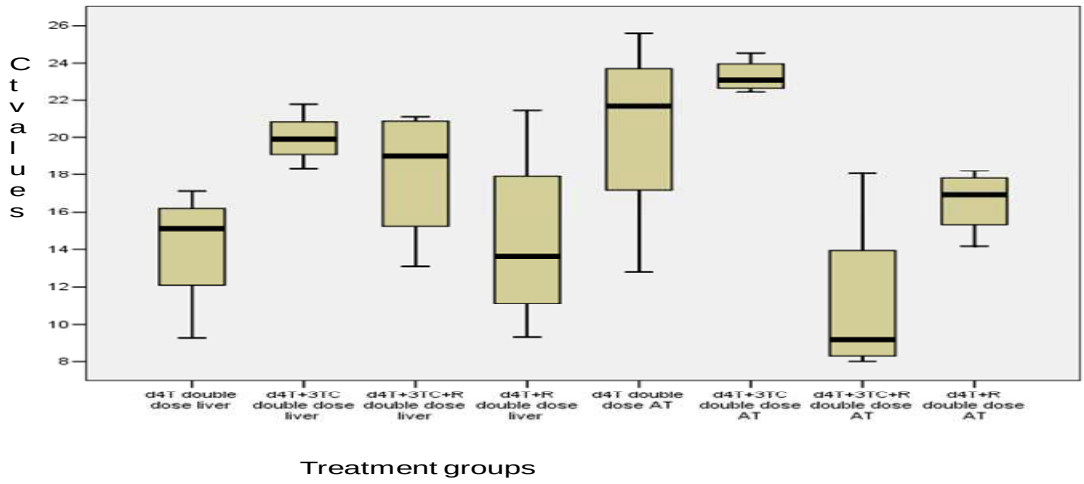


Figure 4.9: The mean ct values for groups treated with double dose stavudine and combination containing double human equivalent dose of stavudine, lamivudine and riboflavin

This group was treated with the double human equivalent dose of stavudine and double human equivalent dose combinations of: stavudine and lamivudine; stavudine, lamivudine and riboflavin; and stavudine and riboflavin. The statistical correlation for liver mtDNA in the group treated with double human equivalent dose of stavudine was 0.656 and group treated with double human equivalent dose combination of stavudine and lamivudine was 0.001. The liver ct values for the group treated double human equivalent dose combination of stavudine and lamivudine was higher than that of the group treated with double human equivalent dose of stavudine alone. However, on addition of riboflavin, the effect was that the ct values for liver decreased. The group treated with double human equivalent dose combination of stavudine and riboflavin recorded lower liver ct values compared with the group treated with double human equivalent dose combination of stavudine, lamivudine and riboflavin. The liver statistical correlation for group treated with double human equivalent dose combination of stavudine, lamivudine and riboflavin was 0.103 and 0.7 for the group treated with double human equivalent dose combination of stavudine and riboflavin. These findings are suggest that riboflavin has ameliorating effect on the liver mtDNA.

The adipose tissue ct values for double human equivalent dose combination of stavudine and lamivudine were comparable to those of group treated with double human equivalent dose of stavudine alone. The statistical correlation for the adipose tissue treated double human equivalent dose of stavudine was 0.221 and that of group treated with double human equivalent dose combination of stavudine and lamivudine was statistically significant at 0.007. The ct values decreased when the riboflavin was added. This is seen in the treatment group with double human equivalent dose combination of stavudine, lamivudine and riboflavin; and double human equivalent dose combination of stavudine and riboflavin. The statistical correlation for the group treated with double human equivalent dose combination of stavudine, lamivudine and

riboflavin was 0.058, and that of double human equivalent dose combination of stavudine and riboflavin was 0.068.

Generally, riboflavin was found to have effect on all treatment groups. The ct values were decreased suggestive of preserved mtDNA.

The comparison between negative (untreated) control and positive (riboflavin) control were correlated. The statistical significance for liver was 0.879 and 0.016 for adipose tissue.

Chapter Five

5.0 Discussion

5.1 Determination of reference range determination

Reference values are required by clinicians and researchers and are used as basis for interpretation of related laboratory data. These values are applicable to the same species and individual groups of animals.

In this study, the reference ranges for haematological and biochemical parameters are reported. These parameters in swiss albino mice are limited in literature although this animal is commonly used in laboratory studies. The few available are not established in controlled experiments.

The haematological profiles and biochemical parameters are variably affected in event of infection, chronic disease, and nutritional deficiencies, and in long term administration of drugs.

The reference limits were defined statistically by applying 95% confidence limits. During data analysis, partitioning was done, distribution of data studied and outliers were identified and removed. The reference range was determined from the parametric data as mean plus (+) or minus (-) two standard deviation. The blood parameters studied were neutrophils, lymphocytes, monocytes, eosinophils and basophils. The percent reference ranges were: neutrophils 8-23%, lymphocytes 67-86%, monocytes 1-9% and eosinophils were upto 5%.

The lipid profile was determined as: total cholesterol (2.5-4.5mmo/L), triglycerides (1.2-3.6mmol/l), high density lipoprotein cholesterol (HDL-C) (upto 2.3mmol/l) and low density lipoprotein cholesterol (LDL-C) (upto to 2.8mmol/l).

The blood glucose was measured three times during the study. The reference range for blood glucose was established as 6.7-7.5mmol/l. The recent data on blood glucose reference ranges was used by Ianello et al., 2005 in establishing

a catabolic response in tissues, such as the liver, muscle, and adipose tissue in severe diabetic swiss albino mouse. However, the blood glucose ranges established by Iannello et al, (2005), (15.4-27.5mmol/L) applied for diabetic swiss albino mice.

The reference range for live weight of swiss albino mice was 28.8-30.8 g while liver was found to have reference range of 0.96-1.14 g.

The above reference ranges were used in interpretation of the toxicity due to the use of nucleoside analogue drugs: stavudine and lamivudine used in treatment of mice in this study. These reference ranges are proposed for application in animal studies of same species.

5.2 Assessment of toxicity during use of nucleoside analogues in mice

It was found that the mice on daily treatment of human equivalent and double human equivalent doses of stavudine or combination of stavudine and lamivudine lived a maximum period of six weeks while those treated daily on half human equivalent of similar drugs lived for four months.

Toxicty was assessed using various parameters. As the study progressed the mice developed wounds, skin rough texture, reddened eyes and lethargy. The animals affected were found to have hepatomegally and depleted adipose tissue. There are no previous studies relating manifestations of ill health on conditions such as hepatomegally and adipose tissue depletion.

It was observed that mice treated with lamivudine only at all dose levels manifested toxic effects of the drugs unlike those treated with riboflavin. These effects include: rough skin texture, sunken eyes and lethargy. They also registered increased weight compared to riboflavin and higher than the reference range (29.8-30.8g). The weight of mice treated with riboflavin was borderline high.

Another notable toxic feature was depletion of adipose tissue. Adipose tissue was significantly depleted in animals treated with single and double human equivalent doses of stavudine. The population treated with human equivalent dose combination of stavudine and lamivudine had enlarged pale liver. However, there were no fat deposits found on the liver. These observations were also reported in the mice treated with double human equivalent dose of stavudine. White patches were found on the liver and spleen for all animals in this group. Fat depletion has been documented in the humans on ARV therapy where there is adipose tissue depletion and fat redistribution (Gaou et al., 2001; Metzler et al., 2001; Roe and Coates, 1995).

Animals treated with half human equivalent dose of stavudine did not have mortalities and adipose tissue was present. Animals treated with half human equivalent dose combination of stavudine and lamivudine did not show any sign of ill health described above. These findings were comparable to groups treated with double human equivalent dose of stavudine and lamivudine but with addition of riboflavin. The groups in which riboflavin was added were found to have body weight within the reference ranges, smooth skin texture, alertness and active. Thus addition of riboflavin in the treatment with nucleoside analogues alleviates the adverse effect of nucleoside analogues.

5.3 Effect of Drug Treatment on Biochemical Parameters

a) Blood glucose Concentration

There was increased blood glucose level in all treatment groups for all dose levels except in the groups treated with combination of stavudine and riboflavin.

The group treated with human equivalent dose combination of stavudine, lamivudine and riboflavin had elevated blood glucose after two weeks of treatment. The rise was reported in the groups treated with human equivalent

dose of stavudine and in the human equivalent dose combination of stavudine and lamivudine, human equivalent dose combination of stavudine, lamivudine and riboflavin. The rise continued with treatment even in the third week. However, the group treated with combination of stavudine and riboflavin was seen to have declining blood glucose levels.

The same trend was observed in the group treated with double the concentration of the drugs mentioned above. It seems inclusion of riboflavin to the high combination of stavudine and lamivudine, has an effect in reducing the effect of these drugs on blood glucose concentration. This protective effect of riboflavin on blood glucose was also reported when it was used with stavudine alone.The inclusion of riboflavin to treatment with stavudine checked the rise in blood glucose.

