TOXICOLOGICAL AND ANTIFERTILITY INVESTIGATIONS OF OLEANOLIC ACID IN MALE VERVET MONKEYS (*Chlorocebus aethiops*)

By

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Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In the Department of Physiological Sciences

University of the Western Cape

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Submitted: **May 2003**

Declaration

I, Mongezi Mdhluli, declare that "**TOXICOLOGICAL AND ANTIFERTILITY INVESTIGATIONS OF OLEANOLIC ACID IN MALE VERVET MONKEYS (*Chlorocebus aethiops*)**" is my own work and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references

Signature: 

Date: 14 May 2003

Dedicated to my wife, our son and my parents

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PREFACE

Toxicology

Natural medicine has been practiced by peoples of all ages and cultures for many centuries throughout the world. Interestingly, the "modern world" is currently experiencing a growing use of herbs or plant extracts as an alternative to conventional medicine, despite technological and scientific advances in the development of pharmaceuticals. However, an increasing recognition of the toxicity of herbs has emphasized the need for toxicity testing. In pharmaceutical companies, safety testing is an essential component of drug discovery before these drugs are made commercially available for human use.

The efficacy of oleanolic acid, a triterpenoid extracted from plants and used in this study, has been well researched and documented, except for its toxicity. Examples of pharmacological effects of oleanolic acid reviewed in chapter 1 include hepatoprotective, anti-inflammatory, anti-tumor, anti-ulcer, anti-microbial and hypoglycemic properties. Additionally, chapter 1 covers information on the effects of several herbs on the cardiovascular, renal, hepatic and hematopoetic systems, tests used to determine toxicity on organ function, as well as the use of non human primates in toxicity testing. Methods and apparatus used to achieve objectives of the study are provided in chapter 2. The results and discussion chapters provide new important information on the effects of oleanolic acid on organ function in male vervet monkeys.

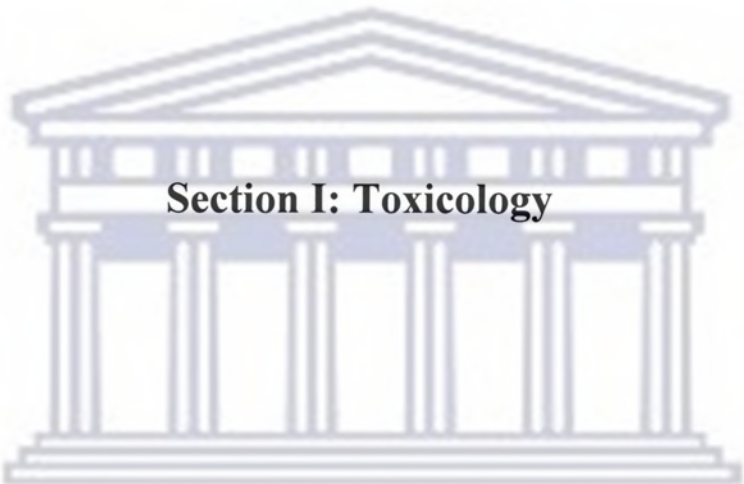
Contraception

Although there are many options for fertility regulation in women, relatively limited contraceptive choices are currently available for men. However, there are recent remarkable advances in research on discovering promising techniques and/or drugs for future male fertility control. Unfortunately, these techniques and/or drugs are currently in their early developmental stages.

Apart from its many pharmacological effects, oleanolic acid also has antifertility properties in male rats with no side effects. These results serve to motivate the researcher to pursue further research on oleanolic acid, using non human primates. Non human primates are closely related to humans in their reproductive function, so the findings of this research could be extrapolated to human medical use.

In this section, background information is provided on the use of contraceptive methods, testicular and epididymal function, sperm morphology, motility and acrosomal integrity, possible strategies for male fertility control and use of non human primates in reproductive research. Chapter 2 outlines methodologies and apparatus used to carry out the aim and objective of this study. The results and discussion chapters provide new findings on the contraceptive effects of oleanolic acid in the male vervet monkey.

Section I: Toxicology



Abstract

Introduction: Plant extracts and herbal preparations are often marketed as natural and safe alternatives to conventional medicines for the prevention and treatment of a variety of ailments, without proof of efficacy and safety. Cardiovascular, hematopoetic, hepatic and renal impairment resulting from the use of conventional drugs is widely acknowledged. However, there is less awareness of the potential toxicity of herbal preparations and other botanicals, many of which are widely perceived by the public as being effective and harmless, and are commonly used for self-medication without supervision. In addition, potential interactions between herbal medicines and conventional drugs may compromise with patient management.

In the safety evaluation of most substances, non human primates are preferred to rodent species for preclinical animal safety studies, because of their biological similarity to humans. They are regarded to be the best metabolic models for humans in a broad range of investigations. Additionally, a disadvantage of using small animal species in toxicological testing is that they require higher doses of drugs and more frequent administrations than in larger species. In light of these considerations, vervet monkeys are used here to investigate toxicity of a plant-derived triterpene, oleanolic acid. The focus is to determine effects of different concentrations of this triterpene on the cardiovascular, hematopoetic, hepatic and renal systems.

Materials and methods: 12 male vervet monkeys used in this study were equally divided into four groups, i.e. three treatment groups (4, 10 and 25 mg/kg bodyweight), and one control group. Each individual in a treatment group received a specified concentration of oleanolic acid in food for 16 weeks. Monkeys in the control group received the vehicle (food) alone. Bodyweight, body temperature, respiratory rate, heart rate, systolic pressure, diastolic pressure, and mean arterial

pressure were recorded from ketamine-anaethetized monkeys at baseline and every second week until week 16. In addition, blood samples were collected at baseline and every fourth week for clinical biochemistry indicators (serum electrolytes, enzymes, proteins, lipids, nitrogenous compounds, bilirubins and glucose) and hematological tests (red cell count and its indices, hemoglobin, haematocrit, white blood cell and differential count and platelet count).

Results: No animal showed deviation from their normal behavioral patterns, food and water intake, was in poor health or died during and after completion of the study. The average bodyweights were not statistically significantly different between controls and the treated groups. The biphasic changes in the average body temperature of treated monkeys were similar to those seen in the control group during the first eight weeks of the study. No statistically significant differences were found in body temperature determinations between controls and the treated groups. Fluctuations observed in the respiratory rates of the treated monkeys were not statistically significantly different from that of the control group. Although not statistically significantly different from the controls, the systolic, diastolic and mean arterial pressures in the group treated with 25 mg/kg oleanolic acid were lower at week 16 compared to baseline, while those of the groups treated with 4 and 10 mg/kg oleanolic acid were relatively unchanged. Except for a reduction in systolic pressure of the control group, other blood pressure parameters were stable. Heart rates in the treated groups were not statistically significantly different from those in the controls. In all groups, except the control, high-density lipoprotein concentrations were higher at week 16 compared to baseline. Fluctuations in low-density lipoprotein and total cholesterol concentrations were similar between controls and the treated groups. The triglycerides were lower at week 16 compared to baseline for all four groups. Upward trends from baseline to the end of the study were observed in creatine kinase concentrations of the controls and the groups that received 4 and 25 mg/kg. Concentrations of this enzyme were unchanged in the group that received 10 mg/kg oleanolic acid between baseline and

the end of the study. No statistically significant differences were found with cholesterol, triglyceride and creatine kinase concentrations between treated groups and the controls.

Serum concentrations of aspartate aminotransferase were unchanged in the controls and the groups treated with 4 and 10 mg/kg oleanolic acid, but changes in this parameter over time were statistically significantly different (P = 0.0452) from the controls in the group that received 25 mg/kg oleanolic acid. Despite wide fluctuations in the alanine aminotransferase concentrations in the groups that received 4 and 25 mg/kg oleanolic acid, no statistically significant differences were found with any of the treated groups compared to the controls. No statistically significantly different changes were seen in alkaline phosphatase activities between controls and the treated groups. Reductions in gamma-glutamyl transferase activities in the groups that received 4 and 25 mg/kg oleanolic acid were not statistically significantly different from concentrations of this enzyme in the controls. In addition, no statistically significant differences were evident between controls and the group that received 10 mg/kg oleanolic acid. There were no statistically significantly different changes in the total and conjugated bilirubin and glucose concentrations between controls and the treated groups. Fluctuations over time in the serum albumin and globulin concentrations were similar between treated groups and the controls, whereas total protein concentrations were relatively constant. Consequently, no statistically significant differences were found between controls and the treated groups.

Wide fluctuations were observed in the creatinine concentrations of the groups that received 4 mg/kg oleanolic acid, while no such changes were encountered in the controls and the group that received 10 and 25 mg/kg oleanolic acid. Serum urea concentrations increased in all groups over time, except for the group that received 10 mg/kg oleanolic acid. Both urea and creatinine concentrations in the treated groups were not statistically significantly different from concentrations in the controls.

Serum concentrations of sodium, chloride, potassium, calcium and magnesium and phosphate in the treated groups were not statistically significantly different from these electrolyte concentrations in the controls.

Decline in red cell and hemoglobin concentrations of the controls and the group that received 25 mg/kg oleanolic acid were not statistically significantly different between these groups. In addition, no statistical significant differences were found in red cell and hemoglobin concentrations between controls and the groups that received 4 and 10 mg/kg oleanolic acid. Controls and the treated groups showed upward trends in haematocrit concentrations. Mean corpuscular volumes were statistically significantly increased; $P = 0.0027$ (4 mg/kg), $P = 0.0010$ (10 mg/kg), and $P = 0.0022$ (25 mg/kg), while mean corpuscular hemoglobin concentrations were statistically significantly reduced; $P = 0.0017$ (4 mg/kg), $P = 0.0004$ (10 mg/kg), $P = 0.0002$ (25 mg/kg) in the treated groups as compared to the controls. No statistically significant differences were evident in the concentrations of mean corpuscular hemoglobin between controls and the treated groups.

White blood cell counts of the treated groups were not statistically significantly different from those of the controls throughout the study period. No statistically significant differences were found in the differential white cells and platelet counts between treated groups and the controls.

Discussions: The results of this study showed that administration of oleanolic acid had no effects on the general wellbeing, bodyweights, body temperature, respiratory and heart rates, and blood pressure of vervet monkeys. A statistically significant increase in the aspartate aminotransferase activity of the group treated with 25 mg/kg oleanolic acid, together with the increase in the alanine aminotransferase levels during the same time period, might indicate oleanolic acid-induced hypersensitivity, and accordingly hepatocellular alteration. However, since serum concentrations of these enzymes returned to baseline levels, as well as the absence of variations over time in other parameters of the hepatic function, particularly alkaline phosphatase activity, it is likely that there

was no underlying subacute liver disease. Serum renal function parameters also appeared to be within normal physiological limits. No pronounced changes were observed in the hematological parameters of monkeys that received oleanolic acid.

Conclusion: This study's results, suggest that oleanolic acid does not produce cumulative liver enzyme alterations, and has no detrimental effects on the renal, hematopoetic and cardiovascular systems of vervet monkeys.

Chapter 1

Introduction

Note: References for section I (toxicology) are in pages 94 – 112

1. General information on oleanolic acid

Oleanolic acid is widely found in many plant species, and occurs in the form of free acid or triterpenoid saponin glycosides. It is believed that many different plant species synthesize triterpenoid saponins as part of their normal programme of growth and development (Haralampidis *et al.* 2002). The stored concentrations of these saponins are either the same or vary between different parts of plants. For example, quantitative determinations have shown that the level of oleanolic acid is similar in shoots, isolated cells and protoplasts of *Calendula officinalis Hohen. Asteraceae* (Auguscinska and Kasprzyk 1982). Based on chemical and spectral data, oleanolic acid structure has been established as 3-ß-hydroxy-olean-12-en-28-oic acid ($C_{30}H_{48}O_3$). The structure illustrates that oleanolic acid is one of the many pentacyclic triterpenoids belonging to the ß-amyrin family of compounds (Figure 1.1). Having similar molecular structure as its isomer, ursolic acid, oleanolic acid differs from the former in the placement of methyl group on the top ring of the structure, i.e. methyl group is positioned at C_{19} for ursolic acid and at C_{20} for oleanolic acid (Li *et al.* 2002).

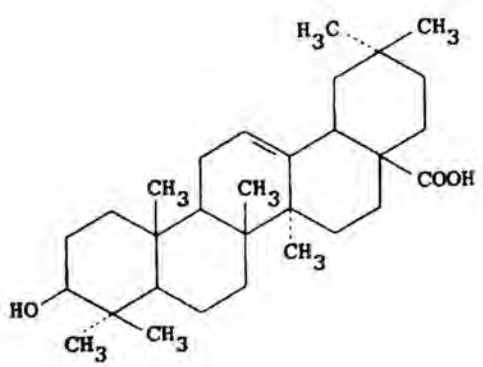


Figure 1.1. The structure of oleanolic acid

1.1. A synopsis of the pharmacology of oleanolic acid

Apart from its wide range of structural diversity, oleanolic acid also exhibits biological activity. The use of oleanolic acid in folk medicine to cure a variety of ailments has since led to scientific discovery of its many pharmacological properties. These properties are briefly described below:

1.1.1. Hepatoprotection

Oleanolic acid has been shown to protect against liver injury caused by a number of hepatotoxins, including carbon tetrachloride, acetaminophen, cadmium, bromobenzene, phalloidin, thioacetamide, furosimide, colchicine and D-galactosamine. In an acetaminophen and cadmium-induced liver injury in mice, pretreatment with oleanolic acid at 25-100 mg/kg (Liu *et al.* 1993) and 100 mg/kg bodyweight ([a]Liu *et al.* 1993) for 3 days respectively, protected animals against this hepatotoxicity, as was indicated by decreases in serum activities of alanine aminotransferase and sorbitol dehydrogenase. Similar protection against carbon tetrachloride-induced hepatotoxicity was observed in rats and mice pretreated with oleanolic acid at 100-400 μmol/kg bodyweight (Liu *et al.* 1998). Hepatoprotection against the latter compound is not mediated through Metallothionein, which is an acute-phase protein proposed to decrease carbon tetrachloride-induced liver injury, but through enhancement of hepatic-glutathione regeneration capacity (Yim *et al.* 2001).

1.1.2. Anti-inflammatory

Oleanolic acid treatment at 60 mg/kg bodyweight produces marked reduction in complement levels and inflammatory effects on carrageenan-induced paw oedema and adjuvant-induced arthritis in rats (Kapil and Sharma 1995). The croton oil ear test showed that oleanolic acid from the leaves of *Thymus willdenowii Boiss. Lamiaceae* (*Labiatae*), a herbal drug used in Moroccan folk medicine, has a topical anti-inflammatory activity in mice (Ismaili *et al.* 2001).

1.1.3. Antitumor effects

Plant extracted triterpenoids have been shown to be valuable as potential cancer chemopreventative agents. Oleanolic acid, isolated from the Chinese medicinal plant (*Glechoma hederaceae L*), has been identified as an inhibitor of 12-O-tetradecanoylphorbol-13-acetate induced Epstein-Barr virus activation in Raji cells (Ohigashi *et al.* 1986, Tokuda *et al.* 1986).

1.1.4. Antiulcer effects

When compared to a known effective antiulcer agent (carbenoxolon-sodium), oleanolic acid and its derivatives were reported to be more effective in response to experimentally induced gastric ulcers in rats (Farina *et al.* 1998, Wrzeciono *et al.* 1985). In a study on indomethacin-induced gastric mucosal lesions and on gastric secretion in pylorus-ligated rats, the two oleanolic acid oligoglycosides, i.e. 3-O-monodesmosides and 28-O-deglucosyl-chikusetsusaponins, produced mucosal and gastro protective effects respectively (Matsuda *et al.* 1998). In the pylorus-ligated rats, oral administration of these active saponins did not decrease the gastric secretion.

1.1.5. Antimicrobial effects

Oleanolic acid has been shown to be effective in inhibiting the synthesis of an insoluble glucan from *Streptococcus mutans* and on the growth of oral bacteria (Kozai *et al.* 1999). Futhermore, oleanolic acid produces preferential growth-inhibitory activity against gram-negative anaerobic periodontal oral pathogens, including *Porphyromonas gingivalis* and *Prevotella intermedia* (Cai and Wu 1996).

1.1.6. Hypoglycemic effects

The hypoglycemic activities of oleanolic acid glycosides, which are not associated with insulin release, are achieved by inhibiting gastric emptying and glucose-uptake in the small intestine

(Yoshikawa and Matsuda 2000). The authors found that capsaicin-sensitive sensory nerves and the central nervous system mediate inhibition of gastric emptying.

1.1.7. Antifertility effects

Oleanolic acid was found to have antifertility effects in male rats (Mdhluli and van der Horst 2002, Rajasekaran *et al.* 1988). See section II (Contraception part) of this thesis for further discussion on this subject.

1.2. Use of herbal medicines

The health care revolution has improved control of disease in humans, and contributed to the social welfare of society. These remarkable developments have been achieved partially by the use of highly effective therapeutic compounds. However, the increasing recognition of the potential toxicity of these compounds is a matter of concern, particularly when a novel compound that is still under the investigation is to be administered to humans for the first time. Therefore, it is expected of any manufacturer developing a new drug to provide supporting data on the safety, apart from the efficacy. It is thought that inclusion of toxicity evaluation further upstream in the drug discovery process adds value by reducing manufacture's effort and resources expended on compounds that are prone to failure during clinical use stage (Dean and Olson 1993).

Many cultures/communities in the developing world have, throughout history, used a variety of dietary supplements, plants and materials derived from plants for the prevention and treatment of different health conditions, including renal diseases, diabetes and hypertension. In recent years, the use of herbal drugs has become well entrenched in Western society and, is no longer confined to traditional medicine practitioners in Asia, Africa and the Middle East. For example, the sale of herbal medicine is a growing industry in Germany, where physicians routinely prescribe these

products, and their annual sales have surpassed two billion American dollars (Harrison 1998). Similarly, the sale of herbal products in the United States has soared from two hundred million in 1988 to over three billion American dollars in 1997 (Mahady 2001). Nonetheless, the perceived health benefits of herbal remedies remain generally unproven and concern about adverse effects is leading to closer scrutiny of these products.

It is a fact that the development of some modern medicines came as the result of the isolation of compounds from herbal medicines, which have served as a source for the synthesis of many important drugs used today. Irrespective of this development, some communities continue to use medicinal herbs in their original form, despite warnings that some may be toxic and should be avoided even though they are readily available. Of concern is the use of medicinal herbs in high doses, as well as their untoward effects in sensitive sub-populations such as the young, elderly and genetically predisposed individuals (Matthews *et al.* 1999).

