EVALUATION OF THE GENOTOXICITY OF ANTIRETROVIRAL DRUGS ON *ALLIUM CEPA* AND MALE FERTILITY IN TWO GENERATIONS OF MICE

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, UNIVERSITY OF LAGOS, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) DEGREE IN CELL BIOLOGY AND GENETICS

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CERTIFICATION

This is to certify that the thesis:

EVALUATION OF THE GENOTOXICITY OF ANTIRETROVIRAL DRUGS ON *ALLIUM CEPA* AND MALE FERTILITY IN TWO GENERATIONS OF MICE

Submitted to the School of Postgraduate Studies, University of Lagos for the award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

is a record of original research carried out

By

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In the Department of Cell Biology and Genetics

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DEDICATION

Dedicated to the Only Wise One of all ages, the Omniscient God, who gave the inspiration, the idea, the strength and the resources to see this programme through. To Him I ascribe all praise, glory and honour for what He has done, for what He is doing and for what He will do! Amen!

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ABSTRACT

The potential of the antiretroviral drugs zidovudine and nevirapine to induce genotoxicity was evaluated using the *Allium cepa* and the mice models. Four doses per antiretroviral were used in the *Allium cepa* chromosomal aberration assay while three doses per antiretroviral were used for the mouse study. The *Allium cepa* study consisted of the 96hr root growth inhibition, the 48hr recovery and the 48hr chromosomal aberration phases to determine the $EC_{50;}$ ascertain if root growth inhibition is reversible, and to observe chromosomal aberrations during mitosis respectively. In mice, the drugs were administered for the 56 days duration of spermatogenesis. Sperm counts, morphology anomalies, DNA content and quality (DNA fragmentation and nicks) were assessed in the parental (at mid- and full-term) and F_1 (at full-term) generations of mice. Dominant lethal assays were done and the birth statistics (gestation, litter size and birth weight) were compared to relate the effects on fertility. Exposure to zidovudine and nevirapine altered the mitotic phase distribution profile in *Allium cepa* with the dividing cells being mostly at telophase and prophase. Sticky chromosomes (23-62%) was the predominant aberration seen, followed by anaphase chromosomal bridges. In parental generation of mice, significant oligospermia (2.9 – 5.4 x 10^6/ml), increased anomalies in sperm morphology (2.1 – 10.6%) and elevated sperm DNA quantity (80.9 – 121.3 µg/ml) were observed. Sperm counts for the treatment groups were generally just above half the negative control value while almost five out of six treatment groups had their sperm anomaly indices at least twice that of the negative control. Fertility was increased about 3 times when both parents were ARV-treated contrasting with reduced fertility when only one parent was on ARVs. Shorter gestation periods (18.8 – 25.0 days) were recorded for all treatment groups compared to the unexposed controls (26 ± 1.4 days). Early foetal loss was evident in the nevirapine groups from the dominant lethal assays.

Across the parental and the F_1 generation, significant reductions in testis weight (p-values = 0.001, 0.006 and 0.011) and severe oligospermia (0.3 – 3.9 x 10^6/ml) were observed and reduced sperm DNA quantity (except for one group; 19.3 – 21.5 µg/ml) was recorded. In the F_1 generation, sperm DNA nicks were reduced 3-5 folds and fertility was reduced by 94.4%. In this study, *Allium cepa* demonstrated some earlier reported genotoxic effects of the ARVs administered, indicating it can be used to investigate their genotoxicity. Fertility in mice was enhanced when both parent received ARVs prior to mating. Mice that received zidovudine and nevirapine treatment prior to mating, whose parents were as well exposed to these drugs, exhibited highly reduced fertility. The reduced sperm counts, high abnormal sperm morphology and altered sperm DNA content amongst other effects observed in this study are attributable to the administration of zidovudine and nevirapine not HIV infection. It is recommended that when evaluating sperm DNA quality, the DNA nicks (via alkaline annealing method) had better correlation with reproductive outcomes and may thus be preferred.

1.0 INTRODUCTION

1.1 Background and Statement of Research Problem

The effectiveness of antiretroviral (ARV) drugs against the human immunodeficiency virus (HIV) for both therapy and in the prevention of mother-to-child transmission (PMTCT) of HIV has been well documented (Walker and Poirier, 2007). In non-breast-fed infants, the use of nucleoside reverse transcriptase inhibitor (NRTI) – based highly active antiretroviral therapy (HAART) has reduced the rate of perinatal HIV-1 transmission to less than 2% (Walker and Poirier, 2007).

However, there are many concerns about the side effects of such exposures to ARVs, particularly the perinatal exposures (Fretheim and Norderhaug, 2006). Reports from clinical studies of the effect on children born to HIV positive mothers have been varied. Culnane *et al.,* (1999) reported that the biometrics of such exposed children were not significantly different from unexposed children. However, Paul *et al.,* (2005) and Briand *et al.,* (2006) found some adverse effects, which were small and faded over time. Sub-clinically, the effects are numerous and include integration of the deoxynucleotides into mitochondrial DNA (mtDNA) / nucleic DNA, alterations in heterochromatin structure, mtDNA structure and number (Walker and Poirier, 2007). These sub-clinical effects may have long-ranging consequences, some of which have not been verified empirically in order to determine the actual risks since ARVs have been in use only since the 1980s.

Despite these observations, ARVs have continued to be used for therapy and to save neonates from HIV infection though possibly at the expense of their future generations. Antiretrovirals (ARVs) are becoming widely available in developing countries for the care and clinical management of HIV-infected persons, though the classes of ARVs in use are limited. Similarly, their use in the PMTCT of HIV is increasing and many children have become perinatally exposed to these drugs. In addition, many HIV-positive women already on ARV therapy (ART) become pregnant unexpectedly and expose their offspring to certain ARVs at very critical stages of pregnancy. Some of these perinatally exposed children have reached their teens, have sexual partners and desire to have children (Birungi *et al.,* 2009). Irrespective of their HIV status, these ARV-treated teenagers could have sexual partners who are either HIV-positive or negative. The cumulative impact of ARV exposures, if any, on their offspring is not known. This research set out to provide some information on these scenarios using animal models.

With the global efforts to make ARVs widely available for the care and treatment of HIV-positive persons, access to ARVs both for therapy and PMTCT is increasing. With the 2010 Nigerian National antenatal HIV sero-prevalence of 4.1% (range: 1.0-12.7%), an estimated 3.1 million people were living with HIV in Nigeria in 2010, of which 1.5 million require ART (FMOH, 2010). The Nigerian government is making efforts to improve access to ART for the estimated 1.3 million adults and 212,720 adolescents and children that require therapy (FMOH, 2010). This is because as at December 2008, only 236,000 people were accessing ART as against an estimated 2.95 million Nigerians living with HIV in 2008 (FMOH, 2008). In view of this, the need arises to investigate

the impact of these ARV exposures, including those given *in utero* and during the first 6 weeks of life for PMTCT, on fertility and other birth statistics.

Furthermore, even though viral replication in blood plasma is suppressed by antiretroviral drugs, virus in the male genital tract is often genetically and phenotypically unique and may not be suppressed (Chan and Ray, 2007). The viral compartmentalization and the varying degrees of ARVs' penetration of the male genital tract affect the effectiveness of ART with particular emphasis on sexual transmission. The incomplete suppression of HIV in the genital tract may yield drug-resistant virus and increase the risk of sexual transmission, thus drug manufacturers are optimizing their drugs to ensure good penetration of the male genital tract (Chan and Ray, 2007). Also Kwara *et al.,* (2008) reported varying concentrations of HAART component drugs in cervicovaginal fluids compared to blood plasma, and showed that some ARVs had high enough concentrations in cervicovaginal fluids to be inhibitory. Similar determinations of the concentration of 7 ARVs in the genital tract, cord blood and amniotic fluid amongst HIV-1 infected women gave amniotic fluid concentrations ranging from ≤ 6 – 100 % that of the blood plasma (Yeh *et al.,* 2009). All these further increase the potential for genotoxic effects due to ART and emphasize the need for this research.

1.2 Aim

The goal of this research was to investigate the impact the administration of zidovudine (ZDV) or nevirapine (NVP) have on mice testicular and sperm parameters, birth

statistics, as well as the intergenerational effects when either a single parent or both parents are receiving ARV drugs. In addition, the suitability of the *Allium cepa* root tip assay in demonstrating the genotoxicity of ZDV and NVP as part of a Reduce-Refine-Replace programme (RRR) was determined.

1.3 Specific Objectives

The specific objectives are to:

i. Assess the effect of administering either ZDV or NVP using the dominant lethal assay and birth statistics in mice, when either the "father-only" or "both-parents" received ARVs.

ii. Determine the impact of administering either ZDV or NVP on the testis and sperm parameters by performing the sperm count, the sperm morphology anomaly assay and by assessing the sperm DNA quality and quantity.

iii. Determine if any genotoxic effect observed resolves or accumulates over two generations of mice

iv. Demonstrate the genotoxicity of ZDV and NVP using the *Allium cepa* root tip assay and determine the assay's utility in elucidating possible cytogenotoxic mechanisms.

1.4 Significance of the Study

This study was designed to clearly ascertain whether any genotoxic effect on the male reproductive system and fertility was due to ARV therapy and not HIV. This distinction has not been possible before now because most research have been in HIV infected persons often on ARV therapy thus making it difficult to equate the toxic effects seen to HIV infection or to ARV therapy. Thus this study was done using mice which cannot be infected with HIV.

Ascertaining the aetiology of the genotoxic effects is further complicated when trying to evaluate the effect in exposed infants or to determine generational effects as the first set of exposed neonates are just reaching their reproductive age. Consequently, the mice model was used because they mature fast and thus will establish if any impact observed resolves or accumulates from generation to generation. Divi *et al.,* (2007) modeled this, using cultured human HeLa cells exposed to zidovudine for up to 77 passages and reported wide-spread mitochondrial morphological damage, mtDNA depletion and loss in membrane potential amongst others. However, Diwan *et al.,* (2000) exposed parental mice to ZDV, only during the critical period of male and female reproductive system development (days 12-18 of gestation) and reported no evidence of developmental reproductive toxicity in offspring up to the F_2 generation. Since this work seeks to investigate any impact on fertility in real life scenarios and ARVs are currently taken for life by HIV-infected persons, a different approach was used.

1.5 List of Abbreviations and Operational Definition of Terms

AIDS Acquired immune deficiency syndrome

AO Acridine orange

ART Antiretroviral (ARV) therapy

ARV Antiretroviral

BMI Body mass index

Cachexia: General ill health with emaciation

CD4 Cluster of differentiation 4 is a glycoprotein expressed on the surface of T helper cells, monocytes, macrophages and dendritic cells.

CMV Cytomegalovirus

COMET Single-cell gel electrophoresis assay

CSF Cerebrospinal fluid

DLA Dominant lethal assay

Double exposure: Both father and mother were treated with ARVs

DSB DNA double strand break

dsDNA Double-stranded DNA

EC_{50} The half maximal effective concentration refers to the concentration of a drug or toxicant which can induce a response halfway between the baseline and maximum after a specified period of time

F_1 The first filial generation which is comprised of offspring(s) resulting from a cross between strains of distinct genotypes. The F_1 generation is the generation resulting immediately from a cross of the first set of parents (parental generation).

F_2 The second filial generation which is comprised of offspring(s) resulting from a cross of the members of F_1 generation. The F_2 generation is the result of a cross between two F_1 individuals.

GPA Glycophorin A

HAART Highly active antiretroviral therapy

HBV Hepatitis B virus

HCV Hepatitis C virus

HIV Human Immunodeficiency Virus

HSV Herpes simplex virus

ICSI Intracytoplasmic sperm injection

IUI Intrauterine insemination

IVF *In vitro* fertilization

LD_{50} The median lethal dose of a toxin, radiation, or pathogen is the dose required to kill half the members of a tested population after specified test duration. LD_{50} Figures are frequently used as a general indicator of a substance's acute toxicity.

MI Mitotic index is the percentage of dividing cells among all cells seen

MIC Minimum inhibitory concentration

MN-RET Micro-nucleated reticulocytes

mtDNA Mitochondrial DNA

N150 Nevirapine 150 milligrams / kilogram dose or group

N5 Nevirapine 5 milligrams / kilogram dose or group

N50 Nevirapine 50 milligrams / kilogram dose or group

NC Negative control

NIMR Nigerian Institute of Medical Research

NNRTIs Non-nucleoside reverse transcriptase inhibitor drugs

NRTI Nucleoside reverse transcriptase inhibitor drugs

NVP Nevirapine

OECD Organization of Economic Cooperation and Development

P Parental generation**:** The first set of parents crossed in which their genotype is the basis for predicting the genotype of their offspring (filial generation). In parental generation, two individuals are mated to determine or predict the genotypes of their offspring.

PC Positive control

PIs Protease inhibitor drug

PMTCT Prevention of mother to child transmission

RBCs Red blood cells

RRR Reduce-Refine-Replace

RT Reverse transcriptase

SCSA Sperm chromatin structural assay

Single exposure: Father was the only parent treated with ARVs

SOPs Standard operating procedures

SSB DNA single strand break

ssDNA Single-stranded DNA

STDs Sexually transmitted diseases

TO Toluidine blue

TUNEL Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

UV Ultraviolet

Z10 Zidovudine 10 milligrams / kilogram dose or group

Z100 Zidovudine 100 milligrams / kilogram dose or group

Z250 Zidovudine 250 milligrams / kilogram dose or group

ZDV Zidovudine

2.0 LITERATURE REVIEW

2.1 HIV and Antiretrovirals (ARVs)

The Human immunodeficiency virus (HIV) is the retrovirus that causes the Acquired immunodeficiency syndrome (AIDS). It was discovered in the early 1980s in the USA. Currently, there is no known curative therapy for HIV/AIDS but several antiretroviral drugs (ARVs) are used in the clinical management. The effectiveness of ARVs against HIV both for therapy and in the prevention of mother-to-child transmission (PMTCT) of HIV has been well documented (Walker and Poirier, 2007). Highly active antiretroviral therapy (HAART) reduces the rates of morbidity and mortality of HIV disease, and has added years and better quality of life for HIV infected persons. Substantial decline in the risk of vertical transmission from 20.7% before ARVs to 6.5% after ARVs use for PMTCT in a 12-years study was reported (Simpson *et al.,* 2000). However, there are concerns about the side-effects of exposures to DNA-reactive ARVs, particularly perinatal exposures (Fretheim and Norderhaug, 2006).

There are different classes of ARVs. These include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, integrase inhibitors, maturation inhibitors and cellular inhibitors. The NRTIs inhibit reverse transcription by competing with the natural deoxynucleotides and become incorporated into the newly synthesized viral DNA strand as a faulty nucleotide. This causes a chemical reaction resulting in DNA chain termination and thus they are classified as competitive substrate inhibitors. The

NNRTIs mimic natural nucleotides and inhibit HIV reverse transcriptase directly by binding allosterically to the enzymes polymerase site and interfere with its function.

2.2 Zidovudine Drug

Most of the information on zidovudine (ZDV) drug was obtained from a review of the drug by manufacturer Merck Pharmaceuticals. The information was last reviewed in January 2010 (http://eglobalmed.com/core/MerckMultimedia/www.merck.com/mmpe/lexicomp/zidovudine.html).

Zidovudine ($C_{10}H_{13}N_5O_4$) is indicated for the treatment of HIV infection in combination with at least two other antiretroviral agents and for the prevention of maternal/foetal HIV transmission (PMTCT). It is also used for HIV post-exposure prophylaxis as part of a multidrug regimen. Zidovudine is a thymidine analog which interferes with the HIV viral RNA-dependent DNA polymerase action, resulting in the inhibition of viral replication. It is a white or brownish powder, sparingly soluble in water and soluble in ethanol. It melts at about 124 °C. It is a nucleoside reverse transcriptase inhibitor with significant penetration into the cerebrospinal fluid (CSF) and crosses the placenta. It has adequate diffusion from blood into CSF, with or without inflammation and exceeding usual minimum inhibitory concentration (MICs). Zidovudine is metabolised in the liver via glucuronidation to inactive metabolites. Its bioavailability ranges from 54% to 74% with terminal half-life elimination of 0.5 - 3 hours and time to peak half-life elimination in serum of 30 - 90 minutes. Orally administered ZDV is excreted in urine as 72 – 74% metabolites and 14 – 18%

unchanged drug while intravenously administered ZDV is excreted in urine as 45 – 60% metabolites and 18 – 29% unchanged drug.

Zidovudine crosses the placenta and adverse events have been observed in some animal reproduction studies. No increased risk of overall birth defects has been observed following first trimester exposure according to data collected by the antiretroviral pregnancy registry. The Perinatal HIV Guidelines Working Group considers ZDV the preferred NRTI for use in combination regimens during pregnancy. In HIV-infected mothers, not previously on antiretroviral therapy and who do not need therapy for their own health, treatment may be delayed until after 10-12 weeks gestation. Cases of lactic acidosis/hepatic steatosis syndrome have been reported in pregnant women receiving nucleoside analogues. It is not known if pregnancy itself potentiates this known side effect; however, pregnant women may be at increased risk of lactic acidosis and liver damage. Hepatic enzymes and electrolytes should be monitored frequently during the 3rd trimester of pregnancy in women receiving nucleoside analogues. Health professionals are encouraged to contact the antiretroviral pregnancy registry to monitor outcomes of pregnant women exposed to antiretroviral medications.

Zidovudine enters breast milk. In infants born to mothers who are HIV positive, HAART during breast-feeding may decrease postnatal infection. Infant prophylaxis with ZDV in combination with nevirapine or nevirapine alone may also decrease the risk of HIV transmission to the infant. However, maternal or infant antiretroviral therapy does not completely eliminate the risk of postnatal HIV transmission. In the

United States where formula is accessible, affordable, safe, and sustainable, complete avoidance of breast-feeding by HIV-infected women is recommended to decrease potential transmission of HIV.

Neonatal dosing of ZDV begins 6-12 hours after birth and continues for the first 6 weeks of life. Maternal oral dosing begins at 14-34 weeks of gestation and continues until start of labour. During labour and delivery, ZDV is administered as an intravenous continuously until the umbilical cord is clamped. ZDV has an LD_{50} of 1920mg/kg in mouse. Oral doses of ZDV are administered around-the-clock to promote less variation in peak and trough serum levels, and may be administered without regard to meals. While administering ZDV intravenously, rapid infusion or bolus injection is avoided. Neonates are often infused over 30 minutes while adults have a loading dose over one hour followed by continuous infusion dose. While on ZDV, the complete blood count and platelet count are monitored at least every 2 weeks, often together with the liver function tests, serum creatinine kinase, CD4 count and HIV viral load. The patients are also closely observed for the appearance of any opportunistic infections and/or for adverse reactions. Adverse reactions reported in adult patients with asymptomatic HIV infection are summarised in Table 1. Their frequency and severity may increase with advanced disease.

Table 1: Adverse Reactions Associated with Zidovudine Therapy

System affected	Adverse effects with frequencies	Adverse effects without frequencies
Central nervous system	Headache (63%), malaise (53%)	Anxiety, chills, confusion, depression, dizziness, fatigue, insomnia, loss of mental acuity, mania, seizure, somnolence, vertigo
Gastrointestinal	Nausea (51%), anorexia (20%), vomiting (17%), Constipation (6%)	Abdominal cramps, abdominal pain, dyspepsia, dysphagia, flatulence, mouth ulcer, oral mucosa pigmentation, pancreatitis, taste perversion
Hematologic	Granulocytopenia (2%; onset 6-8 weeks), anemia (1%; onset 2-4 weeks)	Aplastic anemia, hemolytic anemia, leukopenia, lymphadenopathy, pancytopenia with marrow hypoplasia, pure red cell aplasia
Hepatic	Transaminases increased (1 - 3%);	Hepatitis, hepatomegaly with steatosis, hyperbilirubinemia, jaundice, lactic acidosis
Neuromuscular and skeletal	Weakness (9%)	Arthralgia, back pain, CPK increased, LDH increased, musculoskeletal pain, myalgia, neuropathy, muscle spasm, myopathy, myositis, paresthesia, rhabdomyolysis, tremor
Cardiovascular	-	Cardiomyopathy, chest pain, syncope, vasculitis
Dermatologic	-	Pruritus, rash, skin/nail pigmentation changes, Stevens-Johnson syndrome, toxic epidermal necrolysis, urticarial
Endocrine & metabolic	-	Body fat redistribution, gynecomastia
Genitourinary	-	Urinary frequency, urinary hesitancy
Ocular:	-	Amblyopia, macular edema, photophobia
Otic	-	Hearing loss
Respiratory:	-	Cough, dyspnea, rhinitis, sinusitis
Miscellaneous:	-	Allergic reactions, anaphylaxis, angioedema, diaphoresis, flu-like syndrome, immune reconstitution syndrome

Source: http://eglobalmed.com/core/MerckMultimedia/www.merck.com/mmpe/lexicomp/zidovudine.html

Contraindications for ZDV therapy include life-threatening hypersensitivity to ZDV or any component of the formulation. ZDV is used with caution in patients with the bone marrow compromised. Similarly, dosage adjustment may be required in patients who develop anaemia or neutropenia. ZDV is also used with caution in patients with risk factors for liver disease. ZDV treatment is suspended in any patient who develops clinical or laboratory findings suggestive of lactic acidosis or hepatotoxicity. In addition, ZDV is also administered with caution in patients with severe renal impairment, with some dosage adjustment. Zidovudine has concurrent drug therapy issues with interferon alfa/ribavirin. Thus, it is used with caution in combination with interferon alfa with or without ribavirin in HIV/HBV co-infected patients. These patients are monitored closely for hepatic decompensation, anaemia or neutropenia. Dose reduction or discontinuation of interferon and/or ribavirin may be required if toxicity becomes evident. Pereira *et al.,* (2002) used paired seminal plasma and blood plasma to determine compartmental drug concentration ratios and reported that for ZDV, seminal plasma concentrations approximated blood plasma concentrations early but became greater later in the dosing interval.

Zidovudine in undiluted intravenous formulation vials is stored at 15°C to 25°C and protected from light. When diluted, the solution is physically and chemically stable for 24 hours at room temperature and 48 hours if refrigerated. Zidovudine tablets, capsules and syrup are stored at 15°C to 25°C. The capsules are stored to protect them from moisture.

2.3 Nevirapine Drug

Most of the information on nevirapine (NVP) was obtained from a review of the drug by the manufacturer Boehringer-Ingelheim Pharmaceuticals in 2008. It was downloaded in May 2012 from http://bidocs.boehringer-ingelheim.com/BIWebAccess/ViewServlet .ser?docBase=renetnt&folderPath=/Prescribing+Information/PIs/Viramune/Viramune.p df.

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1. Thus NVP is indicated for combination antiretroviral treatment of HIV-1 infection. Nevirapine binds directly to reverse transcriptase (RT) and blocks the RNA dependent and DNA-dependent DNA polymerase activities by causing a disruption of the enzyme's catalytic site. The activity of NVP does not compete with template or nucleoside triphosphates. HIV-2 RT and eukaryotic DNA polymerases (such as human DNA polymerases α, ß, γ, or δ) are not inhibited by NVP.

Nevirapine ($C_{15}H_{14}N_4O$) has the brand name of Viramune®. It is structurally a member of the dipyridodiazepinone chemical class of compounds. It is a white to off-white crystalline powder with the molecular weight of 266.3g. Nevirapine tablets are used for oral administration. Each tablet contains 200 mg of NVP and other inactive ingredients. Nevirapine suspension is for oral administration in neonates and children. Each 5 ml of

nevirapine suspension contains 50 mg of nevirapine (as nevirapine hemihydrate). The suspension also contains the other excipients and purified water.

In adults, NVP is readily absorbed (greater than 90%) after oral administration in healthy volunteers and in adults with HIV-1 infection. Absolute bioavailability in 12 healthy adults following single-dose administration was 93 ± 9% (mean ± SD) for a 50 mg tablet and 91 ± 8% for an oral solution. Peak plasma NVP concentrations of 2 ± 0.4 µg/ml (7.5 micromolar) were attained by 4 hours following a single 200 mg dose. Following multiple doses, nevirapine peak concentrations appear to increase linearly in the dose range of 200 to 400 mg/day. Steady-state trough (lowest) NVP concentrations of 4.5 ± 1.9 µg/ml (17 ± 7 micromolar, n = 242) were attained at 400 mg/day. NVP tablets and suspension have been shown to be comparably bioavailable and interchangeable at doses up to 200 mg. NVP may be administered with or without food, antacid or didanosine.

Nevirapine is highly lipophilic and is essentially non-ionized at physiologic pH. Following intravenous administration to healthy adults, the apparent volume of distribution of NVP was 1.21 ± 0.09 l/kg, suggesting that NVP is widely distributed in humans. NVP readily crosses the placenta and is also found in breast milk. NVP is about 60% bound to plasma proteins in the plasma concentration range of 1-10 mcg/ml. NVP concentrations in human cerebrospinal fluid were 45% (±5%) of the concentrations in plasma; this ratio is approximately equal to the fraction not bound to plasma protein. *In vivo* trials in humans and *in vitro* studies with human liver

microsomes have shown that nevirapine is extensively bio-transformed via cytochrome P450 (oxidative) metabolism to several hydroxylated metabolites. In the multinational pharmacokinetic sub-study of 1077 subjects that included 391 females; female subjects showed a 13.8% lower clearance of nevirapine than did men. Since neither body weight nor Body Mass Index (BMI) had an influence on the clearance of NVP, the effect of gender cannot solely be explained by body size. NVP pharmacokinetics in HIV-1-infected adults does not appear to change with age (range 18–68 years); however, NVP has not been extensively evaluated in subjects beyond the age of 55 years.

The antiviral activity of NVP has been measured in a variety of cell lines including peripheral blood mononuclear cells, monocyte-derived macrophages, and lymphoblastoid cell lines. In an assay using human embryonic kidney 293 cells, the median EC_{50} value (50% inhibitory concentration) of NVP was 90nM against a panel of 2,923 isolates of HIV-1 that were primarily (93%) clade B clinical isolates from the United States. The 99th percentile EC_{50} value was 470nM in the trial. The median EC_{50} value was 63nM (range 14-302nM, n=29) against clinical isolates of HIV-1 clades A, B, C, D, F, G, and H, and circulating recombinant forms CRF01_AE, CRF02_AG and CRF12_BF. NVP had no antiviral activity in cell culture against group O HIV-1 isolates (n=3) or HIV-2 isolates (n=3) replicating in cord blood mononuclear cells. NVP in combination with efavirenz exhibited strong antagonistic anti-HIV-1 activity in cell culture and was additive to antagonistic with the protease inhibitor ritonavir or the fusion inhibitor enfuvirtide. NVP exhibited additive to synergistic anti-HIV-1 activity in combination with the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, saquinavir and tipranavir; and the NRTIs abacavir, didanosine, emtricitabine,

lamivudine, stavudine, tenofovir and ZDV. The anti-HIV-1 activity of nevirapine was antagonized by the anti-HBV drug adefovir and by the anti-HCV drug ribavirin in cell culture.