Accumulation of glucose in the blood may lead to anaerobic metabolism causing accumulation of lactic acid. The accumulation may be due to loss of liver function, pancreatitis, exocrine pancreas dysfunction (Brinkman et al., 1998) and alteration to oxidative phoshorylation function resulting to rise in mitochondrial redox state and shift to pyruvate/lactate equilibrium (munnich et al., 1992).

b) Lipid Profile

There was no significant derangement on lipid profile among all the groups on treatment with double human equivalent dose of stavudine; double human equivalent dose combination of stavudine and lamivudine and double human equivalent dose combination of stavudine, lamivudine and riboflavin. There was low total cholesterol for the groups treated with human equivalent dose of stavudine and human equivalent dose combination of stavudine and riboflavin.

The groups treated with half human equivalent dose of stavudine and half human equivalent dose combinations of: stavudine, lamivudine and riboflavin were found to have total cholesterol and triglycerides below reference range.

Signaling systems have effect on regulation of body weight and fat accumulation. Leptin, the product of the obese gene, suppresses food consumption and increases energy expenditure. Loss of leptin signaling, caused by mutation of the obese gene or of the leptin receptor leads to massive obesity, hyperglycemia and hyperinsulinemia as in type II diabetes, as well as a 10- to 20-fold over-expression of the leptin message, even though a defective message (Loftus, Kuhajda and Lane, 1998).

Also some metabolic aspects of adipocytes are insulin resistant resulting from defects in the insulin signaling pathway. Further, the adipocytes secrete cachectic factors like TNFα that promote adipocyte loss and oppose insulin action (Weiner, et.al, 1991). In addition to the diabetic state, numerous global hormones such as glucagon, which promotes an increased cyclic AMP (cAMP) level in adipocytes, and glucocorticoids are abnormally elevated (Dubuc et al, 1984). Both elevated cAMP and glucocorticoid promote cell death (Loftus, Kuhajda and Lane, 1998). In this study, the mice treated with single and double human equivalent doses of stavudine, and the group treated with human equivalent dose combination of stavudine and lamivudine developed conditions pointing to obesity: gain in weight, hyperglycaemia and hepatomegally.

c) Coagulation Time

Prothrombin time measures the quality of the extrinsic pathway (as well as the common pathway) of coagulation. The speed of the extrinsic pathway is greatly affected by levels of functional factor VII in the body. Factor VII has a short half-life and the carboxylation of its glutamate residues requires vitamin K. The prothrombin time can be prolonged as a result of deficiencies in vitamin K,

warfarin therapy or lack of intestinal colonization by bacteria (such as in newborns) (Fritsma, 2002).

The activated partial thromboplastin time (APTT) test reflects the activities of most of the coagulation factors, including factor XII and other "contact factors" (pre-kallikrein [PK] and high molecular weight kininogen [HMWK]) and factors XI, IX, and VIII in the intrinsic pro-coagulant pathway, as well as coagulation factors in the common pro-coagulant pathway that include factors X, V, II and fibrinogen (factor I). The APTT also depends on phospholipid (a partial thromboplastin) and ionic calcium, as well as an activator of the contact factors (eg, silica), but reflects neither the extrinsic pro-coagulant pathway that includes factor VII and tissue factor, nor the activity of factor XIII (fibrin stabilizing factor) (Miletich, 1995).

Prolongation of the APTT can occur as a result of deficiency of one or more coagulation factors (acquired or congenital in origin), or the presence of an inhibitor of coagulation such as heparin, a lupus anticoagulant, a nonspecific inhibitor such as a monoclonal immunoglobulin, or a specific coagulation factor inhibitor (Greaves and Preston, 2001). Shortened APTT has no clinical significance (Korte et al., 2000).

The group treated with half human equivalent dose of stavudine the PT and APTT were significantly prolonged PT and APTT. The group treated with half human equivalent dose combination of stavudine and lamivudine showed significantly shortened PT and APTT. Half human equivalent dose combination of riboflavin and stavudine resulted to significantly prolonged APTT. Inclusion of riboflavin to the half human equivalent dose combination of stavudine and lamivudine showed PT fall to within reference range.

The PT and APTT were significantly prolonged for the groups treated with human equivalent dose of stavudine and the combination containing human equivalent dose of stavudine, lamivudine. Inclusion of riboflavin to the human

equivalent dose combination of stavudine and lamivudine prolonged PT and APTT. The APTT and PT were within reference range for the group treated with human equivalent dose combination of stavudine and riboflavin.

The groups treated with double human equivalent dose of stavudine and double human equivalent dose combination of stavudine, lamivudine were found to have PT and APTT significantly prolonged. When the riboflavin was added to the combination PT and APTT were found to be within reference range.

It follows that riboflavin protective effect during antiretroviral therapy was pronounced in coagulation time: PT and APTT. It was noted that as the doses of antiretroviral reduced, the protective effect of riboflavin was diminished.

d) Cell Morphology

Hepatitis may occur with limited or no symptoms, but often leads to jaundice, poor appetite and malaise. Some of the causes of hepatitis include infections, toxic substances (notably alcohol, certain medications, some industrial organic solvents and plants), and autoimmune diseases. Chronic hepatitis may cause nonspecific symptoms such as malaise, tiredness and weakness, and often asymptomatic. On physical examination there may be enlargement of the liver (Fristma, 2002). This is consistent with the observations made in this study. The development of hepatitis in this study is associated with HAART used. Studies have shown that after initiating HAART, the reported incidence of severe liver toxicity ranges from 2 to 18% (Soriano et al, 2008). Hepatic steatosis was also reported in the groups treated with stavudine and a combination of stavudine and lamivudine. It was found that these defective liver morphology was prevented upon treatment with riboflavin.

5.4 Effect of Drug Treatment on Mitochondrial DNA

It was found that the mtDNA short fragment loci amplify under the following conditions: primer (both forward and reverse) concentration of 2mM, taq

polymerase of 1.5mM and mtDNA template of 2mM. The thermocycling profile was established as follows: initial temperature of 95^0c for 2 minutes, 26 cycles of 95^0c for 45 seconds, 57^0c for 45 seconds and 68^0c then final extension of 68^0c for 7 minutes. The mtDNA can be obtained from genomic DNA using specific primers. It was proved that primers specific for the mtDNA are capable of isolating mtDNA from genomic DNA. This was important to establish because the process of mitochondrial isolation from tissues is lengthy and significant amount of mitochondria is lost in the process.

The assessment of mtDNA concentration using PCR then electrophoresis was found to be limited in accuracy. The amplicons in the long fragment were not visible. It may be indicative of inability of taq polymerase to amplify big fragment (8.6kb). However, a short fragment DNA was amplified. The amplicon appeared in the region of 300bp for animals on treatment and the controls-not in any treatment.

Real time PCR is ideal for determining quantities mtDNA. The mtDNA concentration was determined through number of cycle threshold (ct). The concentration of mtDNA was assessed using ct values derived from real time polymerase chain reaction (RT-PCR). The higher the ct value, the lower the mtDNA.

Comparing with the ct values of the negative control, it was noted that the lamivudine treated mice had high values for adipose tissue. The ct values for the same tissue with treatment of riboflavin and control were much lower and comparable. The ct values for liver were high for lamivudine-treated mouse compared to control. The high the ct values, the lower the DNA concentration. It was observed that the mtDNA concentrations in liver and adipose tissue for lamivudine-treated mice were low. This is indicative of low mtDNA concentration. It thus follows that there was mtDNA damage in the liver and adipose tissue. This is consistent with earlier studies which have documented

mtDNA damage in the liver and adipose issue (Mallal and Nolan, 2000). Antiretrovirals of nucleoside analogues have been associated with imparing of mitochondrial function (Barile et al., 1997; Pan-Zhou et al., 2000).

The ct values for the heart, brain and skeletal muscles tissues were comparable in mice treated with lamivudine, riboflavin and the controls. Suggesting that there was minimal mtDNA depletion in the NADH dehydrogenase loci in these tissues. There is no previous information on the mtDNA of heart, brain and skeletal muscles in relation to nucleoside analogue drugs.

It was observed that there was significant depletion of adipose tissue on groups treated with double and human equivalent doses of stavudine. However, there was adipose tissue in the group treated with stavudine half human equivalent dose.

There was improved mtDNA concentration of adipose tissue for combinations containing riboflavin. The group treated with double human equivalent dose combinations of stavudine, lamivudine and riboflavin and those containing human equivalent dose combination of stavudine and riboflavin showed improved mtDNA compared with the group treated with double human equivalent dose combination of stavudine and lamivudine and with double human equivalent dose of stavudine alone. This was through the ct values and is suggestive of more intact mtDNA. Based on ct values, the quality of mtDNA of the adipose tissue for double human equivalent dose for stavudine and riboflavin was higher than for the group treated with double combination of stavudine, lamivudine and riboflavin.