1.2.1. Effects/toxicity of herbs on the cardiovascular system

The growing appeal of herbal remedies to treat cardiovascular related problems is likely to continue. Therefore, this necessitates familiarity of health care professionals with the available information on the cardiovascular effects of herbs. Table 1.1 highlights some results on the effects of herbal remedies that impact on the cardiovascular system.

Commonly used tests to detect the effects of various agents on the cardiovascular system include blood pressure and heart rate measurements, cholesterol and creatine kinase (CK) determinations. The diastolic pressure is described as the main driving force for adequate coronary perfusion (Westerhof and Elzinga 1993), whereas an increase in systolic pressure is regarded as more of a cardiovascular risk than diastolic pressure (Chae and Lloyd-Jones 2002, Kannel 2000). Drug-

induced hypertension is caused by sodium retention and expansion of extracellular fluid or by drugs acting as direct or indirect sympathomimetics (Westerhof and Elzinga 1993).

Table 1.1. Effects of plants/plant extracts on the cardiovascular system

Names of plant /plant extract	Biological activity	References
Andrographis paniculata	Lower systolic blood pressure in stroke-prone spontaneously hypertensive rats (SHR).	Zhang and Tan (1996).
Rauvolfia serpentina	Dose-dependent hypotensive response, without effect on heart rate.	Fahim *et al.* (1995).
Panax ginseng C. A. Meyer	Decreased blood pressure in hypertensive rats.	Jeon *et al.* (2000).
Sho-seiryu-to (TJ-19)	Increased respiratory rate, heart rate, and both systolic and diastolic pressures.	Amagaya *et al.* (2001).
Mentha suaveolens Ehrh	Reduced mean arterial blood pressure and heart rate in normotensive rats (NMR).	Bello *et al.* (2001).
Spergularia purpurea Pers.	Decreased systolic and diastolic pressure in SHR and NMR.	Jouad *et al.* (2001).
Dorstenia psilurus	Reduced systolic blood pressure in fructose-fed hyperinsulinemic and hypertensive rats.	Dimo *et al.* (2001).
Cascara sagrada	Induces portal hypertension.	Nadir *et al.* (2000).
Glycyrrhizin	Induces hypertension by increasing the systolic pressure.	Quaschning *et al.* (2001).
Ephedra sinica	Variable effects on blood pressure and increases heart rate in healthy normotensive adults.	White *et al.* (1997).

Elevated serum levels of total cholesterol and low-density lipoproteins (LDL) are major determinants of coronary heart disease (Kromhout 2001, Kruth 2001), whereas increases in serum levels of high-density lipoproteins (HDL) have the opposite effects. Total cholesterol concentrations increase in diabetes mellitus, cardiovascular disease, nephrosis and hypothyroidism, whereas concentrations decrease in liver disease, hyperthyroidism, fat malabsorption, anemia and severe infections.

CK has three cytoplasmic and two mitochondrial isoenzymes, which provides specific information about injured tissue because of their tissue distribution. The myocardium has substantial amounts of an isoenzyme CK-MB, and a serum measurement of the latter is a sensitive and specific indicator of myocardial degeneration or necrosis (Navin and Hager 1979). Although measurements of different isoenzymes were beyond scope of this study, their determinations are most beneficial, particularly when the total CK is nonspecifically elevated, as with intramuscular injections. Similarly, conditions such as hypothyroidism and hyponatremia add to the increase in the serum CK levels (Finsterer *et al.* 1999, Goldenberg *et al.* 1997).

1.2.2. Hepatotoxicity of herbs

Herbal drugs, many of which are believed to be intrinsically harmless, have been commonly used to treat hepatic dysfunction emanating from hepatotoxic substances. However, there is less awareness of the potential hepatotoxicity of herbal preparations, since some of them often contain highly active pharmacological compounds, such as pyrrolizidine alkaloids (Lin *et al.* 1999). Hepatotoxicity of pyrrolizidine alkaloids ranges from mild liver enzyme alterations to chronic liver disease and liver failure. The varied manifestations of liver injury also include acute and chronic hepatitis, hepatic fibrosis, zonal or diffuse hepatic necrosis, bile duct injury, veno-occlusive disease, and acute liver failure requiring liver transplantation and carcinogenesis (Stedman 2002).

Long term use (over 8 weeks) of extracts from *Echinacea species* have been reported to possibly cause hepatotoxicity (Miller 1998). Hepatotoxicity, manifested by an acute to chronic irreversible liver damage with fulminant hepatic failure has been reported in patients who ingested chaparral (Sheikh *et al.* 1997), a dietary supplement. Pyrrolizidine alkaloids have been found to cause obstruction of the hepatic venous system with possible occurrence of hepatonecrosis.

Examples of biochemical markers for hepatic function are presented in Table 1.2.

1.2.3. Renal toxicity of herbs

The kidney plays an important role in the elimination of numerous potentially harmful substances such as drugs, toxins and endogenous compounds from the body. In many cases, this function renders the kidneys as target organs of adverse effects of drugs, which may induce chronic renal toxicity. Renal toxicity of these compounds is due to the fact that urinary elimination is a main route of excretion, and the proximal tubules are particularly sensitive due to their high reabsorptive activity (Russel *et al.* 2002).

Herbal products have a long history of use for treating problems of the genitourinary tract, including prostatism, infection, stones and impotence (Pinn 2001). However, their effects can be potentially toxic if used incorrectly, and their long-term uses as substitute for conventional medicines may cause disorders of the renal function. The most common drug-induced renal damage is nephrotoxicity, and consumption of different herbs containing cadmium (Subat-Dezulovic *et al.* 2002) or aristolochic acid (Cosyns *et al.* 2001), has been associated with the occurrence of this condition. The aristolochic acid, a naturally occurring nephrotoxin and carcinogen, is implicated in a unique type of renal fibrosis, designated Chinese herb nephropathy that can develop into urothelial cancer. Biochemical markers that assess alterations in the renal function are presented in Table 1.3.

Table 1.2. Biochemical markers which assess alterations in liver function

Test	Properties
Alanine aminotransferase (ALT)	Solely a hepatocellular cytoplasmic enzyme, specific for hepatic necrosis (Sherman 1991).
Aspartate aminotransferase (AST)	Of both cytoplasmic and mitochondrial origin. Greater hepatocellular injury usually releases AST rather than ALT.
Alkaline phosphatase (ALP)	An extracellular enzyme fixed to the plasma membrane glycan (Moss 1997). Its isoenzyme levels are increased with intrahepatic cholestasis and extrahepatic biliary tract disease (Brommage *et al.* 1999).
Gamma-glutamyl transferase (GGT)	A membrane bound enzyme that is prevalent in the liver. Its activity increases with hepatobiliary disease (Artur *et al.* 1984), and is more specific but less sensitive than ALP in cholestasis (Reichling and Kaplan 1988).
Bilirubin	An end product of hemoglobin catabolism. Unconjugated bilirubin is transported in the plasma bound to albumin (Brierley and Burchell 1993). Increased concentrations of total bilirubin may indicate extrahepatic obstruction of bile secretion, hepatic failure or excessive hemolysis of RBC. Increased conjugated form indicates obstructive hepatogenous jaundice, whereas increased unconjugated form indicates hemolytic jaundice.
Proteins: Albumin and globulin	Albumin and globulin are synthesized in the liver. Albumin concentrations are decreased with toxic and trauma stress (Rothschild *et al.* 1988). Globulin concentrations increase in liver disease associated with parenchymal damage (Teloh 1978). Total protein increases with chronic liver disease.
Glucose	Being under control of insulin and glucagon, disturbances of its serum levels are symptomatic of disorders of hormonal production. Apart from diabetes mellitus, glucose concentrations also increase with chronic liver disease.

Table 1.3. Biochemical markers which assess alterations in renal function

Test	Properties
Urea	Formed in the liver from protein catabolism. Increased levels indicate kidney disease or enhanced protein catabolism, and decreased levels are due to high glomerular filtration or protein anabolism rates and severe liver disease (Takenaka 1986).
Creatinine	The end product of creatine metabolism. Reduced renal perfusion increases its serum levels. Together with urea, it is used to detect nephrotoxicity (Stonard 1990).
Sodium	An extracellular cation that is important for nerve impulse conduction (Greene 1986). Its concentrations increase during dehydration and decrease with nephritis.
Potassium	An intracellular cation that is important for nerve impulse conduction and muscle contraction (Greene 1986). Its concentrations increase in chronic renal failure and dehydration.
Chloride	A principal extracellular anion. Hypertension induced by supplemental chloride is mediated by changes in renal function (Greger and Tseng 1993).
Calcium	The most abundant cation in the body (Cashman 2002). Its levels decrease in chronic renal failure.
Magnesium	A common cation in the intracellular fluid (Quamme 1986). Its levels are increased in renal failure.
Phosphate	Constitutes a major buffer system of the body, and its concentrations are increased in renal disease.

1.2.4. Hematotoxicity of herbs

Drug toxicity on the cellular elements of the blood can either be directly by affecting the circulating cells or by interfering with their development. The red blood cells' (RBC) hemoglobin (Hb) may be prevented from transporting oxygen, by its conversion to methemoglobin or sulfhemoglobin. Several compounds may damage or destroy erythrocyte membranes, resulting in hemolytic anemia. The latter is characterized by decreases in RBC counts, hematocrits (Hct) and Hb, and increases in mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentrations (MCHC).

Interaction of herbal medicine with conventional drugs may have negative effects on the hematological processes. During anesthesia, herbs and dietary supplements have inhibitory effects on blood coagulation processes (Cheng *et al.* 2002). Herbal preparations from Latvian plant species are reported to have hemolytic effects in humans (Atroshi *et al.* 2000). Extract of the herb *Tripterygium wilfordii Hook F*, has been document to be hematotoxic, and its use has resulted in cases of leukopenia, thrombocytopenia, and aplastic anemia (Pyatt *et al.* 2000). The essential hematological tests used to determine drug damage and a probable site of action where damage is caused are outlined in Table 1.4.

1.3. The rational use of animals/primates in drug safety testing

In vivo testing is regarded as the first choice in assessing acute and chronic toxicity, effects on fertility, teratogenic and carcinogenic potential of new chemical entities (Ankier and Warrington 1989). Animal use in toxicity studies is based on the assumption that extrapolation of biological data from animals to humans is valid. However, by virtue of the marked species differences that occur in drug metabolism, investigators recommend animal species that are similar to humans in biochemical responses, including absorption, distribution, metabolism and excretion of drugs. Thus, selection of suitable animal species as experimental model will undoubtedly offer the opportunity of valid quantification and prediction of various compounds as potential therapeutic agents or as potential toxicants in humans.

Table 1.4. Hematological indicators

Test	Properties
RBC	Produced in the red bone marrow under the influence of erythropoetin (Tortora and Anagnostakos 1990). Concentrations increase in polycythemia, and decrease in anemia.
Hb	Synthesized inside the immature RBC in the bone marrow. Concentrations increase in polycythemia and congestive heart failure, and decrease in anemia and liver cirrhosis.
Hct	Represents the percentage of RBC's in blood (Kerr 1989). Concentrations increase in polycythemia, severe dehydration and shock, and decrease in anemia and cirrhosis.
Mean corpuscular volume (MCV)	It is a measure of RBC size, and is used to classify anemia as microcytic, normocytic or macrocytic (Irwin and Kirchner 2001). During toxicity testing changes in MCV may indicate the stage of erythropoesis on which drug is acting (Paget 1979).
MCHC	Represents Hb concentration in the blood, and is useful in the diagnosis of iron deficiency or hypochromic anemia.
MCH	Together with MCV and MCHC, it is used to evaluate anemia (Savage 1993).
White blood cells (WBC)	Have two broad classes, which form defense system of the body.
Differentials white cells: Granulocytes (neutrophils, eosinophils and basophils) and agranulocytes (monocytes and lymphocytes)	Granulocytes develop in the red bone marrow. Neutrophils increase in acute infections and inflammation (Ghio *et al.* 1997). Eosinophils and basophils promote allergic inflammation by releasing pro-inflammatory mediators (Bochner and Schleimer 2001). Monocytes arise from the spleen. Monocytosis is associated with stress, infections, hematological disorders, necrosis and hemolysis (Feldman and Ruehl 1984). Lymphocytes arise from the bone marrow. Chronic infections and antigen antibody reactions cause lymphocytosis.
Platelets	Derived from the bone marrow. Thrombocytopenia is the first evidence of drug-induced toxic effects on hematopoesis (Paget 1979).

The use of non human primates as experimental models for man continues to increase in recent years. Yanagita (1973) reported that non human primate species are preferred models for drug safety because of their parallelisms to humans in many respects, including:

(a) Biochemical:- drug metabolism is similar to humans.

(b) Morphology and function of organs: - only kidneys of man and the monkey have a multi-papillar structure, and the occurrence of nephritis is identical to that in humans.

(c) Behaviorally superior (emotional expression and learning ability) over other species: similar attitude to that of humans in drug-seeking behavior and psychological susceptibility to the principal drugs of abuse in humans.

The Old World primates of Africa and Asia are reported to share 92% of their DNA with humans (Sibley and Ahlquist 1987). They are regarded the best metabolic models for human research in a broad range of investigations, including drug toxicological studies. This is because they possess at least five metabolic reactions, which are unique to Old World monkeys and humans (Smith and Caldwell 1976), and they do not demonstrate a species defect in metabolism, as those documented of other mammalian species (Smith and Caldwell 1976). Table 1.5 shows a comparison of the accuracy with which the Old World monkey (rhesus) and the dog would predict metabolic pathways in humans in regard to eleven assorted drugs. Smith and Williams (1974) found that humans and the three Old World monkey species (rhesus, vervets and baboons) have a similar metabolic reaction of conjugation of indoleacetic acid with amino acids to form indoleacetylglutamine, a glutamine conjugate.

Table 1.5. Status of two animal species as metabolic models for humans*

Rating	Percentage of 11 drugs	
	Rhesus monkeys	Dogs
Good	46	46
Fair	36	18
Invalid	18	36

*Data from Smith (1969)

1.3.1. A brief overview: The vervet monkey in biomedical research, its taxonomy and habitat

The vervet monkey has been used in the production of biologicals, drug testing and for research in several diverse disciplines including opthamology and virology (Kushner *et al.* 1982). At the Primate Unit of the Medical Research Council of South Africa, vervet monkeys have been successfully used as an experimental model in the areas of nutrition, reproduction and toxicology.

The vervet monkey is a member of the subfamily *Cercopithecidea,* which includes the baboon and other Old World monkeys. They belong to the genus *Chlorocebus* (Napier and Napier 1967). They are widely distributed throughout much of sub Saharan Africa and inhabit diverse habitats, including semi-arid Savannah, woodlands and rainforest. In the wild, vervet monkeys are able to exploit patchily distributed plants and feed on fruits, leaves, insects, eggs, seafood, grasses, and seeds (Raleigh and Mc Guire 1990). Typically free-ranging, vervet monkeys live in multi-male, multi-female groups of between 6 and 60 individuals per troop.

1.4. Objectives of the study

The purpose of this study was to investigate the possible toxicity of oleanolic acid in male vervet monkeys, by determining changes in the following:

- Behavioral patterns
- Physical and physiological parameters
- Biochemical markers
- Hematological indicators.

Chapter 2

Materials and methods

2.1. Ethical Approval

The Ethics Committee for Research on Animals of the Medical Research Council approved this study. Letter of ethical approval is attached in page 254

2.2. Subjects, housing conditions and diets

12 sexually mature and healthy male vervet monkeys used in this study were maintained indoors at the Primate Unit of the Medical Research Council. The housing and management of this vervet colony have been previously described by Seier (1986). Briefly, all animals were kept under identical housing conditions (temperature 25 - 27°C, humidity 50 – 60%, 12-hour photoperiod, and about 15-20 air changes per hour) in individual stainless steel squeeze-back cages. All individuals selected for this study were identified with numbers in ink tatoo, as well as through cage markings according to the individual's group designation and experimental number. Although individuals were housed singly, they had access to exercise cages and environmental enrichment.

The individuals were fed the same diet, which was sufficient to meet normal daily requirements, throughout the study period. In the morning at about 7H00, each monkey received a mixture of pre-cooked maize meal, vitamin and mineral mixes, trace elements and protein supplement, mixed with water to make a stiff porridge of about 100 grams. At noon, the diet was supplemented with enrichment treats, such as seasonal fruits and vegetables, soaked in biocide to prevent contamination. The afternoon diet (14H00) consisted of pre-cooked maize meal, milled sunflower seeds and maize kernels, mixed with water to make a stiff porridge. Seier (1986) reported that the

diet supported good reproductive performance and breeding at this facility. Drinking water was available *ad libitum* via an automatic watering device.

2.3. Treatments

Individuals were randomly divided into three treatment groups of 4, 10 and 25 mg/kg oleanolic acid and a control group. The choice of doses was based on previous studies on rats, where 16 mg/kg bodyweight oleanolic acid dose showed contraceptive efficacy (Mdhluli and van der Horst 2002, Rajasekaran *et al.* 1988). However, based on information that larger animals do not require huge doses compared to their smaller counterparts, it will be noted that the lowest dose used in this study is four times lower than a dose used in rats.

The availability of animals at the time of the experiment limited the allocation to three individuals per group. The morning diet, as described in the previous section, was used as a vehicle for oleanolic acid administration, and was mixed to a homogenous consistency with oleanolic acid. This treatment was administered daily for 16 weeks in the morning, according to the individual's bodyweight and group designation. In order to maintain relatively constant dosages, bodyweight changes prompted periodical adjustment of oleanolic acid concentrations in the diet. All individuals were monitored daily for changes in food consumption.

2.4. Anesthesia

In preparation for the clinical examinations (bodyweight, body temperature, respiratory and heart rates and blood pressure) and for blood sampling, all monkeys were fasted overnight for a period of 12 hours, but were allowed access to water. The following morning, the individuals were anesthetized by intramuscular injection of ketamine hydrochloride (Anaket-V, Centaur Labs, Bayer Animal Health [Pty] Ltd, Isando) to facilitate handling. The anaesthetic was administered at a dosage of 10 mg/kg bodyweight, using the 23G X ½" hypodermic needles and syringes.

2.5. General observations

To determine effects of oleanolic acid on the general wellbeing of the monkeys, all individuals were observed daily for deviations from normal physical characteristics (see Table 2.1).