Long-term carcinogenicity studies of nevirapine have been carried out in mice and rats (http://bidocs.boehringer-ingelheim.com/BIWebAccess/ViewServlet.ser?docBase =renetnt&folderPath=/Prescribing+Information/PIs/Viramune/Viramune.pdf). Mice were dosed with 0, 50, 375 or 750 mg/kg/day for two years. Hepatocellular adenomas and carcinomas were increased at all doses in males and at the two high doses in females. In studies in which rats were administered NVP at doses of 0, 3.5, 17.5 or 35 mg/kg/day for two years, an increase in hepatocellular adenomas was seen in males at all doses and in females at the high dose. The systemic exposure at all doses in the two animal studies was lower than that measured in humans at the 200 mg twice daily dose. The mechanism of the carcinogenic potential is unknown.

However, in genetic toxicology assays, nevirapine showed no evidence of mutagenic or clastogenic activity in a battery of *in vitro* and *in vivo* studies. These included microbial assays for gene mutation (Ames: *Salmonella* strains and *E. coli*), mammalian cell gene mutation assay (*CHO*/*HGPRT*), cytogenetic assays using a Chinese hamster ovary cell line and a mouse bone marrow micronucleus assay following oral administration. Given the lack of genotoxic activity of nevirapine, the relevance to humans of hepatocellular neoplasms in nevirapine-treated mice and rats is not known. In reproductive toxicology studies, evidence of impaired fertility was seen in female rats at doses providing

systemic exposure approximately equivalent to that provided with the recommended clinical dose of NVP. Animal studies have shown that NVP is widely distributed to nearly all tissues and readily crosses the blood-brain barrier.

No observable teratogenicity was detected in reproductive studies performed in pregnant rats and rabbits. The maternal and developmental no-observable-effect level dosages produced systemic exposures approximately equivalent to or approximately 50% higher in rats and rabbits, respectively, than those seen at the recommended daily human dose. In rats, decreased foetal body weights were observed due to administration of a maternally toxic dose (exposures approximately 50% higher than that seen at the recommended human clinical dose).

There are no adequate and well-controlled trials of NVP in pregnant women. The Antiretroviral Pregnancy Registry, which has been surveying pregnancy outcomes since January 1989, has not found an increased risk of birth defects following first trimester exposures to NVP. The prevalence of birth defects after any trimester exposure to NVP is comparable to the prevalence observed in the general population. Severe hepatic events, including fatalities, have been reported in pregnant women receiving chronic NVP therapy as part of combination treatment of HIV-1 infection. NVP should therefore be used during pregnancy only if the potential benefit justifies the potential risk to the foetus. The Centers for Disease Control and Prevention recommend that HIV-1 infected mothers not breastfeed their infants to avoid risking postnatal transmission of HIV-1. NVP is excreted in breast milk. Because of both the potential for

HIV-1 transmission and the potential for serious adverse reactions in nursing infants, mothers should be instructed not to breastfeed if they are receiving NVP.

Intensive clinical and laboratory monitoring, including liver enzyme tests, is essential at baseline and during the first 18 weeks of treatment with nevirapine. The optimal frequency of monitoring during this period has not been established. In some cases, hepatic injury has progressed despite discontinuation of treatment. Severe, life-threatening skin reactions, including fatal cases, have occurred in patients treated with nevirapine. These have included cases of Stevens-Johnson syndrome, toxic epidermal necrolysis, and hypersensitivity reactions characterized by rash and organ dysfunction. Patients developing signs or symptoms of severe skin reactions or hypersensitivity reactions are discontinued and medical evaluation done immediately. The most serious adverse reactions associated with nevirapine are hepatitis/hepatic failure, Stevens-Johnson syndrome, toxic epidermal necrolysis, and hypersensitivity reactions. Severe, life-threatening, and in some cases fatal hepatotoxicity, including fulminant and cholestatic hepatitis, hepatic necrosis and hepatic failure, have been reported in patients treated with nevirapine. Rash was observed in approximately half of the subjects with symptomatic hepatic adverse events. Fever and flu-like symptoms accompanied some of these hepatic events.

Nevirapine induces hepatic cytochrome P450 metabolic isoenzymes 3A and 2B6. Co-administration of NVP and drugs primarily metabolized by CYP3A or CYP2B6 may result in decreased plasma concentrations of these drugs and attenuate their therapeutic

effects. NVP does not appear to affect the plasma concentrations of drugs that are substrates of other CYP450 enzyme systems.

2.4 ARVs and Genotoxic Potential

The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) have genotoxic potential because they are DNA-reactive and can penetrate the blood-testis barrier in varying degrees (Chan and Ray, 2007). Their mode of operation is that they inhibit the HIV reverse transcriptase and cellular polymerases, notably mtDNA polymerase. And being the first line drugs and the backbone of the 3 drugs used in the HAART used for clinical management and for PMTCT, many get exposed to them. Inhibition of DNA polymerase gamma and other mitochondrial enzymes can gradually lead to mitochondrial dysfunction and cellular toxicity (Kakuda, 2000). Toxicity caused by NRTIs does not always appear to arise through the mitochondrial route. ZDV-related anaemia appears to be related to decreased globin RNA synthesis (Moyle, 2000). The integration of nucleoside analogues into nuclear DNA, best documented with ZDV but likely to occur with other agents, represents an alternative but potentially delayed pathway to cytotoxicity and cell apoptosis (Moyle, 2000). Several physiopathologic mechanisms interact to account for the observed toxicity including oxidative stresses related to HIV infection and NRTIs (Kohler and Lewis, 2007) and HIV itself (Gerard *et al.,* 2006)

2.5 ARVs and Fertility

The demand for assisted reproduction in HIV discordant and concordant couples are expected to rise as life expectancy for patients on HAART improves and vertical transmission risk falls to <1% (Frodsham *et al.,* 2004). Frodsham *et al.,* (2004) reported more HIV-discordant couples (68) seeking assisted fertility than HIV concordant couples (5). Similarly, out of 85 couples seeking assisted reproduction, 71 were HIV-discordant couples while 14 were HIV-concordant couples (Manigart *et al.,* 2006). The rate of pregnancy loss after *in vitro* fertilization (IVF) was elevated in HIV-infected couples (Manigart *et al.,* 2006). In couples affected by HIV, the worst results were obtained when both partners were infected but this may be due to the small sample size and/or a higher mean age of the patients in this cohort compared to the others (Manigart *et al.*, 2006). With oocyte donation in HIV-infected women, these differences disappeared, suggesting that poor IVF results may be due to reduced ovarian response and/or oocyte quality in HIV-positive women (Manigart *et al.,* 2006). Coll *et al.,* (2006) reported that HIV-infected women undergoing IVF have a lower pregnancy rate. The problem appears to be with oocyte quality and as HIV has receptors on the human oocyte surface, a potential hypothesis might be mitochondrial dysfunction as a result of the use of ARVs (Coll *et al.,* 2006). Besides these, HIV-infected women have some prevalent gynaecologic issues such as menstrual anomalies, lower genital tract neoplasia, sexually transmitted infections, the need for gynaecologic surgery, and menopausal issues including osteopenia / osteoporosis (Cejtin, 2008).

2.6 ARVs and Child Anthropometry Information

Culnane *et al.,* (1999) reported that uninfected children born to HIV-infected women receiving ART during pregnancy showed virtually no adverse clinical effects at birth, with median age of children at time of last follow-up visit being 4.2 years (range, 3.2-5.6 years). Physical growth measurements, immunologic parameters, cognitive / developmental function, occurrence of neoplasms, mortality data, baseline echocardiogram and funduscopic evaluation showed no significant differences between children exposed to ZDV and those who received placebo (Culnane *et al.,* 1999).

No adverse effects were observed in HIV-uninfected children with *in utero* and neonatal exposure to ZDV but they suggested continued prospective evaluations to assess the long-term safety of interventions (Culnane *et al.,* 1999). Another study in Thailand used 1408 children in a trial comparing ZDV regimens of different durations and the anthropometric measurements until 18 months of age. Children exposed *in utero* for >7.5 weeks had a slightly lower birth weight ($p = 0.003$; Briand *et al.,* 2006). However, ZDV exposure had no effect on the evolution of Z-scores from 6 weeks to 18 months of age (Briand *et al.,* 2006). Although longer exposure had a negative impact on birth weight; the magnitude of this effect was small and faded over time and neither the total nor the postnatal duration of exposure was associated with changes in infant Z-scores from 6 weeks to 18 months of age (Briand *et al.,* 2006).

2.7 ARVs and Subclinical Manifestations

Though there seems to be lack of clear clinical effects of ARV exposure, yet there have been reports on many subclinical effects. Senda *et al.,* (2007) have reported altered heterochromatin organization of chromosome 1 (1q12) after ZDV therapy. Children of HIV-positive mothers were found to be at risk for mitochondrial damage, which further increased if mothers received ZDV during pregnancy (Poirier *et al.,* 2003). Monkey foetuses exposed to ZDV plus lamivudine sustain a higher level of drug-DNA incorporation and showed evidence of more telomere damage than monkey foetuses exposed to ZDV alone (Olivero *et al.,* 2002). Meng *et al.,* (2007) found low levels of ZDV-DNA incorporation in NRTI-treated children and supports the continued use of ZDV-based therapies during pregnancy but with long-term monitoring of exposed infants for adverse health effects. Moderate to severe mitochondrial morphological damage was observed by electron microscopy in umbilical cords from six of nine infants born to HIV-1-infected mothers taking ZDV plus lamivudine while none of seven unexposed infants showed similar damage (Divi *et al.,* 2004). Compared to unexposed infants, significant mtDNA depletion was observed in umbilical cord (P = 0.006) and cord blood (P = 0.003) from drug-exposed infants (Shiramizu *et al.,* 2003; Divi *et al.,* 2004).

In ZDV-exposed infants, transplacentally micronucleated reticulocyte frequencies (%MN-RET) were evaluated in mother-infant pairs. Tenfold increase in %MN-RET was seen in women and infants who received ZDV-containing ART prenatally but no

increases were detected in three women and infants who received prenatal ART without ZDV (Witt *et al.,* 2007). ZDV-exposed new-borns had %MN-RET decreasing over the first 6 months of life to levels comparable to cord blood controls (Witt *et al.,* 2007). Long-term monitoring of HIV-uninfected ZDV-exposed infants was recommended to ensure their continued health (Witt *et al.,* 2007). The glycophorin A (GPA) somatic cell mutation assay, which screens for large-scale DNA damage in red blood cells (RBCs), showed clear evidence that GPA variants arising from chromosome loss and duplication, somatic recombination, and gene conversion, were significantly elevated in mother-child pairs receiving prepartum ZDV plus lamivudine (Escobar *et al.,* 2007). And the elevations in GPA variants persisted through 1 year of age in NRTIs-exposed children thus justifying their surveillance for long-term genotoxic consequences (Escobar *et al.,* 2007). Similarly, exposure of cells either to relatively high levels of ZDV-lamivudine short-term (100 µM, 3 days), or to peak plasma-equivalent levels of ZDV-lamivudine for an extended period (10 µM, 30 days), resulted in drug-induced mutagenic responses (Torres *et al.,* 2007). Among sets of mice necropsied on days 13, 15, or 21 postpartum, *Hprt* mutant frequencies in T-cells were significantly elevated in the ZDV-only and ZDV-lamivudine groups at 13 days of age (Torres *et al.,* 2007). These results suggest that the mutagenicity by these nucleoside analogues is driven by cumulative dose, and raises the question of whether ZDV-lamivudine has greater mutagenic effects than ZDV alone in prenatally exposed children. (Torres *et al.,* 2007)

2.8 HIV, ART and the Genitourinary system

With the AIDS pandemic, concerns about the sexual transmission of viruses in humans and its health consequences have been reviewed by Dejucq and Je´Gou (2001). Many viruses have been found in the mammalian male urogenital system (Figure 1) and raise a lot of concerns. These concerns range from the transmission of diseases to infertility/sterility resulting from changes in testicular compartments and T-cell-mediated response to spermatozoa due to leukocytes infiltration into the reproductive tract or semen (Dejucq and Je´Gou, 2001). Other concerns include cachexia from reduced testosterone production, the infection of the gametes and embryo resulting in miscarriage, embryonic and foetal abnormalities. This includes the possibility of viral genome integration into germ cell genome and then being transmitted to subsequent generations (Dejucq and Je´Gou, 2001).

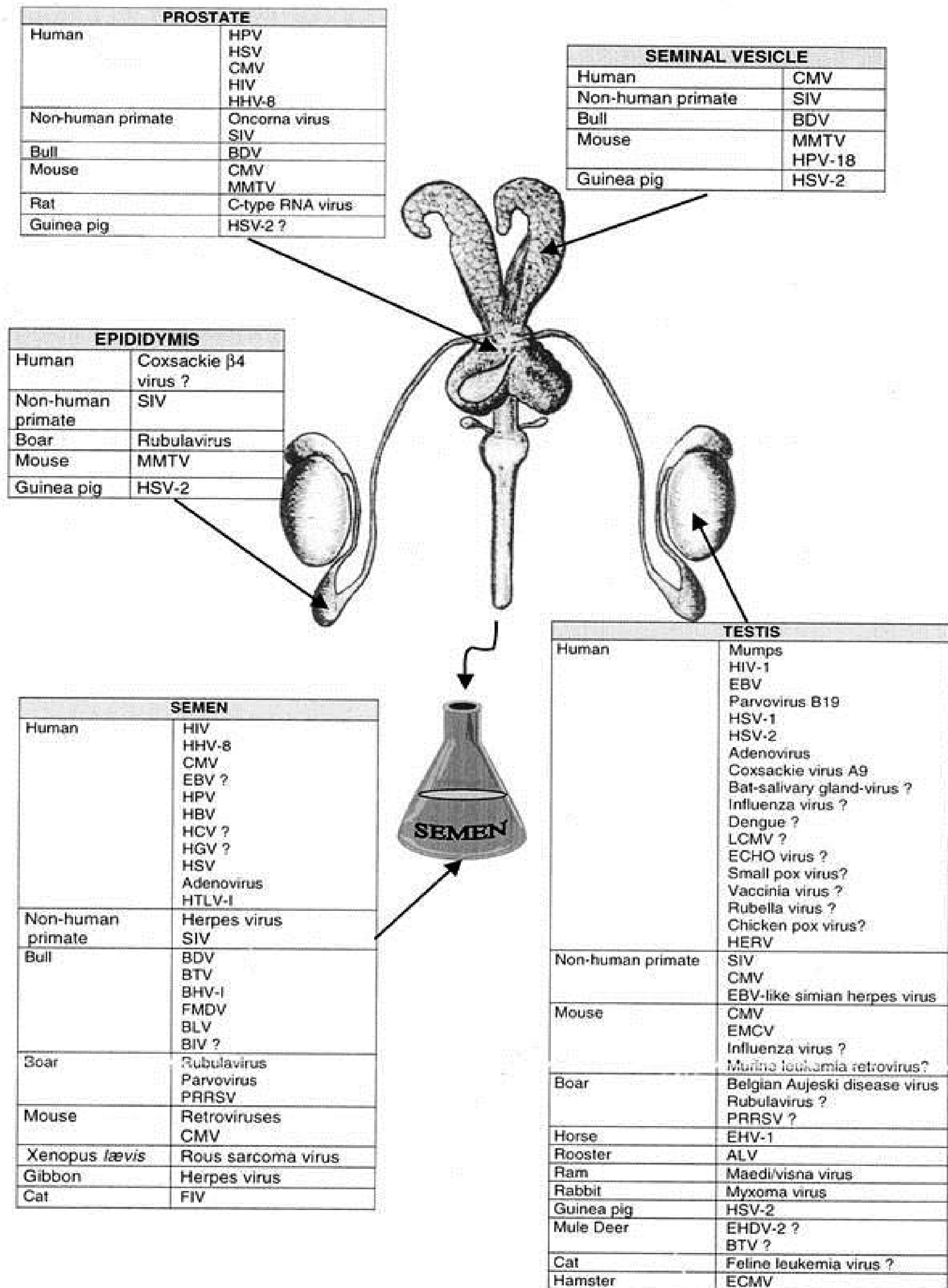

Figure 1: Viruses found in the Genital Tract and Semen of Mammals (Source: Dejucq and Je´Gou, 2001)

The presence of HIV in the semen has been established but the nature of the cells infected remains unclear. It was initially isolated from the mononuclear cell fraction of the semen of two men developing AIDS and from one HIV-1-seropositive man, but has also been shown to be transmitted via the semen of asymptomatic carriers (Dejucq and Je´Gou, 2001). These findings led to the theories that the epididymal epithelial cells may become infected and shed HIV into the epididymal fluid, and/or that the prostate and seminal vesicles may also act as a virus reservoir and shed HIV into the semen. These theories are in addition to the contribution of HIV from macrophages and lymphocytes, which are natural targets for HIV and often present in the semen (Dejucq and Je´Gou, 2001).

The presence of HIV-1 in the spermatozoa themselves is a matter of debate. This would need early resolution since spermatozoa from HIV-positive men cleared of seminal plasma and infected mononuclear cells are used for medically assisted reproduction in serodiscordant couples (Dejucq and Je´Gou, 2001). Thus, although semen washing before artificial insemination may reduce the risk of HIV transmission, HIV may still be detected in the fraction of motile spermatozoa. Electron microscopy and immunocytochemistry studies have provided evidence that HIV-1 can attach to the surface of spermatozoa and enter these cells through the intact plasma membrane; and that spermatozoa from healthy donors may carry HIV-1 on their surface and transmit it to lymphocytes in culture has been demonstrated (Dejucq and Je´Gou, 2001). However, it is unclear if the virus penetrates and replicates in these cells or simply integrates into them. HIV may also infect germ cells early in spermatogenesis, resulting in the clonal transmission of the virus into spermatozoa (Dejucq and Je´Gou, 2001).

The effect of HIV infection on semen characteristics has been investigated. Men with AIDS (advanced disease state) had pyospermia and grossly abnormal spermatozoa but semen from other seropositive men did not differ significantly from those of healthy seronegative donors. No abnormality in sperm count, morphology, number or type of leukocytes in semen, or any other semen characteristic was associated with HIV shedding into semen (Dejucq and Je´Gou, 2001).

Concomitant STDs such as cytomegalovirus (CMV), chancroid, syphilis, gonorrhoea, and Chlamydia infections may affect the level of HIV shedding. Herpes simplex virus (HSV) increases plasma HIV levels several folds and this increase may be reflected in seminal fluid (Dejucq and Je´Gou, 2001). In a study comparing two groups of HIV-1 patients, with dermatological or genital infections (urethritis), they found that the median HIV RNA level in the semen of the group with dermatological infections was one-eighth that of the group with genital infections. In the group with genital infections, the subgroup of patients with gonorrhoea had the highest seminal viral load and the antibiotic treatment of urethritis reduced the viral load in the semen but did not affect the plasma viral load (Dejucq and Je´Gou, 2001). This raises the question of viral compartmentalization in the genital system. The precise identification of the viral reservoir in the body is of the utmost importance for antiretroviral treatment. Thus, although the development of potent treatments raises the hope that HIV-1 eradication might be possible, we do not yet know whether all the compartments in which the virus replicates are accessible to antiretroviral compounds. Replication competent virus was detected in the semen of HIV-infected men receiving antiretroviral treatment, although

there was no detectable virus in the peripheral plasma (Dejucq and Je´Gou, 2001). In light of these studies, it appears that treatment strategies for the complete elimination of HIV-1 from the genital tract should now be a public health priority.

2.9 ARVs and Sperm Parameters

Gresenguet *et al.,* (1992) reported that semen from HIV-1 infected African individuals compared to healthy HIV-1 seronegative individuals, showed oligospermia, azoospermia and asthenospermia. Robbins *et al.,* (2001) followed 26 HIV-infected persons for 12 weeks, assessing sperm parameters at baseline and at different time-points after initiation of ART. Their findings suggested that nucleoside-containing HAART administered via recommended protocols do not induce chromosomal changes in lymphocytes or sperm but may produce improvements in semen quality. However, they followed up for 12 weeks which did not completely cover the duration of spermatogenesis in man (≈ 90 days). In spite of this, they reported a significant treatment-related sperm aneuploidy XY18-18 and the aggregate frequency for the sperm aneuploidy XY18 was twice the mean value reported for healthy men (Robbins *et al.,* 2001).

Semen volumes, percentages of progressive motile spermatozoa and total sperm counts were decreased, while the pH values and spermatozoa multiple anomaly indices were increased in HIV-infected patients (Bujan *et al.,* 2007). La Sala *et al.,* (2007) reported alterations in semen / sperm parameters for HIV-infected persons and attributed the effects to HAART not HIV infection. van Leeuwen *et al.,* (2008) prospectively

followed 34 men about to start combination ART till 48 weeks on therapy and were able to establish that therapy negatively affected the percentage of progressively motile spermatozoa. Whether this reduced motility affects the chances of fathering a child and/or leads to an increased need for assisted reproduction is at present unknown.

2.10 Sperm DNA Abnormalities, Male Fertility and ARV Therapy

To be fertile, the spermatozoon must be capable of undergoing decondensation at an appropriate time in the fertilization process so that the DNA template can be read and transcribed. If sperm DNA is unable to decondense after entering the ooplasma, fertilization may fail or a post-fertilization failure could occur due to the defective sperm DNA. Routine sperm / semen analyses mostly assess the ability of the sperm to reach the oocyte and not necessarily the more important ability to fertilize the oocyte, activate and sustain embryonic growth. Assessing sperm DNA quality would fill this gap in the diagnosis of male infertility (Agarwal and Allamaneni, 2005). Infertile men manifest various alterations in their sperm DNA including an abnormal chromatin structure, chromosomes with microdeletions, aneuploidies and DNA strand breaks (Agarwal and Said, 2003). Saleh *et al.,* (2002) evaluated sperm DNA in infertile men having normal standard sperm parameters and they showed that such men had a significant increase in sperm DNA damage. Therefore it is possible that some cases of unexplained or idiopathic infertility, when the traditional semen analysis falls into normal range and no evident female reproductive system pathologies can be revealed, may be explained by a hidden abnormality of the sperm DNA. Carrell *et al.,* (2003) compared the sperm from male partners of couples with unexplained recurrent pregnancy loss to the general population and to fertile donors and found no significant

difference in sperm concentration and progressively motile sperm counts between them. However, they reported a significant increase in the percentage of sperm with increased aneuploidy, increased DNA fragmentation (via TUNEL assay), and increased tapered morphology in couples with unexplained recurrent pregnancy loss (Carrell *et al.,* 2003). In contrast to the lack of correlation between the aneuploid sperm and any semen quality parameter, they reported a strong correlation between aneuploidy rates and sperm DNA fragmentation and postulated aneuploid sperm might be the result of a defective (leaky) checkpoint mechanism employing cellular apoptosis or another checkpoint after meiosis or spermiogenesis are nearly complete (Carrell *et al.,* 2003). Bellver *et al.,* (2010) similarly evaluated sperm DNA oxidation, fragmentation (via Sperm chromatin dispersion assay) and Y chromosome microdeletions in three cohorts: a) fertile sperm donors; b) men with severe oligozoospermia but without history of recurrent spontaneous abortions; and c) men from couples who had experienced idiopathic recurrent spontaneous abortions. They concluded that features of sperm DNA and Y chromosome microdeletions do not seems to be related to idiopathic recurrent spontaneous abortions and no causative role for sperm DNA fragmentation could be demonstrated (Bellver *et al.,* 2010).

Three main theories have been postulated for the origin of sperm DNA damage. These are defective sperm chromatin packaging while exchanging histones for protamines in sperm DNA; defective apoptosis that results in inability to clear abnormal spermatozoa from the system, and oxidative stress (Agarwal and Said, 2003; Erenpreiss *et al.,* 2006; Tamburrino *et al.,* 2012). A possible fourth mechanism is deficiencies in recombination checkpoints in meiotic prophase where a defective checkpoint may lead to persistent

sperm DNA fragmentation in ejaculated spermatozoa (Erenpreiss *et al.,* 2006). More importantly, there is no consensus on which methodology to use to evaluate sperm DNA damage and many of the methods currently in use do not have clinical correlations with fertility or reproductive outcomes. Many of the currently available methods for assessing sperm DNA quality provide different kinds of information on the sperm DNA status depending on the principle(s) of the method and there is need to identify which of these methods play a crucial role in sperm genomic function (Agarwal and Allamaneni, 2005). The question still remain whether these methods reveal the same type of damage, whether they obtain comparable results and whether they are standardized (Tamburrino *et al.,* 2012). Determining the critical point(s) or activities involving the gamete DNA and relating these to assay principle(s) will help determine which will be more valuable.

For instance, the activation of embryonic genome expression occurs at the 4 – 8 cell stage in human embryos and this suggests that the paternal genome may not be active until that stage (Erenpreiss *et al.,* 2006). Thus evaluating levels of sperm DNA strand breaks and its effect on the decondensation ability of the sperm DNA may be of some importance. The three commonly used methods are terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay (TUNEL), single-cell gel electrophoresis assay (COMET) and Sperm chromatin structural assay (SCSA). The first two are direct methods for assessing fragmented sperm DNA while the third utilizes chromatin structural probes and flow cytometry (Table 2). Thus far, sperm DNA assessment using Sperm Chromatin Structure Assay (SCSA) has proved to be the best predictor of reproductive outcome and some clinical threshold is being established with relation to assisted reproductive technologies (Agarwal and Said, 2003).