The group treated with human equivalent dose combination of stavudine and lamivudine showed declining quality of mtDNA in the liver as treatment progressed. The quality of mtDNA was higher in the groups treated with human

equivalent dose combination of stavudine, lamivudine and riboflavin and human equivalent dose combination of stavudine and riboflavin.

All the results for the groups treated with half human equivalent dose had intact mtDNA for adipose tissue unlike other treatment groups.

The group treated with riboflavin as positive control showed decrease in mtDNA. However, the decrease was insignificant. There was plenty of adipose tissue in this group. The adipose tissue mtDNA was significantly of higher quality after the period of four months for the group treated with half human equivalent dose combination of stavudine, lamivudine and riboflavin and half human equivalent dose combination of stavudine and riboflavin.

It was found that riboflavin had protective effect on the mtDNA of liver and adipose tissue. The results for the ct values across the groups in different dose levels show decline in ct values for groups treated with combination of stavudine and riboflavin. There was presence of adipose tissue in groups which were treated with combinations consisting of riboflavin. It is worthy reporting that stavudine caused depletion of the adipose tissue. The inclusion of riboflavin reversed this adverse effect. In general, there was ameliorating effect of riboflavin on adipose tissue.

Nucleoside reverse transcriptase inhibitors (NRTI) are associated with inhibition of DNA ploymrase γ leading to mtDNA depletion and/or impairment in the production of mitochondrial enzymes and proteins involved in oxidative phosphorylation (Kohler and Lewis, 2007). Stavudine (d4T), can induce lipoatrophy, fatty liver, hyperlactataemia and abnormal liver biochemistry. NRTI toxicity is usually ascribed to mitochondrial DNA (mtDNA) depletion and impaired mitochondrial respiration. Stavudine overdosage triggers fat wasting, leptin insufficiency and mild liver damage, without causing respiratory chain dysfunction (Igoudjil, et.al 2007).

The common trend in handling incidences of mitochondrial toxicity applied in medicine is early detection and cessation of antiretrovirals causing it (White, 2001). This is expected to give the mitochondria time to rejuvenate and heal.

There are no previous studies on effects of riboflavin directly on mtDNA. There has been speculation whether the use of riboflavin can reserve the mitochondria capacity (Stankov et al., 2010) when mitochondria toxicity is reported.

5.5 Impact of riboflavin

The mtDNA results for groups treated with combination of antiretroviral drugs containing riboflavin indicated low ct values indicating an increase in mtDNA MtDNA was deranged in all groups treated with stavudine and lamivudine without riboflavin.

In addition, the blood glucose levels were found to be within reference ranges where riboflavin was used as compared to groups treated with stavudine which developed hyperglycaemia. Adipose tissue was also preserved in groups treated with riboflavin. Stavudine was found to deplete adipose tissue. Riboflavin also was found to reduce PT and APTT.

There has been speculation that riboflavin can reduce lactate levels (Bernsen et al., 1993) and check myopathy caused by complex I deficiency (Ogle et al., 1997) and increase the capacity of mitochondria (Stankov et al., 2010) in patients on antiretroviral therapy.

In this study, it was found that the riboflavin has ameliorating effect on the mtDNA and biochemical profiles. Therefore, inclusion of riboflavin in the regimen for treatment of HIV when antiretroviral of nucleoside analogue is being administered should be considered.

Studies have reported riboflavin as an essential vitamin that is required for normal cellular functions, growth and development in all aerobic forms of life. Its forms- flavin adenine mononucleotide (FMN) and flavin adenine dinucleotide (FAD) participate in various metabolic redox reactions including electron transport chain in mitochondria and also as a prosthetic group of many enzymes like glutathione reductase and succinate dehydrogenase (Jazzer and Naseem, 1996; De Souza et.al, 2006). It can undergo photolysis and photoaddition leading to generation of various free radicals having potential to impair the macromolecules *in vitro* and *in vivo* (Ahmad, et.al, 2006).

It is a photo-sensitizer, used in photodynamic therapy (PDT) and ribo-phototherapy (RPT) for treatment of various diseases including cancer (Zhang, Gorner, 2009; Bareford et.al, 2008).

However, it was found that mtDNA decreased in cases where riboflavin was administered alone. The coagulation time (PT and APTT) were prolonged. The blood glucose levels in this group were found to be declining. This suggests that if riboflavin is used alone, it can induce toxicity.

5.6 Conclusion and Recommendation

Conclusion

- This study has established reference ranges for live weight, liver weight, biochemical and haematological parameters in normal swiss albino mice that can be used for future related studies.
- Experimental drugs: stavudine and lamivudine were confirmed to induce a range of toxicities: MtDNA derangement, elevation of blood glucose, prolonged PT and APTT, hepatomegally and liver morphology in groups treated with stavudine, combination of stavudine and lamivudine both human and double human doses

- Half human equivalent dose of stavudine and combination of stavudine and lamivudine showed minimal toxicity based on biochemical and haematological parameters, cell morphology and mtDNA derangement.
- Riboflavin was shown to ameliorate these effects.
- Thus the hypothesis that mitochondrial toxicity can be minimized by riboflavin has been proved to true.

Recommendations

- Coagulation profile should be monitored in patients being treated with HAART: stavudine and lamivudine
- Riboflavin should be considered for inclusion in treatment where stavudine and lamivudine are being administered particularly human equivalent dose.
- Studies on riboflavin should be done on human subjects to confirm its efficacy and safety.
- These findings suggest need for clinical trials with human participants.
- The efficacy of half human equivalent dose for stavudine in HIV should be investigated as it was found to be safe in these experiments.

Chapter Six

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Appendices

Appendix 1: Mouse Husbandry

PURPOSE

This Standard Operating Procedure (SOP) describes the basic procedures for routine care and husbandry of mice.

RESPONSIBILITY

Animal care staff, Project supervisors, Principal Investigator

MATERIALS

Cage cards
Rodent diet (within 6 months of the milling date)
Clean or sterilized water bottles
Clean or sterilized water valves
Clean or sterilized cages with bedding
Clean or sterilized micro-barrier tops and wire-bar lids
Clean or sterilized environmental enrichment devices
Clidox disinfectant

Procedures

Observation of animals:

Observe all rodents daily for illness, injury and general condition, and to ensure they have sufficient food and water, including weekends and holidays.

Emergency and holiday care:

Provide routine care and husbandry for all rodents on weekends and holidays.

Animal identification methods:

Identify all cages with a cage card.

Include, at a minimum, the following information:

Investigator

Protocol number

Species

Strain

Sex

Number of animals in the cage

Identify individual animals as needed in accordance with SOP. Examples ofindividual identification methods include: ear punching and ear tags.

Food and water:

Feed animals with recommended pellets for mice/rodent nutrition.

Food storage:

Store feed on plastic pallets off the floor and 4-6 inches away from walls, in a vermin-proof storeroom.

Store feed bags in leak-proof containers with tightly fitting lids.

Maintain temperature and humidity controls in the storeroom. Avoid temperatures above 21°C and extremes in humidity. Use feed within 6 months of milling date.

Stack feed in a manner that allows easy reading of the milling date.

Check watering devices daily for proper operation and cleanliness.

Replace empty bottles with clean ones rather than refilling them.

Monitor water quality routinely to ensure that it is free of contaminants that could potentially expose animals to chemical or infectious agents.

Breeding program:

House breeders in monogamous pairs and trios groups.

Remove the pregnant female from the breeding group and put her in her own cage before giving birth.

Wean litters before the next litter is born to prevent over-crowding in the cage.

Social and environmental enrichment:

Group-house rodents whenever possible.

Provide environmental enrichment devices for all rodents.

Examples of enrichment include, but are not limited to, the following: cellulose-based shelters, PVC pipe, Nestlets®, corn husks, autoclaved blocks of wood.

Quarantine procedures:

Quarantine rodents from unapproved vendors for up to eight weeks depending on the status of both the vendor and the facility where they will be housed. The

veterinarian will determine the quarantine requirements for each in-coming rodent.

Quarantine of rodents from approved vendors is not required.

Euthanasia and disposal of dead animals:

Ensure that euthanasia is conducted by trained and experienced staff.

Dispose of dead animals by incineration.

Vets will be consulted while these steps are observed.

Vermin control

The animal house and store will be vermin-proof.

The animal house will be kept under surveillance routinely.

The cages where mice will be kept are lockable.

Environment and environmental control:

Maintain room temperatures between 18 and 24° C.

Maintain relative humidity between 30% and 70%.

Maintain a daily log of room temperature and humidity.

Bedding:

Use wood chips, shredded paper products or chopped corn cobs as bedding materials.