Table 2.1. Clinical symptoms

Observation	Symptoms
Behavior	Unusual vocalization, restlessness, depressed, fearful, aggressive, irritable, excited, and passive.
Gastrointestinal	Fecal consistency and color, hemorhagic and mucoid stool.
Discharge	From eyes, nose, ears, rectum and penis.
Coat condition	Lustre, color, integrity and alopecia.
Movements	Twitch, tremor, sluggishness, confused, disorientated, alert and co-ordinated.
Penile	Prolapse.

2.6. Physical and physiological parameters

These were determined every second week, and include the parameters below.

2.6.1. Bodyweight

Bodyweight measurements were determined with a Berkel scale (Holland), and readings were recorded in kilograms (kg).

2.6.2. Body temperature

A glass thermometer was inserted into the rectum for approximately 30 seconds and the temperature was recorded in degree Celsius (°C).

2.6.3. Respiratory rate

Respiratory rates were determined by counting the number of breaths over a period of one minute. Respiration rate was recorded as breaths per minute (b/min).

2.7. Blood pressure and heart rate

Subjects were placed in a dorsal recumbency position and a neonatal blood pressure cuff #4, connected to the Dinamap XL vital signs monitor (Johnson & Johnson Medical Inc, Tampa, Fl) with screw-on hose, was attached to the upper arm. Systolic, diastolic and mean arterial pressures were recorded in millimeters of Mercury (mmHg) and heart rates as beats per minute (bpm). At each sampling period, three readings were recorded for each individual, and expressed as a mean for that parameter.

2.8. Blood collection, hematological and biochemical parameters

2.8.1. Blood collection and analyses

All blood samples were collected between 8H30 and 11H00 from each of the twelve monkeys on five occasions at time intervals of four weeks. Blood samples were collected aseptically by femoral venipuncture using 21GX½" vacutainer needles (Becton Dickson AG, Plymouth, UK) and Polypropylene tube holders (Terumo Corp, Tokyo, Japan). Analyses of the full blood count, electrolytes and creatine kinase were performed at a large commercial laboratory (PathCare, Cape Town, South Africa), which is accredited by the South African National Accreditation Systems (SANAS). The analytical chemistry laboratory of the Nutritional Intervention Programme of the Medical Research Council did the rest of the biochemical tests. These tests represent hepatic, renal and hematological functions.

2.8.2. Biochemical analyses

The specimens for serum glucose analysis and other biochemical tests were collected into sodium fluoride and SST tubes respectively. Blood samples were analyzed with an automated analyzer (Technicon RA-1000 autoanalyzer). Methods in Table 2.2 were used to determine concentrations of the following serum parameters:

(a) Enzymes: ALT, AST, ALP, GGT and CK.

(b) Proteins: Total protein, albumin and globulin.

(c) Lipids: Total cholesterol, HDL, LDL and triglycerides.

(d) Nitrogenous compounds: Urea and creatinine.

(e) Electrolytes: Sodium, potassium, chloride, calcium, magnesium and phosphate.

(f) Bilirubins: Total bilirubin and conjugated bilirubin.

(g) Glucose.

2.8.3. Hematological analyses

Blood for hematological assays were collected into vacutainer collection tubes with powdered (EDTA) anticoagulant (Becton-Dickson Vacutainer Systems). After being maintained at room temperature, specimens were tested within two hours of collection using the fully automated analyzer (Beckman Coulter STAKS S). Table 2.2 lists methods used for determination of the following hematological parameters:

(a) RBC, Hb, Hct, MCV, MCH, and MCHC.

(b) WBC, neutrophils, eosinophils, basophils, lymphocytes and monocytes.

(c) Platelets.

Table 2.2. Analytical methods

Test	Method
Red cell count	Impedence
Hemoglobin	Spectophotometric
Haematocrit	Impedence
Red blood cell indices	Impedence
White cell count	Impedence
Differential count	Volume conductivity scatter
Platelets	Impedence
Electrolytes	Indirect iron selective electrode
AST	NADH-NAD
ALT	NADH-NAD
ALP	p-Nitrophenyl phosphate
GGT	ϒ-glutamyl-p-nitro-anilide
CK	CK-NAC serum start
Urea	Urease
Creatinine	Picric acid
Glucose	Sodium hydroxy-4-aminoantipyrine
Total bilirubin	Diazo sulfanilic acid
Conjugated bilirubin	Diazo sulfanilic acid
Total cholesterol	Cholesterol esterase
HDLc	HDL cholesterol precipitating reagent
LDLc	Formula (LDLc = total cholesterol – HDLc)
Triglycerides	Biuret
Total protein	Biuret
Albumin	Bromocresol-green
Globulin	Formula (Glob = T. Prot – albumin)

2.9. Statistical analysis

All parameters were subjected to Repeated Measures Analysis of variance using the computer software SAS (Version 8). A statistical significance refers to both time-group interactions, and $P < 0.05$ was regarded as significant.

Chapter 3

Results

Note:

- Since inclusion of standard deviations in the graphs would have made visualization impossible, they are tabulated in Appendix A (pages 114 – 125).
- Statistical data (*P*-values) provided in this study represent changes observed between controls and the treated groups during the entire period of the study, and not at a particular time point.

3.1. General observations

None of the treated monkeys showed any noticeable deviation from their normal behavior, locomotor activity or food and water intake. All animals remained healthy during the study period and a year after completion.

3.2. Physical and physiological parameters

3.2.1. Bodyweight

The bodyweight of the controls and the group that received 25 mg/kg oleanolic acid were about five percent below baseline values at the end of the study (Figure 3.1). Reductions in the other treated groups were about two percent. No statistically significant differences were observed between controls and the treated groups.

3.2.2. Body temperature and respiratory rate

Clinical evaluations of body temperature and respiratory rates are depicted in Figures 3.2 and 3.3 respectively. The figures show similar fluctuations between controls and the treated groups in both parameters, except for the decline in body temperature of the controls during the second half of the study. None of the changes observed in both parameters in the treated groups were statistically significantly different from those in the controls.

Legends to figures

Note: The apparent huge peaks in some figures have to be considered against the background of a very small scale.

- Data in Figures 3.1 - 3.7 were obtained every second week, and in Figures 3.8 - 3.43 every fourth week.
- Figures 3.1 - 3.3 are parameters to monitor the physical conditions of male vervet monkeys.
- Figures 3.4 - 3.7 are physiological parameters to measure effects of oleanolic acid on cardiovascular function in male vervet monkeys.
- Figures 3.8 - 3.30 are clinical chemistry indicators measured to monitor toxicity of oleanolic acid on the cardiovascular, hepatic and renal function in the male vervet monkeys.
- Figures 3.31 – 3.43 are hematological indicators to monitor effects of oleanolic acid on the hematopoetic function in male vervet monkeys.

Explanations for unit abbreviations in Figures 3.1-3.43

Unit	Explanation
kg	kilogram
°C	degree Celsius
mmHg	millimeters of mercury
mmol/L	millimol per litre
U/L	units per litre
µmol/L	micromol per litre
g/L	gram per litre
g/dL	gram per decilitre
fl	femtolitre
pg	picogram

Figure 3.1. Bodyweight

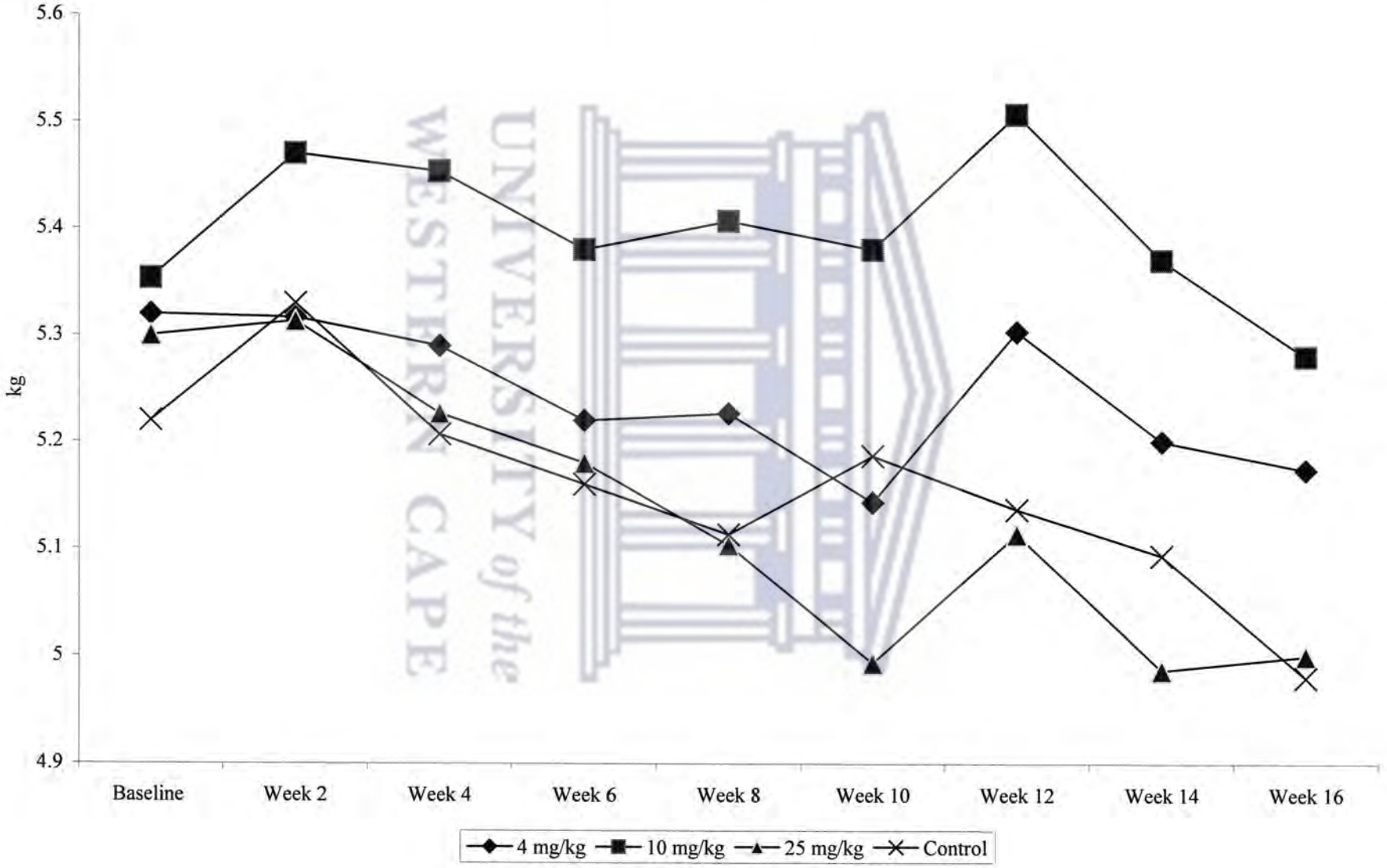


Figure 3.2. Body temperature

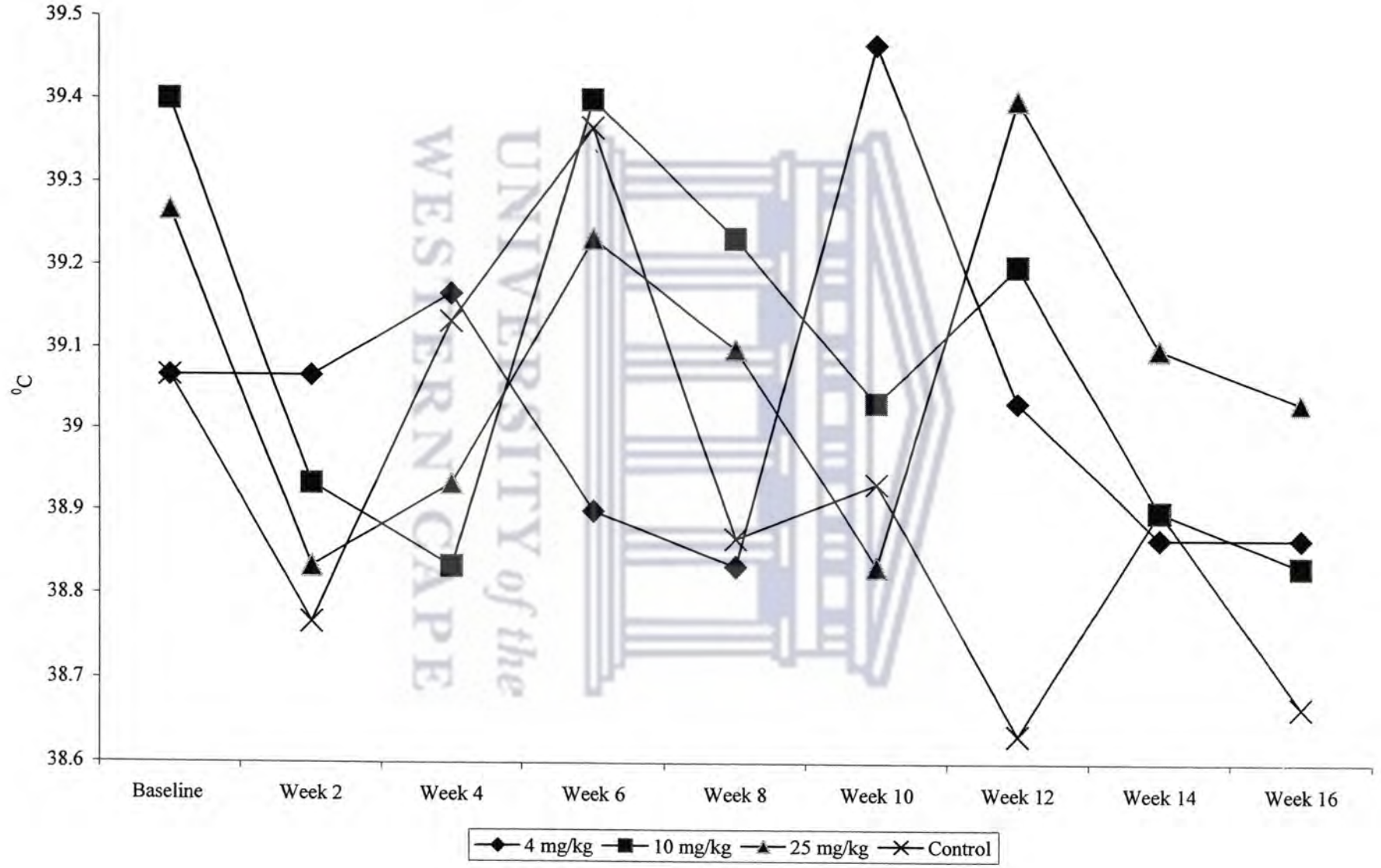
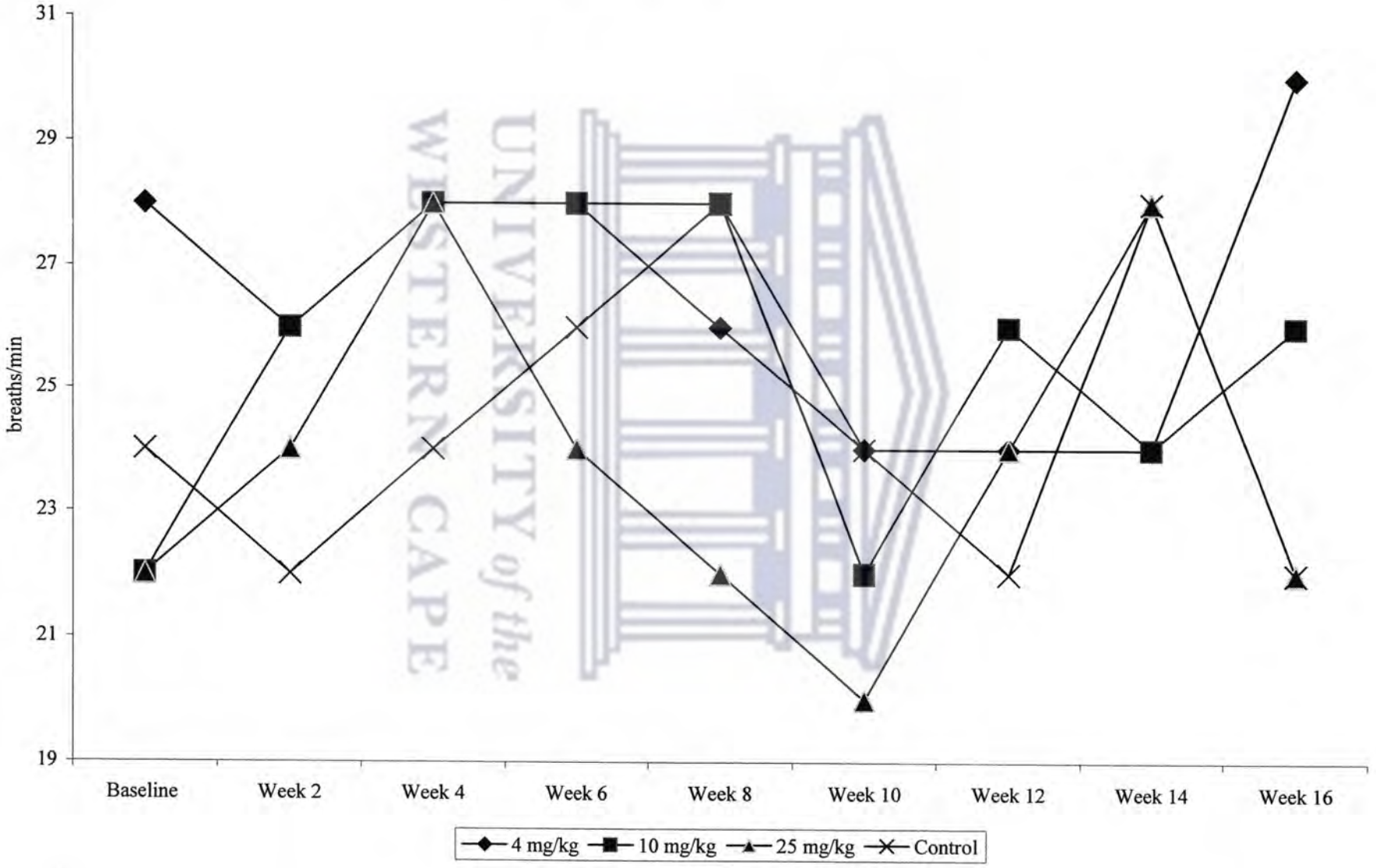


Figure 3.3. Respiratory rate



3.3. Cardiovascular function

As from week two, there were decreases in systolic, diastolic and mean arterial pressures in the group that received 25 mg/kg oleanolic acid, which did not return to baseline levels (Figures 3.4-3.6). Except for a linear decline in the systolic pressure of the controls, patterns similar to those of the diastolic and mean arterial pressures were not observed in the remaining treated groups or in the controls. Heart rates of monkeys in the control group and the group that received 4 mg/kg oleanolic increased during the first four weeks, but declined to near baseline levels by the end of the study (Figure 3.7). Similar changes were observed in the groups that received 10 and 25 mg/kg oleanolic, however, these values were much higher at week 16 compared to those at baseline. However, no statistically significant differences between controls and the treated groups were found in all four parameters.