Table 2: Methods and Principles Used to Assess Sperm Chromatin / DNA Integrity

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clinical correlation
TUNEL assay	Quantifies the incorporation of dUTP at breaks in double-stranded DNA; Catalyzed by terminal deoxynucleotidyl transferase	Bright field microscopy; Fluorescence microscopy; Flow cytometry	Sensitive exclusively for DNA DSBs and SSBs; Correlates well with other assays like SCSA, TB and COMET	Relatively expensive, labour consuming, High intra-assay variability and inter-lab variability	no
COMET assay	Quantifies DNA SSBs and DSBs using electrophoresis of DNA-fluorochrome-stained single sperm cells	Fluorescence microscopy	High level of sensitivity	Time consuming; Needs computer-assisted image analysis; High inter-assay variability, different inter-lab protocol options and high variability in data report format; Difficult to standardize	No
a) Alkaline COMET assay (pH ≥ 12)	Denatures sperm DNA and therefore identifies both DNA SSBs and DSBs	Fluorescence microscopy	High sensitivity as identifies both DNA SSBs and DSBs	Possible overestimation of DNA breaks due to induced conversion of alkali-labile sites into breaks	No
b) Neutral COMET assay (pH < 9)		Fluorescence microscopy	Specific for the detection of DNA DSBs	Low sensitivity	No
SCSA	Measures *in situ* DNA susceptibility to the acid-induced conformational helix-coil transition by AO fluorescence staining	Flow cytometry	Quantitative detection of DNA breaks and sperm with nuclear immaturity; Extensively standardized; High statistical robustness; High intra- and inter-lab repeatability	Need flow cytometer and dedicated software	Yes

COMET ≈ single-cell gel electrophoresis assay; SCSA ≈ sperm chromatin structural assay; TUNEL ≈ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay; DSB ≈ double strand break; SSB ≈ single strand break; TB ≈ toluidine blue; AO ≈ acridine orange.
Source: Erenpreiss *et al.,* 2006.

Establishing clinical threshold becomes relevant as it has been shown that spermatozoa with high DNA damage contribute to successful fertilization and *in vitro* development; however the percentage of such sperm in the semen is crucial (Erenpreiss *et al.,* 2006). Studies have now provided consistent evidence that sperm containing fragmented DNA can be alive, motile, morphologically normal and able to fertilize an oocyte (Tamburrino *et al.,* 2012). However different data/thresholds exist in the literature for the levels of sperm DNA fragmentation in fertile and subfertile men, and the lack of agreement among the different studies evaluating their impact on assisted reproductive outcomes reflects how different methods may affect the results (Tamburrino *et al.,* 2012). A systemic meta-analysis of papers reporting the relationship between sperm DNA damage and reproductive outcomes shows that when data are pooled according to the method (TUNEL and SCSA) employed in the study, some conclusions could be drawn (Li *et al.,* 2006; Tamburrino *et al.,* 2012). Li *et al.,* (2006) reported that sperm DNA damage assessed by the TUNEL assay significantly decreases only the chance of IVF clinical pregnancy, but not that of either IVF fertilization or ICSI fertilization or ICSI clinical pregnancy. However, sperm DNA damage assessed by the SCSA had no significant effect on the chance of clinical pregnancy after IVF or ICSI treatment (Li *et al.,* 2006). Since the techniques reveal different types of damage, the results from studies detecting sperm DNA damage with different methods are not comparable except when pooled by the assay method used.

Sperm DNA damage has a negative correlation with embryo quality following IVF and ICSI ($r = \pm 0.70$; $p = 0.03$) (Saleh *et al.*, 2003), but whether DNA-damaged spermatozoa

can impair the process of embryo development remains unclear. Nonetheless, it has been reported that damage to sperm DNA may be linked to an increase in early embryonic death (Agarwal and Said, 2003; Carrell *et al.,* 2003). In addition, another potential consequence of sperm DNA damage is infertility in the offspring of the next generation (Agarwal and Said, 2003). Deletions in the Y-chromosome that leads to male infertility was postulated to have arisen *de novo* in the germ line of the patient's father as the fathers of the affected patients did not have these deletions (Agarwal and Said, 2003). It has been proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization while the oocyte attempts to repair DNA damage prior to the initiation of the first cleavage (Erenpreiss *et al.,* 2006). Mutations occurring at the first cleavage may become fixed in the germline and may be responsible for infertility, childhood cancer and a high risk of imprinting diseases in the offspring (Erenpreiss *et al.,* 2006).

There is now some evidence that the oocyte is able to repair DNA damage but the extent of this repair depends on the type of DNA damage present in the sperm and on the quality of the oocyte (Ménézo *et al.,* 2010; Tamburrino *et al.,* 2012). However, whether all types of DNA damage can be repaired is not clear as double-stranded DNA breaks appear to be less repairable than single stranded breaks (Tamburrino *et al.,* 2012). Within the fertilized oocyte, sperm DNA damage can be repaired during the period between sperm entry into the cytoplasm and the beginning of the next S phase, by virtue of pre- and post-replication mechanisms (Agarwal and Said, 2003). However, the biological impact of abnormal sperm chromatin structure depends on the combined effects of the level of chromatin damage in the spermatozoa and the capacity of the

oocyte to repair that pre-existing damage (Agarwal and Said, 2003). As fertilisation may occur normally even when sperm DNA damage is present, any sperm DNA damage that cannot be effectively repaired by the oocyte may affect the subsequent post-fertilisation steps, such as embryonic and foetal development. Therefore, the assessment of sperm DNA status may be of particular significance in cases in which fertilisation is normal but implantation fails or early miscarriage occurs. Currently, it appears that those methods that directly evaluate the occurrence of DNA strand breaks (TUNEL and COMET) better define the relationship between sperm DNA fragmentation and assisted reproductive techniques outcomes (Tamburrino *et al.,* 2012).

It remains to be established whether sperm DNA damage can be decreased by some treatment modalities, such as the administration of antioxidants, leading to improved reproductive outcomes either naturally or through assisted reproductive technologies. Published studies on *in vivo* treatments aimed at reducing sperm DNA fragmentation have shown few beneficial effects (Tamburrino *et al.,* 2012). There could be several reasons for the limited efficacy of oral anti-oxidants. These include biases in selection of study patient, length of therapy, type of antioxidant (single or cocktail), effective absorption of the drugs in the reproductive tract and intra-individual variability of DNA fragmentation (Tamburrino *et al.,* 2012).

La Sala *et al.,* (2007) examined the efficacy and effect of HAART in HIV-1-infected men undergoing assisted reproductive procedures and reported a significant upward shift in mitochondrial DNA in spermatozoa from the HAART treatment group

compared to the HAART-untreated group or the HIV-1-uninfected group. The mtDNA copy numbers of the HIV-1 untreated men were not significantly different from that of uninfected control group (La Sala *et al.,* 2007). However, they did not detect any case of apoptosis-associated sperm DNA fragmentation (La Sala *et al.,* 2007). The finding of increased mtDNA might be correlated with the results of van Leeuwen *et al.,* (2008), who found a decrease in motile spermatozoa as early as 4 weeks after initiating therapy. Earlier, White *et al.,* (2001) demonstrated the propagation of multiple mtDNA deletions as a result of long-term HAART in sperm samples. They proposed that these deletions may arise through damage to the mitochondrial genome of the precursor spermatogonial cells, resulting in the amplification of these deleted mtDNA molecules (White *et al.,* 2001). In addition, abnormal spermatozoa may be the consequence of an abortive mitochondrial-mediated apoptotic event at the stem cell level, hence the mixed presence of the abnormal and normal populations of mature sperm cells in the samples analysed (White *et al.,* 2001).

2.11 Synopsis on Allium cepa Assay and the Three Rs Initiative

The *Allium cepa* test system was first used in 1938 to examine the effect of colchicine and has received much attention since that time (Grant, 1982). In the *Allium cepa* test, both macroscopic and microscopic effects are observed and the microscopic examination allows assessment of chromosome damage and cell division disturbances, providing information on the severity or toxic mechanism. The system has a wide range of applications, such as for testing chemicals, drinking water, and industrial waste. The system operates over a wide pH range (3.5-11.0) without any obvious effects upon the growth of the root systems. Disadvantages of the assay include concerns associated with

the state of compounds (powder or liquids) being tested and the presence of insoluble compounds in test solvent such as industrial effluent. The *Allium cepa* test is highly sensitive and positive toxic effects may result for compounds which may not be harmful when tested in other systems. However, false negatives have been shown to rarely occur in the *Allium cepa* test (Ennever et al., 1988), thus any compound giving a negative result can be reliably considered non-mutagenic. This test has shown good agreement with results from other test systems, even those using prokaryotic and eukaryotic organisms (Fiskesjö, 1985). However, extrapolating results from one test system to another should be based on the results of a battery of tests and with due consideration to the metabolic pathways of the compound tested.

The Reduce-Refine-Replace initiative (3Rs) was first articulated by British scientists WMS Russell and RL Burch in 1959 because of the extensive use of animal models in research. The first R, Reduce, was to encourage scientists to **reduce** the number of animal models being used for research. As they reduce the number, they were further encouraged to **refine** the methodologies they use for crucial animal research and ultimately to **replace** the animal models with other models such as the plant *Allium cepa* model used here. Though it is a plant model, it results have correlated with results from other test systems (Fiskesjö, 1985). The *Allium cepa* model can thus be used to further elucidate the mechanisms of any genotoxic response observed as its large chromosome sizes could be very useful.

3.0 MATERIALS AND METHODS

This study was carried out in two phases. The first phase was to investigate the occurrence of genotoxicity after the administration of ZDV or NVP using the standard *Allium cepa* root tip assay and to elucidate some of the cytogenotoxic mechanisms. The second phase was a two-generational study using albino mice exposed to either ZDV or NVP.

3.1 Major Materials Used for the Phase I and Phase II Studies

Locally available *Allium cepa* (purple variety) bulbs were purchased from the Mile 12 market in Lagos, Nigeria. Two hundred and four (204) medium-sized bulbs (42 – 53 mm) were used for the phase I study. Albino mice (*Mus musculus*) aged 6 weeks and above were obtained from the Animal House at the College of Medicine, University of Lagos. Six hundred and twenty (620) albino mice were used. Syrup formulations (concentration 10mg/ml) of Zidovudine (ZDV) and Nevirapine (NVP) were obtained from the Nigerian Institute of Medical Research (NIMR), Lagos antiretroviral clinic pharmacy. Maleic hydrazide was used as positive control in the *Allium cepa* study while ethylmethylsulfonate (EMS) was used as positive control for the mice study.

3.2 Methods

3.2.1 Methods for Allium cepa Study

3.2.1.1 The 96 hr Root Growth Inhibition Test (Odeigah et al., 1997)

The 96 hr root growth inhibition was performed first to determine the EC_{50} and thus obtain a good range for the test organism. The half maximal effective concentration (EC_{50}) refers to the concentration of a drug or toxicant which can induce a response halfway between the baseline and maximum after a specified period of time. With respect to this assay, the EC_{50} would represent the concentration of the drug that would allow 50 % of the root growth achieved by the untreated controls. This was determined as 96 hr semi-static exposure test using 6 concentrations of each drug (Table 3 and Figure 2). The test drug solutions (dilutions) were prepared using deionized water and stored at 2-8°C in aliquots sufficient for each day's work. Deionized water was thus used as the negative control. Aliquots were allowed to reach room temperature before use each day. The bulbs were grown away from direct sunlight in an inner laboratory using on glass bottles each holding about 50 ml of solvent. At the end of the 96 hr exposure period, one *Allium cepa* bulb (out of six) with the poorest root growth was discarded and the length of the root bundle of the remaining five *Allium cepa* bulbs was measured. This was done to reduce outlier effect and increase the chances that an effect seen is due to the chemical administered. The effect of the ARVs' on root growth was investigated by comparing the concentrations and their corresponding root length and then expressed as a percentage of the root length of the negative control. The EC_{50} was then calculated for each drug.

Table 3: The Concentrations of Test Drugs Used for the 96hrs *Allium cepa* Root Growth Assay

Concentrations	Zidovudine [µM]	Nevirapine [µM]
1	10	10
2	100	100
3	200	200
4	400	400
5	800	800
6	1200	1200

At the therapeutic dose, the zidovudine and nevirapine human plasma levels are equivalent to 10µM. This helped select the starting dose for the 6 molar concentrations used per drug (molar weight ZDV = 267.24g and NVP = 266.3g).

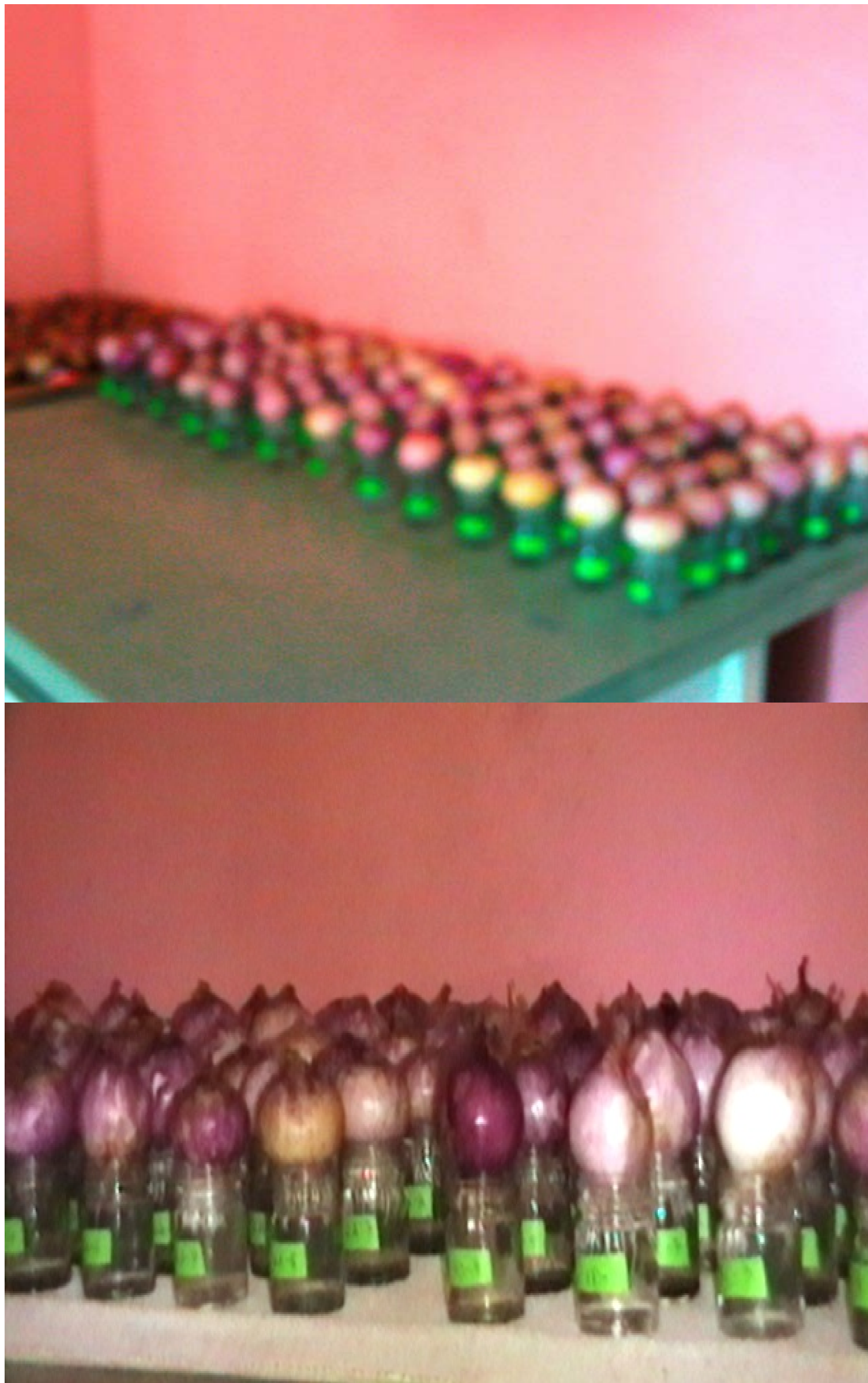

Figure 2: Laboratory Set-up of the *Allium cepa* Model
(Bulbs were grown in equal-sized glass bottles away from direct sunlight)

3.2.1.2 Allium cepa Recovery Studies (48 hours)

After the root length measurements at 96 hours, the test drug solutions were removed, replaced with deionized water and the five measured *Allium cepa* bulbs per concentration were grown in the medium water. All liquids were changed again at 24 hr interval. After another 48 hr, the root length measurements were repeated for all the *Allium cepa* bulbs, including those in the control solutions. These are to determine if the changes at 96 hr are reversible and if the *Allium cepa* roots can recover and continue growing.

3.2.1.3 Allium cepa Chromosomal Aberration Test (48 hours)

This assay was performed according to Odeigah *et al.,* (1997) with 4 concentrations per drug: 10 %, 25 %, 50 % and 100 % of the EC_{50} concentration per drug. Deionized water was used as solvents and as the negative control while 5 mg/l maleic hydrazide was used as the positive control. All *Allium cepa* bulbs were first grown in deionized water for 24 hr before transfer to the drug solutions. The test solutions were changed every 24 hr and the *Allium cepa* bulbs were exposed to the drugs for a total period of 48 hr. Twelve *Allium cepa* bulbs were exposed to each concentration but the two with the poorest growth were excluded for each concentration. The remaining ten *Allium cepa* bulbs were then prepared for microscopy using the conventional aceto-orcein squash technique (Odeigah *et al.,* 1997). Two to three root tips from one *Allium cepa* bulb were harvested, fixed, and macerated in a solution of 9 parts 45% acetic acid and 1 part 1N HCI for 5 min. Aceto-orcein stain was then dropped on the squashed material. The

cover slip was used to press down and spread the squashed materials. The edges of the coverslip were then sealed with nail varnish to reduce fluid evaporation. Ten slides per concentration were examined blind and scored for mitotic index and for chromosomal aberrations according to Odeigah *et al.,* (1997).

3.2.2 Methods for the Albino Mouse Model Study

3.2.2.1 Ethical Approval

Ethical clearance was obtained from the Institutional Review Board (IRB) of the Nigerian Institute of Medical Research (NIMR) prior to commencing the study (Appendix 1). And the "International Guiding Principles for Biomedical Research Involving Animals" (CIOMS, 1985) was adhered to.

3.2.2.2 Animal Husbandry

The albino mice were maintained in NIMR's animal house, subjected to the natural light-dark cycle. They received food and tap water *ad libitum* and were housed in small groups (except during mating) with wood chippings as bedding. To ensure successful mating, the mice were co-housed in male: female ratio of 1:1 (dominant lethal assay) or 1:5 for P and F_1 harems for one week or 10 days respectively. All animals were handled in accordance with the "International Guiding Principles for Biomedical Research Involving Animals" (CIOMS, 1985) which prescribes procedures for the humane treatment of research animals. A combination of chloroform and cervical dislocation was the euthanasia method applied.

3.2.2.3 Design and Critical Assumptions

This phase evaluated the effect of the administration of ZDV or NVP on the male reproductive system by performing the dominant lethal assay and by evaluating the sperm counts, morphology and the DNA quantity and quality of the sperm cells. Parental generations (P) of mice were dosed once daily for 8 weeks to cover one complete spermatogenic cycle which is approximately 56 days in mice (OECD guideline 416). Test drugs were administered by gavage. After mating, the females continued to receive the drugs during and after pregnancy until the offspring (F_1) were weaned. Offspring were weaned at 4 weeks and the sexes separated. At age 6 weeks and above, the F_1 offspring from the least dose of both ZDV and NVP were directly exposed to the same dose of ARVs by gavage and then were mated to give rise to the F_2 generation.

3.2.2.4 Drug Administration and Sampling

The three different doses of each of the drugs were administered once daily. For zidovudine 10, 100 and 250 mg/kg were administered while for nevirapine 5, 50 and 150 mg/kg were administered. The average weight of each weight-matched group was used to calculate the weekly or fortnightly volume administered using the syrup formulations of ZDV and NVP. For pregnant dams, individual weights were ascertained weekly and the group average weight was used to determine the drug volume administered. For the non-pregnant mice, individual weights were initially determined weekly for the first 2 weeks and thereafter weights were ascertained fortnightly. The

group average weight was used to determine the drug volume administered to each group. The dams continued to receive their doses during pregnancy and lactation.

Each dosage was administered daily to 16 male and 10 virgin female mice for 8 weeks. At the end of week 3, six of the males were randomly selected and mated (1:1) to 6 untreated virgin females (cohabitation for one week). The males continued to receive their ARV drug doses until week 4. At the end of week 4 of drug administration and after one week of co-habitation, the mated females were separated and used for the dominant lethal assay (Appendix 2) according to OECD guideline 478 and Odeigah (1997). The 6 male mice were subsequently euthanized and cauda epididymis obtained to access the sperm for the determination of the sperm count, morphology and DNA quantity and quality according to Odeigah (1997) and Georgiou *et al.,* (2009) respectively.

At the end of week 7, another set of six males were selected and mated (1:1) to 6 untreated virgin females (cohabitation for one week). These males continued to receive their doses of the ARV drug until week 8. At the end of week 8 of drug administration and after one week of co-habitation, the mated females were separated and used for the DLA. The 6 male mice were euthanized and cauda epididymis obtained to access the sperm for the determination of the sperm count, morphology and DNA quantity and quality according to Odeigah (1997) and Georgiou *et. al.* (2009). At the end of week 8 of drug administration, of the 4 male mice remaining, 2 were mated (1:5) to 10 untreated females while the other 2 mated (1:5) to the 10 treated females (co-

administered ARV for 8 weeks). The treated females received the same drug and dosing that their male partners' received. These treated (10) and untreated (10) females produced the F_1 generation. Thus they provided models of ARV exposure demonstrating either "both-parents" (double) exposure or "father-only" (single) exposure respectively. In addition, mating and sampling at week 4 represent mid-term effects while mating and sampling at week 8 represent effects at full-term. The negative (no administration of ARVs) and positive controls (480 mg/kg ethyl methanesulfonate) were subjected to the same mating procedures at week 3 and 7, and sperm analysis at week 4 and 8. A diagrammatic representation of the study design is attached as Appendix 3.

The F_1 generations of ZDV 10mg/kg and NVP 5mg/kg were weaned at 4 weeks, the sexes separated and a two - week's interval given for all dams to wean. At age 6 weeks and above, the 8 weeks-dosing procedure commenced for the F_1 generation. From the F_1 generation obtained from the treated females, 16 males and 10 females were selected and the same procedures and interventions (DLA was not done) used to dose and mate the P generation were repeated on them at weeks 7 and 8 only. Similarly 16 males and 10 females were selected from the F_1 generation born to the untreated females, and the same procedures and interventions (DLA was not done) used to dose and mate the P generation were repeated on them at weeks 7 and 8 only. For any group with less than 16 males and 10 females' offspring, the available number of offspring was used. Birth statistics in each generation and male fertility data were collected. Birth statistics include the sexes, the number of live and dead pups per litter, period of gestation, birth weights per pup, average birth weight per litter and any physical anomalies observed.

3.2.2.5 Parental Generation Recovery Mating

The 4 males that were used to produce the F_1 generation were kept for another 8 weeks (56 days) and the drugs were withdrawn. They were then mated to the same set of treated and untreated females to ascertain if the effects of the drug administration could resolve after the drug was withdrawn. Birth statistics were collected and compared to each group's previous birth statistics. At this point, the males were not currently on ARVs and only the dams were currently ARV-treated (pseudo-single exposure).

3.2.2.6 Sperm DNA Extraction and Analyses

The extraction of sperm DNA was done using Qiagen DNA mini kit and their protocol on "isolation of genomic DNA from sperm: protocol 2 (QA04 Jul-10)". The extraction SOP is attached as Appendix 4. The quantification and evaluation of DNA fragmentation and nicks were done according to Georgiou *et al.,* (2009) protocols for (1) quantification of DNA; (2) quantification of DNA fragmentation and; (3) quantification of DNA nicks. The standard operating procedures (SOPs) used are attached as Appendices 5, 6 and 7. The Georgiou *et al.,* (2009) methods utilize Picogreen dye and fluorometry and is preferred because it is the most sensitive compared to UV 260/280 spectrometry, Nanodrop, and Hoechst dye determinations (GenVault, 2005; Holden *et al.,* 2009; Georgiou *et al.,* 2009).

The Georgiou *et al.,* (2009) protocol for DNA quantification optimized the Picogreen quantification method by first fragmenting the DNA samples up to 70% using Proteinase K and then quantifying by comparing to a standard curve made using similarly fragmented DNA standard. This was done because Picogreen quantification is dependent on the degree of fragmentation (not on fragment size) in the DNA sample and this could underestimate DNA concentration by up to 70% if the DNA was totally fragmented (Georgiou *et al.,* 2009).

The Georgiou *et al.,* (2009) protocol for quantifying DNA fragmentation is an ultrasensitive and quantitative complement of the qualitative and subjective COMET assay. It measures irreparable DNA damage. It quantifies DNA fragments in the 0-23kb range and cannot quantify fragmented DNA above 23kb. DNA sizes above 23kb are considered "intact" DNA. The Georgiou *et al.,* (2009) protocol for the quantification of nicks is a complement of the TUNEL assay (Georgiou *et al.,* 2009) and measures repairable DNA damage. It is also 10,000 fold more sensitive than the currently available Hoechst-based method, estimating nicks in as low as 15 picograms of DNA (Georgiou *et al.,* 2009). However, it cannot discriminate between single-strand and double-strand DNA nicks (Georgiou *et al.,* 2009). The principle is based on the determination of the annealing degree of DNA under alkaline conditions ($pH > 11$), which depends on the number of nicks present in DNA (Georgiou *et al.,* 2009). The more nicks in double-stranded DNA (dsDNA), the easier it is annealed to single-stranded DNA (ssDNA) at alkaline pH. This differential annealing is quantified by the differential binding of PicoGreen to dsDNA instead of ssDNA

The methodology for quantifying the DNA involves first digesting the DNA with DNAse I for 3 hours to convert it to totally fragmented DNA. This is to reduce variability as PicoGreen binding to dsDNA depends on its fragmentation degree and not dependent on fragment size (Georgiou *et al.,* 2009). The digested DNA was then diluted, PicoGreen was added and the fluorescence (FU) measured using a TBS-380 Turner Biosystems mini-fluorimeter. The FU is then converted to DNA concentration using the totally fragmented DNA standard curve obtained using similarly digested ʎ-DNA standard. The full procedures are attached as Appendices 4, 5 and 6.

The degree of DNA fragmentation in each sample was determined by adding PicoGreen to an aliquot of same dilution used for the DNA quantification. The FU of the undigested DNA sample was measured. The FU of the digested and undigested aliquots were compared to show whether the DNA consists only of intact DNA or is totally fragmented. If the DNA is partially fragmented, mathematical treatment using the formulae according to Georgiou *et al.,* (2009) yielded the percentage fragmentation. The full procedures are attached as Appendices 4, 6 and 7.