Use enough bedding to keep the rodents clean and dry until the next scheduled change.

Note: Cedar shavings are not recommended due to the harmful presence of aromatic hydrocarbons.

Lighting:

Provide a regular diurnal lighting cycle.

Note: Lights are controlled by timers set at a photoperiod of 12 to 14 hours of light. Check the timer performance routinely.

Space requirements:

Provide cages that are appropriate in size for the number of rodents housed in them.

Cleaning of cages:

Change ventilated cages once every two weeks and static cages once every week, at a minimum.

Change cages more frequently if needed to keep the animals clean and dry and provide a healthy environment.

Supply fresh bedding with each cage change with the exception of the sentinel cages.

Cleaning and sanitation of housing room and equipment:

Weekly:

Flush the floor drains.

Change/clean pre-filters in changing stations/biosafety cabinets

Check light timers.

Mop floors.

Monthly:

Wipe down all surfaces such as sinks, doors, door jams and doorknobs, windows, cart tops, shelves, etc. with the disinfectant solution.

Clean changing stations/biosafety cabinets: remove filters, vacuum and drain.

Clean room exhaust grills.

Bi-annually:

Wash all walls with disinfectant solution.

Wash racks.

Transportation:

Transport mice being purchased from International Livestock Research Institute (ILRI) in filtered commercial rodent transport boxes.

Transport mice within animal house-Chiromo in either filter cages or unfiltered cages wrapped in a material which prevents animal allergens from escaping into the environment. Cages needed to be covered by opaque material.

Preparation of disinfecting solution:

Wear personal protective equipment when preparing and using disinfectant solution.

Dilute according to manufacturer's recommendation.

Clean surfaces prior to disinfection to remove any organic material.

Apply disinfectant and allow for adequate contact time.

Waste Management:

Deposit non-regulated or non-infectious Medical Waste and soiled materials in waste bin.

Regulated or infectious Medical Waste is processed by the School of Biological Sciences University of Nairobi Waste Management program.

SAFETY

Working with mice can trigger related allergies. Consult with the Vet regarding requirements for personal protective equipment.

For animal related injury, Vets will be consulted.

SOP Title: Animal Area Monitoring Sheet: Rodents									SOP 1							
Effective Date: 01/09/2002																
	Item	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D	Time—A.M./ P.M.															
D	Room Temp.—High															
D	Room Temp.—Low															
D	Health Check															
D	Animals Fed & Watered															
D	Enrichment Provided															
D	Room Lights Checked															
2X	Water Bottles Changed															

2X	Pans/ Tubs Changed															
W	Cages Changed															
W	Trash Emptied															
W	Feed Mill Date Checked															
W	Floor Cleaned															
W	Doors/ Door Frames Cleaned															
W	Anteroom/Hallway Stocked & Cleaned															
W	Paper Shoe Covers Sorted															
W	Curtains Cleaned															
W	Air Filters Checked															
BW	Cage Lids Changed															
BW	Feeders Changed															
BW	Racks Changed or Disinfected															
M	Enrichment Changed															
M	Room Disinfected															
M	Air Vents Cleaned															
M	Drains Flushed															
M	Protocol Expiration Date Checked															
Q	Sentinels Submitted															
	Initials															

KEY: D = Daily, 2X = Twice a week, W = Weekly, BW = Every other week, M = Monthly, Q = Quarterly

Notes/Remarks/General Comments:

_____
_____
_____
_____
_____
_____
_____

Checked by_____ Date:_____

SOP Title: Incident Reports	SOP 1
Effective Date: 01/09/2012	

ALL INCIDENTS SHOULD BE REPORTED TO THE PRINCIPAL INVESTIGATOR

Seek the category from the following choices: Building Function, Construction, Pest Control, Husbandry Issues, Animal Welfare, or SOP Compliance

Date	Category	Location	Reported By	Event	Immediate Response

Checked By:_____Date_____

SOP Title: Daily Cage Tally	SOP 1
Effective Date: 01/09/2012	

Cage no:_____

Date	Routine Check (Time)	No. of Mice	Wt/Mice	No./Sex		Status	Project Status	Health Mice	Mortality	Overall Comment	Sign

Confirmed By:_____ Date_____

SOP Title: Mice Weight Monitoring	SOP 1
Effective Date: 01/09/2012	

Date	Mice (Tag) No	Weight (g)			

Confirmed By:_____Date_____

Appendix 2: Standard operating procedures for sample collection from mice

Purpose

This standard operating procedure (SOP) defines the specimen collection guidelines for tissues: blood, liver, skeletal muscles, adipose tissue, heart and brain. These guidelines shall apply during sacrifice, tissue harvesting, aliquoting, storage, transport and analysis of the aforementioned specimen of mice at all times whenever this procedure is necessary.

Objective

This SOP is developed to ensure acceptable specimen of high quality have been collected for generation of high quality laboratory results. Good laboratory practices for dealing with specimen shall be observed.

Responsibility

Principal investigator, Supervisors and Technical Staff

Introduction

Mice shall be slaughtered and tissues harvested. These tissues include blood, liver, skeletal muscles, adipose tissue, heart and brain. The specimen shall be used for lipid profile, mitochondrial DNA analysis, complex I electron transport chain enzyme assay and histology.

Personnel Responsibilities

Principal Investigator

The Principal Investigator shall be responsible for implementation of these guidelines.

Supervisors

The supervisors shall approve these guidelines and shall give their advice from time to time.

Technical Staff

During sacrifice and tissue harvesting there shall be three technical staff. One shall be an expert in mouse slaughter and tissue harvesting. He/she shall ensure ethical handling of the animals throughout the process. He/she shall help in sacrifice and tissue harvesting.

One of the three technical members will be assigned the duty of placing specimen in the appropriate specimen collection containers. The containers must be prepared, labeled and set before the slaughter. He/she shall be responsible for aliquoting and immediate treatment of the specimen such as placing them in ice.

The other technical person shall be responsible for preparing slaughter equipments surface cleaning and sterilization, carcass disposal, disinfection and preparation of the next animal for sacrifice.

Sacrifice

The surface for sacrifice shall be sterilized using ethanol (70% volume). Surgical board, foil paper, pairs of scissors and cotton wool shall be provided.

Pre-identified mice shall be slaughtered for tissue harvesting. The label of the mouse shall be noted. Preliminary observation shall be made and any features outside normal shall be noted. These features include skin (rough or smooth), eyes and general physical appearance.

The sacrifice shall involve three categories of mice: those on drugs (lamivudine and riboflavin) and those not on any treatment to serve as control. Equal number of mice from each category shall be slaughtered. Same type and number of tissues shall be harvested from all these mice.

Tissue Harvesting, Container and Label
The following tissues shall be harvested: blood, liver, skeletal muscles, adipose tissue, heart and brain.

Labels on the specimen shall include letters and figures. The first letter shall be either T for mice on lamivudine treatment or B for those on riboflavin or C for mice not on any drugs. The second two numbers separated by a dot shall indicate cage and mouse number. For instance, 1.1 represent first cage for first digit and first mouse for second one after the dot. The middle letter will represent specimen e.g. S for serum, L for liver, M for skeletal Muscle, A for adipose tissue, H for heart and B for brain. The next set of digits will represent the date e.g. 1010 means October 2010. There shall be last digit to indicate the test; D for DNA analysis, E for enzyme assay, H for histology and L for lipid profile. Thus a label such as T1.1S1010L stands for:

T- Lamivudine treated mouse
cage 1, first mouse
S- Serum
1010-October 2010

L-lipid Profile
Liver from first mouse in cage number one for enzymatic assay from the riboflavin treated group collected in October 2010 shall have a label:
B1.1L1010E

Blood
Blood will be collected through cardiac puncture. This specimen shall be placed in cryovials. A total of 1mL blood shall be collected and used for lipid profile. This shall be done immediately before the heart collapses. Serum shall be separated from the whole blood and the cryovials labeled appropriately.

Liver

The liver will be collected after opening the rib cage. This specimen shall be used for enzyme assay, histology and mitochondrial DNA analysis. Immediately after extraction, it shall be aliquoted into four portions. Two aliquots shall be used for mitochondrial DNA analyses: one neat tissue (macerated) and another mitochondrial extract.

Skeletal Muscle

This will be extracted from the limbs. It will be used for mitochondrial DNA analyses. It shall be aliquoted into two portions. One will be macerated and the other mitochondria will be extracted.

Adipose Tissue

This will be extracted from the under skin. It will be used for mitochondrial DNA analyses. It shall be aliquoted into two portions. One will be macerated and the other mitochondria will be extracted.

Heart

This will be extracted from the rib cage. It will be used for mitochondrial DNA analyses. It shall be aliquoted into two portions. One will be macerated and the other mitochondria will be extracted.