Despite lack of statistically significant differences, serum concentrations of HDL in the treated groups, but not the controls, were higher at week 16 compared to baseline (Figure 3.8). There were similar fluctuations in LDL (Figure 3.9) and total cholesterol (Figure 3.10) concentrations between controls and the treated groups throughout the study period. The triglycerides of all groups, except the group that received 25 mg/kg oleanolic acid started with an increase, and then declined to levels below the baseline at the end of the study (Figure 3.11). No statistical significant differences were found between controls and the treated groups in the LDL, total cholesterol and triglyceride concentrations.

Despite huge differences in CK concentrations between treated groups (4 and 25 mg/kg oleanolic acid) and the controls (Figure 3.12) over time, no statistically significant differences were found in this parameter. Similar statistical results were observed between controls and the group that received 10 mg/kg oleanolic acid.

Figure 3.4. Systolic pressure

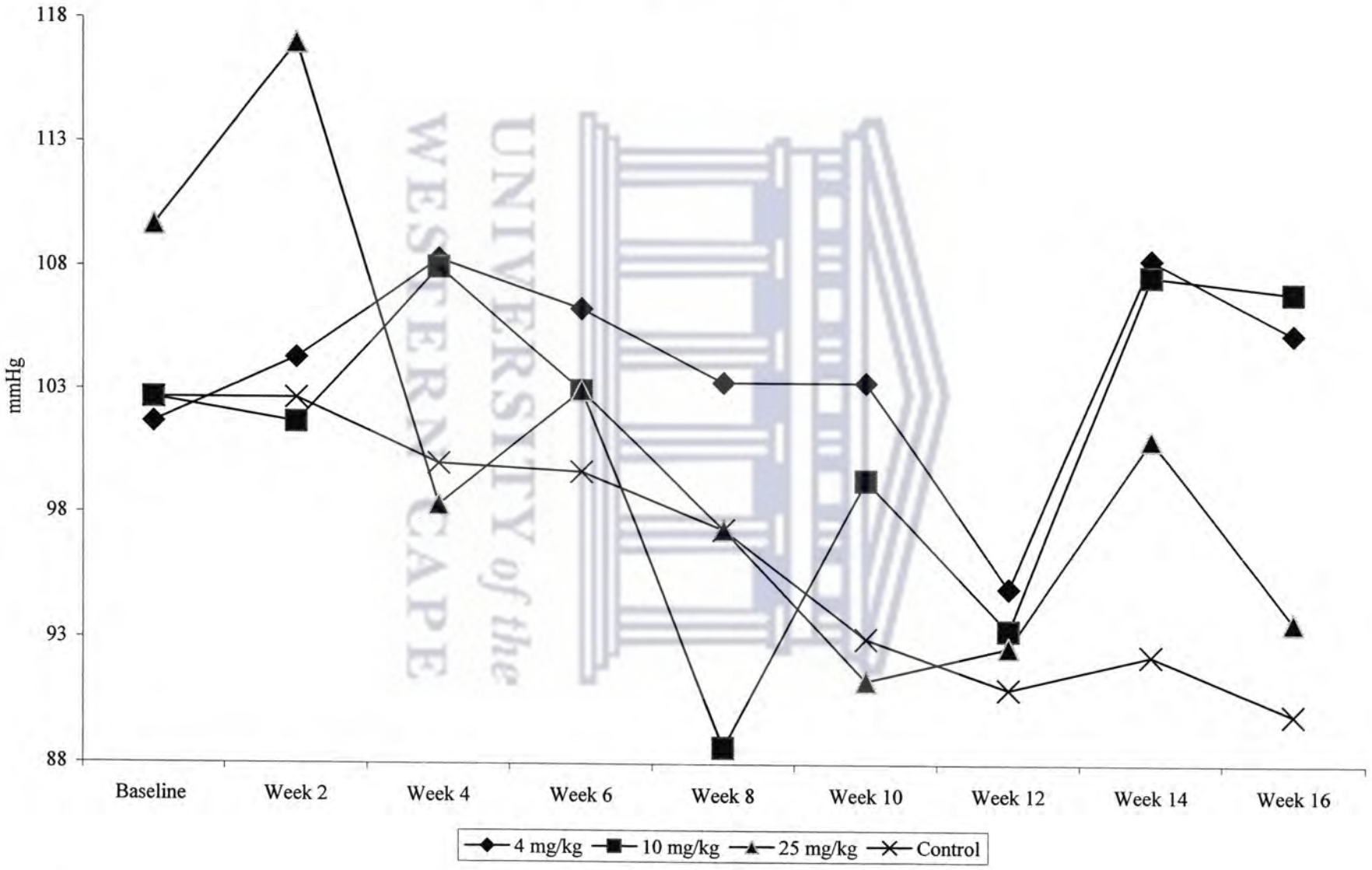


Figure 3.5. Diastolic pressure

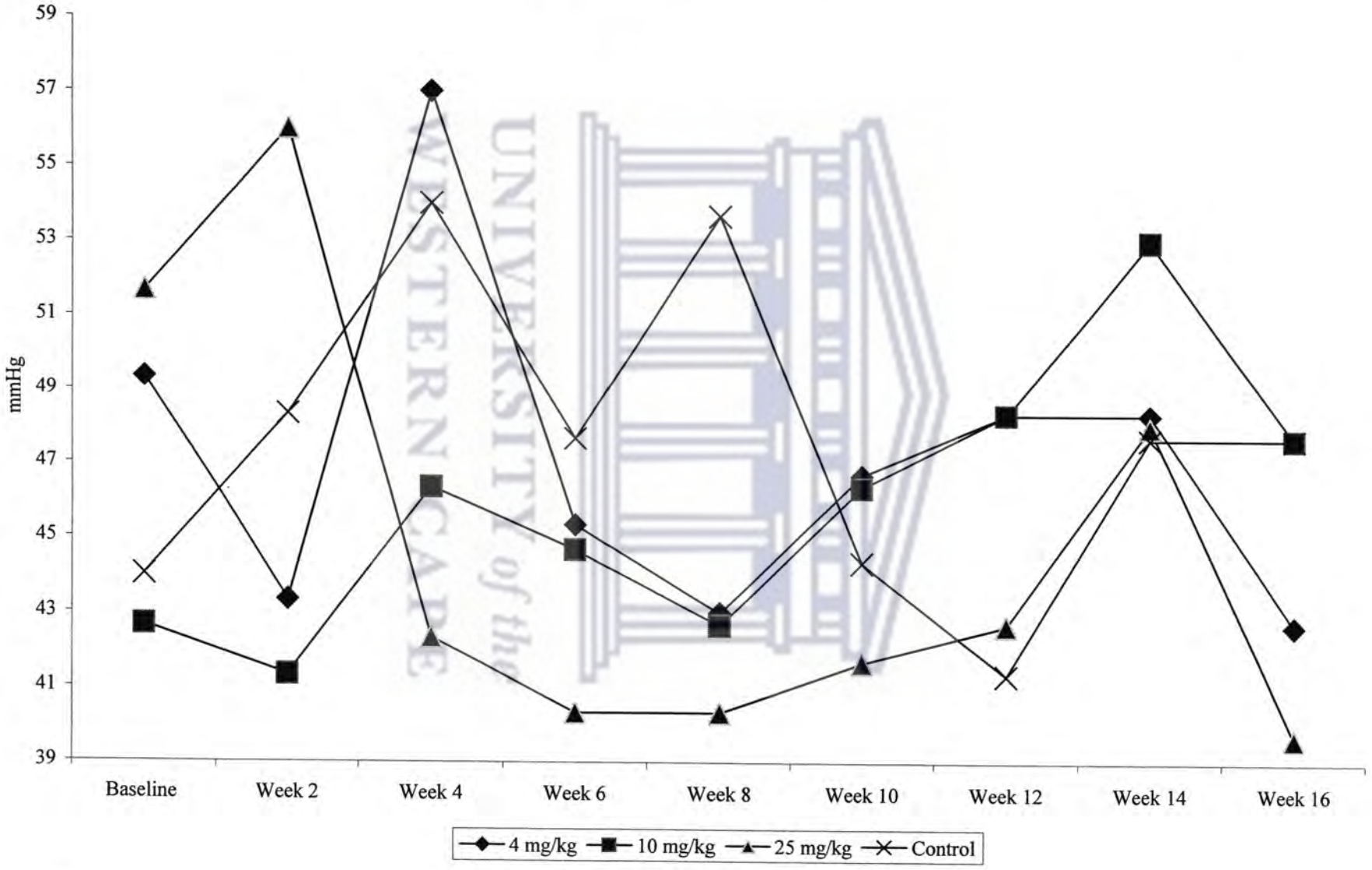


Figure 3.6. Mean arterial pressure

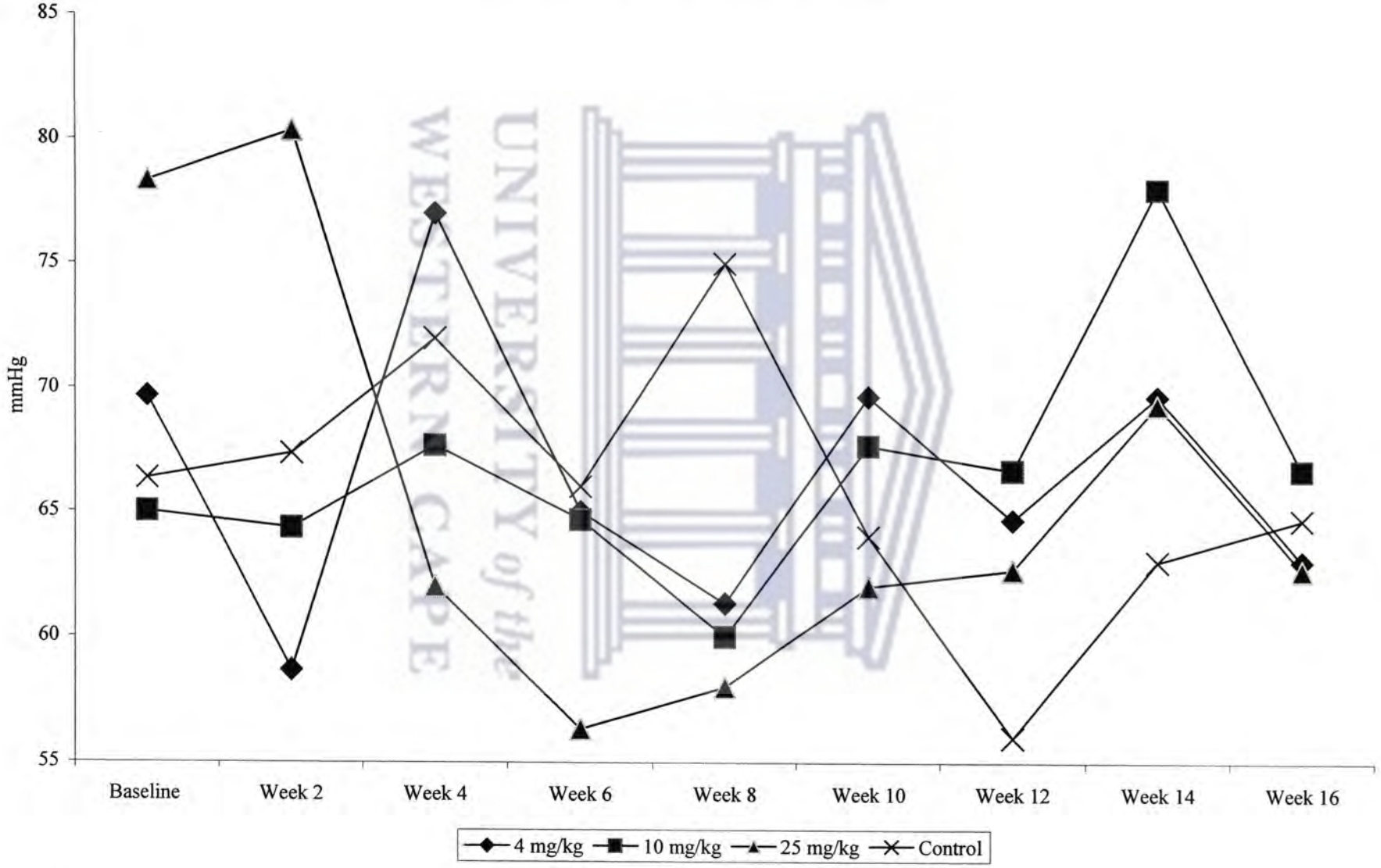


Figure 3.7. Heart rate

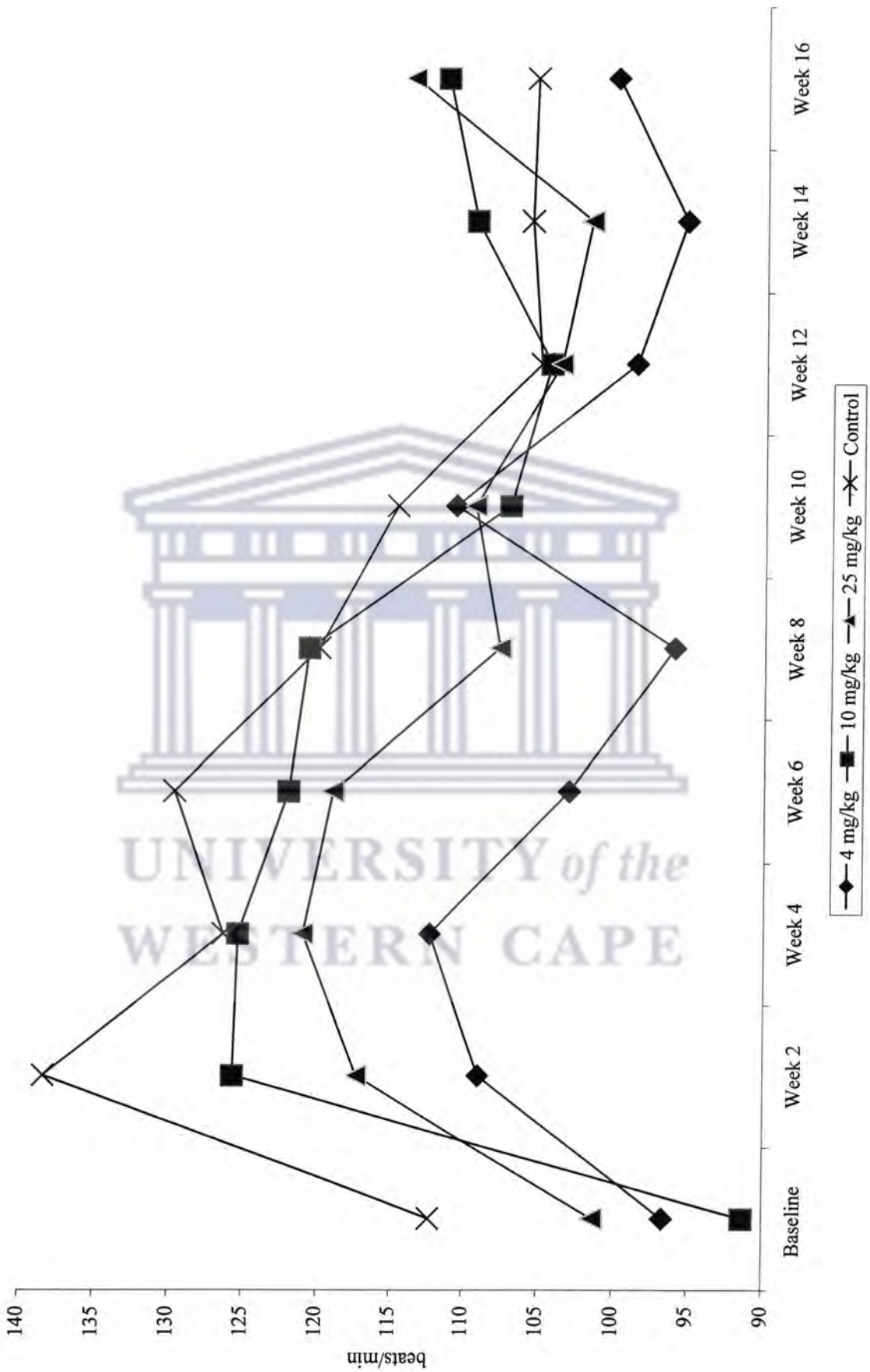


Figure 3.8. High-density lipoproteins