The quantification of the DNA nicks present involved preparing 3 aliquots of same dilution. Then the first was treated with equal volumes of equimolar (0.05N) HCl and NaOH (not annealed sample), the second was treated first with NaOH at room temperature for 5 min before neutralizing with equal volume of equimolar HCl (partially annealed sample) and the third was treated first with NaOH at 80°C for 5 min before neutralizing with HCl (completely annealed DNA sample). PicoGreen was then

added to all 3 aliquots and their FU measured. Mathematical treatment of the FU values according to the formula by Georgiou *et al.,* (2009) yielded the percentage of nicked DNA in each sample. The nicks method is based on determining the annealing degree of DNA under alkaline conditions as the more nicks are present in dsDNA, the easier it is annealed to ssDNA at alkaline pH. The full procedures are attached as Appendices 4 and 8.

3.2.2.7 Testis / Epididymis Weights, Sperm Count and Morphology Analyses

Testes and epididymis dissected out were placed on Whatman No. 1 filter paper and weighed rapidly using a Shimadzu AUX 220 (d=0.1 mg) weighing balance before putting them into phosphate buffered saline (PBS) or 0.05% PBS-Triton-X100.

Neubauer improved haemocytometer was used to count the sperm cells after preparation. Preparation involved cutting one of the cauda epididymis into tiny bits using fine scissors, mixing well, diluting 10 times and sieving to remove debris. 10 µl of sperm suspension was pipetted into the haemocytometer set-up and left to settle for 3-5 min before counting. Spermatozoa in four corners squares and one middle square were counted in both haemocytometer chambers. Only a 20% difference in counts was acceptable between both chambers, else it was repeated. Real sperm count was calculated as the mean of the haemocytometer chamber values multiplied by the dilution factor and divided by the volume of the counting chamber. The full procedure is attached as Appendix 9.

The second cauda epididymis was cut into tiny bits using fine scissors, mixed well, diluted 2 times and sieved. The debris was allowed to settle. Handling it gently, aqueous Eosin (20 µl of 1% stock) was added to the sperm suspension (200 µl) and left to stain for 30 min. Thereafter 2 smears were made on slides and allowed to air-dry. Thereafter, they were coded and evaluated. The full procedure is attached as Appendix 10 and the job aid for identification of aberrations as Appendix 11.

3.2.3 Laboratory and Data Analyses

All slide examination and analyses were performed under blind code (the samples were coded and there was no prior knowledge of the results from the other analysis). The microscopic analysis includes the scoring of mitotic index and the chromosomal aberrations in *Allium cepa* mitotic cells. The mitotic index (MI) was determined by the examination of 40 – 100 cells/slide and calculated as mitotic index per 400 – 1000 cells. Mitotic index (MI) = (Dividing cells [all stages] / total cells counted multiplied by 100). At least 40 dividing cells were scored for aberrations per slide, making a total of 400 – 800 cells examined per concentration. The sperm counts and morphology analysis were done according to Odeigah (1997). The SOPs and the pictorial bench aid for the morphology analysis used are attached as Appendices 9, 10 and 11. Multiple tally counters were used for sperm counts and morphology analysis. The dominant lethal assay was done according to OECD guide 478 and Odeigah (1997). The SOP is attached as Appendix 2.

Data obtained were collated and analyzed using Microsoft Excel 2010 and Epi Info (CDC, version 3.5.3). Graphs were drawn using Microsoft Excel 2010. Average group values are given except when otherwise stated such as when the median values were preferred because of high intra-group variability. The Student's t-test and Analysis of Variance (ANOVA) were the statistical tests applied and a p-value less than 0.05 was regarded as being significant. Univariate (correlation) and multivariate (regression) analyses comparing the parameters were done using Statgraphics Centurion XVI (version 16.1.11). The best linear regression model had p-values for both the model and each independent variables being less than 0.05.

4.0 RESULTS

The results from the phase I study using the *Allium cepa* assay include results from the 96 hours root growth inhibition, the 48 hours recovery assays and the 48 hours chromosome aberration assay. The results from the phase II study using the albino mouse model are results from evaluating the testis-body weight ratios, the sperm counts and morphology, quantification of sperm DNA quantity, fragmentation and nicks, the dominant lethal assays, gestation period, birth statistics, litter size and weights and pup survival for the ZDV (Z10, Z100, and Z250) and NVP (N5, N50 and N150) groups.

4.1 The 96 hours Allium cepa Root Inhibition and the 48 hours Recovery Assays

Figure 3 shows the root length growth in relation to the different concentrations of ARVs. ZDV caused more root growth inhibition and was more toxic than NVP, especially at the higher concentrations. A lower EC_{50} of 65 µM was obtained for ZDV compared to 92.5 µM for NVP. The relationship between concentrations and root growth was not linear for ZDV (Figure 3).

During the 48 hours recovery assay, there was some additional growth (between 0.7 – 47% of their initial growth) for all the concentrations but the cumulative root growths for all were less than the root growth recorded for the NC, with most being less than half of the NC's root growth (Figure 4). The additional growth during this assay showed no consistent pattern, but both test drug groups had their highest additional growths in the 400µM concentrations (22% and 47% for NVP and ZDV respectively; Figure 4).

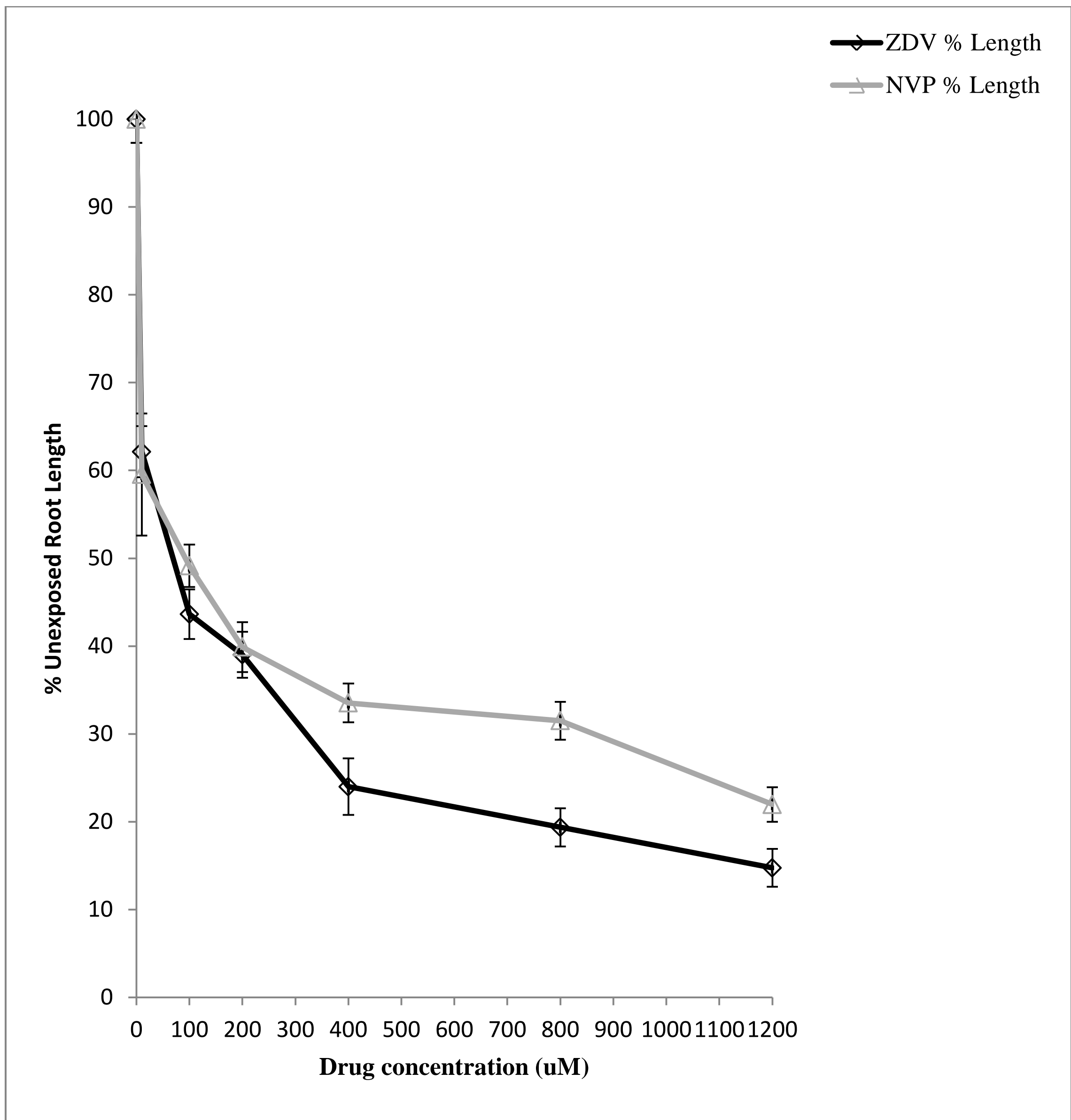

Figure 3: Growth Curve for *Allium cepa* Roots Grown for 96 hr in 0 to 1200 µM ZDV (black line) or NVP (grey line). Root length is expressed as percentage of unexposed control (34.6 ± 5.9mm; n=5 bulbs/group). ZDV ≈ Zidovudine and NVP ≈ Nevirapine. The half maximal effective concentration (EC_{50}) for ZDV was 65.0µM and for NVP was 92.5µM. NVP root length for the three highest concentrations were significantly longer than their corresponding ZDV root length.

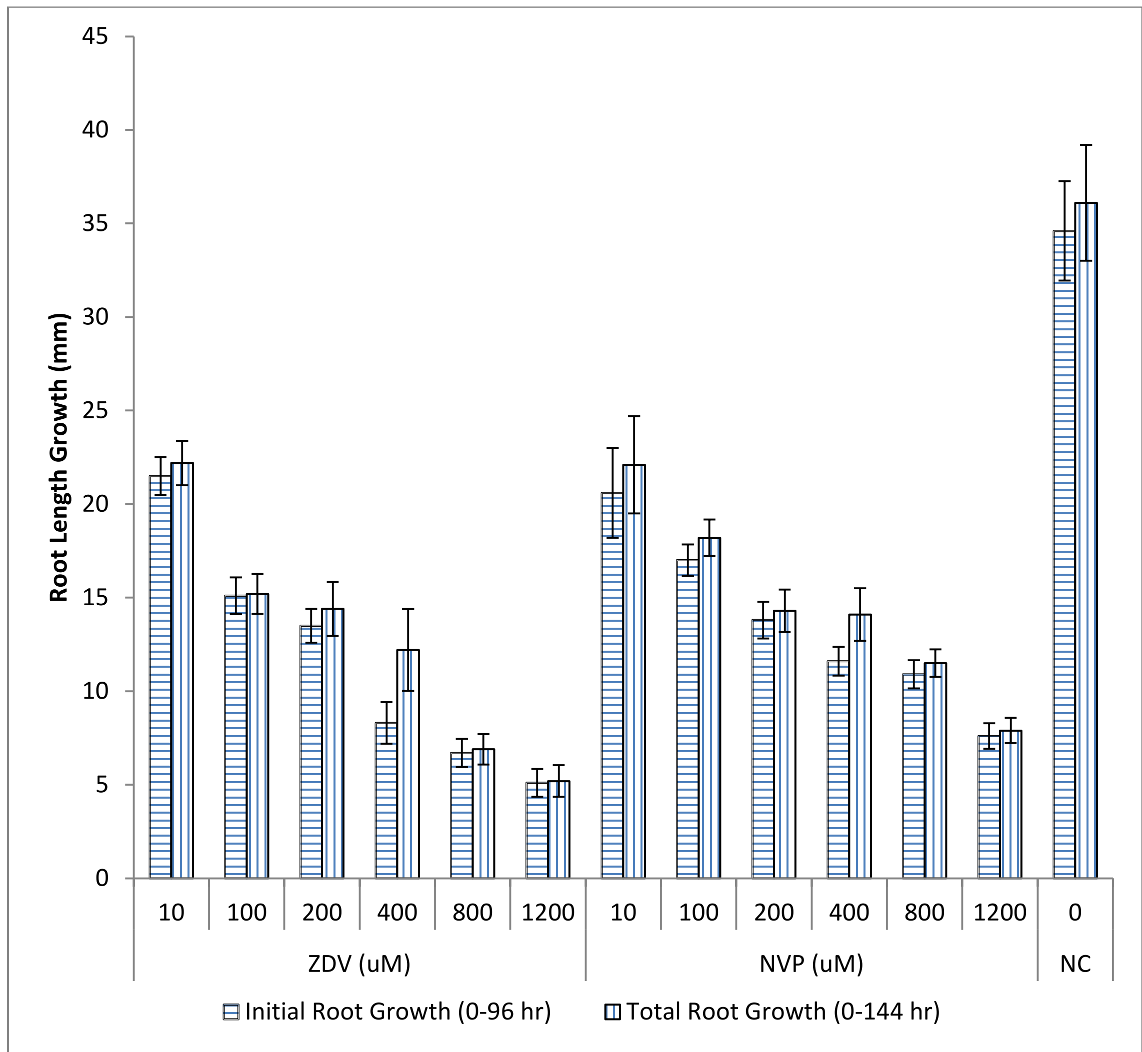

Figure 4: Root Length (mm) During the Initial 96hr (bars with horizontal stripes) in which Bulbs were Grown in Water (NC = negative control), ZDV or NVP. From 96-144hr (bars with vertical stripes), all Bulbs were Grown in Water without Drug. ZDV ≈ Zidovudine (µM) and NVP ≈ Nevirapine (µM). Values shown are mean ± Standard error (n = 5 per concentration). NC did not have much additional growth as it seems to have reached its peak root length.

4.2 The 48 hours Chromosome Aberration Assay

The root growth inhibition during the 48 hours aberration assay was also recorded and is shown in Figure 5. The results confirm that the relationship between concentration and root growth is not linear for ZDV. All test ARVs dosage groups did not have up to 800 “intact” cells for the evaluation of genotoxic effects on mitosis because some had cells with disrupted plasma membranes (Table 4). All treatment groups had mitotic index (MI; 4.2 – 7.6%) higher than the NC’s 4.1%, except for ZDV 100% EC_{50} group which had an MI of 2.8%. The higher mitotic indexes for most test groups were not significant as only ZDV 50% EC_{50} value was significantly higher than the NC’s mitotic index (Table 4).

The mitotic index was non-linear for both the ZDV and NVP test groups. The NC had the highest percentage of dividing cells in telophase and in metaphase (Table 4). For the ZDV test groups, the 2 highest concentrations had the highest percentage of dividing cells being in telophase and in prophase. ZDV 25% EC_{50} concentration had equivalent percentage of dividing cells in telophase, metaphase and prophase while ZDV 10% EC_{50} concentration had the highest percentage of dividing cells in prophase and in telophase (Table 4). The highest chromosomal aberration amongst the ZDV groups was seen at 100% ZDV EC_{50} concentration (Table 5). For NVP test groups, the 3 highest concentrations had the highest number of dividing cells being in telophase and in prophase (Table 4) while the NVP 10% EC_{50} concentration closely resembled the NC.

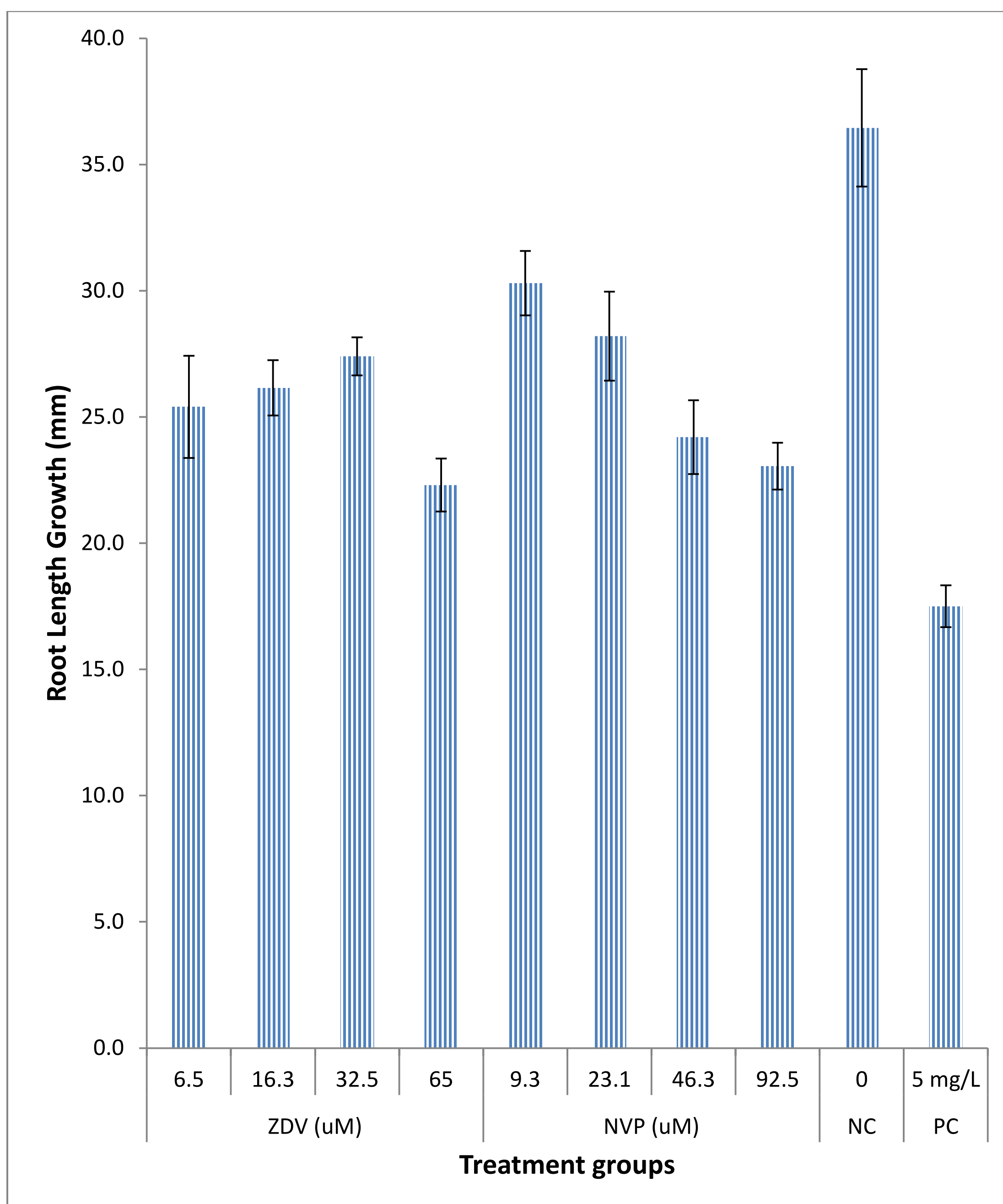

Figure 5: Root Growth Curve of *Allium cepa* Bulbs During the 48 hr Chromosomal Aberration Assay. ZDV ≈ Zidovudine, NVP ≈ Nevirapine and PC ≈ Positive control (Maleic hydrazide).

Table 4: Impact of ZDV and NVP on Mitotic Index and Cell Cycle Phases in *Allium cepa* Root Cells[a] Exposed for 48 hr

Chemical Exposure (μM)	EC_{50} (%)	Cells evaluated (n)	Dividing Cells (n)	Percentage of cells in				
				Mitosis	Prophase[c]	Metaphase[c]	Anaphase[c]	Telophase[c]
None	-	1000	41	4.1 ± 2.3	17.1	34.1	12.2	36.6
6.5 ZDV	10	736	48	6.5 ± 3.3	39.6	10.4	22.9	27.1
16.3 ZDV	25	744	43	5.5 ± 2.6	27.9	27.9	16.3	27.9
32.5 ZDV	50	747	57	7.6 ± 3.4[b]	24.6	22.8	17.5	35.1
65.0 ZDV	100	690	19	2.7 ± 2.1	15.8	5.3	0.0	78.9
9.3 NVP	10	503	25	5.3 ± 5.4	16.0	28.0	4.0	52.0
23.1 NVP	25	361	15	4.1 ± 4.8	26.7	6.7	6.7	60.0
46.3 NVP	50	429	21	2.9 ± 5.2[b]	38.1	0.0	0.0	61.9
92.5 NVP	100	720	37	5.0 ± 3.5	37.8	2.7	0.0	59.5

[a] Each experimental group contained 10 bulbs and 2-3 root tips from each bulb were squashed together on one slide. On each slide 40-100 cells were examined. Values shown are means ± standard deviation of the mean.
[b] For the percentage of cells in mitosis: for unexposed vs.32.5 μM ZDV, $p = 0.02$; for ZDV 50% of EC_{50} vs. NVP 50% of EC_{50}, $p = 0.03$. All other associations were statistically non-significant. [c]Data for these phases were collated from the ten bulbs per concentration.

Table 5: Percentage of *Allium cepa* cells with Various Chromosomal Aberrations after Exposure for 48 hr to ZDV or NVP

Chemical Exposure (µM)	EC_{50} (%)	Cells evaluated (n)	Sticky chromosomes (%)	Chromatin bridges (%)	Vagrant forms (%)	Binucleate (%)	Multipolar mitosis (%)	Chromosomal Fragments (%)	Total Aberration (%)
None	-	1000	0	0	0	0	0	0	0
6.5 ZDV	10	736	25.0	12.5	6.3	4.2	-	6.3	54.3
16.3 ZDV	25	744	23.3	7.0	16.3	2.3	2.3	-	51.2
32.5 ZDV	50	747	24.6	8.8	12.3	3.5	1.8	3.5	54.5
65.0 ZDV	100	690	57.9	-	-	5.3	-	-	63.2
9.3 NVP	10	503	36.0	-	20.0	-	-	-	56.0
23.1 NVP	25	361	53.3	-	6.7	-	-	-	60.0
46.3 NVP	50	429	61.9	-	-	4.8	-	-	66.7
92.5 NVP	100	720	56.8	-	2.7	5.4	-	-	64.9

ZDV = Zidovudine and NVP = Nevirapine. *Sticky chromosomes* refer to the tendency of chromosome arms or entire chromosomes to stick together. *Chromatin bridges* occur in mitosis when the telomeres of sister chromatids fuse together and fail to completely segregate into their respective daughter cells. *Chromosomal fragments* refer to fragments of a chromosome that may be lacking a centromere and so is often lost when the cell divides. *Vagrant forms* refer to chromosomal formations different from the normal formation during mitosis. *Binucleate* occurs when a cell has more than one nucleus. *Multipolar mitosis* occurs when the chromosomal material is pulled to more than two poles, resulting in the formation of a corresponding number of nuclei.

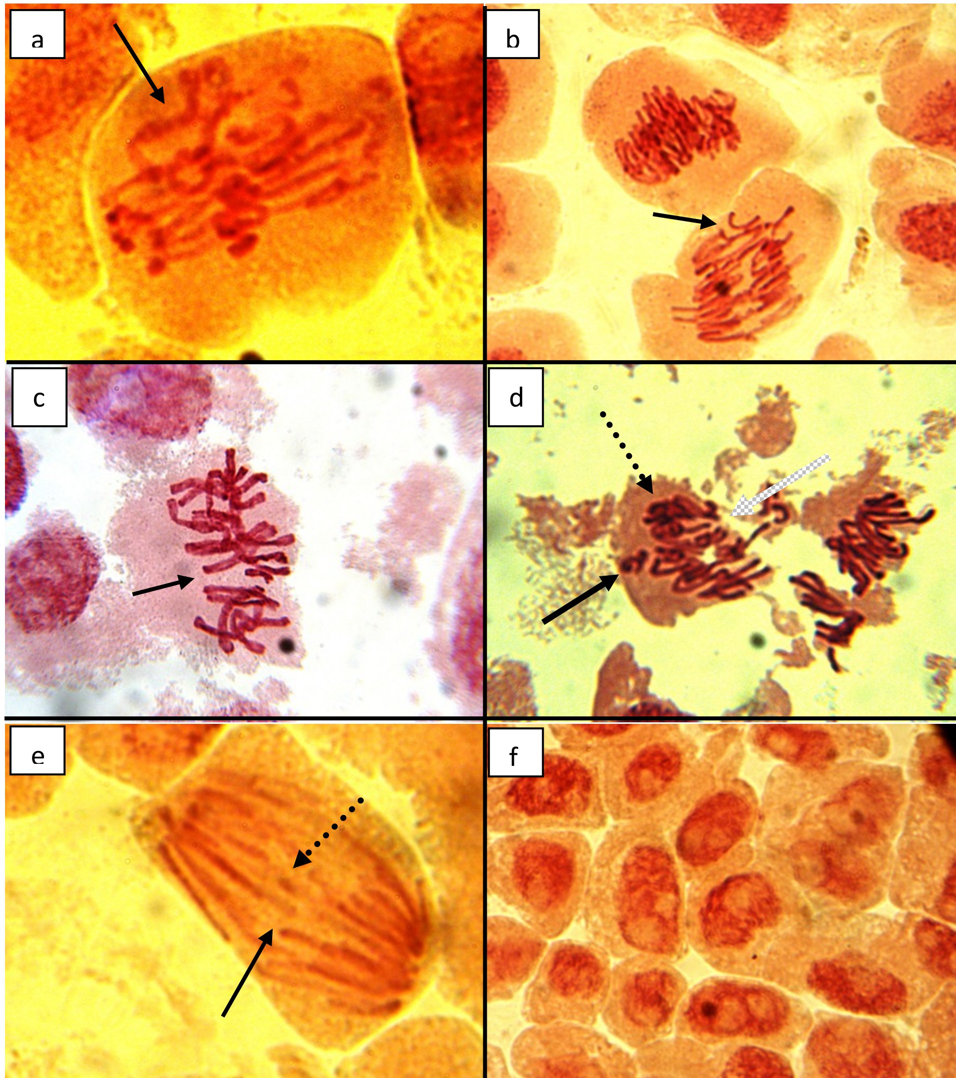

Plate 1: Micrographs of chromosomal aberrations seen in bulbs treated for 48 hr with zidovudine (1000X magnification)

a ≈ Cell exposed to 65µM zidovudine (ZDV) showing sticky metaphase chromosomes
b ≈ Cell exposed to ZDV 6.5µM showing anaphase bridges (arrowed)
c ≈ Vagrant metaphase form with misaligned chromosomes (exposed to 32.5µM ZDV)
d ≈ Multipolar aberration (more than 2 poles) in cell exposed to ZDV 16.3µM
e ≈ Cell exposed to 6.5µM ZDV; arrows indicate uneven breaks (black) and chromosomal fragment (dashed)
f ≈ Interphase cells exposed to 6.5µM ZDV indicating interphase arrest

Generally, the NVP groups had little or no cells at metaphase and anaphase especially for the three highest concentrations. The highest chromosomal aberration amongst the NVP groups was at 50% EC_{50} concentration (Table 5). Stickiness accounted for 23 – 62 % of the chromosomal aberrations recorded (Table 5). This was mainly sticky telophase and few sticky anaphases. For ZDV groups, in addition to the stickiness, there were bridges, vagrant and binucleate aberrations; while the NVP groups had vagrant and binucleate aberrations in addition to the stickiness (Table 5).