Brain

This will be extracted from the skull. It will be used for mitochondrial DNA analyses. It shall be aliquoted into two portions. One will be macerated and the other mitochondria will be extracted.

Table 1: A list of specimen, label, legend, analyses and storage temperature

Specimen	No.	Label	Legend	Analyses	Temp
Blood		T3.1S1010L	3TC, cage 3, 1st mouse, serum, Oct 2010, lipid profile	Lipid profile	25^0C. immediate analysis
		T3.2S1010L	3TC, cage 3, 2nd mouse, serum, Oct 2010, lipid profile		
		B3.1S1010L	Riboflavin, cage 3, 1st mouse, serum, Oct 2010, lipid profile		
		B3.2S1010L	Riboflavin, cage 3, 2nd mouse, serum, Oct 2010, lipid profile		
		C1S1010L	Control, 1st mouse, serum, Oct 2010, lipid profile		
		C2S1010L	Control, 2nd mouse, serum, Oct 2010, lipid profile		
Liver	1	T3.1L1010E	3TC, cage 3, 1st mouse, liver, Oct 2010, enzyme assay	Enzyme assay	-80^0C
	2	T3.2L1010E	3TC, cage 3, 2nd mouse, liver, Oct 2010, enzyme assay		
	3	B3.1L1010E	Riboflavin, cage 3, 1st mouse, liver, Oct 2010, enzyme assay		
	4	B3.2L1010E	Riboflavin, cage 3, 2nd mouse, liver, Oct 2010, enzyme assay		
	5	C1L1010E	Control, mouse 1, liver, Oct 2010, enzyme assay		
	6	C2L1010E	Control, mouse 2, liver, Oct 2010, enzyme assay		
		T3.1L1010H	3TC, cage 3, 1st mouse, liver, Oct 2010, Histology	Histology	25^0C, preserve in formalin
		T3.2L1010H	3TC, cage 3, 2nd mouse, liver, Oct 2010, Histology		
		B3.1L1010H	Riboflavin, cage 3, 1st mouse, liver, Oct 2010, Histology		
		B3.2L1010H	Riboflavin, cage 3, 2nd mouse, liver, Oct 2010, Histology		
		C1L1010H	Control, mouse 1, liver, Oct 2010, Histology		
		C2L1010H	Control, mouse 2, liver, Oct 2010, Histology		
	7	T3.1L1010D	3TC, cage 3, 1st mouse, liver, Oct 2010, MtDNA	Mitocho	-80^0C

	8	T3.2L1010D	3TC, cage 3, 2nd mouse, liver, Oct 2010, MtDNA		-80^{0}C
	9	B3.1L1010D	Riboflavin, cage 3, 1st mouse, liver, Oct 2010, Mt. DNA		
	10	B3.2L1010D	Riboflavin, cage 3, 2nd mouse, liver, Oct 2010, MtDNA		
	11	C1L1010D	Control, mouse 1, liver, Oct 2010, MtDNA		
	12	C2L1010D	Control, mouse 2, liver, Oct 2010, MtDNA		
Skeletal Muscle	13	T3.1M1010D	3TC, cage 3, 2nd mouse, skeletal muscle, Oct 2010, MtDNA		
	14	T3.2M1010D	3TC, cage 3, 2nd mouse, skeletal muscle, Oct 2010, MtDNA		
	15	B3.1M1010D	Riboflavin, cage 3, 1st mouse, skeletal muscle, Oct 2010, Mt DNA		
	16	B3.2M1010D	Riboflavin, cage 3, 2nd mouse, skeletal muscle, Oct 2010, MtDNA		
	17	C1M1010D	Control, mouse 1, skeletal muscle, Oct 2010, Mt DNA		
	18	C2M1010D	Control, mouse 2, skeletal muscle, Oct 2010, Mt DNA		
Heart	19	T3.1H1010D	3TC, cage 3, 1st mouse, heart, Oct 2010, Mt DNA		
	20	T3.2H1010D	3TC, cage 3, 2nd mouse, heart, Oct 2010, Mt DNA		
	21	B3.1H1010D	Riboflavin, cage 3, 1st mouse, heart, Oct 2010, Mt DNA		
	22	B3.2H1010D	Riboflavin, cage 3, 2nd mouse, heart, Oct 2010, Mt DNA		
	23	C1H1010D	Control, mouse1, heart, Oct 2010, Mt DNA		
	24	C2H1010D	Control, mouse 2,heart, Oct 2010, Mt DNA		
Brain	25	T3.1B1010D	3TC, cage 3, 1st mouse, brain, Oct 2010, Mt DNA		
	26	T3.2B1010D	3TC, cage 3, 2nd mouse, brain, Oct 2010, Mt DNA		
	27	B3.1B1010D	Riboflavin, cage 3, 1st mouse, brain, Oct 2010, Mt DNA		
	28	B3.2B1010D	Riboflavin, cage 3, 2nd mouse, brain, Oct 2010, Mt DNA		
	29	C1H1010D	Control, mouse1, brain, Oct 2010, Mt DNA		

	30	C2H1010D	Control, mouse 2,brain, Oct 2010, Mt DNA		
Adipose Tissue	31	T3.1A1010D	3TC, cage 3, 1st mouse, adipose tissue, Oct 2010, Mt DNA		
	32	T3.2A1010D	3TC, cage 3, 2nd mouse, adipose tissue, Oct 2010, Mt DNA		
	33	B3.1A1010D	Riboflavin, cage 3, 1st mouse, adipose tissue, Oct 2010, Mt DNA		
	34	B3.2A1010D	Riboflavin, cage 3, 2nd mouse, adipose tissue, Oct 2010, Mt DNA		
	35	C1A1010D	Control, mouse 1,adipose tissue, Oct 2010, Mt DNA		
	36	C2A1010D	Control, mouse 2,adipose tissue, Oct 2010, Mt DNA		

Specimen Distribution and Storage

The specimen shall be distributed as follows:

All specimens for mitochondrial analysis shall be stored at -800C till the time of analysis.

The specimen for histology shall be stored in formalin and delivered to Anatomical Pathology Unit of University of Nairobi for analysis within 7 hours of collection.

Specimen for enzyme assay shall be stored at -800C till the time of analysis.

Lipid profile specimen shall be delivered to Clinical Chemistry Laboratory of the University of Nairobi for analysis within 7 hours of collection.

Approved:
Sign: _____Date: _____

Appendix 3: Preparation of mitochondria from a mouse

Procedure

1. Remove the liver from decapitated mice (previously starved).
2. Wash in cold (0-4°C) 0.25M sucrose until the wash is clear (two washes of 20ml sucrose). Remove the gall bladder and any connective tissues.
3. Mince liver in 30ml 0.25M sucrose with sharp scissors.
4. Homogenize this mince in a Potter Elvehjem homogenizer being careful to keep it in ice bath.
5. Dilute the slurry with sucrose to yield a 10% homogenate.
6. Distribute the homogenate among 40ml plastic centrifuge tubes and centrifuge at 600g x 10 minutes at 0-4°C.
7. Pour the supernatant into a clean cold beaker. The fluffy layer should not enter the beaker.
8. Re-centrifuge this supernatant in clan tubes at 1200g x 10 minutes. Discard the supernatant along with any pink microsomal layer.
9. The pellet should be a tan tightly packed entity. This should be suspended in 5ml 0.25M sucrose with a smaller homogenizer.
10. Dilute the suspension with 0.25M sucrose and centrifuge at 8000g x 10 minutes.
11. Re-wash the pellet and centrifuge as 10 above. Suspend the pellet in 2-3ml 0.25M sucrose.
12. Maintain this suspension in ice.

Appendix 4: Purification of Total DNA from Animal Blood or Cells

This protocol is designed for high-throughput purification of total DNA from animal blood (with nucleated or non-nucleated erythrocytes) or from cultured animal or human cells.

Important points before starting

1. All centrifugation steps are carried out at room temperature (15–25°C).
2. PBS is required for use in step 1 (see page 14 for composition). Buffer ATL is not required in this protocol.
3. Ensure that ethanol has not been added to Buffer AL.
4. Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy 96 Blood & Tissue Kit.

Things to do before starting

Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C for 5 min until the precipitates have fully dissolved.
Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
Mix Buffer AW1 before use by inverting several times.
Preheat an incubator to 56°C for use in step 2.

Procedure

1. **For blood with non-nucleated erythrocytes, follow step 1a; for blood with nucleated erythrocytes, follow step 1b; for cultured cells, follow step 1c.**

   Blood from mammals contains non-nucleated erythrocytes. Blood from animals such as birds, fish, or frogs contains nucleated erythrocytes.

1a. Non-nucleated: Pipet 20 µl proteinase K into each collection

microtube. Add 50–100 µl anticoagulated blood per collection microtube. Use a 96-Well-Plate Register (provided) to identify the position of each sample. Adjust the volume to 220 µl each with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 5 min at room temperature before continuing with step 2.