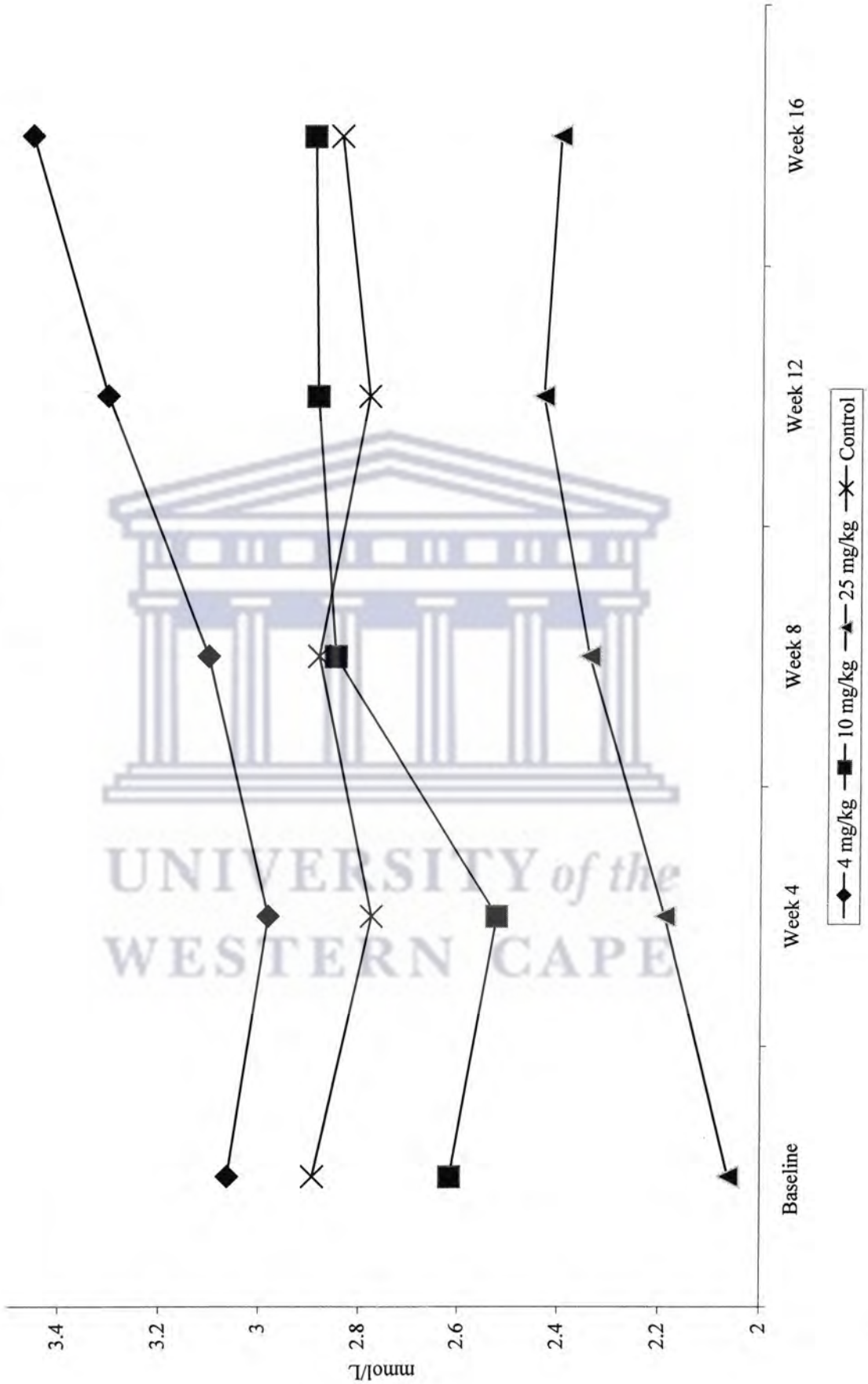


Figure 3.9. Low-density lipoproteins

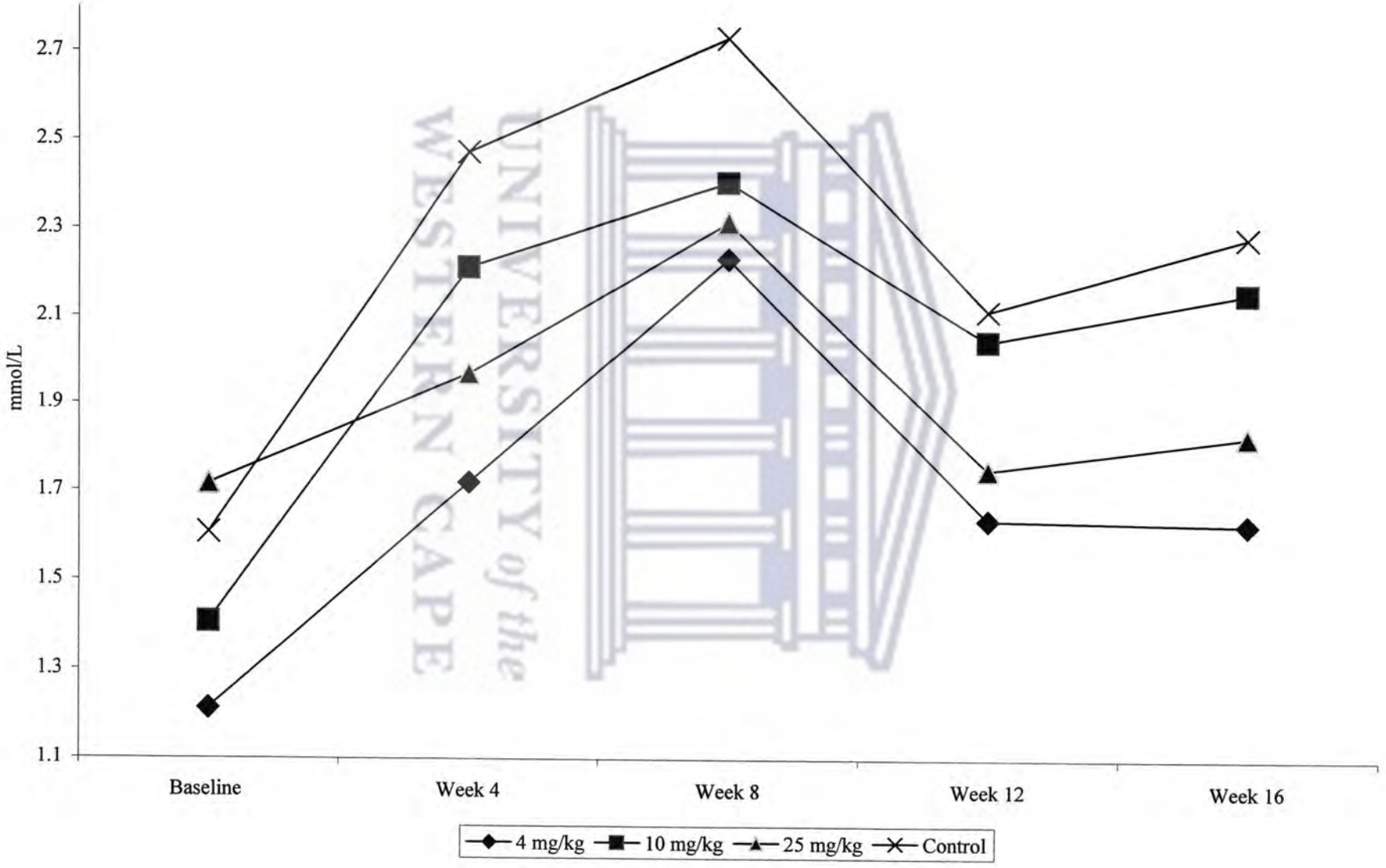


Figure 3.10. Total cholesterol

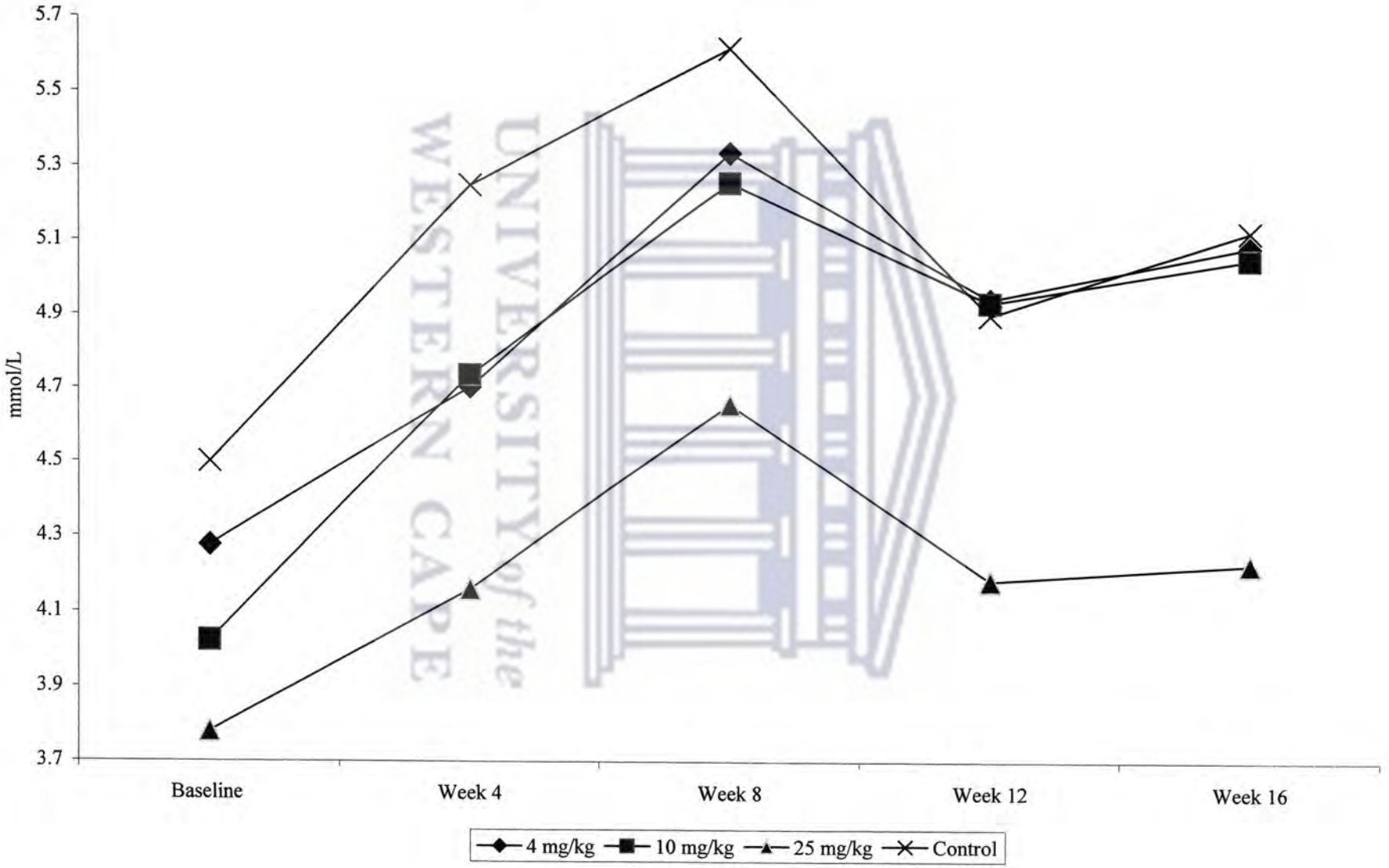


Figure 3.11. Triglycerides

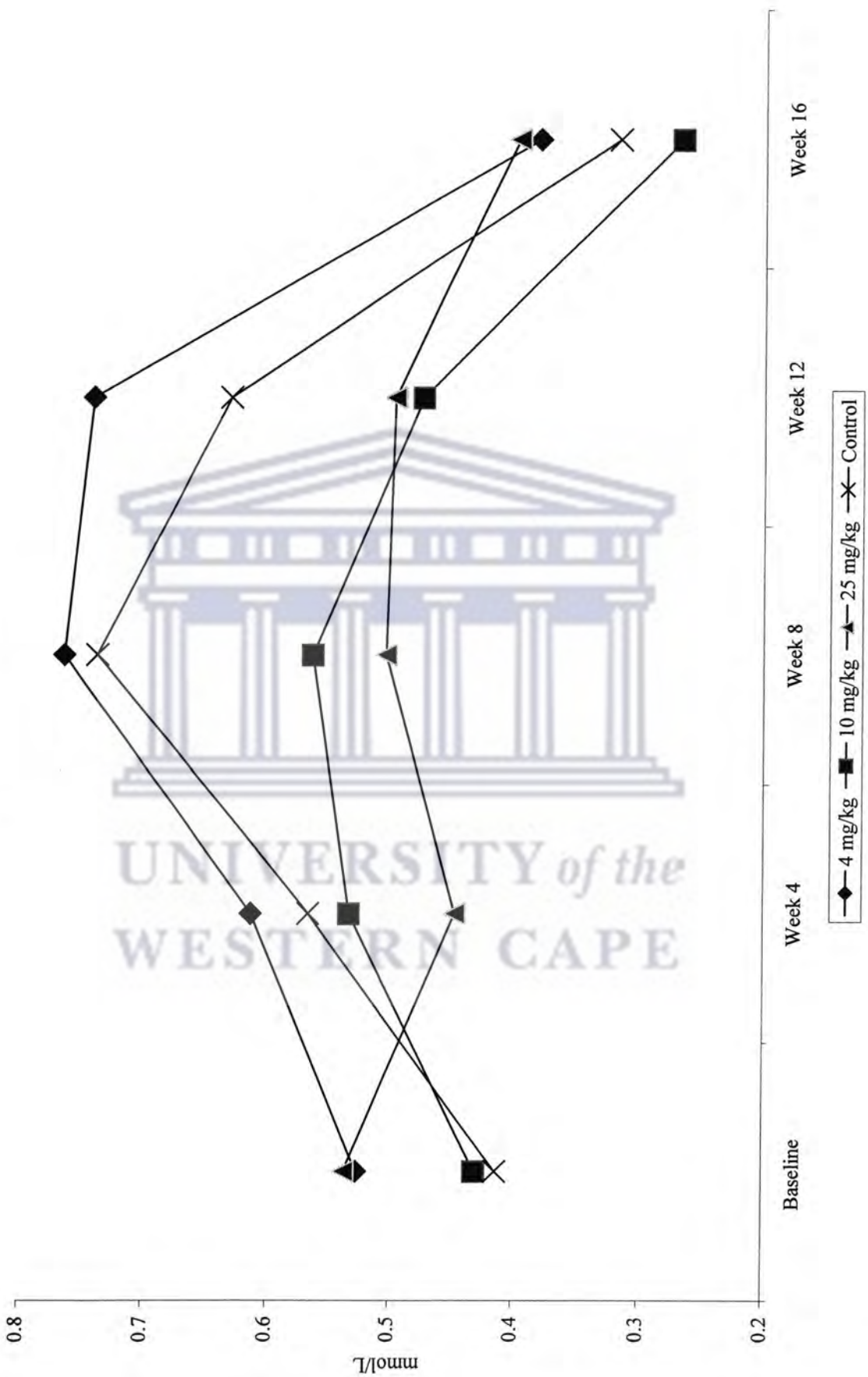
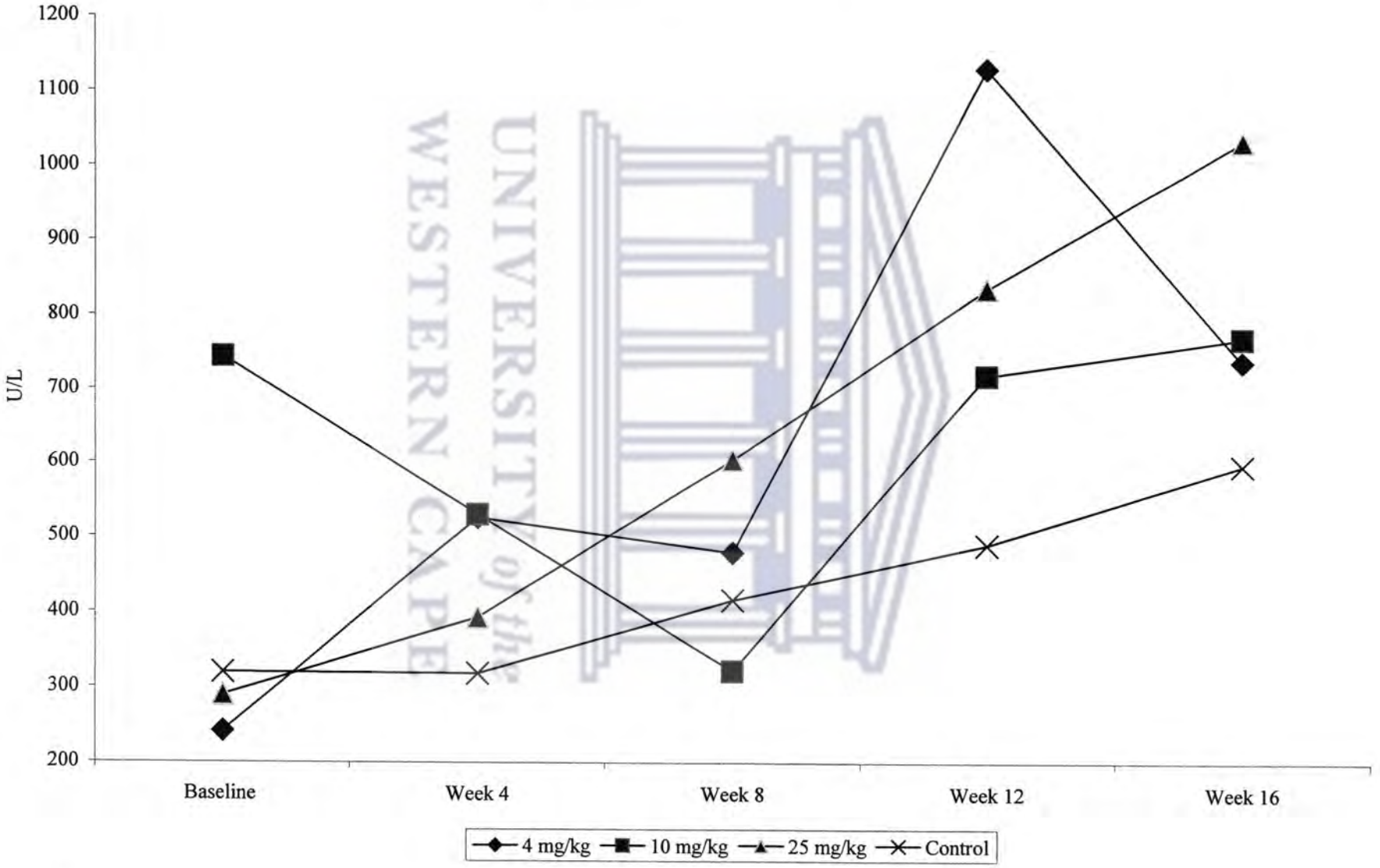


Figure 3.12. Creatine kinase



3.4. Hepatic function

Activities of serum AST (Figure 3.13) and ALT (Figure 3.14) in the groups that received 4 and 10 mg/kg oleanolic acid were slightly higher at week 16 compared to baseline. Although both enzymes had almost similar patterns in the group that received 25 mg/kg oleanolic acid, a statistically significant difference ($P = 0.0452$) could only be found for AST in this group when compared with the controls.

The ALP concentrations in the treated groups had negligible fluctuations (Figure 3.15), and thus, were not statistically significantly different from the controls. Serum concentrations of GGT in the groups that received 4 and 25 mg/kg oleanolic acid were considerably lower at week 16 compared to baseline (Figure 3.16). Although there were wide fluctuations in GGT concentrations in the group that received 10 mg/kg oleanolic acid, concentrations of this enzyme returned to baseline levels at the end of the study. However, these changes were not statistically significantly different from those of the controls.