4.3 Testicular Size, Sperm Count and Head Anomaly in the Parental Generation

The median testis-body weight ratio of the negative control (NC) was 0.344 and 0.366 at mid- (week 4) and full-term (week 8) respectively. The median sperm count of the NC was 3.6 x 10^6/ml and 8.0 x 10^6/ml at mid- and full-term respectively. At mid-term, the testis-body weight ratios of all treatment groups were not significantly different from the NC but at full-term, the testis-body weight ratio of NVP group N5 was the only one significantly lower than the NC's value (Tables 6 and 7).

On comparison, both the testis-body weight ratios and the body weights of the different N5 mice at mid- and full-term were significantly different (Table 8). Though the full-term N5 mice weighed significantly higher (p=0.002), they however had significantly lower testis-body weight ratios (p=0.009).

Table 6: Mid-Term Median Testis-Body Weight Ratios and Sperm Counts of the Parental Generation

Groups	Males (n)	Testis-Body weight ratio ± SD	P-values	Sperm Count x 10^3/ml ± SD	P-values
NC	6	0.344 ± 0.037	-	3,600 ± 1067.4	-
PC	6	0.344 ± 0.078	0.475	2,000 ± 1379.3	0.063
Z10	6	0.371 ± 0.052	0.863	2,830 ± 792.6	0.051
Z100	6	0.342 ± 0.056	0.416	4,480 ± 2139.2	0.872
Z250	6	0.313 ± 0.055	0.168	4,025 ± 2312.7	0.619
N5	6	0.393 ± 0.045	0.182	5,615 ± 1487.5	**‡0.031**
N50	*6 (5)	0.333 ± 0.095	0.436	2,280 ± 1096.1	**‡0.014**
N150	*6 (4)	0.333 ± 0.076	0.238	5,995 ± 3051.2	0.521

*Number of males in group (number of males whose sperm counts were measurable); SD ≈ Standard deviation; ‡ ≈ significant P-values

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

Table 7: Full-Term Median Testis-Body Weight Ratios and Sperm Counts of the Parental Generation

Groups	Males (n)	Testis-Body weight ratio ± SD	P- values	Sperm Count x10^3/ml ± SD	P-values
NC	6	0.366 ± 0.038	-	8,055 ± 1179.8	-
PC	6	0.341 ± 0.045	0.205	4,825 ± 2416.2	**‡0.045**
Z10	6	0.337 ± 0.038	0.314	5,425 ± 1412.8	**‡0.003**
Z100	6	0.357 ± 0.034	0.398	4,140 ± 1871.7	**‡0.002**
Z250	6	0.355 ± 0.043	0.392	4,815 ± 1595.8	**‡0.003**
N5	6	0.300 ± 0.047	**‡0.038**	4,375 ± 1577.1	**‡0.001**
N50	6	0.324 ± 0.041	0.193	2,915 ± 1335.7	**‡0.000**
N150	6	0.389 ± 0.057	0.750	3,855 ± 1035.8	**‡0.000**

SD ≈ Standard deviation; ‡ ≈ significant P-values

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

Table 8: Comparison of the Testis-Body Weight Ratios and Body Weights amongst the Nevirapine 5mg/kg Group Parental Generation at Mid- and Full-Term

Males	N5 Week 4 testis-body weight ratio	N5 Week 8 testis-body weight ratio	N5 Week 4 body weight	N5 Week 8 body weight
1	0.442	0.340	26.4	26.9
2	0.448	0.387	25.5	29.5
3	0.370	0.305	24.0	29.8
4	0.416	0.295	24.8	27.6
5	0.351	0.270	24.7	29.3
6	0.346	0.264	24.6	26.4
	P- values =	**‡0.009**		**‡0.002**

‡ ≈ significant P-values

N5 Week 4 ≈ Nevirapine 5 mg/kg group sampled at mid-term of drug administration

N5 Week 8 ≈ Nevirapine 5 mg/kg group sampled at full-term of drug administration

Furthermore, at mid-term, two NVP groups (N5 and N50) had sperm counts differing significantly from the NC's, with N5's count being higher while N50's count was lower (Table 6). At mid-term, the sperm counts of some individual males in groups N50 (one male) and N150 (two males) were very low and could not be determined. However at full-term, all treatment groups had significantly lower sperm counts when compared to the NC (Table 7).

The median sperm anomaly in the negative control (NC) was 1.89% and 2.77% at mid- and full-term respectively. The anomalies in sperm morphology recorded for the different treatment groups were varied (Plate 2) but increased with the increasing drug concentrations, except for intermediate groups Z100 and N50 having more anomalies than their highest concentration (Table 9). Group Z100 had some mice with very high sperm anomalies. Only 3 and 5 mice could be evaluated for sperm anomalies at mid-term for groups N50 and N150 respectively because of their low sperm counts. Similarly at full-term, only 5 and 4 mice could be evaluated for sperm anomalies for groups Z100 and Z250 respectively because of their low sperm counts.

Table 9: Median Anomalies (%) in Sperm Morphology amongst the Parental Generation Sampled Mid- and Full-Term of Drug Administration

Groups	Mid – term (week 4)			Full – term (week 8)		
	Males (n)	Sperm Anomaly (%) ± SD	P-values	Males (n)	Sperm Anomaly (%) ± SD	P-values
NC	4[†]	1.89 ± 1.78	-	6	2.77 ± 1.87	-
Z10	6	3.02 ± 3.97	0.165	6	4.98 ± 12.62	0.260
Z100	6	12.49 ± 34.67	0.110	5*	8.47 ± 19.20	0.158
Z250	6	7.32 ± 5.37	**‡0.017**	4*	10.57 ± 10.53	0.105
N5	6	4.29 ± 2.66	0.054	6	2.05 ± 5.58	0.622
N50	3*	30.66 ± 14.82	0.101	6	4.85 ± 6.94	0.239
N150	5*	19.58 ± 12.11	**‡0.031**	6	4.38 ± 6.50	0.259

[†]4 randomly evaluated; *Number used due to low sperm counts.
‡ ≈ significant P-values; SD ≈ Standard deviation

NC ≈ Negative Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

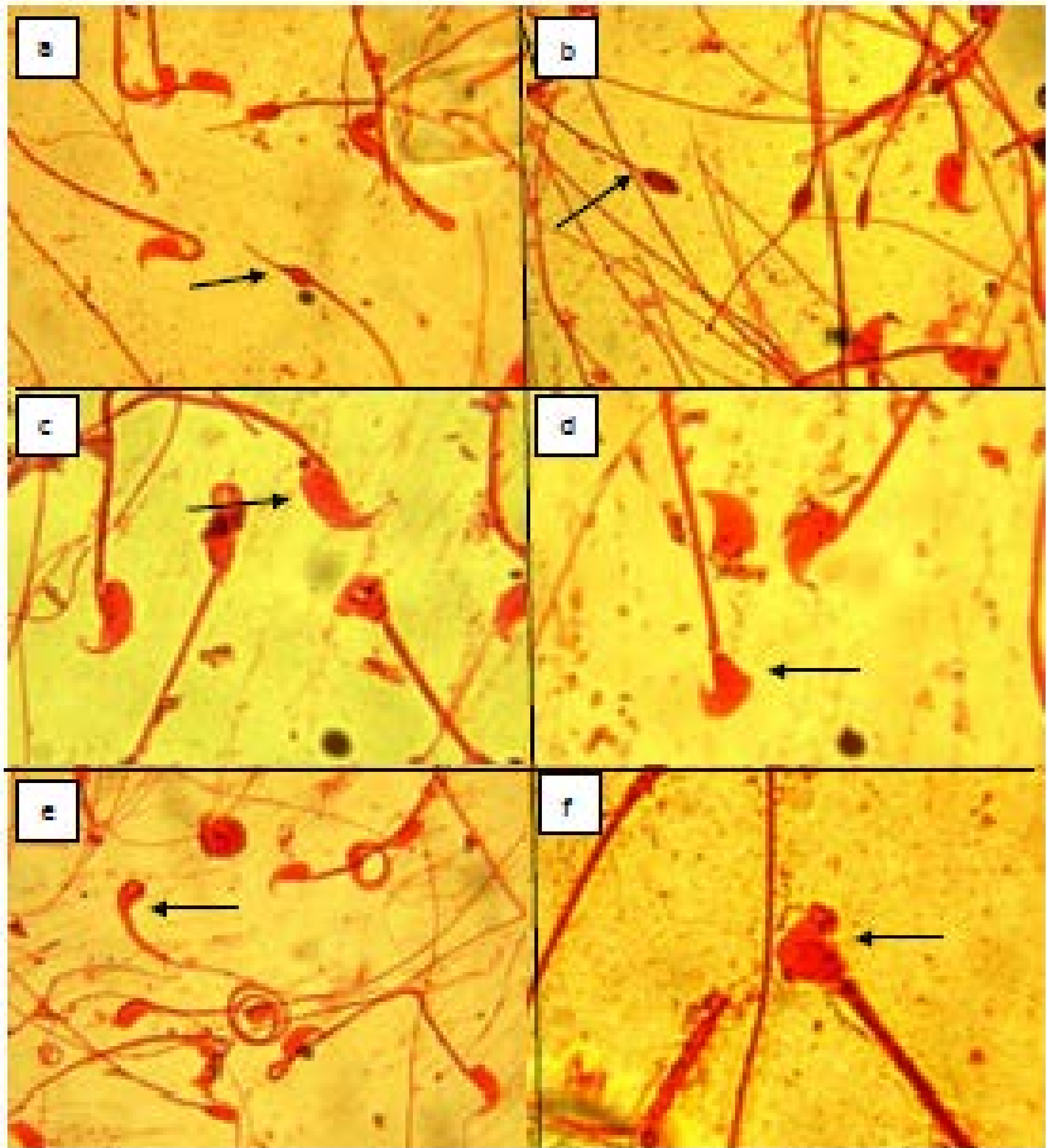

Plate 2: Micrographs showing some Sperm Morphology Anomalies Seen in this Study (1000X magnification)

a ≈ Spermatozoon (arrowed) showing pin-head anomaly
b ≈ Spermatozoon showing knobbed (arrowed) anomaly
c ≈ Spermatozoon (arrowed) exhibiting normal sperm morphology
d ≈ Spermatozoon (arrowed) exhibiting an amorphous head anomaly
e ≈ Spermatozoon (arrowed) illustrating the knobbed head anomaly
f ≈ Spermatozoon (arrowed) illustrating the hammer-head anomaly

4.4 Sperm DNA Quantity, Fragmentation and Nicks in the Parental Generation

At mid-term, the median sperm DNA concentration, percentage DNA fragmentation and DNA nicks for the NC was 52.9µg/ml, 97.7% and 51.2% respectively (Table 10) while at full-term they were 78.9µg/ml, 81.3% and 25.5% respectively (Table 11). Each group's full-term values were compared to that of only the fertile NC males to determine whether they were significantly different.

Amongst the NC group, the only mouse that had implants at mid-term had higher sperm DNA quantity, less fragmentation and more nicks compared to the infertile NC males and the median group values (Table 10). At mid-term, there was no significant difference between the NC and ARV test groups in the median DNA quantity per sperm but almost all the test groups had significantly reduced sperm DNA fragmentation. Similarly, all the test groups had lower DNA nicks compared to the NC but only 3 (Z10, N5 and N150 groups; Table 10) were significantly lower.

Amongst the NC group, the 4 male mice that sired implants in the dams at full-term had higher sperm DNA quantity and more nicks compared to the infertile NC males and the median group values (Table 11). At full-term, all the test groups had significantly higher median DNA quantity per sperm compared to the values for the fertile NC mice, however there was no significant difference in the degree of DNA fragmentation and nicks present (Table 11). The DNA fragmentation values of the test groups were similar to that of the NC but there was more variation in the degree of DNA nicks seen.

Table 10: Median Sperm DNA Quantity, Fragmentation and Nicks in the Parental Generation Sampled Mid-Term of Drug Administration

Group	Mice (n)	DNA Conc. (µg/ml)	P-Value	Frag DNA (%)	P-Value	Nicked DNA (%)	P-Value
NC_All	6	52.9 ± 18.1	-	97.7 ± 3.9	-	51.2 ± 11.7	-
NC_fertile	1	65.5	-	95.7	-	56.9	-
NC_Infertile	5	44.9 ± 19.5	-	98.1 ± 4.2	-	47.7 ± 12.7	-
PC	6	61.1 ± 10.7	0.286	77.7 ± 4.3	‡**0.000**	25.5 ± 8.4	‡**0.002**
Z10	6	62.7 ± 10.2	0.536	96.2 ± 9.0	0.501	33.6 ± 7.4	‡**0.028**
Z100	6	51.3 ± 3.9	0.146	92.0 ± 4.1	‡**0.008**	38.1 ± 10.2	0.084
Z250	6	53.3 ± 4.9	0.204	82.8 ± 6.2	‡**0.000**	41.0 ± 8.3	0.070
N5	6	85.8 ± 27.3	0.628	64.3 ± 7.1	‡**0.000**	27.3 ± 5.8	‡**0.002**
N50	6	52.2 ± 9.9	0.174	74.8 ± 7.7	‡**0.000**	50.6 ± 4.2	0.871
N150	6	69.5 ± 27.6	0.921	69.9 ± 8.7	‡**0.000**	37.3 ± 10.0	‡**0.049**

± SD ≈ Standard deviation; ‡ ≈ significant P-values

NC_All ≈ Average of all negative control group mice
NC_fertile ≈ Average of only the negative control mice that were fertile
NC_Infertile ≈ Average of only the negative control mice that were infertile
PC ≈ Positive Control group
Z10 ≈ Zidovudine 10 mg/kg group
Z100 ≈ Zidovudine 100 mg/kg group
Z250 ≈ Zidovudine 250 mg/kg group
N5 ≈ Nevirapine 5 mg/kg group
N50 ≈ Nevirapine 50 mg/kg group
N150 ≈ Nevirapine 150 mg/kg group

Table 11: Median Sperm DNA Quantity, Fragmentation and Nicks in the Parental Generation Sampled Full-Term after Drug Administration

Group	Mice (n)	DNA Conc. (µg/ml)	P-Value	Frag DNA (%)	P-Value	Nicked DNA (%)	P-Value
NC_All	6	78.9 ± 29.1	-	81.3 ± 9.3	-	25.5 ± 11.8	-
NC_fertile	4	78.4 ± 35.4	-	81.3 ± 9.8	-	32.0 ± 12.1	-
NC_Infertile	2	79.3 ± 21.7	-	85.3 ± 11.9	-	18.5 ± 12.1	-
PC	4	91.2 ± 84.4	0.726	84.8 ± 3.7	0.883	22.2 ± 15.3	0.467
Z10	6	96.8 ± 32.3	0.333	80.4 ± 6.4	0.739	13.9 ± 7.3	0.063
Z100	6	121.3 ± 34.9	0.126	82.3 ± 4.1	0.872	28.2 ± 9.5	0.515
Z250	6	119.9 ± 28.2	0.139	86.5 ± 5.4	0.757	16.9 ± 8.8	0.183
N5	6	80.9 ± 12.7	0.814	86.0 ± 3.5	0.595	44.1 ± 14.7	0.164
N50	6	121.3 ± 56.8	0.132	85.2 ± 6.9	0.599	18.2 ± 13.1	0.270
N150	6	93.6 ± 36.2	0.226	89.2 ± 6.2	0.471	15.3 ± 3.6	0.064

± SD ≈ Standard deviation; ‡ ≈ significant P-values

NC_All ≈ Average of all negative control group mice
NC_fertile ≈ Average of only the negative control mice that were fertile
NC_Infertile ≈ Average of only the negative control mice that were infertile
PC ≈ Positive Control group
Z10 ≈ Zidovudine 10 mg/kg group
Z100 ≈ Zidovudine 100 mg/kg group
Z250 ≈ Zidovudine 250 mg/kg group
N5 ≈ Nevirapine 5 mg/kg group
N50 ≈ Nevirapine 50 mg/kg group
N150 ≈ Nevirapine 150 mg/kg group

4.5 Results of the Dominant Lethal Assay (DLA) of the Parental Generation

The number of implants and other details recorded for the ZDV and NVP groups mating at mid-term (week 4) and full-term (week 8) are shown in Tables 12 and 13 respectively.

At mid-term, only 4 out of the 8 groups had implants but there was no consistent pattern between the number of pregnancy/implants recorded and the drug concentrations (Table 12). At full-term, all the groups had implants except for the NVP groups. The three NVP groups had no implants at full-term. At full-term, only the group Z100 had one pregnancy showing both some dead and some reabsorbed implants while group Z250 had reabsorbed implants in one mouse. At full-term, two out of the three ZDV groups (Z100 and Z250 groups) had higher average number of implants per pregnancy compared to the NC (Table 13).

In some test groups where no implants / pregnancies were recorded, heavy arterial lining and/or uterine fluids were observed in the female parent. Counting dams with these observations among the pregnancies for each group, the Tables showing the DLA at mid- and full-term were modified (Tables 14 and 15). The modified Table 15 showed all groups as having fertile mating that could become implants at week 8. However nevirapine groups mated at full-term (week 8) had all implants aborted early.

Table 12: Mid-Term Mating Results from the Dominant Lethal Assay of the Parental Generation

Group	Dams (n)	Pregnancy n (%)	Implants (n)	Dead (%)	Resorbed (%)
NC	6	1 (16.7)	8.0	0	0
PC	6	-	-	-	-
Z10	6	-	-	-	-
Z100	6	3 (50.0)	8.3	0	0
Z250	6	-	-	-	-
N5	6	3 (50.0)	6.0	0	0
N50	6	-	-	-	-
N150	6	1 (16.7)	7.0	0	0

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

Table 13: Full-Term Mating Results from the Dominant Lethal Assay of the Parental Generation

Group	Dam (n)	Pregnancy n (%)	Implants (n)	Dead (%)	Resorbed (%)
NC	6	4 (66.7)	6.8	0	0
PC	6	2 (33.3)	8.0	0	50.0
Z10	6	2 (33.3)	6.0	0	0
Z100	6	3 (50.0)	8.3	4.0	4.0
Z250	6	3 (50.0)	9.0	0	14.8
N5	6	-	-	-	-
N50	6	-	-	-	-
N150	6	-	-	-	-

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

Table 14: Mid-Term Dominant Lethal Assay Results of the Parental Generation When Dams with Heavy Arterial Linings on the Uterus are added to the Count

Group	Pregnancy (n)	Pregnancy (%)	Implants (n)	Dead (%)	Resorbed (%)
NC	1	16.7	8.0	0	0
PC	*0/1	*0/16.7	0	0	0
Z10	-	-	-	-	-
Z100	3	50.0	8.3	0	0
Z250	-	-	-	-	-
N5	3	50.0	6.0	0	0
N50	*0/1	*0/16.7	0	0	0
N150	*1/2	*16.7/33.3	7.0	0	0

*value before / value after adding arterial linings to the count

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

Table 15: Full-Term Dominant Lethal Assay Results of the Parental Generation When Dams with Heavy Arterial Linings on the Uterus are added to the Count

Group	Pregnancy (n)	Pregnancy (%)	Implants (n)	Dead (%)	Resorbed (%)
NC	4	66.7	6.8	0	0
PC	2	33.3	8.0	0	50.0
Z10	*2/4	*33.3/66.7	6.0	0	0
Z100	*3/4	*50/66.7	8.3	4.0	4.0
Z250	3	50.00	9.0	0	14.8
N5	*0/2	*0/33.3	0	0	0
N50	*0/2	*0/33.3	0	0	0
N150	*0/3	*0/50.0	0	0	0

*value before / value after adding arterial linings to the count

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

4.6 Gestation Period and Birth Statistics of the Parental Generation

The NC had an average gestation period of 26 days and shorter gestation periods were observed for the treatment groups (Table 16). At the initial mating, all ZDV groups had shorter gestation periods but none was significant (Table 16). For the NVP groups at initial mating, 4 out of 5 groups with births had significantly shorter gestation periods (Table 16). There was no significant difference in gestation period obtained after initial mating and after the recovery mating for the groups that had births at both the initial and recovery mating.

The NC had an average of 4 births from 10 dams. The number of births recorded for the 20 dams (10 "father-only" treated [S] and 10 "both-parents" treated [D]) per group is shown in Figure 6. Generally, dams with "both-parents" being ARV-treated (double exposure) had more births compared to the dams with the "father-only" being ARV-treated (single exposure), and there was significantly more births at initial mating than at the recovery mating [p=0.044]. During labour after recovery mating, one dam each died from two groups (NVP 5mg/kg double exposure [N5-D] and NVP 50mg/kg single exposure [N50-S]) and thus were not counted (asterisked in Figure 6).

For the ZDV "father-only" treated groups, there was no significant difference between the number of births recorded at the initial and at the recovery mating, while for the "both-parents" treated groups there were generally more births at the initial mating than at the recovery mating (Figure 6 and Table 17).

Table 16: Duration of Gestation Observed for each Parental Generation Group

Group	Gestation @ Initial mating (days) ± SD	P-values	Gestation @ Recovery mating (days) ± SD	P-values	Initial versus Recovery mating p-values
NC	26.0 ± 1.4	-	-	-	-
PC-S	22.4 ± 2.3	**‡0.025**	20.0†	-	-
PC-D	24.4 ± 4.2	0.463	24.0 ± 4.4	0.514	0.902
Z10-S	20.7 ± 2.9	0.068	17.5 ± 0.7	**‡0.001**	0.243
Z10-D	23.1 ± 2.8	0.051	20.5 ± 4.9	0.353	0.336
Z100-S	22.0 ± 4.2	0.401	20.5 ± 0.7	**‡0.004**	0.671
Z100-D	17.0†	-	18.0†	-	-
Z250-S	22.0†	-	20.0†	-	-
Z250-D	25.0 ± 3.6	0.630	20.5 ± 2.1	0.120	0.186
N5-S	21.5 ± 3.5	0.306	24.5 ± 2.1	0.489	0.412
N5-D	18.8 ± 1.0	**‡0.000**	-	-	-
N50-S	22.0 ± 0.0	**‡0.011**	21.4 ± 4.5	0.084	0.866
N50-D	19.8 ± 4.4	**‡0.032**	20.5 ± 3.0	**‡0.027**	0.796
N150-S	-	-	22.5 ± 2.1	0.213	-
N150-D	18.9 ± 1.9	**‡0.000**	20.5 ± 3.5	0.252	0.362

SD ≈ Standard Deviation; ‡ ≈ significant P-values
†Single birth recorded thus p-value and SD could not be calculated
NC ≈ Negative control group
PC-S ≈ Positive control "father-only" treated sub-group
PC-D ≈ Positive control "both-parents" treated sub-group
Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group
Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group
Z100-S ≈ Zidovudine 100 mg/kg "father-only" treated sub-group
Z100-D ≈ Zidovudine 100 mg/kg "both-parents" treated sub-group
Z250-S ≈ Zidovudine 250 mg/kg "father-only" treated sub-group
Z250-D ≈ Zidovudine 250 mg/kg "both-parents" treated sub-group
N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group
N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group
N50-S ≈ Nevirapine 50 mg/kg "father-only" treated sub-group
N50-D ≈ Nevirapine 50 mg/kg "both-parents" treated sub-group
N150-S ≈ Nevirapine 250 mg/kg "father-only" treated sub-group
N150-D ≈ Nevirapine 250 mg/kg "both-parents" treated sub-group

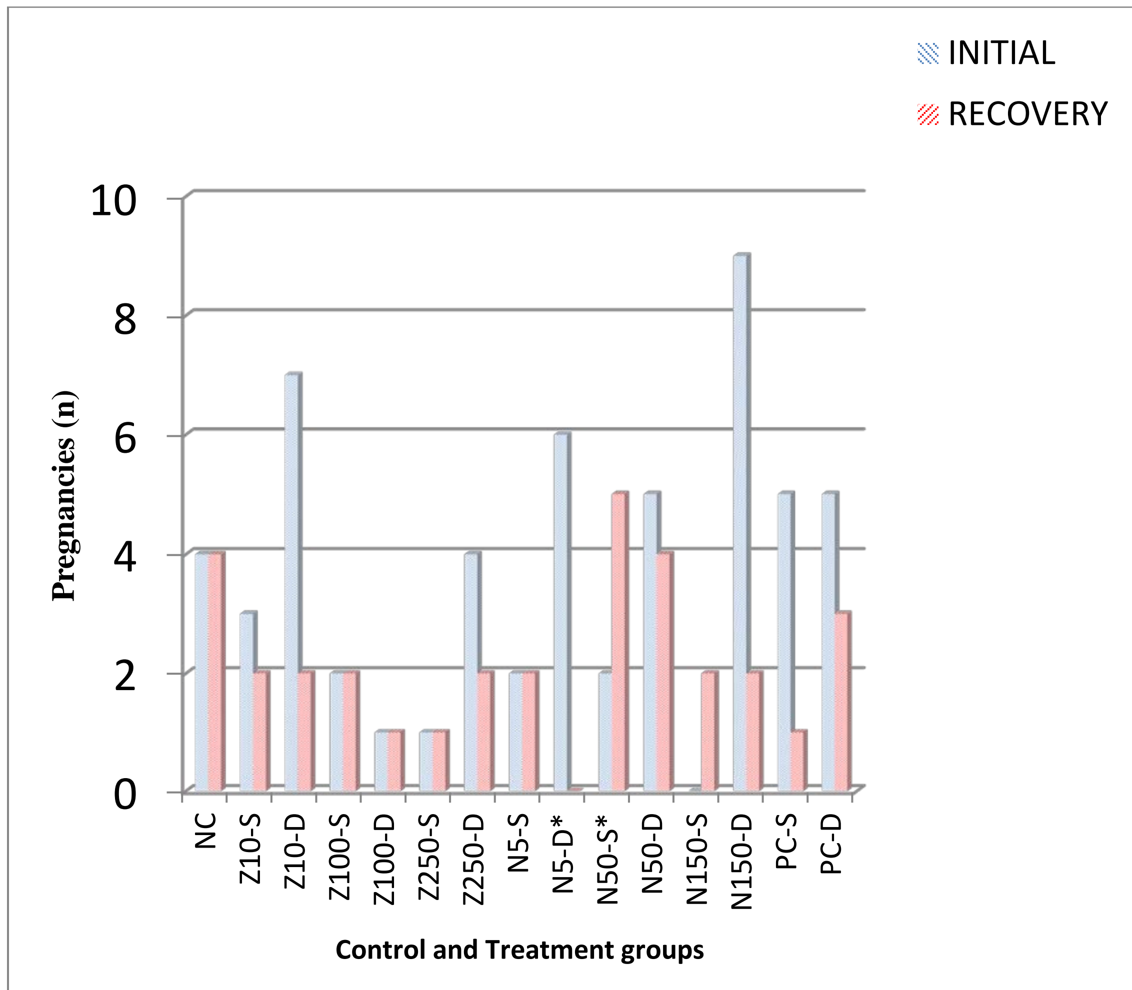

Figure 6: Pregnancies Recorded for the Parental Generation at the Initial and Recovery Mating. At recovery, the asterisked groups each lost one dam during labour and were not counted.