Keep the clear covers from the collection microtube racks for use in step 3.

1b. Nucleated: Pipet 20 µl proteinase K into each collection microtube. Add 5–10 µl anticoagulated blood. Use a 96-Well-Plate Register (provided) to identify the position of each sample. Adjust the volume to 220 µl each with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 5 min at room temperature before continuing with step 2.

Keep the clear covers from the collection microtube racks for use in step 3.

1c. Cultured cells: Centrifuge the appropriate number of cells (maximum 5 x 106 each) for 5 min at 300 x g. Use a 96-Well-Plate Register (provided) to identify the position of each sample. Resuspend the pellets in 200 µl PBS each. Add 20 µl proteinase K each. Continue with step 2.

When using a frozen cell pellets, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube.

Ensure that an appropriate number of cells is used in the procedure. For cell lines with a high degree of ploidy (e.g., HeLa cells), it is recommended to use less than the maximum number of cells listed in Table 1, page 16.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml). Seal the collection microtubes properly using the caps provided, mix by vortexing, and incubate for 5 min at room temperature before continuing with step 2.
Keep the clear covers from the collection microtube racks for use in step 3.

2. Add 200 µl Buffer AL (without added ethanol) to each sample.
   Ensure that ethanol has not been added to Buffer AL.

3. Seal the collection microtubes properly using the caps provided. Place a clear cover (saved from step 1) over each rack of collection microtubes, and shake the racks vigorously up and down for 15 s. To collect any solution from the caps, centrifuge the collection microtubes. Allow the centrifuge to reach 3000 rpm, and then stop the centrifuge.
   Do not prolong this step.

IMPORTANT: The rack of collection microtubes must be vigorously shaken up and down with both hands to obtain a homogeneous lysate. Inverting the rack of collection microtubes is not sufficient for mixing. The genomic DNA will not be sheared by vigorous shaking. The lysate and Buffer AL should be mixed immediately and thoroughly to yield a homogeneous solution.
Keep the clear covers from the collection microtube racks for use in step 6.

Incubate at 56°C for 10 min. Place a weight on top of the caps during the incubation. Mix occasionally during incubation to disperse the sample, or place on a rocking platform.

Note: Do not use a rotary- or vertical-type shaker as continuous rotation may release the caps. If incubation is performed in a water bath make sure that the collection microtubes are not fully submerged and that any remaining water is removed prior to removing the caps in step 5.

4. Carefully remove the caps, and add 200 µl ethanol (96–100%) to each sample.
5. Seal the collection microtubes properly using the caps provided. Place a clear cover over each rack of collection microtubes, and shake the racks vigorously up and down for 15 s. To collect any solution from the caps, centrifuge the collection microtubes. Allow the centrifuge to reach 3000 rpm, and then stop the centrifuge.

Do not prolong this step.

IMPORTANT: The rack of collection microtubes must be vigorously shaken up and down with both hands to obtain a homogeneous lysate. Inverting the rack of collection microtubes is not sufficient for mixing. The genomic DNA will not be sheared by vigorous shaking. The lysate and ethanol should be mixed immediately and thoroughly to yield a homogeneous solution.

6. Place two DNeasy 96 plates on top of S-Blocks (provided). Mark the DNeasy 96 plates for later sample identification.
7. Remove and discard the caps from the collection microtubes. Carefully transfer the lysis mixture (maximum 900 µl) of each sample from step 6 to each well of the DNeasy 96 plates.

Take care not to wet the rims of the wells to avoid aerosols during centrifugation.
Do not transfer more than 900 µl per well.

Note: Lowering pipet tips to the bottoms of the wells may cause sample overflow and cross-contamination. Therefore, remove one set of caps at a time, and begin drawing up the samples as soon as the pipet tips contact the liquid. Repeat until all the samples have been transferred to the DNeasy 96 plates.

8. Seal each DNeasy 96 plate with an AirPore Tape Sheet (provided). Centrifuge for 4 min at 6000 rpm.

AirPore Tape prevents cross-contamination between samples during centrifugation.
After centrifugation, check that all of the lysate has passed through the membrane in each well of the DNeasy 96 plates. If lysate remains in any of the wells, centrifuge for a further 4 min.

9. Remove the tape. Carefully add 500 µl Buffer AW1 to each sample.

Note: Ensure that ethanol has been added to Buffer AW1 prior to use.

10. Seal each DNeasy 96 plate with a new AirPore Tape Sheet (provided). Centrifuge for 2 min at 6000 rpm.
    Remove the tape. Carefully add 500 µl Buffer AW2 to each sample.
    Note: Ensure that ethanol has been added to Buffer AW2 prior to use.

11. Centrifuge for 15 min at 6000 rpm.

Do not seal the plate with AirPore Tape.

The heat generated during centrifugation ensures evaporation of residual ethanol in the sample (from Buffer AW2) that might otherwise inhibit downstream reactions.

12. Place each DNeasy 96 plate in the correct orientation on a new rack of Elution Microtubes RS (provided).
13. To elute the DNA, add 200 µl Buffer AE to each sample, and seal the DNeasy 96 plates with new AirPore Tape Sheets (provided). Incubate for 1 min at room temperature (15–25°C). Centrifuge for 4 min at 6000 rpm.

200 µl Buffer AE is sufficient to elute up to 75% of the DNA from each well of the DNeasy 96 plate.
Elution with volumes less than 200 µl significantly increases the final DNA concentration of the eluate but may reduce overall DNA yield. For samples containing less than 1 µg DNA, elution in 50 µl Buffer AE is recommended.

14. Recommended: For maximum DNA yield, repeat step 15 with another 200 µl Buffer AE.

A second elution with 200 µl Buffer AE will increase the total DNA yield by up to 25%. However due to the increased volume, the DNA concentration is reduced.
If a higher DNA concentration is desired, the second elution step can be performed using the 200 µl eluate from the first elution. This will increase the yield by up to 15%.
Use new caps (provided) to seal the Elution Microtubes RS for storage.

Appendix 5: Specimen Label and Identity

Standard operating procedures

No.	Label	Legend: treatment, mouse number and tissue name
1	T1L	3TC + d4T human equivalent dose mouse 1 liver
2	T1A	3TC + d4T human equivalent dose mouse 1 adipose tissue
3	T2L	3TC + d4T human equivalent dose mouse 2 liver
4	T2A	3TC + d4T human equivalent dose mouse 2 adipose tissue
5	T3L	3TC + d4T human equivalent dose mouse 3 liver
6	T3A	3TC + d4T human equivalent dose mouse 3 adipose tissue
7	T4L	3TC + d4T human equivalent dose mouse 4 liver
8	T4A	3TC + d4T human equivalent dose mouse 4 adipose tissue
9	T5L	3TC + d4T human equivalent dose mouse 5 liver
10	T5A	3TC + d4T human equivalent dose mouse 5 adipose tissue
11	T6A	3TC + d4T human equivalent dose mouse 6 adipose tissue
12	T6L	3TC + d4T human equivalent dose mouse 6 liver
13	T7L	3TC + d4T human equivalent dose mouse 7 liver
14	T7A	3TC + d4T human equivalent dose mouse 7 adipose tissue
15	T7L1	3TC + d4T human equivalent dose mouse 8 liver
16	T7A1	3TC + d4T human equivalent dose mouse 8 adipose tissue
17	D1L	d4T human equivalent Mouse 1 liver
18	D2L	d4T human equivalent Mouse 2 liver
19	D2A	d4T human equivalent Mouse 2 adipose tissue
20	D3L	d4T human equivalent Mouse 3 liver
21	D3A	d4T human equivalent Mouse 3 adipose tissue
22	D4L	d4T human equivalent Mouse 4 liver
23	D4A	d4T human equivalent Mouse 4 adipose tissue
24	D5L	d4T human equivalent Mouse 5 liver
25	D5A	d4T human equivalent Mouse 5 adipose tissue
26	D6L	d4T human equivalent Mouse 6 liver
27	D6A	d4T human equivalent Mouse 6 adipose tissue
28	D7L	d4T human equivalent Mouse 7 liver
29	R6A	Riboflavin+d4T+3TC human equivalent mouse 6 liver
30	D7A	d4T human equivalent Mouse 7 adipose tissue