Since total bilirubin concentrations of all groups, including controls, followed near similar trends (Figure 3.17), no statistically significant differences could be found between treated groups and the controls. Conjugated bilirubin concentrations were considerably reduced over time in the controls, while baseline levels of this parameter were relatively unchanged in the treated groups at week 16 (Figure 3.18). No statistically significant differences were found between controls and the treated groups.

Glucose concentrations in monkeys in the control and treated groups were lower at week 16 compared to baseline (Figure 3.19). Therefore, no statistically significant differences were observed between controls and the treated groups.

As the study progressed, an increase in albumin concentrations in all groups (Figure 3.20) was followed by a decrease in globulin concentrations (Figure 3.21). Although not statistically significantly different from the controls, magnitudes of fluctuations in these parameters were greater in the treated groups compared to the controls. Fluctuations in total protein concentrations of the treated groups were not statistically significantly different from those of the control group (Figure 3.22).

Figure 3.13. Aspartate aminotransferase

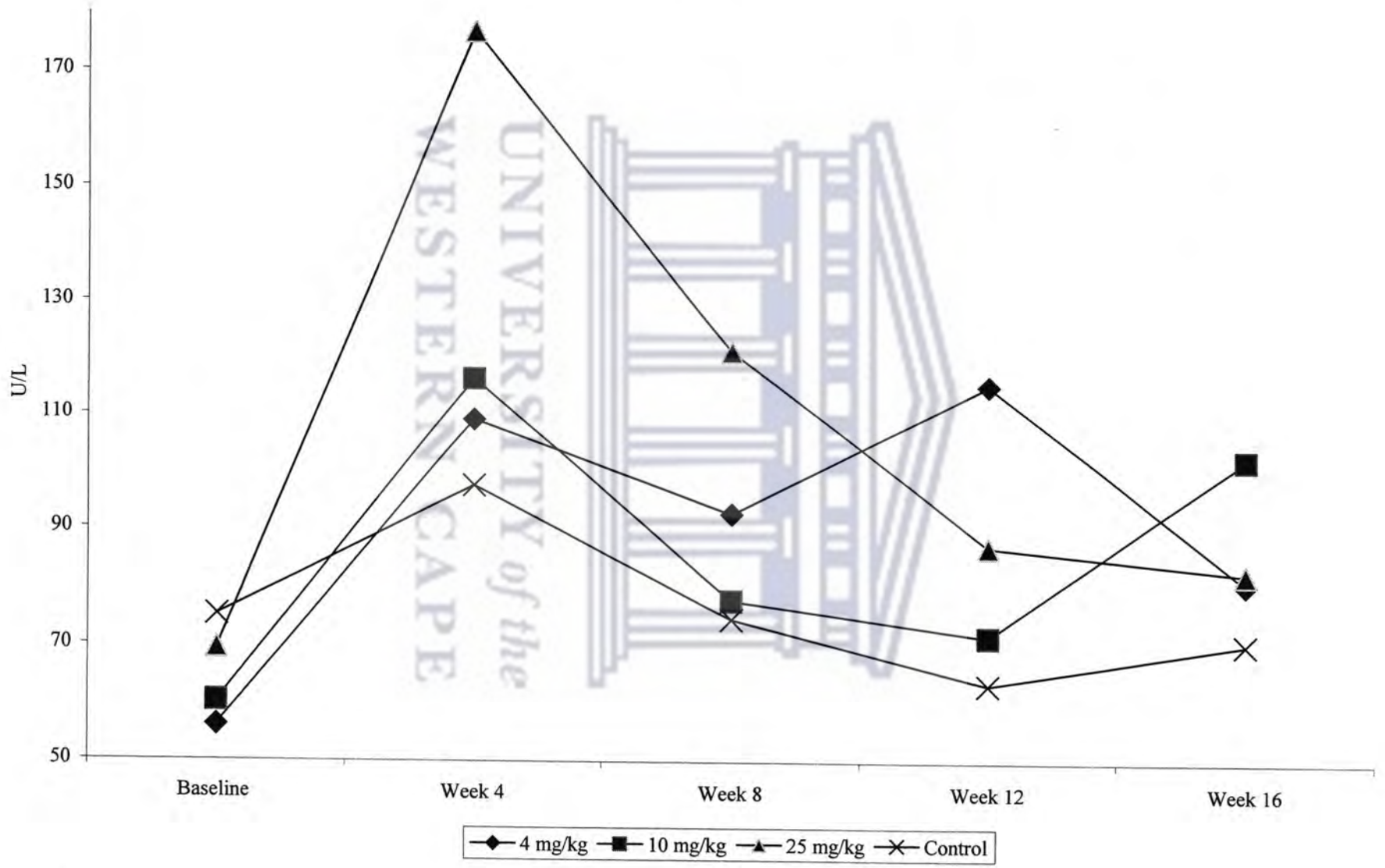


Figure 3.14. Alanine aminotransferase

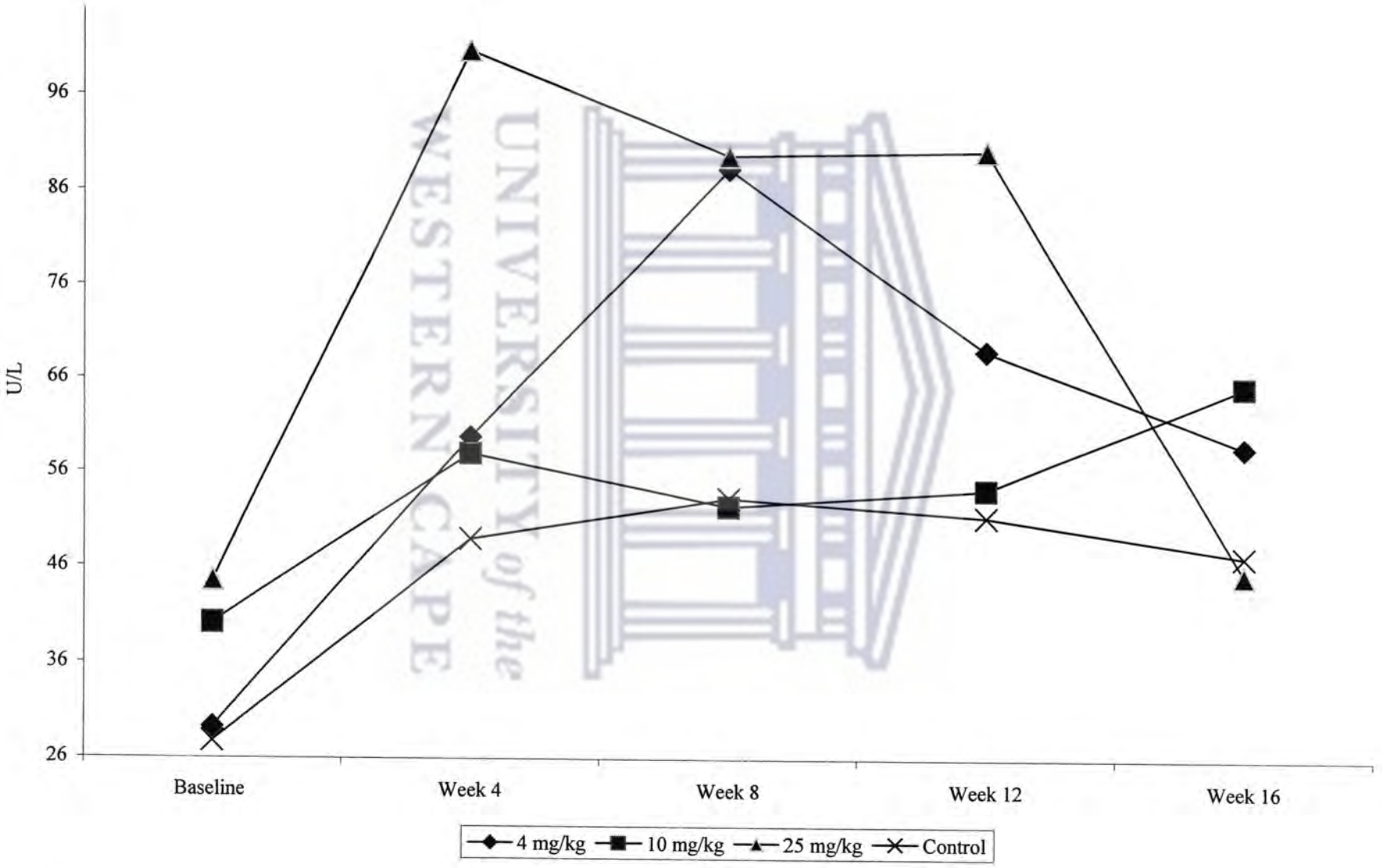


Figure 3.15. Alkaline phosphatase

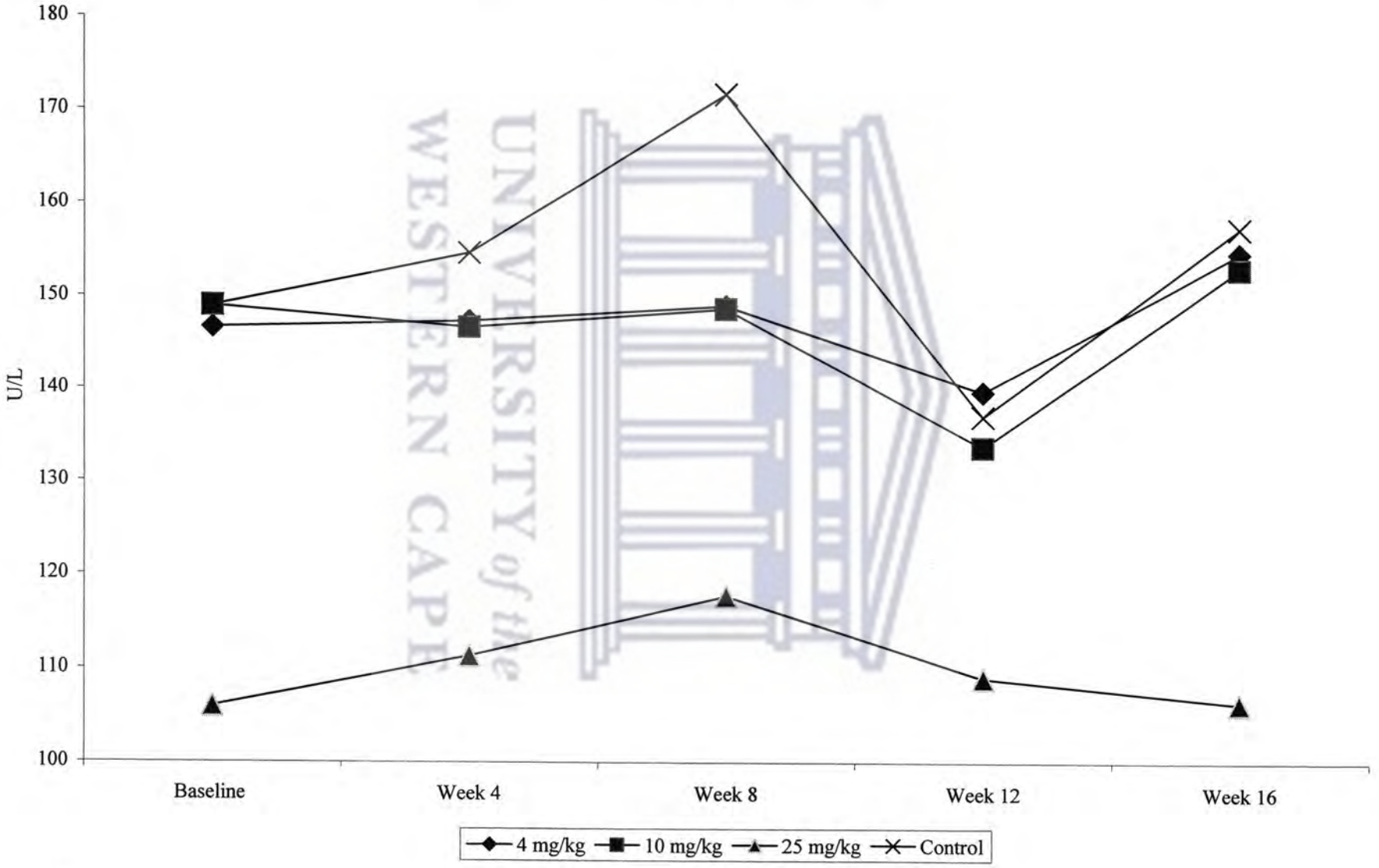


Figure 3.16. Gamma-glutamyl transferase

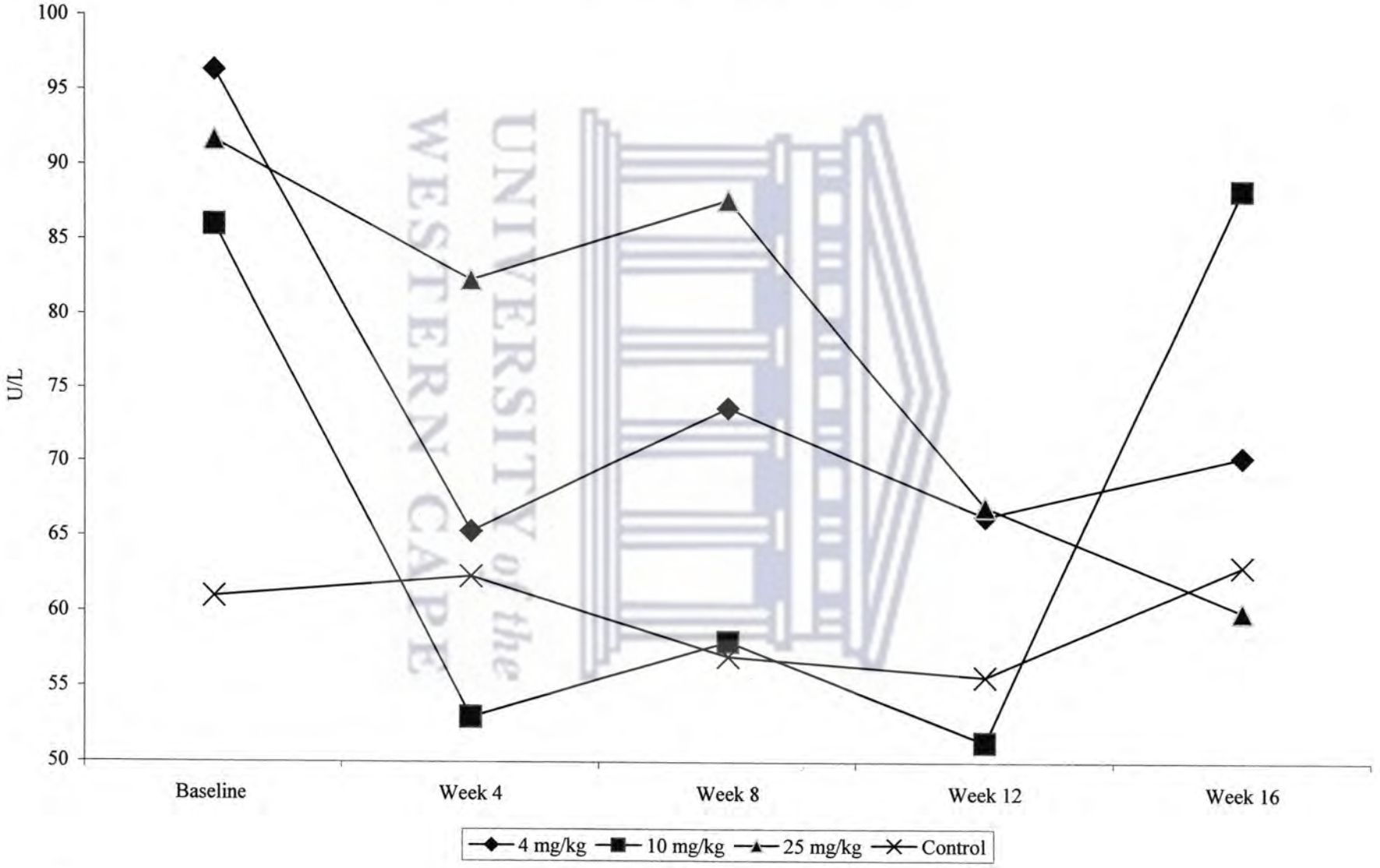


Figure 3.17. Total bilirubin

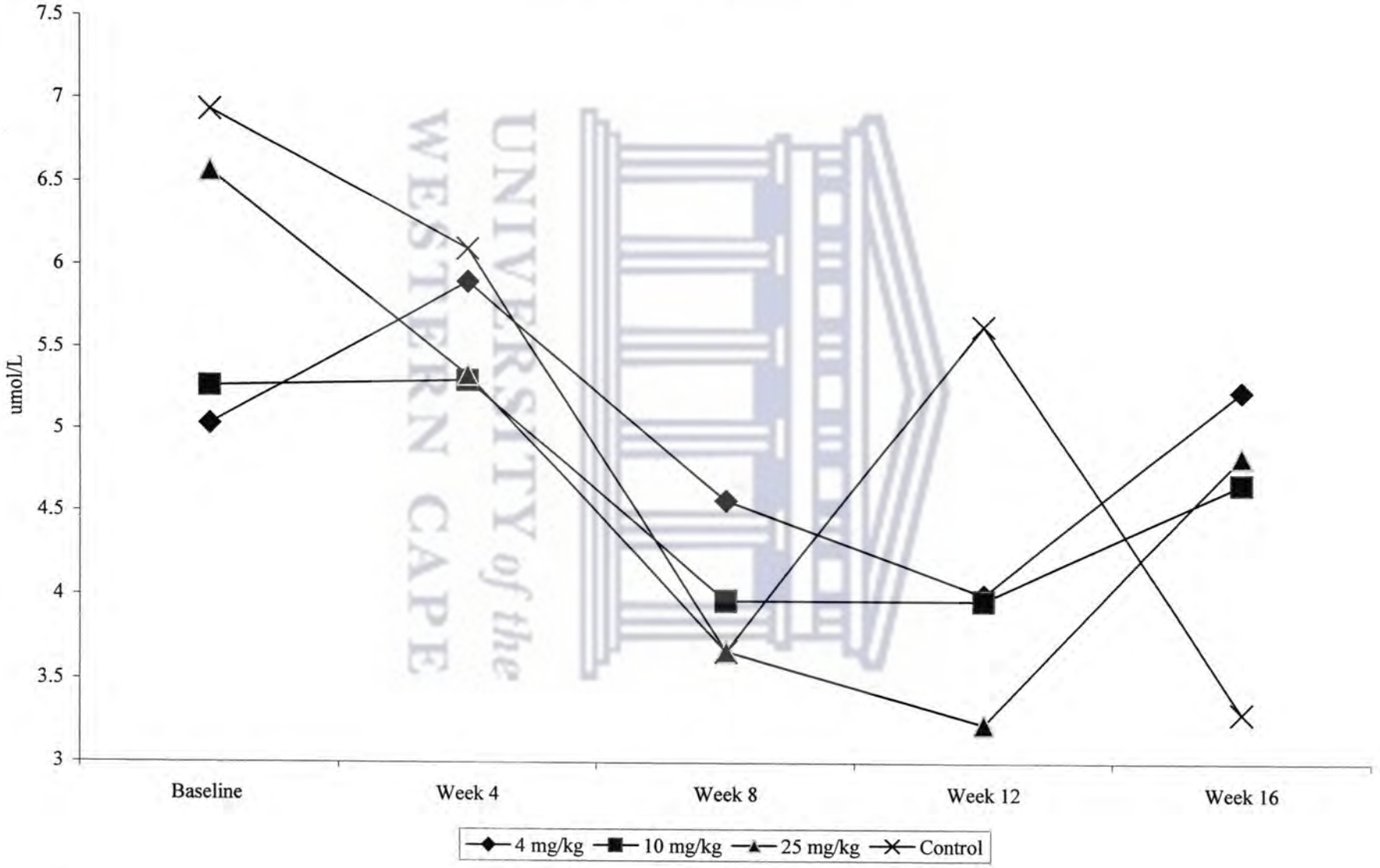


Figure 3.18. Conjugated bilirubin

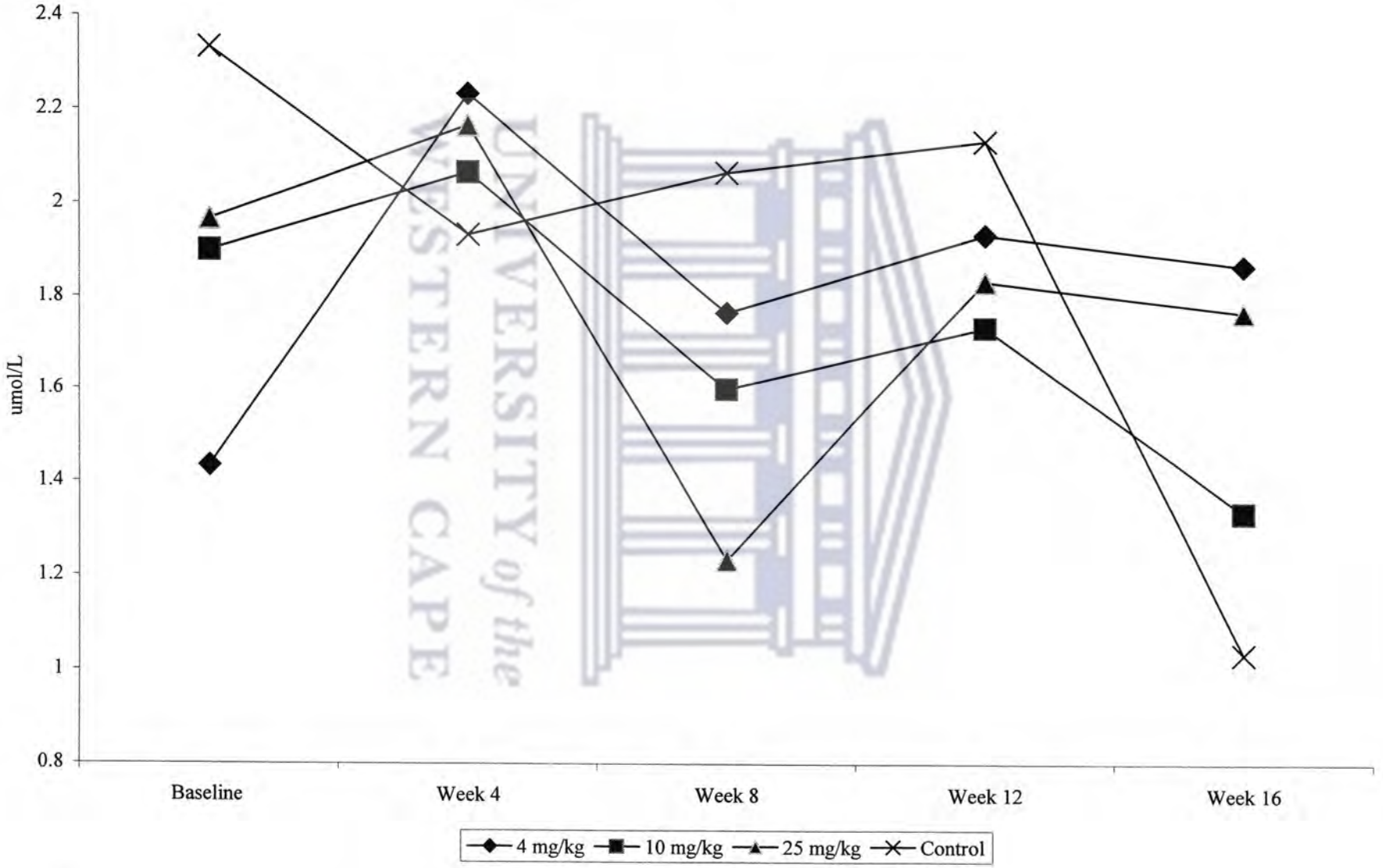


Figure 3.19. Glucose

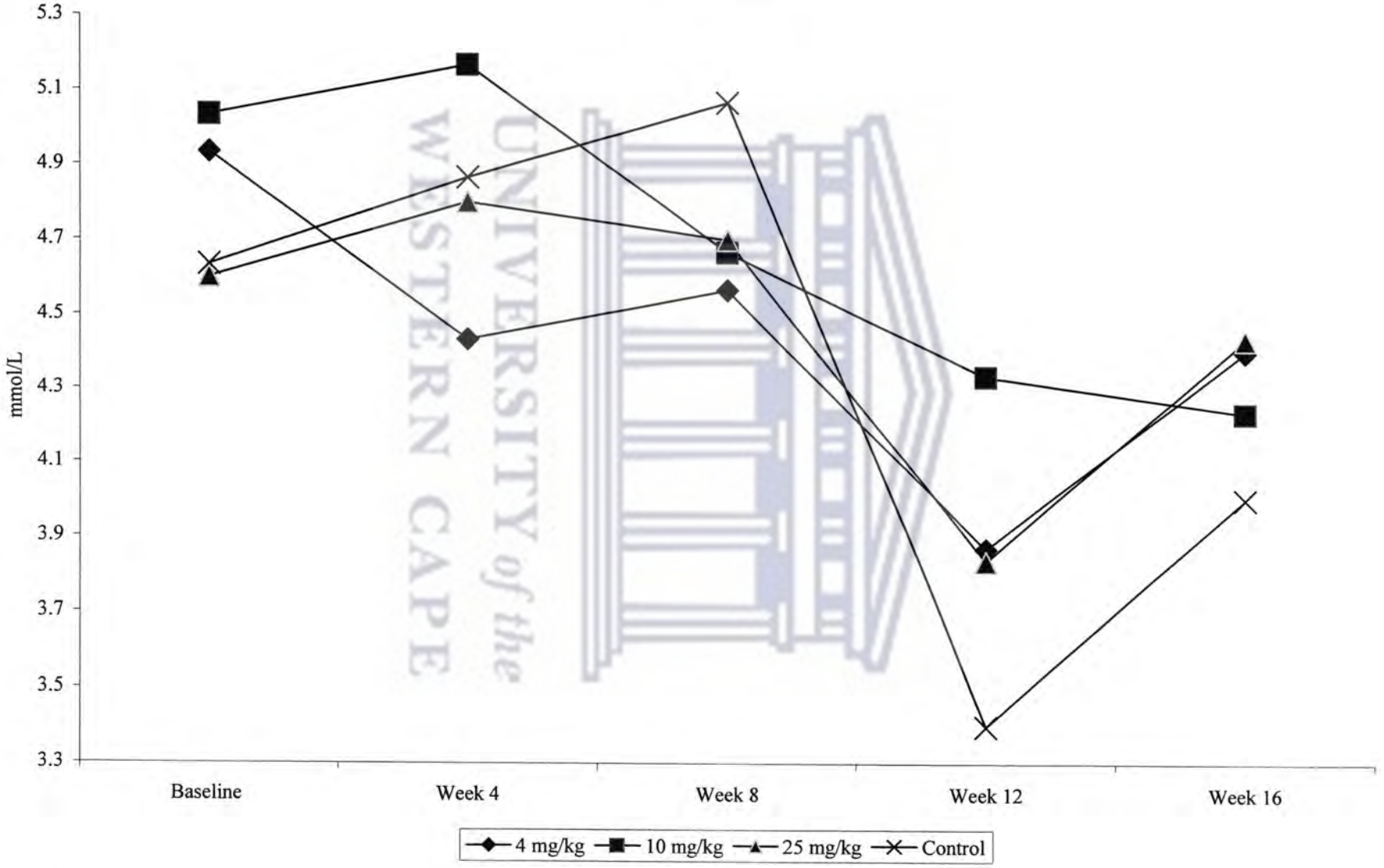


Figure 3.20. Serum albumin

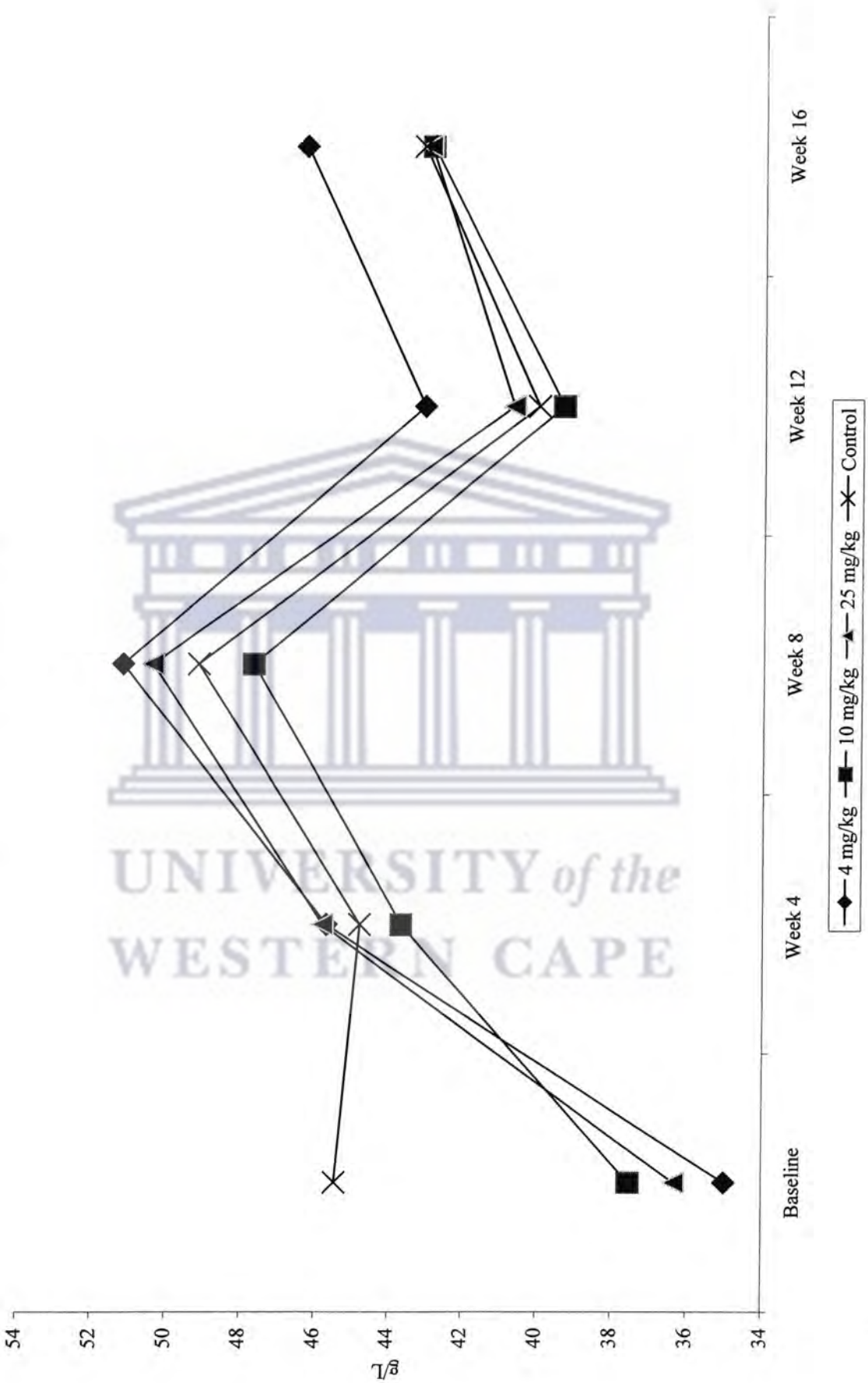


Figure 3.21. Serum globulin

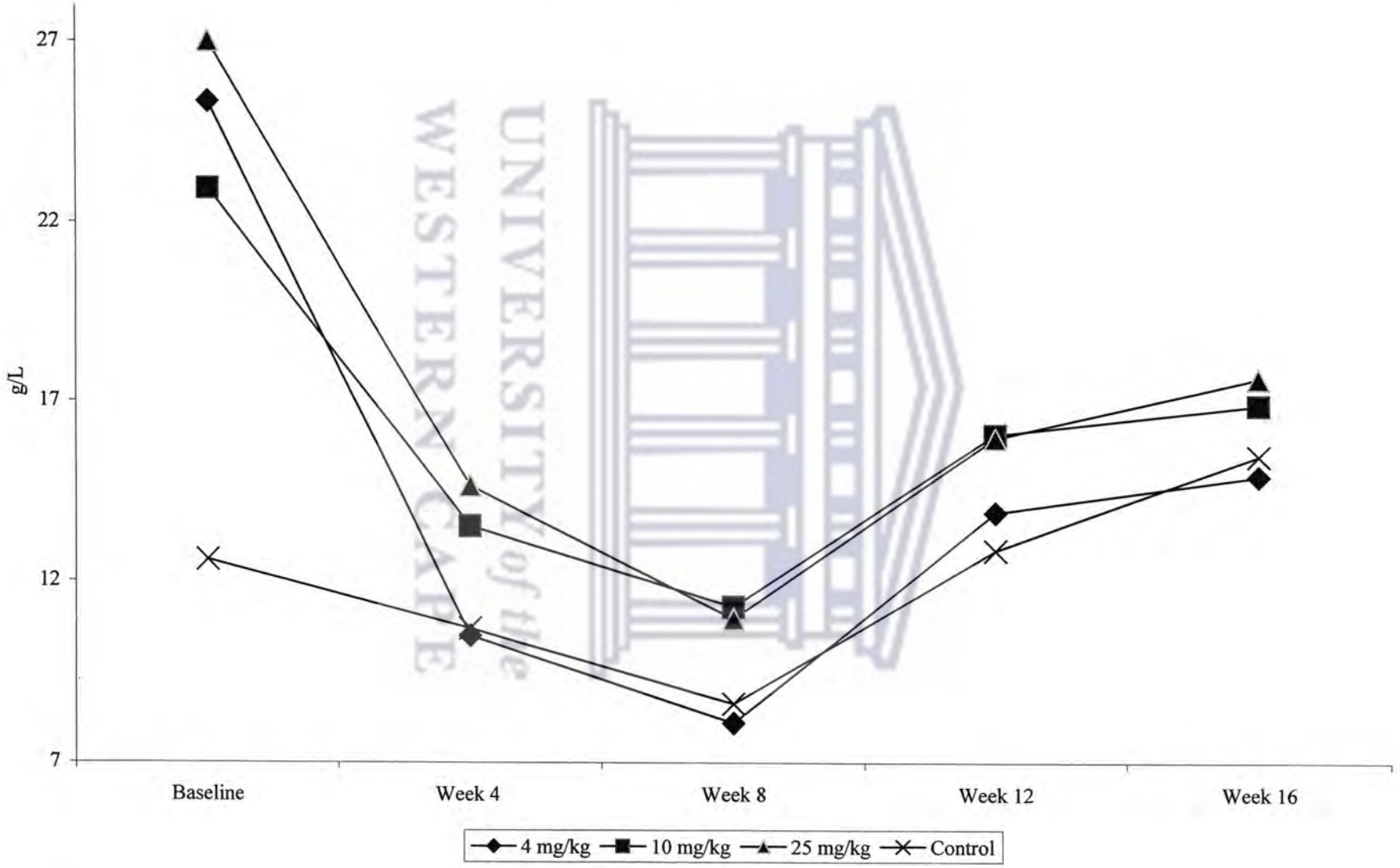
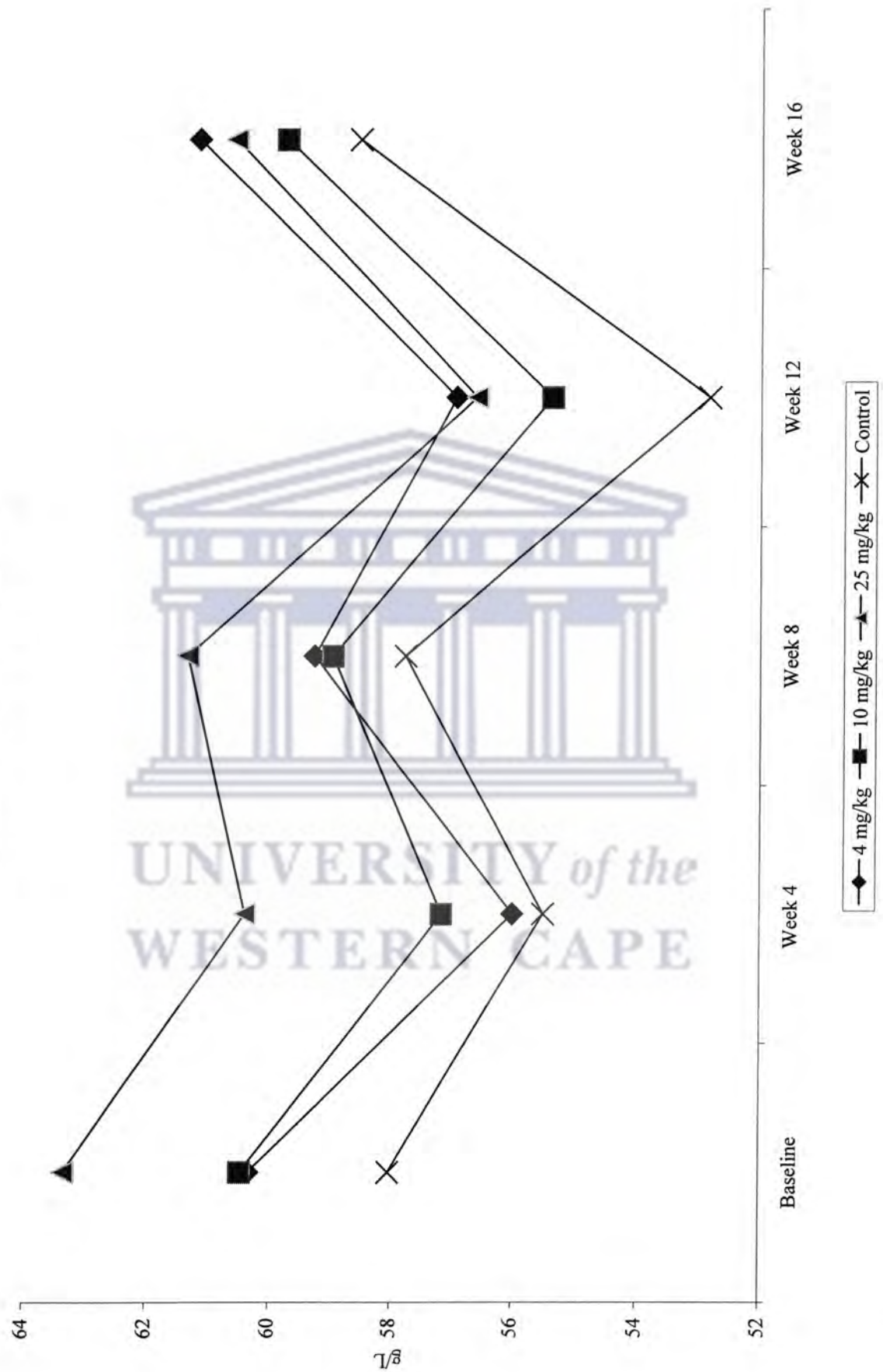


Figure 3.22. Total serum proteins



3.5. Renal function

There were no wide fluctuations in creatinine concentrations of all groups throughout the study period, except for the group that received 4 mg/kg oleanolic acid (Figure 3.23). The latter peaked sharply at week 12, which however, returned to within baseline levels by the end of the study. Corresponding changes in serum urea concentrations were seen between controls and the treated groups (Figure 3.24). None of the changes observed over time in both parameters of the treated groups were statistically significantly different from those of the controls.