NC ≈ Negative control group
PC-S ≈ Positive control "father-only" treated sub-group
PC-D ≈ Positive control "both-parents" treated sub-group
Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group
Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group
Z100-S ≈ Zidovudine 100 mg/kg "father-only" treated sub-group
Z100-D ≈ Zidovudine 100 mg/kg "both-parents" treated sub-group
Z250-S ≈ Zidovudine 250 mg/kg "father-only" treated sub-group
Z250-D ≈ Zidovudine 250 mg/kg "both-parents" treated sub-group
N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group
N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group
N50-S ≈ Nevirapine 50 mg/kg "father-only" treated sub-group
N50-D ≈ Nevirapine 50 mg/kg "both-parents" treated sub-group
N150-S ≈ Nevirapine 250 mg/kg "father-only" treated sub-group
N150-D ≈ Nevirapine 250 mg/kg "both-parents" treated sub-group

Table 17: Fertility Rates in Relation to Exposure and Mating in the Parental Generation

Groups	Fertility (%) at Initial mating		Fertility (%) at Recovery mating	
	Single	Double	Single	Double*
Z10	30	70	20	20
Z100	20	10	20	10
Z250	10	40	10	20
N5	20	60	20	0
N50	20	50	50	40
N150	0	90	20	20
Single versus Double P-value		**‡0.020**	0.535	†0.814
Single vs Single; Double vs Double P-value			0.364	**‡0.025**

Single ≈ "father-only" treated. Double ≈ "both-parents" treated. *Double exposure at recovery mating was pseudo-single exposure as only the dam was currently on ARVs.
†Single exposure at first mating versus double exposure at recovery mating.
‡Significant P-values

NC ≈ Negative Control group

PC ≈ Positive Control group

Z10 ≈ Zidovudine 10 mg/kg group

Z100 ≈ Zidovudine 100 mg/kg group

Z250 ≈ Zidovudine 250 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

N50 ≈ Nevirapine 50 mg/kg group

N150 ≈ Nevirapine 150 mg/kg group

For the NVP “father-only” treated groups, more births were recorded at the recovery mating than at the initial mating, while for the “both-parents” treated groups the reverse was the case with more births recorded at the initial mating than at the recovery mating (Figure 6 and Table 17).

At the initial mating after 8 weeks of drug administration, the “both-parents” treated groups had significantly higher number of births (p=0.020) than the “father-only” treated groups (Table 17). However, there was no significant difference (p=0.535) in the number of births between the “father-only” and the “both-parents” treated groups at the recovery mating. In addition, there was no significant difference (p=0.814) in the number of births at initial mating from the “father-only” treated groups when compared to the number of births at recovery mating from the “both-parents” treated groups. The “both-parents” treated groups at recovery mating can be regarded as pseudo-single exposure as only the dam was currently on ARVs while the male have been left for 8 weeks without ARVs prior to recovery mating

4.7 Litter Sizes, Birth Weights and Pup Survival amongst the Parental Generation

The average litter size for the NC was 6 pups and the average birth weight for each NC pup was 1.41 grams. The number of pups per litter was varied across the control and treatment groups but the difference between groups at initial and recovery mating was not significant (p=0.90).

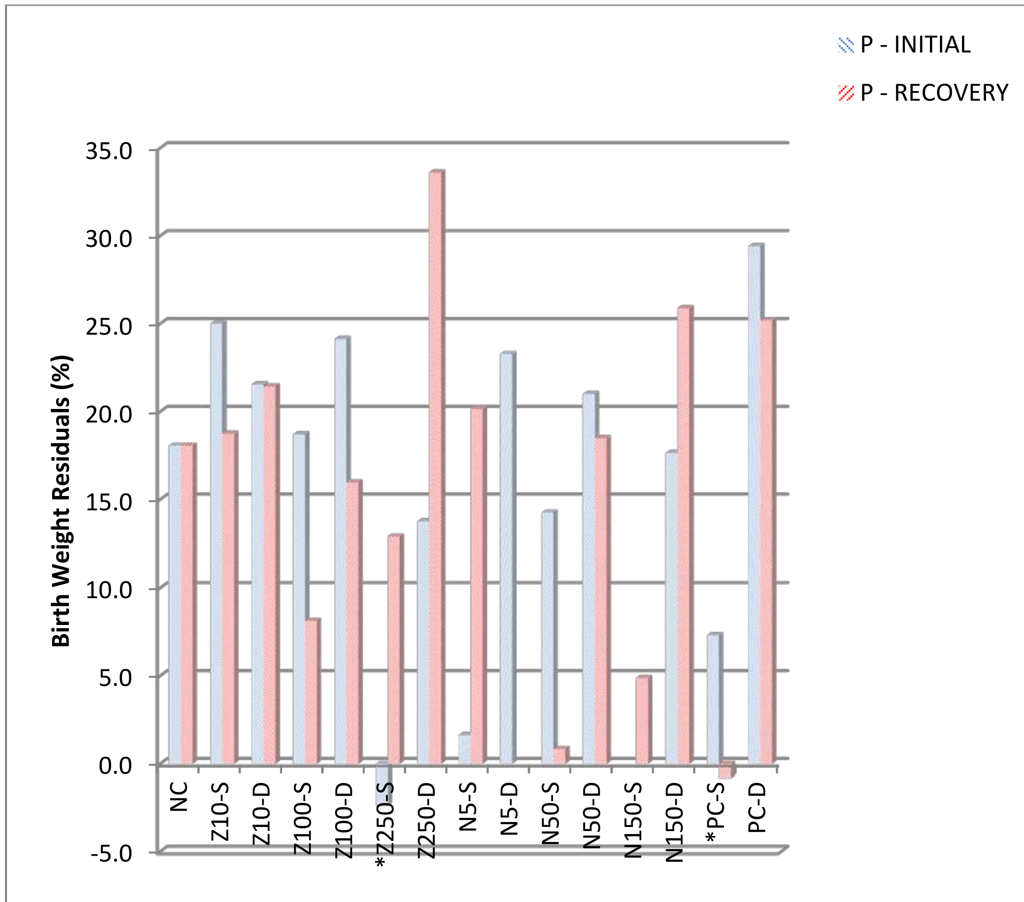

Figure 7: Birth Weight Residuals (%) from the Expected Birth Weights amongst the Parental Generation (Asterisked groups had negative residuals).

NC ≈ Negative control group
PC-S ≈ Positive control "father-only" treated sub-group
PC-D ≈ Positive control "both-parents" treated sub-group
Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group
Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group
Z100-S ≈ Zidovudine 100 mg/kg "father-only" treated sub-group
Z100-D ≈ Zidovudine 100 mg/kg "both-parents" treated sub-group
Z250-S ≈ Zidovudine 250 mg/kg "father-only" treated sub-group
Z250-D ≈ Zidovudine 250 mg/kg "both-parents" treated sub-group
N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group
N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group
N50-S ≈ Nevirapine 50 mg/kg "father-only" treated sub-group
N50-D ≈ Nevirapine 50 mg/kg "both-parents" treated sub-group
N150-S ≈ Nevirapine 250 mg/kg "father-only" treated sub-group
N150-D ≈ Nevirapine 250 mg/kg "both-parents" treated sub-group

Similarly, average birth weights per pup was varied across the control and treatment groups but the difference between groups at initial and recovery mating was not significant (p=0.92). Birth weights being a function of the litter size, residuals (group average birth weight minus the expected as a percentage of the expected) were obtained from the expected birth weights (Enzmann and Crozier, 1935) for particular litter size. The residuals of each group's average birth weight from the expected birth weight per litter size were still not significant at initial and recovery mating (p=0.85). However two groups (Z250-S and PC-S), both with the "father-only" treated, had negative residuals from the expected (Figure 7).

The pups born to the NC had a survival rate of 70.8%. The pups born to the different drug exposure groups exhibited varying rates of survival (0 – 100%) determined at weaning (Table 18). Their sex ratios were also determined at weaning (Table 18). Both the average and median survival rates of the pups born to the "both-parents" treated groups (50.4 and 47.7 respectively) were higher than those of the "father-only" treated groups (41.0 and 31.8 respectively) but the difference was not significant (p=0.821). The "both-parents" treated groups had higher survival rates than the corresponding "father-only" treated groups except for the N50 group (Table 18). The pup deaths were due to a variety of reasons. Some pups were cannibalised while others died without any signs of cannibalism. Pup deaths in ZDV group Z250 were due to cannibalism within days from birth, while deaths at Z10 and N5 were mainly deaths without any bodily signs of cannibalism.

Table 18: Survival Rates and Sex Ratios of the F_1 Generation Pups at Weaning from the Parental Generation Dams

Groups	"Father-only" treated				"Both-parents" treated			
	Pups born (n)	**Pups Survived (n)**	**Survival rate (%)**	**Female to Male ratio**	**Pups born (n)**	**Pups Survived (n)**	**Survival rate (%)**	**Female to Male ratio**
NC	24	17	70.8	8:9	-	-	-	-
Z10	22	7	31.8	2:5	50	27	54.0	14:13
Z100	10	8	80.0	5:3	7	7	100.0	5:2
Z250	4	0	0.0	-	28	0	0.0	-
N5	10	2	20.0	0:2	39	14	35.9	8:6
N50	15	11	73.3	9:2	29	12	41.4	7:5
N150	-	-	-	-	52	37	71.2	22:15
Total (n)	**61**				**205**			
Average			41.0				50.4	
Median			**31.8**				**47.7**	

NC ≈ Negative Control group
PC ≈ Positive Control group
Z10 ≈ Zidovudine 10 mg/kg group
Z100 ≈ Zidovudine 100 mg/kg group
Z250 ≈ Zidovudine 250 mg/kg group
N5 ≈ Nevirapine 5 mg/kg group
N50 ≈ Nevirapine 50 mg/kg group
N150 ≈ Nevirapine 150 mg/kg group

Figure 8 shows two litters born 2 days apart and illustrates their growth difference. It further shows cannibalism involving one pup from the litter exhibiting abnormal growth as evidenced by their fur hairs. Furthermore, it could not be ascertained if exposure to ARV may predispose the dams to producing more female pups as only few groups differed significantly from the expected 1:1 sex ratio (Table 18). The increased fertility observed for the "both-parents" treated groups is also estimated to be about 3.3 times higher than fertility observed for the "father-only" treated groups (from the total number of pups born to each exposure arm – 205/61; Table 18).

4.8 Results of Testis Size, Sperm Count and Anomaly in the F_1 Generation

The number of F_1 generation mice was small for some groups due to the small number of pups born to the P generation that survived to adulthood and produced offspring. The patterns of the testis-body weight ratios and sperm counts from the F_1 generation are varied and are presented in Table 19 according to the drug exposure of their parental generation. Three out of four of the F_1 test groups had significantly smaller testis-body weight ratios. The F_1 generation males were observed to generally have smaller testis sac, especially amongst the ZDV groups.

In addition, sperm counts were severely reduced amongst the F_1 generation (Table 19) such that the highest group sperm count (3,950 x 10^3/ml) recorded for the F_1 generation was still less than half the negative control sperm count (8,055 x 10^3/ml). And 3 out of the 4 groups had significantly lower sperm counts when compared to the NC.

Figure 8: Abnormal looking pup (arrowed) cannibalised by the mother

Table 19: Median Testis-Body Weight Ratios and Sperm Counts obtained for F_1 Generation Males after 8 weeks of Drug Administration

Groups	Males (n)	Testis-Body weight ratio ± SD	P-value	Sperm Count $x10^3$ /ml ± SD	P-values
NC	6	0.366 ± 0.038	-	8055 ± 1179.8	-
Z10-S	4*	0.270 ± 0.036	**‡0.006**	2570 ± 983.1	**‡0.000**
Z10-D	6	0.178 ± 0.075	**‡0.001**	320 ± 412.8	**‡0.000**
N5-S	2*	0.313 ± 0.025	0.105	3950 ± 2636.0	0.340
N5-D	6	0.290 ± 0.049	**‡0.011**	2945 ± 1622.1	**‡0.000**

*Smaller sample sizes due to the small number of surviving pups; SD ≈ Standard deviation; -S ≈ descendent from "father-only" treated and –D ≈ descendent from "both-parents" treated; ‡ ≈ significant P-values

NC ≈ Negative control group

Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group

Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group

N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group

N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group

Furthermore, elevated sperm morphology anomalies were prevalent amongst the F_1 generation with the Zidovudine 10mg/kg "both-parents" treated (Z10-D) group having the highest anomalies (Table 20). However, only that of Z10-D group was significant when compared to the NC (Table 20).

4.9 Sperm DNA Quantity, Fragmentation and Nicks in the F_1 Generation

The F_1 generation values were compared to those of the full-term P generation fertile NC males to determine if they were significantly different. The F_1 generation all had significantly lower sperm DNA quantity (µg/ml) compared to the NC (Table 21).

In addition, their DNA fragmentations (%) were not significantly different from the NC except for N5 group descendent from the "both-parents" treated P generation (N5-D) which had significantly reduced DNA fragmentation. However, the entire F_1 group had lower sperm DNA nicks, with that of Z10-D being significantly smaller (Table 21). The ZDV group Z10 mice descendent from the "both-parents" treated P generation had both significantly lower sperm DNA quantity and DNA nicks (Table 21).

Table 20: Median Sperm Anomalies obtained for F_1 Generation Males after 8 Weeks of Drug Administration

Groups	Males (n)	Sperm anomalies ± SD	P- values
NC	6	2.77 ± 1.87	-
Z10-S	4*	10.99 ± 18.00	0.205
Z10-D	5*	57.21 ± 18.08	**‡0.005**
N5-S	2*	24.28 ± 18.92	0.357
N5-D	6	14.85 ± 29.71	0.091

SD ≈ Standard deviation; ‡ ≈ significant P-values

NC ≈ Negative control group

Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group

Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group

N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group

N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group

Table 21: Median Sperm DNA Quantity, Fragmentation and Nicks obtained For F_1 Generation Males after 8 Weeks of Drug Administration

Group	Mice (n)	DNA Conc. (µg/ml)	P-Value	Frag DNA (%)	P-Value	Nicked DNA (%)	P-Value
NC_All	6	78.9 ± 29.1	-	81.3 ± 9.3	-	25.5 ± 11.8	-
NC_fertile	4	78.4 ± 35.4	-	81.3 ± 9.8	-	32.0 ± 12.1	-
NC_Infertile	2	79.3 ± 21.7	-	85.3 ± 11.9	-	18.5 ± 12.1	-
Z10-S	4	19.9 ± 1.5	**‡0.005**	69.6 ± 19.8	0.163	14.8 ± 7.5	0.051
Z10-D	6	19.3 ± 2.5	**‡0.004**	89.0 ±15.7	0.834	3.5 ± 3.2	**‡0.015**
N5-S	2	20.4 ± 0.1	**‡0.005**	34.6 ± 49.0	0.384	11.2 ± 9.3	0.113
N5-D	6	21.5 ± 1.9	**‡0.005**	60.2 ± 14.0	**‡0.019**	15.5 ± 6.7	0.052

± Standard deviation; -S ≈ descendent from "father-only" treated and –D ≈ descendent from "both-parents" treated; ‡ ≈ significant P-values

NC_All ≈ Average of all negative control group mice

NC_fertile ≈ Average of only the negative control mice that were fertile

NC_Infertile ≈ Average of only the negative control mice that were infertile

Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group

Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group

N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group

N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group

4.10 Results of the Dominant Lethal Assay of the F_1 Generation

The DLA assay was performed mid-term of drug administration (week 4) only for the ZDV F_1 generation groups. All the 6 female mice per group had no implants. Hence it was discontinued for the nevirapine groups.

4.11 Gestation Period, Birth Statistics, Litter Size and Birth Weights amongst the F_1 Generation

Only one birth was recorded for the F_1 Z10 and N5 groups. This was from the N5 group descendent from the "both-parents" treated P generation that also had "both-F_1 parents" exposed to ARVs (Table 22). Its gestation period was 24 days. It had 3 pups with an average birth weight of 1.57 g and a positive residual of 27.6% from the expected weight.

4.12 Inter-Generational Variations between the Parental and F_1 Generations

On comparing the ZDV P and F_1 generations testis-body weight ratios individually (Z10-S p=0.021; Z10-D p=0.003) and collectively (p = 0.001), the F_1 generation had significantly smaller ratios (Table 23). However, for the NVP parental and F_1 generations, testis-body weight ratios were not significantly different when compared individually (N5-S p=0.900; N5-D p=0.470) and collectively (p = 0.563).

Table 22: Number of Births Obtained From the F_1 Groups

P generation exposure →	**Descendent from "Father-only"**		**Descendent from "Both-parents"**	
F_1 generation exposure →	Father-only	Both-parents	Father-only	Both-parents
Groups ↓	Number of births (dams)			
Z10	0 (10)	0 (2)	0 (10)	0 (10)
N5	0 (10)	-	0 (10)	1 (8)

Z10 ≈ Zidovudine 10 mg/kg group

N5 ≈ Nevirapine 5 mg/kg group

Table 23: Inter-Generational Comparison of Values between the Parental (P) and F_1 Generations

Parameter	Z10 Median values					N5 Median values				
	P Z10	F_1 Z10-S	P-value	F_1 Z10-D	P-value	P N5	F_1 N5-S†	P-value	F_1 N5-D	P-value
Testis-Body weight ratios	0.34	0.27	**‡0.021**	0.18	**‡0.003**	0.30	0.31	0.900	0.29	0.470
Sperm counts (10^3/ml)	5425	2570	**‡0.005**	320	**‡0.000**	4375	3950	0.852	2945	0.184
% Sperm head anomaly	4.98	10.99	0.479	57.21	**‡0.005**	2.05	24.28	0.370	14.85	0.105
DNA quantity	96.8	19.9	**‡0.002**	19.3	**‡0.002**	80.9	20.4	**‡0.000**	21.5	**‡0.000**
DNA (pg) / Sperm	20.6	6.4	**‡0.004**	56.4	0.065	18.9	9.3	0.333	7.1	**‡0.003**
Sperm DNA Fragments %	80.4	69.6	0.194	89.0	0.976	86.0	34.6	0.370	60.2	**‡0.006**
% Sperm DNA Nicks	13.9	14.8	0.745	3.5	**‡0.009**	44.1	11.2	**‡0.033**	15.5	**‡0.002**

† N5-S group values from only 2 surviving males; ‡ ≈ significant P-values

Z10-S ≈ Zidovudine 10 mg/kg "father-only" treated sub-group
Z10-D ≈ Zidovudine 10 mg/kg "both-parents" treated sub-group
N5-S ≈ Nevirapine 5 mg/kg "father-only" treated sub-group
N5-D ≈ Nevirapine 5 mg/kg "both-parents" treated sub-group

Similarly, when the ZDV P and F_1 generations sperm counts were compared individually (Z10-S p=0.005; Z10-D p=0.000) and collectively (p = 0.000), the F_1 generation had significantly reduced sperm counts. However, the NVP P and F_1 generations sperm counts were not significantly different when compared individually (N5-S p=0.852; N5-D p=0.184) and collectively (p = 0.254) though they had lower counts.

In addition, when sperm morphology anomalies amongst the ZDV P and F_1 generations were compared individually (Z10-S p=0.479; Z10-D p=0.005) and collectively (p = 0.019), the F_1 generation had higher anomalies with some being significant (Table 23). Sperm anomalies increased amongst the NVP F_1 generation compared to their P generation but were not significant individually (N5-S p=0.370; N5-D p=0.105), only being significantly higher collectively (p = 0.043).

For both the ZDV and NVP F_1 groups, the testis-body weight ratios and sperm counts were least for the "both-parent" treated groups. In summary, most of the F_1 groups (3 out of 4 F_1 generation groups; Z10-S, Z10-D, N5-D) had significantly smaller testis-body weight ratios compared to only the N5 group amongst the P generation (Table 23).

The DNA quantity per sperm was different between the Z10 P generation and the Z10 F_1 group descendent from the "father-only" treated group, with the F_1 value being significantly smaller (Table 23). The Z10 F_1 group descendent from the "both-parents"

treated group had increased DNA quantity / sperm with it median value being greater than twice the already elevated Z10 P generation value (Table 23). However, the difference was not significant (p=0.065). Both the N5-S and N5-D F_1 groups had lower DNA quantity per sperm compared to the N5 P generation value but only that of the N5-D F_1 group was significant lower (p=0.003). However, these reduced DNA quantity per sperm were now within the range of values for the unexposed controls (9.3 ± 3.9 pg).

The degree of sperm DNA fragmentation of the ZDV F_1 groups was not significantly different from that of their P generation values. Amongst the NVP F_1 groups, only the N5-D group had significantly decreased DNA fragmentation (p=0.006). Furthermore, the sperm DNA nicks was significantly reduced 3-5 folds in the F_1 generation of Z10-D, N5-S and N5-D groups when compared to their P generation (Table 23). In the parental generation both Z10 groups had a total of 10 pregnancies while the N5 group had a total of 8 pregnancies. In the F_1 generation, the Z10 groups had no pregnancy while the N5 groups recorded only one. This gives a total of 18 pregnancies in the P generation compared to one in the F_1 generation. This represents a 94.4% reduction in fertility (Figure 6 and Table 22).

Using the week 4 and week 8 data of the P generation in univariate correlation analysis, the testis weight correlated positively with the sperm count and sperm DNA quantity while it had negative correlation with the DNA quantity per sperm and the DNA nicks (Table 24).

Table 24: Correlation Coefficients between the Variables Using the Parental Generation Data

	Testis Weight	Sperm Count	Sperm Anomaly	DNA Qty	DLA Preg-Individual	DLA Preg-Group	Preg-S	Preg-D	Preg-T
Testis Weight	--	0.53	-0.23*	--	--	--	--	--	--
Sperm Count	0.53	--	-0.33	--	0.25	--	--	--	--
Sperm Anomaly	-0.23*	-0.33	--	--	--	--	--	-0.29	-0.33
DNA Qty	0.44	0.31	--	--	0.20*	--	--	--	--
DNA / Sperm	-0.26	-0.71	0.31	--	--	--	--	--	--
DNA Frag	--	-0.21*	--	--	--	--	--	--	--
DNA Nicks	-0.45	-0.35	--	-0.58	--	--	--	--	--
Dose	--	--	--	--	--	0.79	-0.84	--	-0.46

P-value less than 0.05 is regarded as significant.
*Asterisked coefficients not significant but p-values between 0.05 and 0.09
DNA Qty ≈ DNA quantity (µg/ml)
DLA Preg-Individual ≈ Dominant lethal assay pregnancies when compared individually
DLA Preg-Group ≈ Dominant lethal assay pregnancies when compared group-wise
Preg-S ≈ Pregnancies or births amongst the Father-only treated groups
Preg-D ≈ Pregnancies or births amongst the Both-parents treated groups
Preg-T ≈ Sums of pregnancies or births to both groups

Testis weight also had a non-significant negative correlation with the sperm anomalies seen. The sperm count was negatively correlated with the sperm anomalies and DNA nicks and positively correlated with the DNA quantity. The sperm anomalies had a positive correlation with the DNA quantity per sperm while the DNA quantity was negatively correlated with DNA nicks (Table 24).

The number of pregnancies recorded in the dominant lethal assay was positively correlated with sperm count when compared individually but not as a group. Number of pregnancies amongst the father-only treated groups had no significant correlation with the sperm parameters measured. The number of pregnancies observed for the both-parent treated groups had a negative correlation with only the sperm anomaly. When pregnancy data was combined for both the father-only and both-parents treated groups and analysed, only the negative correlations with the sperm anomalies and dosage administered were significant (Table 24).

The use of the linear regression model to predict pregnancy / fertility gave varied results for the father-only and both-parents treated groups at full-term. The best regression model for the father-only treated groups explained 83% of the variation while for the both-parents treated groups, the best model only explained 28% of the variation (Table 25). The best regression model predicting pregnancies amongst the "father-only", the "both-parents" and all treated groups are shown graphically in Figure 9. The DNA quantity (μg/ml sperm) and the DNA nicks (%) were the two variables retained in the

best model for both treatment groups. The variables included in the best regression to predict pregnancy in both treatment arms were the sperm anomalies, DNA quantity, DNA nicks and dose (Table 25) and these explained 41% of the variation seen. The coefficients for the best regression models predicting pregnancies amongst the “father-only” and “both-parents” treated groups are shown in Tables 26 and 27 respectively, while the coefficients for the best model predicting pregnancies in both groups together are shown in Table 28.