31	D8L	d4T human equivalent Mouse 8 liver
32	D8A	d4T human equivalent Mouse adipose tissue
33	R1L	Riboflavin+d4T+3TC human equivalent mouse 1 liver
34	RIA	Riboflavin+d4T+3TC human equivalent mouse 1 adipose tissue
35	R2L	Riboflavin+d4T+3TC human equivalent mouse 2 liver
36	R2A	Riboflavin+d4T+3TC human equivalent mouse 2 adipose tissue
37	R3L	Riboflavin+d4T+3TC human equivalent mouse 3 liver
38	R3A	Riboflavin+d4T+3TC human equivalent mouse 3 adipose tissue
39	R4L	Riboflavin+d4T+3TC human equivalent mouse 4 liver
40	R5L	Riboflavin+d4T+3TC human equivalent mouse 5 liver
41	R4A	Riboflavin+d4T+3TC human equivalent mouse 4 adipose tissue
42	R5A	Riboflavin+d4T+3TC human equivalent mouse 5 adipose tissue
43	R6A	Riboflavin+d4T+3TC human equivalent mouse 6 adipose tissue
44	R7L	Riboflavin+d4T+3TC human equivalent mouse 7 liver
45	R7A	Riboflavin+d4T+3TC human equivalent mouse 7 adipose tissue
46	R8L	Riboflavin+d4T+3TC human equivalent mouse 8 liver
47	R8A	Riboflavin+d4T+3TC human equivalent mouse 8 adipose tissue
48	R9L	Riboflavin+d4T+3TC human equivalent mouse 9 liver
49	R9A	Riboflavin+d4T+3TC human equivalent mouse 9 adipose tissue
50	C1L	No treatment mouse 1 liver
51	C1A	No treatment mouse 1 adipose tissue
52	C2L	No treatment mouse 2 liver
53	C2A	No treatment mouse 2 adipose tissue
54	C3L	No treatment mouse 3 liver
55	C3A	No treatment mouse 3 adipose tissue
56	D2L1	d4T half human equivalent Mouse 1 liver
57	D2A1	d4T half human equivalent Mouse 1 adipose tissue
58	D2L2	d4T half human equivalent Mouse 2 liver
59	D2A2	d4T half human equivalent Mouse 2 adipose tissue
60	D2L3	d4T half human equivalent Mouse 3 liver
61	D2L4	d4T half human equivalent Mouse 4 liver
62	D2A4	d4T half human equivalent Mouse 4 adipose tissue
63	D2L5	d4T half human equivalent Mouse 5 liver

64	D2A5	d4T half human equivalent Mouse 5 adipose tissue
65	D2L6	d4T half human equivalent Mouse 6 liver
66	D2A6	d4T half human equivalent Mouse 6 adipose tissue
67	D2L7	d4T half human equivalent Mouse 7 liver
68	D2A7	d4T half human equivalent Mouse 7 adipose tissue
69	D2L8	d4T half human equivalent Mouse 8 liver
70	D2A8	d4T half human equivalent Mouse 8 adipose tissue
71	D2L9	d4T half human equivalent Mouse 9 liver
72	D2A9	d4T half human equivalent Mouse 9 adipose tissue
73	D2L10	d4T half human equivalent Mouse 10 liver
74	D2A10	d4T half human equivalent Mouse 10 adipose tissue
75	T2L1	d4T+ 3TC half human equivalent mouse 1 liver
76	T2A1	d4T+ 3TC half human equivalent mouse 1 adipose tissues
77	T2L2	d4T+ 3TC half human equivalent mouse 2 liver
78	T2A2	d4T+ 3TC half human equivalent mouse 2 adipose tissue
79	T2L3	d4T+ 3TC half human equivalent mouse 3 liver
80	T2A3	d4T+ 3TC half human equivalent mouse 3 adipose tissue
81	T2L4	d4T+ 3TC half human equivalent mouse 4 liver
82	T2A4	d4T+ 3TC half human equivalent mouse 4 adipose tissue
83	T2L5	d4T+ 3TC half human equivalent mouse 5 liver
84	T2A5	d4T+ 3TC half human equivalent mouse 5 adipose tissue
85	T2A6	d4T+ 3TC half human equivalent mouse 6 adipose tissue
86	T2L6	d4T+ 3TC half human equivalent mouse 6 liver
87	T2L7	d4T+ 3TC half human equivalent mouse 7 liver
88	T2L8	d4T+ 3TC half human equivalent mouse 8 liver
89	T2A8	d4T+ 3TC half human equivalent mouse 8 adipose tissue
90	T2L9	d4T+ 3TC half human equivalent mouse 9 liver
91	T2A9	d4T+ 3TC half human equivalent mouse 9 adipose tissue
92	D2X1L	d4T+ 3TC + Riboflavin double human equivalent mouse 1 liver
93	D2XL1	d4T+ 3TC + Riboflavin double human equivalent mouse 1 liver
94	D2XL2	d4T+ 3TC + Riboflavin double human equivalent mouse 2 liver

95	D2XL3	d4T+ 3TC + Riboflavin double human equivalent mouse 3 liver
96	D2X6L	d4T+ 3TC + Riboflavin double human equivalent mouse 6 liver
97	D2X8L	d4T+ 3TC + Riboflavin double human equivalent mouse 8 liver
98	D2X1A	d4T+ 3TC + Riboflavin double human equivalent mouse 1 adipose tissue
99	D2X3A	d4T+ 3TC + Riboflavin double human equivalent mouse 2 adipose tissue
100	D2X2A	d4T+ 3TC + Riboflavin double human equivalent mouse 2 adipose tissue
101	D2X6A	d4T+ 3TC + Riboflavin double human equivalent mouse 6 adipose tissue
102	D2X7L	d4T+ 3TC + Riboflavin double human equivalent mouse 7 liver
103	D2X7A	d4T+ 3TC + Riboflavin double human equivalent mouse 7 adipose tissue
104	D2X8A	d4T+ 3TC + Riboflavin double human equivalent mouse 8 adipose tissue
105	DTRL1	d4T+ Riboflavin half human equivalent mouse 1 liver
106	DTRA1	d4T+ Riboflavin half human equivalent mouse 1 adipose tissue
107	DTRL3	d4T+ Riboflavin half human equivalent mouse 3 liver
108	DTRA3	d4T+ Riboflavin half human equivalent mouse 3 adipose tissue
109	DTRL4	d4T+ Riboflavin half human equivalent mouse 4 liver
110	DTRA4	d4T+ Riboflavin half human equivalent mouse 4 adipose tissue
111	DRL1	d4T+ 3TC double human equivalent mouse 1 liver
112	DRA1	d4T+ 3TC double human equivalent mouse 1 adipose tissue
113	DTRL2	d4T+ Riboflavin half human equivalent mouse 2 liver
114	DRL2	d4T+ 3TC double human equivalent mouse 2 liver
115	DRL3	d4T+ 3TC double human equivalent mouse 3 liver
116	DRA3	d4T+ 3TC double human equivalent mouse 3 adipose tissue
117	DRL4	d4T+ 3TC double human equivalent mouse 4 liver
118	DRA4	d4T+ 3TC double human equivalent mouse 4

		adipose tissue
119	DRL5	d4T+ 3TC double human equivalent mouse 5 liver
120	DTL1	d4T+ Riboflavin human equivalent mouse 1 liver
121	DTA1	d4T+ Riboflavin human equivalent mouse 1 adipose tissue
122	DTL2	d4T+ Riboflavin human equivalent mouse 2 liver
123	DTA2	d4T+ Riboflavin human equivalent mouse 2 adipose tissue
124	DTL3	d4T+ Riboflavin human equivalent mouse 3 liver
125	DTA4	d4T+ Riboflavin human equivalent mouse 4 adipose tissue
126	DRL6	d4T+ 3TC double human equivalent mouse 6 liver
127	DRA6	d4T+ 3TC double human equivalent mouse 6 adipose tissue
128	DRL7	d4T+ 3TC double human equivalent mouse 7 liver
129	DRA7	d4T+ 3TC double human equivalent mouse 7 adipose tissue
130	DRA3	d4T+ 3TC double human equivalent mouse 3 adipose tissue
131	DTL4	d4T+ Riboflavin human equivalent mouse 4 liver
132	CTL1	No treatment mouse 1 liver
133	CTL2	No treatment mouse 2 liver
134	CTA2	No treatment mouse 2 adipose tissue
135	CTA1	No treatment mouse 1 adipose tissue
136	CTL3	No treatment mouse 3 liver
137	CTA3	No treatment mouse 3 adipose tissue
138	T2A7	d4T+ 3TC half human equivalent mouse 7 adipose tissue
139	R2L1	Riboflavin human equivalent mouse 1 liver
140	R2A1	Riboflavin human equivalent mouse 1 adipose tissue
141	R2L2	Riboflavin human equivalent mouse 2 liver
142	R2A2	Riboflavin mouse human equivalent 2 adipose tissue
143	R2L3	Riboflavin mouse human equivalent 3 liver
144	R2L4	Riboflavin mouse human equivalent 4 liver
145	R2A3	Riboflavin mouse human equivalent 3 adipose tissue
146	R2A4	Riboflavin mouse human equivalent 4 adipose tissue
147	C2L1	No treatment mouse 1 liver
148	C2A1	No treatment mouse 2 adipose tissue
149	C2L2	No treatment mouse 2 liver
150	C2L3	No treatment mouse 3 liver
151	C2L4	No treatment mouse 4 liver

152	C2A4	No treatment mouse 4 adipose tissue
153	C2L5	No treatment mouse 5 liver
154	T6A	d4T+ 3TC half human equivalent mouse 6 adipose tissue

Appendix 6: Oil Red O staining Method for Lipids

(Bancroft and Stevens, 1977)

Preparation of stain

The working solution should be prepared an hour in advance by mixing three parts of stock solution of Oil Red O (saturated in 99% isopropanol) with two parts of distilled water and filtered before use.