Table 25: Comparing the Regression Models (Variables and Importance) Predicting Fertility amongst the Parental Generation at Full Term

	Regression Model	Independent variables included	ANOVA p-value	R-Squared (%)	R-Squared *df* adjusted (%)
Preg-S	Best	Testis weight, DNA quantity, DNA nicks and Dose	0.0000	85.4	83.0
	Second	Testis weight, DNA quantity, DNA nicks, Dose and ***DNA Fragmentation**	0.0000	86.8	84.1
	Third	Testis weight, DNA quantity, DNA nicks, Dose, ***Sperm Count** and ***DNA Fragmentation**	0.0000	86.9	83.5
Preg-D	Best	Sperm Anomaly, DNA quantity and DNA nicks	0.0054	34.9	28.2
	Second	Sperm Anomaly, DNA quantity, DNA nicks and ***Body weight**	0.0090	37.3	28.4
	Third	Sperm Anomaly, DNA quantity, DNA nicks and ***Body weight, *testis weight, *Epididymis weight, *Sperm count** and ***Sperm count per epididymis weight**	0.0441	44.8	26.3
Preg-T	Best	Sperm Anomaly, DNA quantity, DNA nicks and Dose	0.0007	48.7	41.4
	Second	Sperm Anomaly, DNA quantity, DNA nicks, Dose, ***Body weight, *Epididymis weight, *sperm count** and ***sperm count per epididymis weight**	0.0064	55.0	40.0
	Third	Sperm Anomaly, DNA quantity, DNA nicks, Dose and ***Body weight**	0.0017	49.3	39.9

*Asterisked variables are not significant in the regression equation (individual $p \geq 0.05$)
Preg-S ≈ Pregnancies or births amongst the Father-only treated groups; Preg-D ≈ Pregnancies or births amongst the Both-parents treated groups; Preg-T ≈ Sums of pregnancies or births to both groups; *Df* ≈ Degrees of freedom; ANOVA ≈ Analysis of variance; R-squared ≈Correlation coefficient squared to show variation explained.

Table 26: Regression Coefficients for the Best Regression Model Predicting Pregnancy amongst the "Father-only" Treated Groups

Coefficients[a]					
Model	Unstandardized Coefficients		Standardized Coefficients	t	P-value
	B	Std. Error	Beta		
(Constant)	2.186	0.409		5.346	0.000
AvWtTestis	11.919	4.541	0.254	2.625	0.015
SpDNAQty	-0.003	0.002	-0.193	-2.061	0.050
SpDNANick	-0.017	0.004	-0.374	-4.428	0.000
Dose	-0.007	0.001	-1.002	-11.427	0.000

a. Dependent Variable: PregS

AvWtTestis = Testicular weight (g)

SpDNAQty = Sperm DNA Quantity (µg/ml)

SpDNANick = Sperm DNA Nicks (%)

Dose = Dose of drug received

PregS = Pregnancies amongst the "Father-only" treated groups

Table 27: Regression Coefficients for the Best Regression Model Predicting Pregnancy amongst the "Both-Parents" Treated Groups

Coefficients[a]					
Model	Unstandardized Coefficients		Standardized Coefficients	t	P-value
	B	Std. Error	Beta		
(Constant)	11.687	1.763		6.630	0.000
SpHdAbn	-0.106	0.035	-0.469	-2.981	0.006
SpDNAQty	-0.028	0.011	-0.417	-2.574	0.015
SpDNANick	-0.084	0.029	-0.483	-2.860	0.008

a. Dependent Variable: PregD

SpHdAbn = Sperm Head Anomalies (%)

SpDNAQty = Sperm DNA Quantity (µg/ml)

SpDNANick = Sperm DNA Nicks (%)

PregD = Pregnancies amongst the "Both-parents" treated groups

Table 28: Regression Coefficients for the Best Regression Model Predicting Pregnancy amongst Both the "Father-only" and the "Both-Parents" Treated Groups

Coefficients[a]					
Model	Unstandardized Coefficients		Standardized Coefficients	t	P-value
	B	Std. Error	Beta		
(Constant)	13.722	1.524		9.007	0.000
SpHdAbn	-0.083	0.031	-0.393	-2.726	0.011
SpDNAQty	-0.025	0.009	-0.384	-2.597	0.015
SpDNANick	-0.083	0.025	-0.506	-3.280	0.003
Dose	-0.012	0.004	-0.403	-2.820	0.009

a. Dependent Variable: PregT

SpHdAbn = Sperm Head Anomalies (%)

SpDNAQty = Sperm DNA Quantity (µg/ml)

SpDNANick = Sperm DNA Nicks (%)

Dose = Dose of the drug received

PregT = Pregnancies amongst both the "Father-only" and the "Both-parents" treated groups

5.0 DISCUSSION

The *Allium cepa* 96 hr Root Growth Inhibition and the 48 hr Recovery Assays

The 96 hr *Allium cepa* root growth inhibition assay suggests that zidovudine is more toxic than nevirapine especially at the higher concentrations of 400-1200 µM. The lower EC_{50} obtained for zidovudine supports this. The relationship between drug concentrations and root growth does not appear to be linear for zidovudine. This could mean that there are different toxic mechanism(s) operating at different concentrations. However, nevirapine had a linear relationship between its concentrations and the length of root growth they allowed. The 400 µM concentration was a threshold beyond which the higher concentrations had only little additional growth after the drugs were withdrawn. This suggests that the genotoxic effects particularly at those concentrations may not be reversible.

The 48 hr recovery study further suggests that when the drugs are withdrawn there would be a semblance of recovery at all concentrations. However, the root growth inhibition effect could not be fully reversed as most groups still had cumulative root growths less than half the root growth exhibited by the negative control. This finding from the *Allium cepa* model is contradictory to earlier reports about recovery from the effects of ARVs in exposed children. Briand *et al.,* (2006) worked with children exposed *in utero* to zidovudine for longer than 7.5 weeks and found they had slightly lower birth weight, a small difference which faded before they were 18 months old. However, the monitoring period of 18 months was small. Culnane *et al.,* (1999) found no significant difference in several parameters after monitoring zidovudine-exposed

children between 3.2 – 5.6 years and yet suggested continued monitoring and evaluations. This study using *Allium cepa* bulbs show there will be some recovery as evidenced by the additional root growths. However, the *Allium cepa* bulbs treated with the least concentrations of zidovudine or nevirapine achieved only two-thirds of the growth the untreated bulbs attained. And the least concentrations used here were equivalent to the peak blood levels of these drugs at the therapeutic dose.

The *Allium cepa* 48 hr Chromosomal Aberration Assay Results

All treatment groups had mitotic index higher than the negative control, except for the zidovudine 100% EC_{50} group, suggesting the administration of the drugs may have made the root cells mitogenic. This mitogenic activity could be a compensatory response mechanism for the cells being destroyed or compromised. This explanation is plausible as it would agree with the finding of tenfold increase in micro-nucleated reticulocyte frequencies among mother-child pairs that received zidovudine prenatally (Witts *et al.,* 2007). Micro-nucleated reticulocytes often occur as a result of damage to the reticulocytes and the body being in a hurry to replace the damaged cells, releases cells not fully matured into the blood stream. At maturity, mammalian red blood cells lose their nuclei. Hence the postulation that increased mitogenic activity in ARV-exposed *Allium cepa* root cells might be a compensatory mechanism.

The inconsistency of mitotic phase distribution patterns with dose observed in the zidovudine groups may suggest that different toxic mechanism(s) may be at work at different concentrations. Stickiness was the major chromosomal anomaly recorded and

more aberrations were seen at higher concentrations though not significantly higher. Stickiness during cell division could lead to aneuploidy and/or polyploidy. Stickiness combined with other aberrations seen, such as bridges and vagrant chromosome formations, increases the potential to induce micro or macronuclei formation, aneuploidy and/or polyploidy. Escobar *et al.,* (2007) screened for DNA damage in reticulocytes from mother-child pairs exposed to zidovudine using the glycophorin A (GPA) somatic cell mutation assay. They clearly showed that GPA variants arising from chromosome loss, duplication and recombination were significantly elevated in the zidovudine-exposed pairs (Escobar *et al.,* 2007). Torres *et al.,* (2007) similarly reported ARV-induced mutagenic responses after evaluating *Hprt* mutant frequencies in T-cells. In this study, the stickiness and bridges observed could produce uneven breaks and loss of fragments. Crasta *et al.,* (2012) tracked newly formed micronuclei and showed evidence that errors in mitosis generate DNA breaks and whole-chromosome containing micronuclei may form. These micronuclei undergo defective and asynchronous DNA replication that extensively fragment the DNA, persist in cells over several generations and can be distributed to daughter nuclei (Crasta *et al.,* 2012). They thus showed how chromosome segregation errors or aberrations can lead to mutations and chromosomal rearrangements that can integrate into the genome and persist over generation (Crasta *et al.,* 2012). Consequently, the DNA damage and aberrations in cell division induced by zidovudine and nevirapine tested in this study may persist, could become transferred to offspring and could be involved in tumorigenesis.

The Genotoxic Effects Exhibited Confirm the Utility of the *Allium cepa* Model

It has been reported in the literature that zidovudine arrests HeLa cells in S-phase (Olivero *et al.,* 2005) while nevirapine induces premature senescence and accumulate HeLa cells in G1 phase (Landriscina *et al.,* 2005; Stefanidis *et al.,* 2008). The findings of this study agree with these earlier reports because many cells were synchronised in interphase, suggesting the cells were arrested in interphase. Furthermore, the nevirapine-treated *Allium cepa* root cells had few or no cells in metaphase and anaphase as most cells were in telophase. This alteration in the mitotic phase distribution agrees with the induction of premature senescence. It would appear that nevirapine-treated cells released from the interphase arrest into mitosis may have had their metaphase and anaphase period shortened. Thus most cells in mitosis amongst the NVP groups were congregated at telophase. If this proves to be the case, it would have dire consequences if important cell division checkpoints are skipped. Errors could accumulate to the detriment of future generations as viability and fertility may be compromised.

The results suggest that the genotoxicity of zidovudine or nevirapine can be demonstrated in the *Allium cepa* model. In our setting at least, this could enhance the Reduce-Refine-Replace (3Rs) initiative. Often resource-limited settings lack capacity for cell-lines research and have to use mostly animal models for research. As the 3Rs initiative seeks to reduce the extensive use of animal models in research, scientists are encouraged to reduce the number of animal models used, to refine their methodologies and ultimately to replace the animal models with other models such as the plant *Allium*

cepa model used here. Though it is a plant model, it has been able to demonstrate the genotoxicity of zidovudine and nevirapine consistent with earlier reports. The *Allium cepa* model can thus be used to further elucidate the mechanisms of these genotoxic effects and their large chromosomes might make it easier to elucidate the mechanism(s).

Effect on Mice Testicular Size

After administering the ARVs for the entire period of spermatogenesis, some treatment groups exhibited significantly lowered testis-body weight ratio, with its frequency of occurrence increasing across the generations. The effect of this on spermatic parameters, function and fertility remains to be ascertained. Two genetic studies working with mutant or knockout mice reported reduced sperm counts as a result of reductions in testis weight when certain genes were turned off but this did not affect the mice fertility (litter size or frequency) and the sperm DNA quality (Schürmann *et al.,* 2002; Santti *et al.,* 2005). Thus the reason(s) for the significant reductions in sperm counts observed in this study may be connected with testicular weight but might not affect fertility.

Mice Testicular Weight and Sperm Counts

Reduced testicular weight observed suggests impaired testis development. It is worthy of note that in this study, the 5mg/kg nevirapine group that had smaller testis also had lower sperm counts at full term while fertility in the dams sired by them ranged from 20 – 60%. Bujan *et al.,* (2007) earlier reported that semen volumes, percentages of progressive motile spermatozoa and total sperm counts decreased, while the pH values

and spermatozoa multiple anomaly indices were increased in HIV-infected patients. In this study, we can attribute the reductions in mice sperm counts and increased sperm head anomalies to the administration of ARVs.

Testicular Weight and Reproductive Outcomes

The testicular weight had significant correlations with the sperm counts, sperm DNA quantity and sperm DNA nicks. These indicate that testis weight affects these parameters which are relevant to fertility. Thus testicular size may directly or indirectly affect fertility. In addition the testis weight was a significant variable in the regression equation predicting fertility amongst the "father-only" treated groups. Their regression equation explained the highest variability in reproductive outcomes (83%). However, in the multivariate analysis predicting fertility in all treatment groups using data from both the "father-only" and "both-parents" treated groups, testis weight was not selected statistically for inclusion into any of the top 3 models. This suggests that when acting alone, testis weight influences fertility but when other confounding factors are involved its effect diminishes. Other confounding factors here suggested include the repair of sperm genomic DNA damage in the oocyte. In this study, the repair of DNA damage in the oocyte is postulated to have improved fertility in the "both-parents" exposed groups as the oocyte DNA repair machinery may be ready and more effective having had to repair its own DNA damage after ARV treatment.

Sperm DNA Quantification and Quality in Relation to Reproductive Outcomes

Since the sperm counts and morphology results alone could not fully explain the fertility profile seen, the sperm DNA quantity and quality were evaluated. Saleh *et al.,* (2002) had earlier shown that there was a significant increase in DNA damage in sperm from infertile men with normal standard sperm parameters. In this study, after 8 weeks of administrating ARVs, the sperm DNA quantity of the treatment groups were all higher than that of the untreated control in the parental generation. The DNA damage and chromosomal aberrations induced by the ARVs administered could be responsible for the higher sperm DNA quantity amongst the treatment groups. In the *Allium cepa*, sticky chromosomes were crucial in the genotoxicity of zidovudine or nevirapine. If the high frequency of stickiness seen in the *Allium cepa* model were replicated during spermatogenesis in the mouse, this could result in abnormal DNA quantity (higher or lower) in some spermatids.

There was no significant difference in sperm DNA fragmentation between the treatment groups and the untreated control. Similarly, there was no significant difference in the percentage nicks in sperm DNA between the treatment groups and the negative control. However, the treatment group had reduced nicks in sperm DNA. From the multivariate regression analysis, the nicks in sperm DNA played a role in determining fertility and not the sperm DNA fragmentation that is frequently used as a marker of sperm DNA damage. Since sperm DNA fragmentation has been earlier reported to be an unreliable predictor of reproductive outcomes (Li *et al.,* 2006; Tamburrino *et al.,* 2012) as was the

case in this study, determining sperm DNA nicks might better predict reproductive outcomes.

For the nevirapine-treated groups, the induction of premature senescence associated with this drug might aggravate the DNA damage if cell division is hastened to its completion and cellular check points were skipped. These increase the potential for the uneven distribution and/or loss of nuclear material. And if the chromosomes were sticky as observed in *Allium cepa*, there may be fewer fragmentation and nicks in the sperm DNA. These could affect their sperm DNA quantity, the DNA fragmentation and the DNA nicks much more when compared to the zidovudine-treated groups. The observation of lower DNA quantity, DNA fragmentation and DNA nicks amongst some NVP F_1 groups point to this.

The effects on fertility amongst the NVP groups were varied. If important cell division checkpoints were skipped, unviable embryo or zygote may result causing nature to abort them. This may be why early foetal loss was prevalent across the NVP groups from the dominant lethal assay. Another possible explanation could be that the “sticky” genome did not decondense for subsequent transcription when needed for development, hence the early abortions. These might also be implicated in the lower pup survival rates for the NVP-treated groups if the genome could not be accessed to support development. More importantly, the inclusion of the sperm DNA quantity and the percentage nicks as significant determinants of fertility in all groups in this study supports these postulations. In their review Tamburrino *et al.,* (2012) had concluded that in humans,

DNA fragmentation is associated with alterations in embryo quality, leading to a decreased implantation rate or an increase in early miscarriage after assisted reproductive technologies have been used.

Tamburrino *et al.,* (2012) further clarified that those methods directly evaluating the occurrence of DNA strand breaks (TUNEL and COMET) better define clear and direct relationship between sperm DNA fragmentation and assisted reproductive outcomes. The methods by Georgiou *et al.,* (2009) used in this study for quantifying DNA fragmentation and DNA nicks are ultrasensitive and quantitative complements of the COMET (measures irreparable DNA damage) and TUNEL (measures repairable DNA damage) assays respectively. This may explain why the findings from this study agree with the conclusion of Tamburrino *et al.,* (2012). The greater relevance of the DNA nicks to reproductive outcomes may be because it measures reparable DNA damage.

Dominant Lethal Assay Results

The result of the modified dominant lethal assay showed early foetal loss was an effect of administering zidovudine or nevirapine, particularly for the nevirapine-treated groups. Early foetal loss was observed in the two highest doses of zidovudine and in all doses of nevirapine administered in the mice study. The nevirapine-treated groups had no implant but had an average of 3 dams from each group losing their pregnancies. It is possible fertile mating occurred and may have been aborted early for example at implantation (Tamburrino *et al.,* 2012). This further agrees with the findings of Barreiro *et al.,* (2006) that the rate of foetal death was higher in HIV-positive (23%) women than

in HIV-negative (4.7%) women (odds ratio = 6.1, 95% CI: 1.02 to 46.68; P value = 0.02). This study thus implicates the administration of zidovudine and nevirapine in early foetal loss in mice.

Birth Statistics Data and Fertility

The birth statistics data showed that fertility amongst the "both-parents" treated groups increased while fertility was reduced amongst the cases where only "one-parent" was ARV-treated. When birth statistics from the initial and "recovery mating" were compared, it became evident that the increase in fertility only obtains if both parents are *currently* on ARVs therapy. The mechanism(s) for these observations is not clear. It has been shown that the oocyte can repair DNA damage in sperm (Ménézo *et al.,* 2010). It is therefore possible that the extent of the repair is higher than earlier postulated under certain conditions. In this study, fertility was increased 3 times amongst the "both-parents" treated groups and oocyte repair of DNA damage in the sperm is postulated to be responsible for this. Oocyte repair of sperm DNA damage will yield viable embryo and offspring, reducing early foetal loss and improving pup survival (Ménézo *et al.,* 2010). If the female received antiretroviral drugs, it may suffer DNA damage which its system will attempt to repair. Hence the DNA repair mechanism in the female would already be activated, efficient and effective in repairing DNA damage. If sperm exhibiting DNA damage fertilises an oocyte from such dams, the DNA repair mechanism may quickly repair the damage, may be beyond what would have been possible if the dam had not received the DNA damaging drugs.

Two key findings from the multivariate regression analysis support this explanation. First, sperm DNA nicks, a marker for repairable DNA damage, was significant and included in the best model predicting fertility in the “father-only” treated groups, the “both-parents” treated groups and their combination. Secondly, in this study, 3 doses of zidovudine (10, 100 and 250 mg/kg) and nevirapine (5, 50 and 150 mg/kg) were administered. For the “father-only” treated groups, dose was a significant variable in the top three multivariate regression models predicting fertility. However, for the “both-parents” treated groups, dose was not included at all in the top three multivariate regression models predicting fertility. For dose, with more than tenfold difference between the least and highest concentrations, not to influence fertility means another mechanism was at work voiding its impact. That mechanism might be the DNA damage repairing effect of the oocyte fertilized (Ménézo *et al.,* 2010). It is possible that when the female is also exposed to the ARVs causing DNA damage, the oocytes may have been predisposed to DNA repair (maybe from repairing its own ARVs induced DNA damage), facilitating its ability to do more repairs on the sperm genome. This may be why the best model predicting fertility for the “both-parents” treated groups explained only 28% of the variability compared to 83% explained by the best model for the “father-only” treated groups.

Moreover, the few or no pregnancies recorded in the dominant lethal assay (DLA) for the parental and F_1 generations respectively may be additional proof that the DNA repair predisposed oocyte is able to do DNA repairs and increase fertility. This is because for the DLA assessments, only the father was ARV-treated and mated to virgin untreated females.

Human Studies show Enhanced Fertility when Both Couples are on HAART

There are indications that the enhanced fertility observed in this mice study when "both-parents" are ARV-treated also occurs in humans. First, it is mainly HIV sero-discordant couples that present at fertility clinics and fertility treatment is ethically only with pre-exposure ARV prophylaxis for the uninfected spouse prior to intercourse. Thus Vernazza (2008) reported that in 22 couples having unprotected intercourse with ***a short pre-exposure prophylaxis for the woman while the male partner was under completely suppressive HAART***, pregnancy rates were still surprisingly high being more than 50% after three timed intercourses. In addition, Myer *et al.,* (2010) in a review of 11 programs across seven African countries reported that the ***rate of new pregnancies was significantly higher among women receiving ART*** compared to women not on ART. Myer *et al.,* (2010) stated that other factors independently associated with increased incident pregnancy included ***having a male partner enrolled*** and called for further investigations to unravel the biomedical mechanism(s) underlying these associations.

Thus it remains to elucidate the mechanism(s) and to ascertain whether DNA repair by the oocyte as postulated here is implicated. This fertility enhancing effect when "both-parents" are ARV-treated may explain why fertility effects noticed in humans could not be clearly attributed to either HIV or to the ARVs. The results of this study further suggest that offspring born to the "both-parents" treated groups had higher survival rate than those born to the "father-only" treated groups. This could still be related to the

DNA repair by the oocytes resulting in the offspring from the “both-parents” treated groups having healthier DNA genome complement.

Pup Survival and Causes of Mortality

Many of the pups that died in the higher dose groups, particularly the zidovudine 250mg/kg group, were mainly from cannibalism within days from birth. Zidovudine is known to induce post-natal stress in women and this might be related to the induction of post-natal stress in the dams by zidovudine. Thus there was no survivor from 32 pups born to the dams treated with the highest zidovudine concentration (250mg/kg). Furthermore, literature cites congenital defects as a possible reason for cannibalism by the dams as they can detect which pup has congenital defects. In this study, the congenital deformities would most likely be genome-based as no physical deformities were observed in the pups at birth. Death of pups from the least doses administered (zidovudine 10mg/kg and nevirapine 5 mg/kg) maybe due to congenital defects as there were no bodily signs of cannibalism on the dead pups. It appears that different genotoxic mechanism(s) become operational depending on the drug concentration.

Gestation Periods, Litter Sizes and Birth Weights

In this study, gestation periods shorter than that of the negative control were observed for all treatment groups, though not all were significant. This agrees with the report of Sauer *et al.,* (2009) from studies in humans. In a review of their 10 years’ experience at providing fertility care to HIV-positive men with HIV-negative partners, Sauer *et al.,* (2009) reported a high rate of the infants was born premature (preterm delivery ≈ 43%).

In the *Allium cepa* study, it was found that the ARVs altered mitotic phase distributions, hastening cells through cell division. The administration of the ARVs in mice might be resulting in shorter gestation periods in the treatment groups through a similar mechanism. It appears the ARVs by some mechanism(s) hastened the cellular processes such as mitosis towards their conclusion.

There was no significant difference between the negative control and the treatment groups in the litter sizes (number of pups) and in the pup birth weights. This may indicate zidovudine and nevirapine does not have a genotoxic impact on litter size and birth weights.

Inter-Generational Comparisons

Compared to the P generation, fertility was reduced in the F_1 generation. This suggests that children infected with HIV from birth who are also perinatally exposed to ARVs and are now on therapy for their HIV infection may have reduced fertility. For such male children, there may be reduction in testis weight which may in turn affect their sperm counts particularly if they were exposed to zidovudine. However, the effect of "both-parents" being ARV-treated may not only be beneficial such as the enhanced fertility aforementioned. Amongst the F_1 groups, the "both-parents" treated groups had the least testis weight and sperm counts. The sperm DNA quantity of the F_1 generation was not significantly different from the negative control except for that of nevirapine 5 mg/kg "both-parents" treated group which was significantly lower. The F_1 groups had significantly less DNA nicks except for zidovudine 10 mg/kg "father-only" treated

group that was not significant. The reduction in DNA nicks may be due to DNA damage such as chromosome stickiness and could be implicated in the reduced fertility observed in the F_1 generation. The genome needs to decondense to enable transcription (Agarwal and Allamaneni, 2005) and the presence of nicks enhances decondensation though a threshold may apply. It may follow that some level of nicks may be required (possibly arising during protamination of the sperm DNA) to ensure the genome can be read when the need arises. Defective protamination, the replacement of histones in sperm DNA by protamines, have been postulated as one of the aetiologies of sperm DNA damage (Agarwal and Said, 2003; Erenpreiss *et al.,* 2006; Tamburrino *et al.,* 2012).

Deductions from the Correlation Analysis of the Variables

The correlation analysis showed a positive relationship between testis weight and both the sperm counts and DNA quantity. Thus the groups with reduced testis weight may also have reduced sperm counts and DNA quantity. The sperm count had negative correlation with both sperm anomalies and DNA nicks. This may indicate that when spermatogenesis is impeded, sperm head anomalies and DNA nicks will likely increase. Sperm DNA quantity had a good negative correlation with sperm DNA nicks. This predicts that sperm with higher DNA quantity may be more tightly packed. An overtly tight packing might cause the genome to fail to decondense when the need arises during development (Agarwal and Allamaneni, 2005).

6.0 SUMMARY OF FINDINGS / CONCLUSION

The key findings from the different aspects of this research are summarized in Table 26. Each research objective is matched to their key findings to show how the objective has been accomplished.

Table 29: Summary of Findings

Objectives		Summary of findings
To assess the effect of administering either zidovudine or nevirapine on the dominant lethal assay and birth statistics in mice, when either the "father-only" or "both-parents" received ARVs.	1	Singular parent being ARV-treated led to lower fertility while there was increase in fertility when both-parents were currently ARVs treated
	2	Fertile mating occurred but early abortions or foetal loss was evident, and particularly with the drug Nevirapine
	3	Shorter gestation periods were recorded for all treatment groups.
To determine the impact of administering either zidovudine or nevirapine on the testis and sperm parameters by performing the sperm count, the sperm morphology anomaly assay and by assessing the sperm DNA quality and quantity	1	Significant oligospermia was seen after administering ARVs through one full spermatogenic cycle.
	2	Reduced testis size was observed for one group, which might be indicative of impaired testis growth.
	3	Anomalies in sperm morphology increased
	4	Sperm DNA quantity and nicks were key variables in predicting fertility. Assessing them to predict reproductive outcomes is preferable to assays evaluating sperm DNA fragmentation often used.

Objectives		Summary of findings
To determine if any genotoxic effect observed resolves or accumulates over two generations of mice	1	Severely reduced fertility was observed between the P and F_1 generations. This indicates detrimental effects could accumulate across generations
	2	The occurrence of significant reductions in testis weight was higher in the F_1 generation compared to the P generation. This suggests that genotoxic effects accumulates across generations
	3	Significant and severe oligospermia together with reduced sperm DNA quantity and nicks was prevalent in the F_1 generation compared to the P generation. This supports the accumulation of genotoxic effects.
To demonstrate the genotoxicity of zidovudine and nevirapine using the *Allium cepa* root tip assay and determine the assay's utility in elucidating possible cytogenotoxic mechanisms.	1	*Allium cepa* model can be used to demonstrate some earlier reported genotoxic effects of zidovudine and nevirapine
	2	Alters the mitotic phase distribution profile
	3	Stickiness was the major chromosomal aberrations seen. It can lead to the formation of micro/macro nuclei, aneuploidy and/or polyploidy
	4	Withdrawal of the ARVs gave varied recovery rates but most were less than half of the growth exhibited by the negative control

7.0 CONTRIBUTIONS TO KNOWLEDGE

1. Administering zidovudine or nevirapine to both the male and female mice prior to mating increased their fertility.

2. Offspring descendent from mice exposed to zidovudine or nevirapine, and who further received these drugs themselves prior to mating exhibit severely reduced fertility.

3. *Allium cepa* model can be used to investigate the genotoxicity of zidovudine or nevirapine. The sticky chromosomal aberration is crucial in the genotoxic mechanism(s) and effects of zidovudine and nevirapine.

4. Significantly reduced sperm counts, high abnormal sperm morphology and alterations in sperm DNA quantity observed in this study are attributed to the administration of zidovudine and nevirapine.

5. In mice, foetal loss when the "father-only" is ARV exposed, and preterm deliveries are associated with the administration of zidovudine or nevirapine prior to mating, especially for those who received nevirapine.

8.0 SUGGESTIONS FOR FUTURE RESEARCH / RECOMMENDATION

1. Further elucidation of the mechanism(s) boosting fertility when both parents were treated with antiretroviral drugs
2. Elucidation of the structural presentation of the increased sperm DNA quantity, whether it is micro/macronuclei, aneuploidy or polyploidy, and more importantly whether it is transcriptionally active
3. More studies to ascertain if evaluating sperm DNA nicks and/or sperm DNA quantity is more predictive of reproductive outcomes.

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APPENDICES

Appendix 1: Ethical Approval / Waiver Document

INSTITUTIONAL REVIEW BOARD

NIGERIAN INSTITUTE OF MEDICAL RESEARCH
6, Edmond Crescent Off Murtala Muhammed Way, P. M. B. 2013 Yaba, Lagos.
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Secretariat: Room 207, Biochemistry Division, Research Block, NIMR

15th Oct. 2009

PROJECT TITLE: **GENOTOXICITY OF ARFVS ACROSS GENERATIONS: ASSESSING THE IMPACT ON MICE BIRTH STATISTICS AND MALE FERTILITY WHEN EITHER A SINGLE OR BOTH PARENTS ARE RECEIVING ZIDOVUDINE OR NEVIRAPINE**

WAIVER LETTER

The above named proposal has been adequately reviewed; the protocol and safety guidelines satisfy the conditions of NIMR IRB, policies regarding waiver.

Therefore the study under its reviewed state is hereby approved by for waiver Institutional Review Board, NIMR.

DR. P. U. AGOMO .... 07/01/10 ....
Name of vice IRB chairman *Signature & Date of IRB vice Chairman*

DR. A.A.ADEIGA .... 8/1/10 ....
Name of IRB Member *Signature & Date of IRB Member*

This approval is given with the investigator's Declaration as stated below;
By signing below I agree/certify that:

1. I have reviewed this protocol submission in its entirety and that I am fully cognizant of, and in agreement with, all submitted statements.
2. I will conduct this research study in strict accordance with all submitted statements except where a change may be necessary to eliminate an apparent immediate hazard to a given research subject.
   - I will notify the IRB promptly of any change in the research procedures necessitated in the interest of the safety of a given research subject.
   - I will request and obtain IRB approval of any proposed modification to the research protocol or informed consent document(s) prior to implementing such modifications.

3. I will ensure that all co-investigators and other personnel assisting in the conduct of this research study have been provided a copy of the entire current version of the research protocol and are fully informed of the current (a) study procedures (including procedure modifications); (b) informed consent requirements and process; (c) potential risks associated with the study participation and the steps to be taken to prevent or minimize these potential risks; (d) adverse event reporting requirements; (e) data and record-keeping; and (f) the current IRB approval status of the research study.

4. I will respond promptly to all requests for information or materials solicited by the IRB or IRB Office.

5. I will submit the research study in a timely manner for IRB renewal approval.

6. I will not enroll any individual into this research study until such time that I obtain his/her written informed consent, or, if applicable, the written informed consent of his /her authorized representative (i.e., unless the IRB has granted a waiver of the requirement to obtain written informed consent).

7. I will employ and oversee an informed consent process that ensures that potential research subjects understand fully the purpose of the research study, the nature of the research procedures they are being asked to undergo, the potential risks of these research procedures, and their rights as a research study volunteer.

8. I will ensure that research subjects are kept fully informed of any new information that may affect their willingness to continue to participate in the research study.

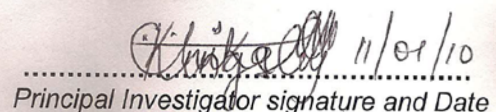
9. I will maintain adequate, current, and accurate records of research data, outcomes, and adverse events to permit an ongoing assessment of the risks/benefit ratio of research study participation.

10. I am cognizant of, and will comply with, current federal regulations and IRB requirements governing human subject research including adverse event reporting requirements.

11. I will make a reasonable effort to ensure that subjects who have suffered an adverse event associated with research participation receive adequate care to correct or alleviate the consequences of the adverse event to the extent possible.

12. I will ensure that the conduct of this research study adheres to Good Clinical Practice guidelines.

MR. ONWUAMAH CHIKA
Principal Investigator Name

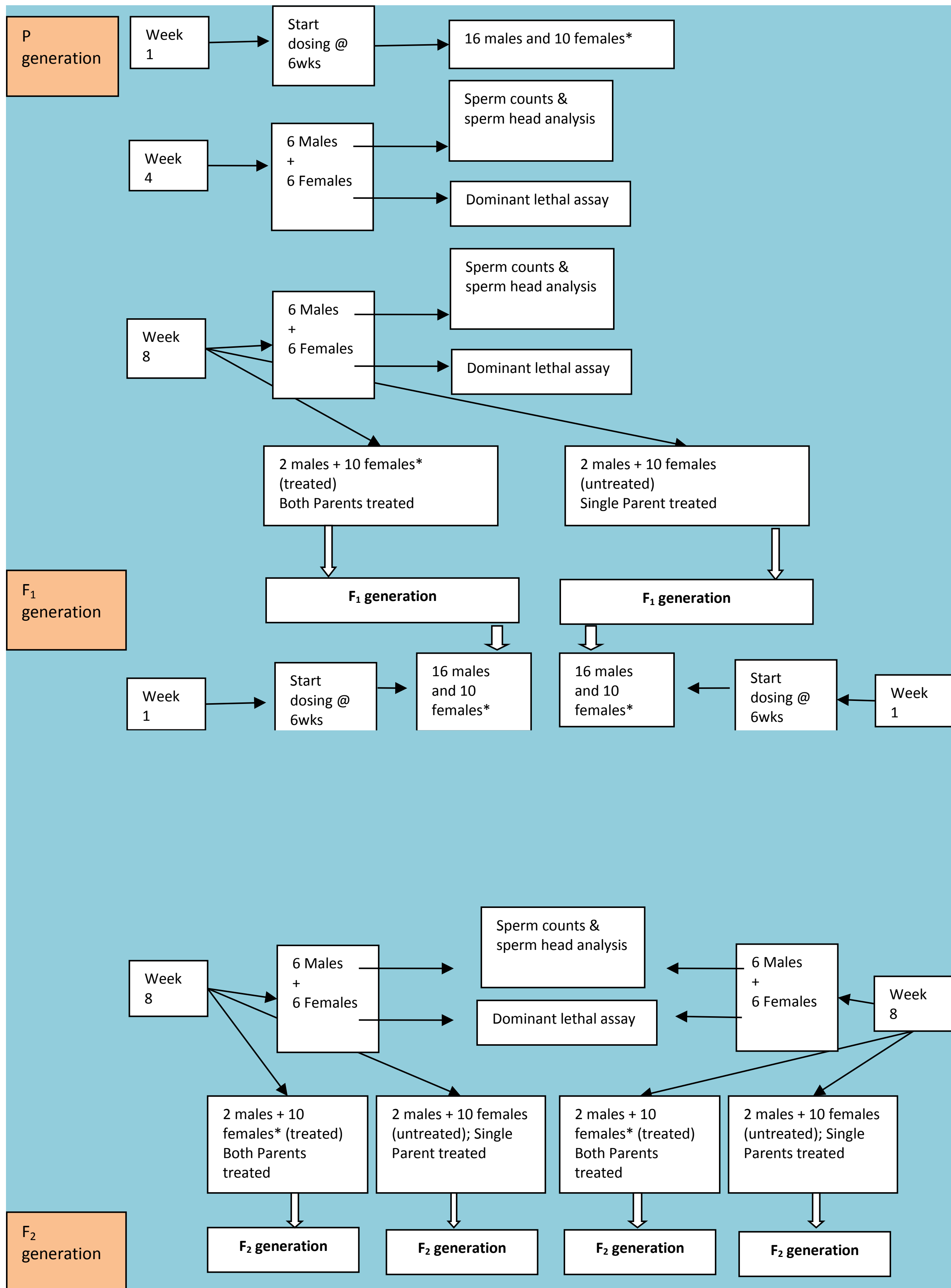


Principal Investigator signature and Date

Appendix 2: Standard Operating Procedure for Performing the Dominant Lethal Assay in Mice

1. At the end of the one week co-habitation (mating) of virgin females to treated males (1:1), the females are separated and kept in another cage.
2. The females will be sacrificed in the early second half of gestation (13 days from the mid-week of mating)
3. The dams are euthanized; the uterus is removed and weighed. Record as gravid uterine weight
4. The uterine contents are then exposed.
5. The number and uterine position of any re-absorptions and/or fetus (live or dead) are recorded.
6. The total number of implants is recorded.

Appendix 3: Diagrammatic Representation of the Phase II Mouse Study Design



Appendix 4: Standard Operating Procedure for Isolation of Sperm DNA (Qiagen User Protocol 2)

1. Prepare buffer X2 (20mM Tris-Cl [pH8.0], 20mM EDTA, 200 mM NaCl and 4% SDS). Store at 2-8°C. Prior to use, bring to room temperature.
2. Add 80 mM DTT and 250 µg/ml Proteinase K to buffer X2
3. Add 100µl buffer X2 to 100µl sperm in a 1.5ml tube. Vortex to mix
4. Incubate at 55°C on the thermomixer for 1 hour 30 min at 800rpm.
5. Add 200µl of buffer AL, vortex for 15 s to mix and incubate at 70°C for 10min.
6. Spin down the tube, add 200µl of ethanol (96–100%) and vortex for 15 s to mix
7. Spin down the tube to remove drops from inside the lid.
8. Carefully apply the mixture from above (including any precipitate) to the spin column in a 2 ml collection tube without wetting the rim.
9. Close the cap and centrifuge at 8000 rpm for 2 min.
10. Place the spin column in another clean 2 ml collection tube.
11. Repeat centrifugation if all solution has not passed through membrane
12. Carefully open the spin column and add 500 µl Buffer AW1. Do not wet the rim.
13. Close the cap and centrifuge at 8000 rpm for 2 min.
14. Centrifuge again at a higher speed until all the solution has passed through.
15. Place the spin column in a clean and labelled 1.5ml eppendorf tube.
16. Elute the DNA in buffer AE.
17. Carefully open the spin column and add 200µl buffer AE.
18. Incubate at room temperature for 5mins and centrifuge at 8000rpm for 2 min.
19. Repeat step 15-16
20. Add all 200µl eluates to one tube, vortex to mix and spin down
21. Ensure tube is labelled appropriately and store the DNA.

Appendix 5: Standard Operating Procedure for Establishing the DNA (Unsheared and Fragmented) Concentration Standard Curve using Picogreen and TBS-380 Mini-Fluorimeter

Lamda (ʎ) DNA standard (100µg/ml) and TE buffer from Invitrogen molecular probes were used. These came together with the Picogreen reagent. The TE buffer came as a 20X concentrate that was diluted to working 1X solution.

The totally fragmented DNA standard solution (fluorescence 70% lower than same quantity of intact DNA) was prepared by digesting 80µl of 20µg ʎ DNA with 10µl DNAse I at room temperature for 3 hours. The working 14.5 U DNAse I solution was prepared fresh from 10mg/ml DNAse I stock, 50mM $MnCl_2$ stock and 9mg/ml BSA stock to finally contain 9mM $MnCl_2$ and 0.9 mg/ml BSA. The digestion was stopped by the addition of 10 µl of 50mM EDTA. Working Picogreen solution was prepared fresh by adding 10µl of the commercial reagent to 990µl of TE buffer.

A) Construction of totally fragmented DNA standard curve

(i) Prepare a series of final volume 225 µl of TE solutions containing different concentrations of fragmented DNA according to the Table below

(ii) First from the 80 µl digested 20µg DNA standard, prepare 300 µl of 2000 ng (30µl "20 µg" DNA plus 270 µl TE buffer) and 200 µl of 200 ng (2 µl "20 µg" DNA plus 198 µl TE buffer) DNA solutions for use according to the Table

(iii) To each of them add 75 µl of PicoGreen working solution. Vortex and incubate in the dark for 2 min.

(iv) After incubation spin down, make triplicates of 100µl each in the mini-cell cuvettes and measure their fluorescence on the TBS-380 Mini-Fluorimeter.

(v) Select the BLUE channel. Calibrate the instrument with TE buffer blank and the most fluorescent sample (the 1000ng/ml sample).

(vi) Measure the fluorescence of the triplicates for each DNA dilution. The TBS-380 mini-fluorometer will give a direct concentration read out, and data may be used to generate a standard curve of reading versus DNA concentration.

DNA Dilutions for the standard curve

DNA Stock [ng/ml] to use	Vol of TE Buffer (µl) to add	Vol of DNA Stock (µl) to add	Total vol (µl)	DNA Conc [ng/ml] in final dilution	Working PG vol [µl] to add
2000	112.5	112.5	225	1000	75
2000	168.8	56.2	225	500	75
2000	202.5	22.5	225	200	75
2000	213.8	11.2	225	100	75
200	168.8	56.2	225	50	75
200	202.5	22.5	225	20	75
200	213.8	11.2	225	10	75
200	219.4	5.6	225	5	75
200	222.8	2.2	225	2	75
200	223.9	1.1	225	1	75
0	225	0	225	0	75

B) Construction of unsheared DNA standard curve

i. Repeat the procedure A above now using unsheared DNA standard (100µg) to make the 2000 ng and 200 ng solutions needed for the DNA dilutions

Appendix 6: Standard Operating Procedure for Quantification of DNA Sample of Unknown Concentration

1 **Note**: Put the fluorimeter on to warm for 5 min before use. Select its BLUE channel. After adding any reagent, vortex for 15 s to mix. Diluent is 1X TE buffer. Always prepare fresh working PicoGreen (wPG) by adding 10 µl stock to 990 µl 1X TE buffer. Vortex to mix and keep in the dark

2 Make a 1:10 dilution of the sperm DNA in a Sarstedt 2.0 ml tube

3 Vortex and spin down briefly. Aliquot 80 µl into a fresh tube for each sample.

4 Add 10 µl of fresh DNAse I and digest at room temperature for 3 hours.

5 Add 10 µl 50 mM EDTA to stop the digestion.

6 Prepare the BLANK by adding 100 µl TE buffer to the tube

7 Prepare the DNA calibrator by adding100 µl 1000ng/ml DNA standard to a tube

8 Make a 1:4 dilution of the digested test DNA (50 µl to 150 µl TE buffer)

9 Add 100 µl of wPG to the blank and to the DNA. Incubate in the dark for 2 min. Add 100 µl into the mini-cell cuvettes and calibrate the instrument

10 Prepare samples in batches of 12. Add 100 µl wPG to each

11 Incubate in the dark for 2 min. Add 100 µl into three mini-cell cuvettes and read

12 Record your FU readings on an appropriate record form

13 Calculate the DNA concentration in the sample by converting using the fragmented DNA standard curve equation ($y = 0.0058x$ where y is your average FU reading; $R^2 = 0.9996$)

14 Multiply by 40 the dilution factor to give concentration in nanograms / ml

Appendix 7: Standard Operating Procedure for Quantifying DNA Fragmentation

1. **Note**: Put the fluorimeter on to warm for 5 min before use. Select its BLUE channel. After adding any reagent, vortex for 15s to mix. Use 1X TE buffer as diluent.
2. Always prepare fresh working PicoGreen (wPG) by adding 10µl stock to 990µl 1X TE buffer. Vortex to mix and keep in the dark.
3. It is critical that the dilution of the DNA sample be the same as used in the quantification above. It should contain at least 5pg of DNA.
4. Using the undigested DNA samples, make a 1:40 dilution in a Sarstedt tube
5. Vortex to mix, spin down and designate it as 'DNA sample B (undigested)'.
6. Measure its fluorescence units in triplicate and designate them as $FU_{undigested}$.
7. Compare the resulting $FU_{digested}$ from the DNA quantification above and the $FU_{undigested}$ obtained here to determine the fragmentation status of the DNA sample as follows:

   a. If $FU_{digested}$ and $FU_{undigested}$ are equal (with s.e. ±3%), the DNA sample is totally fragmented.

   b. If $FU_{digested}$ and $FU_{undigested}$ differ by 67–73%, the DNA sample is composed only of intact DNA.

   c. If $FU_{digested}$ and $FU_{undigested}$ differ by below 70%, the DNA sample is partially fragmented. Thus a mixture of fragmented and intact fractions.

   d. Quantify the intact and fragmented fractions as follows: multiply the $FU_{digested}$ value by 3.333 and set the resulting value as 100%.

e. Then use the resulting value (m) to convert the $FU_{undigested}$ value to percentage and designate as y% (y% = [$FU_{undigested}$ * 100%]/m).

f. Enter this y% value in the equation x% = (100% – y%)/0.7

8 This equation calculates the concentration of the fragmented DNA fraction expressed as x% of the average total DNA concentration.

9 Thus 100 - x% is the concentration of the intact DNA fraction

Appendix 8: Standard Operating Procedure for Quantifying Sperm DNA Nicks

1. **Note**: Perform after quantifying the DNA concentration as it needs at least 15 pg of DNA. Put the fluorimeter on to warm for about 5 min before use. Vortex for 15s to mix after adding each reagent
2. Label three 2.0ml Sarstedt tubes as tubes A, B and C for each DNA sample.
3. Add 75µl of DNA sample to the labelled tubes. Collate the tubes A, B and C on separate tube racks.
4. Prepare fresh a NaOH/HCl mixture containing equal volumes of 0.05N NaOH and 0.05N HCl sufficient for the number of tests (150 µl per sample needed)
5. To each tube labelled A, add 150 µl of the NaOH/HCl mixture.
6. Add 75µl of working PicoGreen (wPG) (10µl stock to 990µl 1X TE buffer).
7. Incubate for 2 min in the dark and spin down briefly. Add 100 µl of each sample to 3 mini-cell cuvettes used in the TBS-380 fluorimeter.
8. Blank with TE buffer and calibrate with 200ng DNA standard.
9. Record the FU of the 3 replicates per sample and designate their average as FU_A.
10. To each tube labelled B, add 75µl of 0.05N NaOH. Incubate at room temperature for exactly 5 min and then add 75µl of 0.05N HCl.
11. Add 75µl wPG. Incubate for 2 min in the dark and spin down briefly
12. Add 100 µl of each sample to 3 mini-cell cuvettes.
13. Blank with TE buffer and calibrate with 200ng DNA standard.
14. Record the FU of the 3 replicates per sample and designate their average as FU_B.
15. To each tube labelled C, add 75µl of 0.05N NaOH, incubate at 80°C for 7 min
16. Add 75 µl of 0.05N HCl. Then add 75 µl of wPG solution.
17. Incubate for 2 min in the dark and spin down briefly

18 Add 100 µl of each sample to 3 mini-cell cuvettes.

19 Blank with TE buffer and calibrate with 200ng DNA standard.

20 Record the FU of the 3 replicates per sample and designate their average as FUc.

21 Calculate the percentage of nicked DNA using the formula

$1 – F = (FU_B – FU_C) / (FU_A – FU_C)$.

22 Specifically, 100 multiply (1 – F) represents the percentage nicked in the total DNA and 100 multiply F represents the percentage of unnicked DNA.

Appendix 9: Standard Operating Procedure for Performing the Sperm Counts

1. Euthanize the mouse, remove the right epididymis
2. Excise the cauda epididymis, weigh and record.
3. Place the cauda epididymis in a petri dish containing 1ml PBS-Triton-X100 (0.05%)
4. Cut into bits with fine scissors and mix by vigorous pipetting 20X
5. Transfer the suspension to a tube and make up the volume to 10ml with PBS
6. Vortex for 20 s to mix suspension properly
7. Sieve through 80µm mesh and allow debris to settle for 3-5mins.
8. ***Store at 4˚C pending analysis (pause point)***
9. Put haemocytometer on flat surface with the cover slip in place and fill the haemocytometer chamber by transferring about 10µl sperm suspension into both chambers of the haemocytometer
10. Place the chamber in a covered Petri dish with moistened filter paper for 3-5mins for the spermatozoa to settle.
11. Count sperm heads in the four corner squares and the middle square in each chamber (In regard to the heads on the lines of the squares, count only those on the left and top lines of each square. Do not include those on the right and bottom lines).
12. Record counts obtained for the two haemocytometer chambers
13. The haemocytometer counts are acceptable if there is less than 20% difference (as percentage of the mean) between different chamber counts
14. Sperm per cauda epididymis = (mean count multiplied by dilution volume)/volume of counting chamber
15. Sperm per gram cauda epididymis = sperm count per cauda /cauda weight (grams)

Appendix 10: Standard Operating Procedure for Preparing Slides for Sperm Morphology Analysis

1 Dissect the mouse, remove the left epididymis

2 Excise and weigh the cauda epididymis and record

3 Put this in a petri dish containing 1ml of PBS

4 Mince the epididymis with fine scissors and pipette up and down vigorously 20X using a Pasteur pipette

5 Transfer the suspension to a tube and make up the volume to 2ml with PBS

6 Remove the epididymal fragments by filtering through an 80µm mesh

7 Allow debris to settle for 3-5mins

8 The samples can be kept in this state for more than 24hrs at 4˚C (pause point)

9 To make smears, an aliquot of 200µl is added to a tube

10 Stain with 20µl of 1.0% aqueous Eosin-Y by mixing (10:1 - aliquot:eosin-Y)

11 Leave for 30 mins and then make 4 smears – 2 smears per slide

12 Make smear by applying one drop of suspension to clean slide

13 Allow to air dry, put on a cover-slip and seal with nail varnish (2nd pause point)

14 Code and randomise the slides

15 View in bright field at 1000X and score the sperm head abnormalities seen

16 Examine and score 800 – 1000 sperm cells / mouse

Appendix 11: Sperm Morphology Analysis Bench Aid

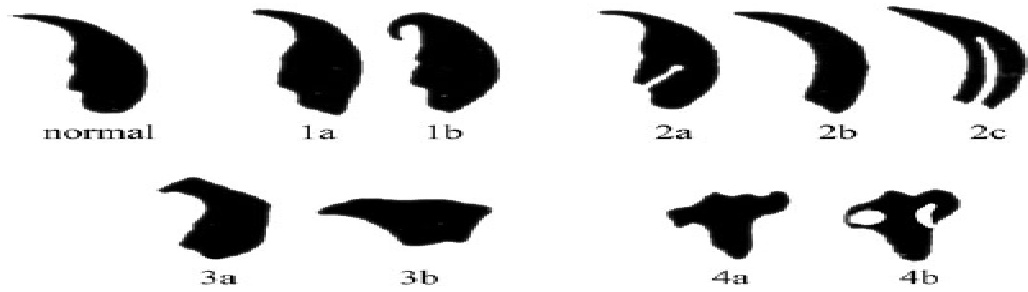


Fig. 2. Typical examples of sperm head abnormalities (according to Buda and Krzanowska, 1978). *Four classes of abnormal spermatozoa heads were distinguished by Krzanowska on the basis of microscopic observation of sperm in adult male mice. These are Type 1 (almost normally developed head with slightly distorted acrosome (Type 1b), head with slightly changed distal part (Type 1a); Type 2 (basicly narrow head, frequently contains canal with low stainability and Type 3 (considerably misshapen head, mainly in the apical or distal partseriously misshapen head, some of them possessing vacuoles or canals with low stainability).*

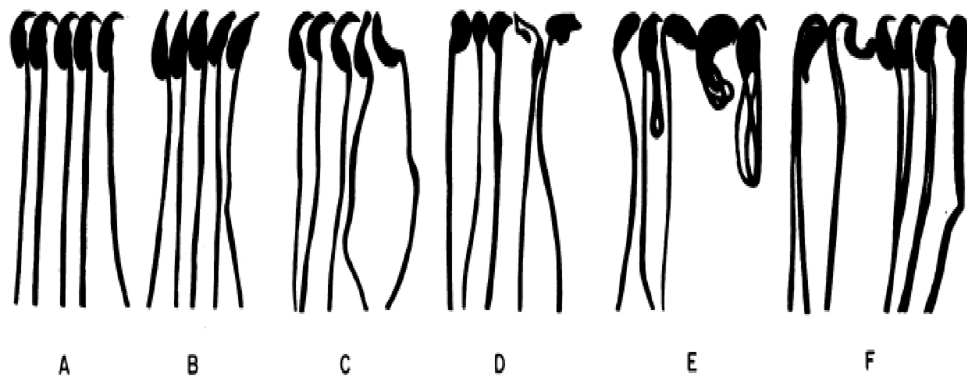
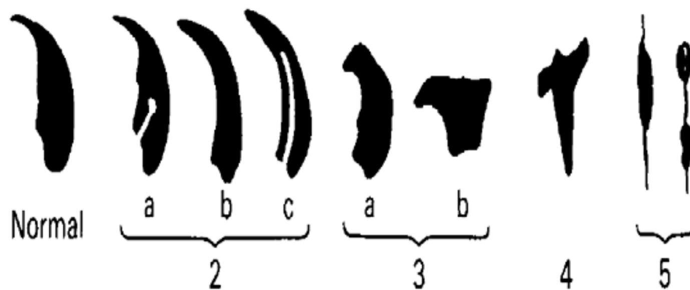


FIG. 1. The shape of A is normal whereas B to F are abnormal murine sperm. Sperm in B lack the usual hook, C have a banana-like form, D are amorphous, E are folded on themselves, and F possess two tails (×1,000).



Text-fig. 1. Shapes of normal and abnormal mouse sperm heads according to the arbitrary Classes 2–5 and subclasses (see text).