Method

1. Dry sections of the tissue onto slides and rinse in 60% isopropanol.
2. Stain for 15min in Oil Red O.
3. Differentiate in 60% isopropanol until a delipidised control section appears colourless.
4. Wash in water and counter-stain nuclei with Mayer's Haemalum for 3min.
5. Wash well in tap water.
6. Wash in distilled water and mount in glycerine jelly.

Results

Unsaturated hydrophobic lipids that are insoluble I the dye bath and mineral oils are stained red. Some phospholipids appear pink.

Appendix 7: Leishman's stain for blood cells and parasites

(*J. V. Dacie, S.M. Lewis. Practical Haematology, fifth edition, Curchill Livingston, 1975 p72-73)*

Prepare cytospin slide or smear of sample.

- **Either** spray the wet preparation with Smear Fix©, leave for 1-2 mins, wash off the fixative with distilled water and drain....
- **Or** lower the slide gently into a coplin jar of acetic alcohol (3% acetic acid in 95% methanol), fix for 1 minute, wash off the fixative with distilled water and drain.
- Put slides on a rack and cover with 1ml of Leishman stock - 20 seconds.
- Add 2ml of pH6.8 buffer and tip the rack up and down to mix the solutions, stain for 7 minutes.
- Rinse quickly in distilled water then treat with pH6.8 buffer - 2 minutes.
- Rinse quickly in distilled water, shake off the excess and dry on a warm (50°C) hotplate, or carefully blot dry with fibre-free blotting paper.
- Clear and mount.
- Red blood cells - red to yellowish red
  Neutrophils - dark purple nuclei, pale pink cytoplasm, reddish-lilac small granules
  Eosinophils - blue nuclei, pale pink cytoplasm, red to orange-red large granules
  Basophils - purple to dark blue nucleus, dark purple, almost black large granules
  Lymphocytes - dark purple to deep bluish purple nuclei, sky blue cytoplasm
  Platelets - violet to purple granules
  Parasites (Leishmania, malaria, etc.) - dark blue-black.

- **Leishman stain**
- 0.15% Leishman powder in 100% methanol. Use after 24hrs.

- **Phosphate buffer (Sorensen)**
- Stock A: 0.2M sodium di-hydrogen orthophosphate (mw 156).
  To prepare dissolve 3.12g in 100ml distilled water.
- Stock B: 0.2M di-sodium hydrogen orthophosphate (mw 142).
  To prepare dissolve 2.83g in 100ml ditilled water.

- For pH 6.8 add 25.5ml of A to 24.5ml of B and make up to 100ml with distilled water.

Procedure for Leishman Staining and Differential WBC Count

- Let thin blood film to air dry
- Then flood the slide with Leishman stain solution
- Wait for 8 minutes. Then flood off with distilled water in 2 – 3 seconds. Longer washing will remove stain
- Wait for 20 minutes. Observe the appearance of metallic cum that float on the surface of the slide.
- Then wash with tap water. Stand in a rack to drain and air dry.
- Using a low-power lens (10 x objectives) on the microscope, check the approximate differential cell distribution.
- Using the 40x objective high-power lens, perform a 200 cell differential count.
- 100x objective oil immersion lens should be used for examining fine intracellular details of the cells.
- Move the slide along the stage of the microscope in a broad battlement track running transversely across the body of the film, avoiding the edges completely.
- Record the numbers of each type of white cell. Calculate the percentage of each of the five basic leucocytes.

Appendix 8: Determination of Lipid Profile

(a): Determination of total cholesterol

Specimen: serum, heparinised plasma or EDTA plasma

Wavelength: 500nm

Temperature: 25°C or 37°C

Pipette into tubes	Reagent blank (RB)	Sample or STD
Sample/STD	-	10µL
Reagent (RGT)	1000µL	1000µL
Mix and incubate for 10min. at 20-25°C or 5min. at 37°C. Measure the absorbance of the sample / STD (ΔASTD) against the reagent blank within 60min.		

Calculation of Cholesterol Concentration

$$C = 5.17 \times \frac{\Delta A_{sample}}{\Delta ASTD} \text{ (mmol/L)}$$

(b) Determination of HDL cholesterol

Specimen: serum, heparinised plasma or EDTA plasma

Wavelength: 500nm

Temperature: 25°C or 37°C

1. Precipitant (PREC)

Pipette into tubes	Macro	Semi-micro
Sample	500µL	200µL
PREC a	1000µL	-
PREC b	-	500µL
Mix well, incubate for 10min. at room temperature. Centrifuge for at least 2min at 10000g, alternative for 10min at 4000g.		

After centrifugation separate the clear supernatant from then precipitate within 1hour and determine the cholesterol concentration as follows:

2. Cholesterol Determination

Pipette into cuvette	Reagent blank	STD	Sample
Distilled water	100µL	-	-
STD	-	100µL	-
HDL-supernatant	-	-	100µL
Reagent	1000µL	1000µL	1000µL
Mix, incubate for 5min at 37°C or 10 min at 20-25°C. Measure the absorbance of the sample and the STD, respectively against the reagent blank within 60min (ΔA).			

Calculation of HDL-cholesterol Concentration

$$C = 2.28 \times \frac{\Delta A_{sample}}{\Delta ASTD} (mmol/L)$$

Calculation of LDL-cholesterol Concentration

The LDL-cholesterol concentration (LDL-C) is calculated from the total cholesterol concentration (TC), the HDL Cholesterol concentration (HDL-C) and the triglycerides concentration (TG) according to Friedewal *et al.*

$$LDL\text{-}C = TC - HDL\text{-}C - \frac{TG}{2.2} (MMOL/l)$$

(c) Determination of Triglycerides

Specimen: serum, heparinised plasma or EDTA plasma

Wavelength: 500nm

Temperature: 25°C or 37°C

Pipette into cuvette	Reagent blank (RB)	Sample or STD
Sample/STD	-	10µL
Reagent (RGT)	1000µL	1000µL
Mix and incubate for 10min. at 20-25°C or 5min. at 37°C. Measure the absorbance of the sample (ΔA_{sample}) and the standard (ΔASTD) against the reagent blank within 60min.		

Calculation of Triglycerides Concentration

$$C = 2.28 \times \frac{\Delta A_{sample}}{\Delta ASTD} \text{ (mmol/L)}$$

Appendix 9: Protocol for Coagulation time determination

Prothrombin Time Test

The test was done to demonstrate the competency of extrinsic/ tissue factors pathway.

Reagents:

Commercial thromboplastin reagents that are mixed with calcium chloride are used.

Procedure

Patient plasma (0.1ml) was placed into10mm x 75mm glass test tube and incubated for one minute. Thromboplastin reagent (0.2ml) was added to the patient plasma and stop watch was set simultaneously. The tube containing the mixture was rocked in quick moments and time was noted when the clot formed. The test was repeated for plasma control.

Sample was prepared as whole blood with anticoagulant in 3.2 % (aq) trisodium citrate in the ratio of 1 volume to 9 volumes of blood.

Quality control

Blood sample was analysed one hour after collection. grossly haemolysed and clotted samples were not analysed. Plasma control was analysed alongside the patient specimen.

Results

The mean of duplicate readings in seconds taken and expressed as:

Prothrombin time in seconds

Activated Partial Thromboplastin Time (Aptt) Procedure

(Dacie and Lewis, 2000)

Procedure

The tubes were prepared containing calcium chloride and kept at 37^0c for 5 minutes before use. Control plasma (0.1ml) was placed in glass tube. The APTT reagent was added into the plasma. The mixture was incubated for 5 minutes at at 37^0c then, 0.1ml of calcium chloride was added to the mixture and stop watch started simultaneously. Careful observation was made to time the formation of clot. The test was performed in duplicate. The results for control plasma were recorded and the procedure was repeated with test sample.

Sample was prepared from whole blood with anticoagulant in 3.2% (aq) trisodium citrate in the ratio of 1 volume to 9 volumes of blood.

Quality control

Precaution was observed to analyse samples in less than two hours. Grossly haemolysed samples and clooted ones were not analysed. Temperature for analysis and incubation was maintained at 37^0c.

Results

The results were expressed in seconds. The results of the normal plasma in seconds are compared with those of the patient's plasma in seconds.