Fluctuations in sodium (Figure 3.25), chloride (Figure 3.26), potassium (Figure 3.27), calcium (Figure 3.28) and magnesium (Figure 3.29) concentrations of the treated groups were relatively similar to those of the controls. Therefore, no statistically significant differences could be found between treated groups and the controls. Baseline phosphate concentrations in the groups that received 4 and 25 mg/kg oleanolic acid were increased by an average of six percent at week 16, whereas that of a group that received 10 mg/kg oleanolic acid were reduced by same percentage (Figure 3.30). Nonetheless, these changes were not statistically significantly different from those in the controls.

Figure 3.23. Serum creatinine

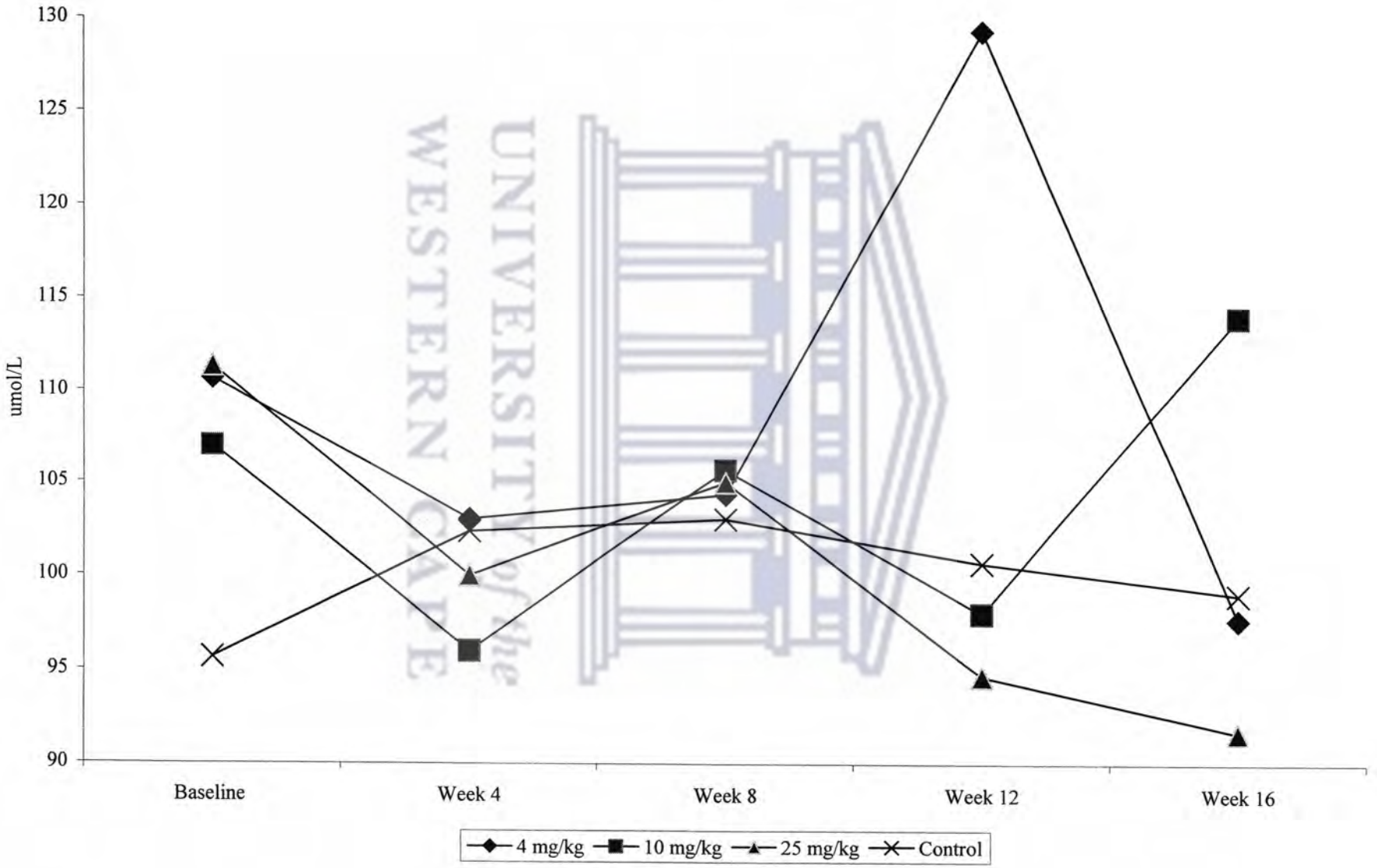


Figure 3.24. Serum urea

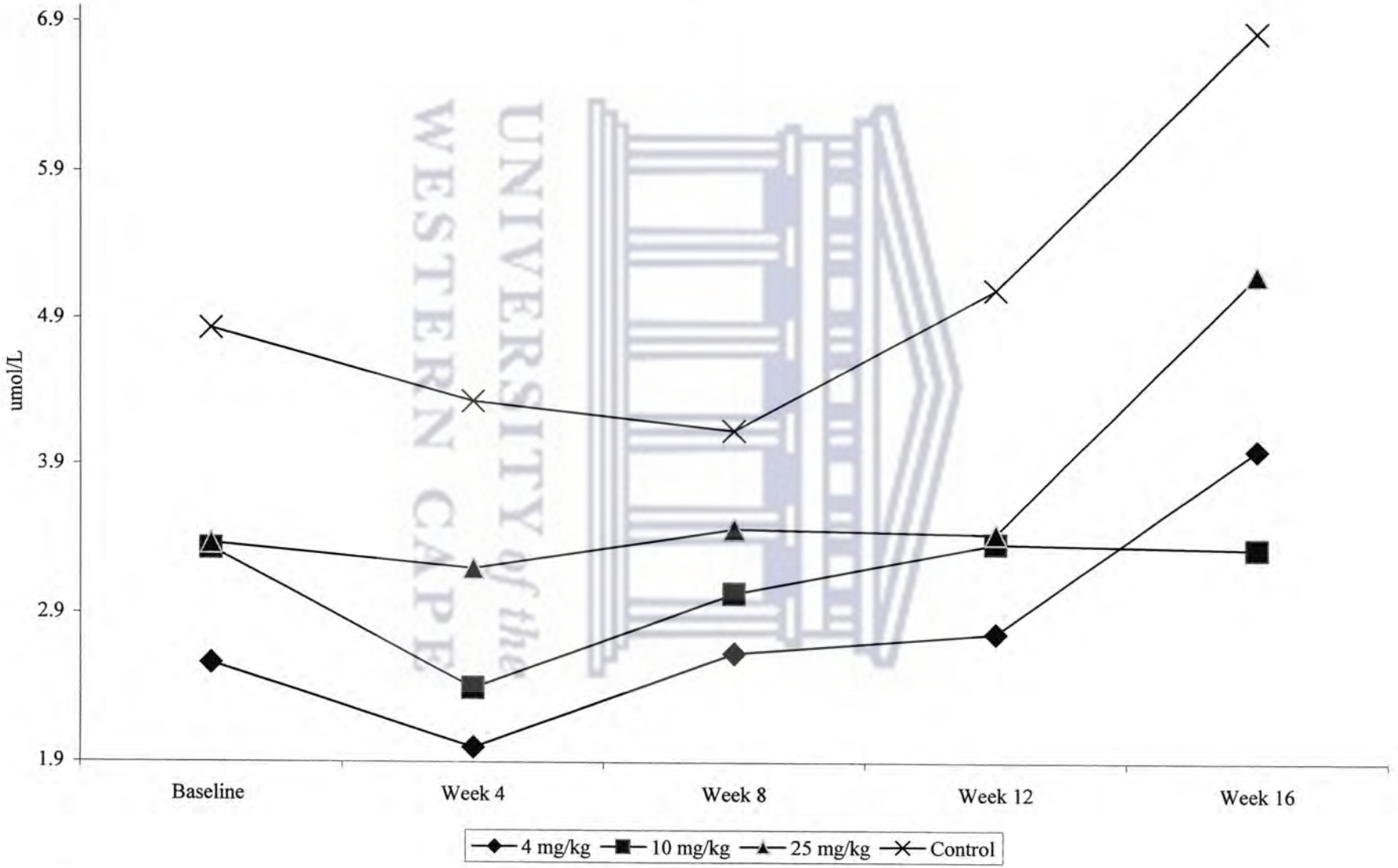


Figure 3.25. Sodium

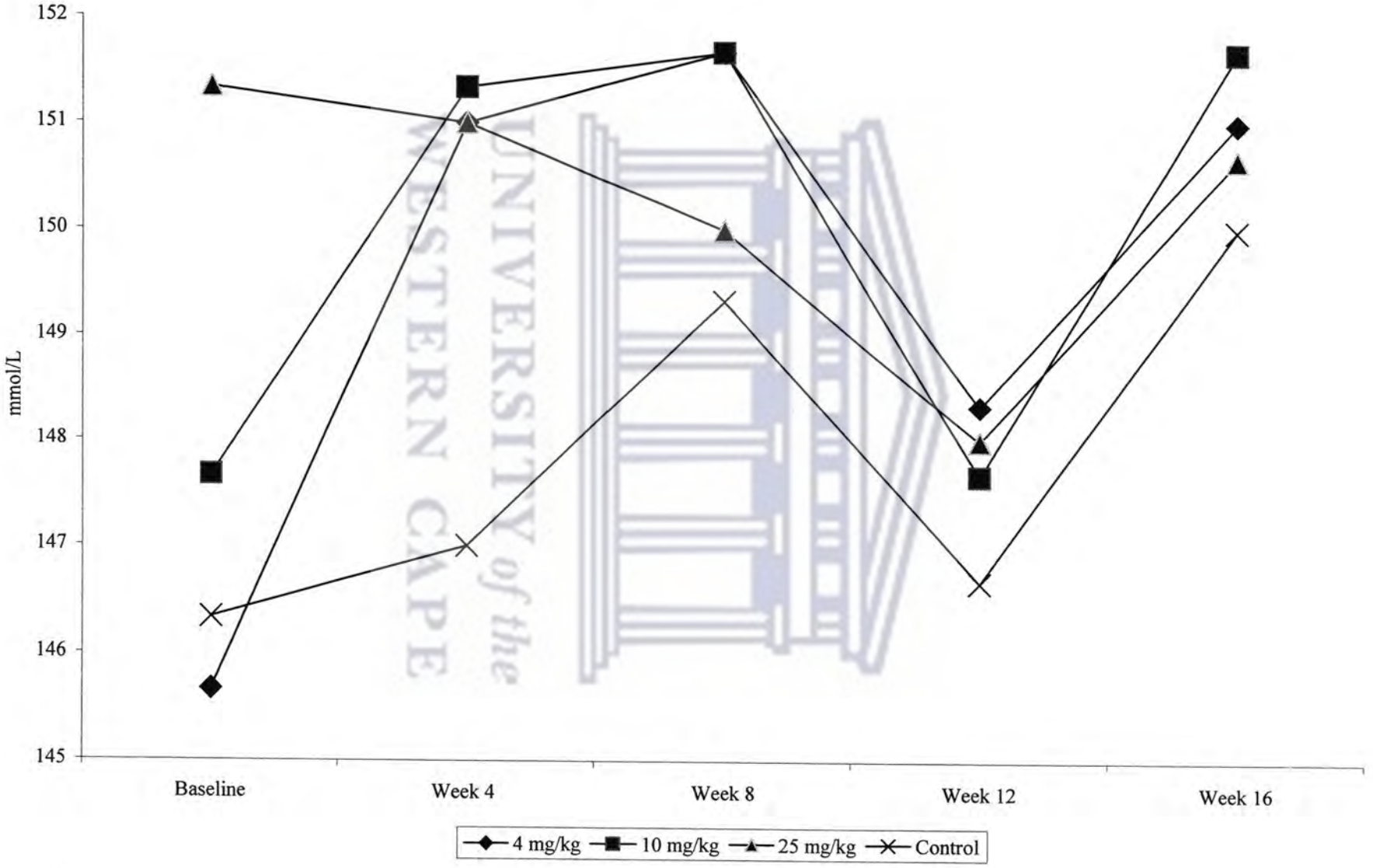


Figure 3.26. Chloride

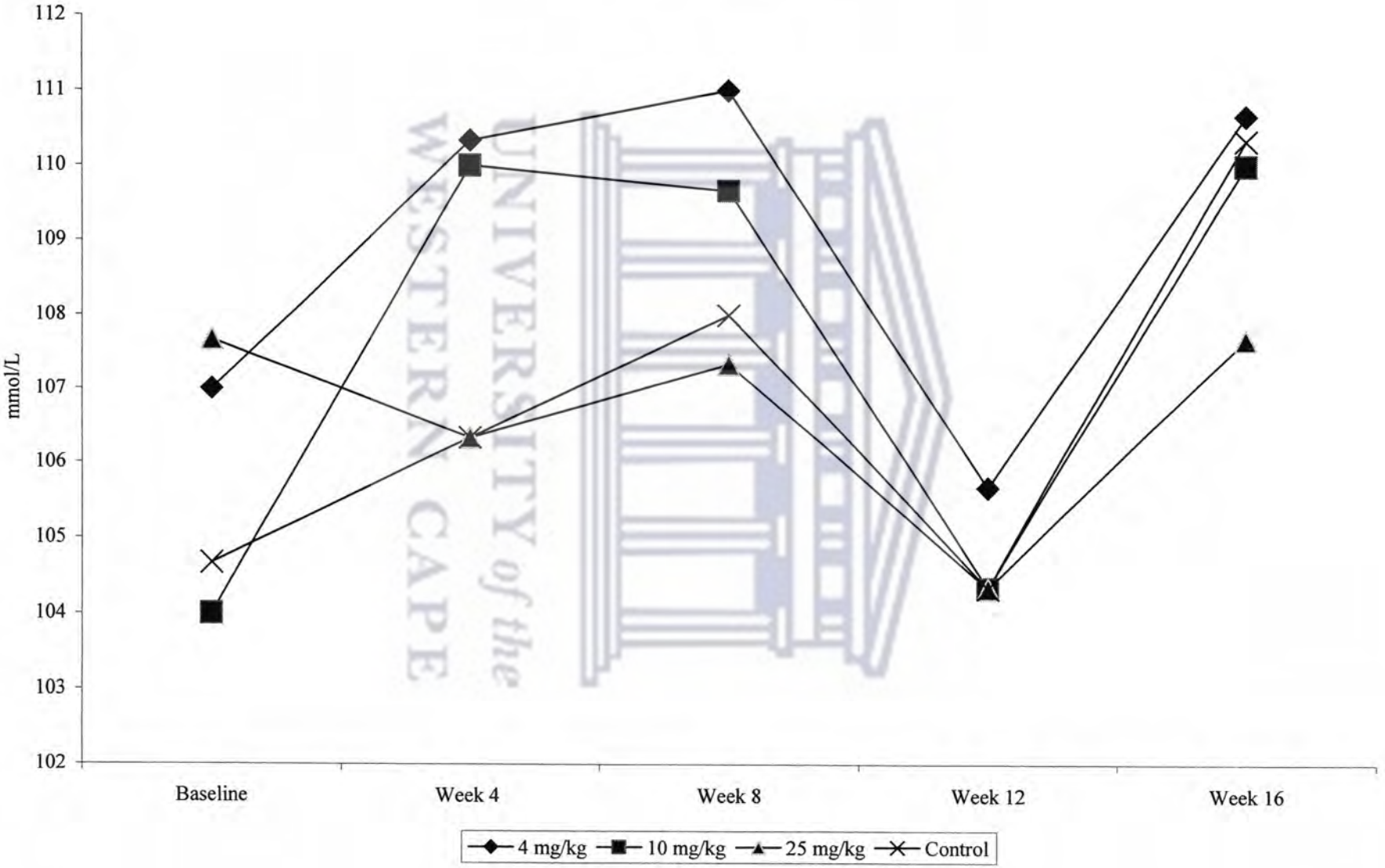


Figure 3.27. Potassium

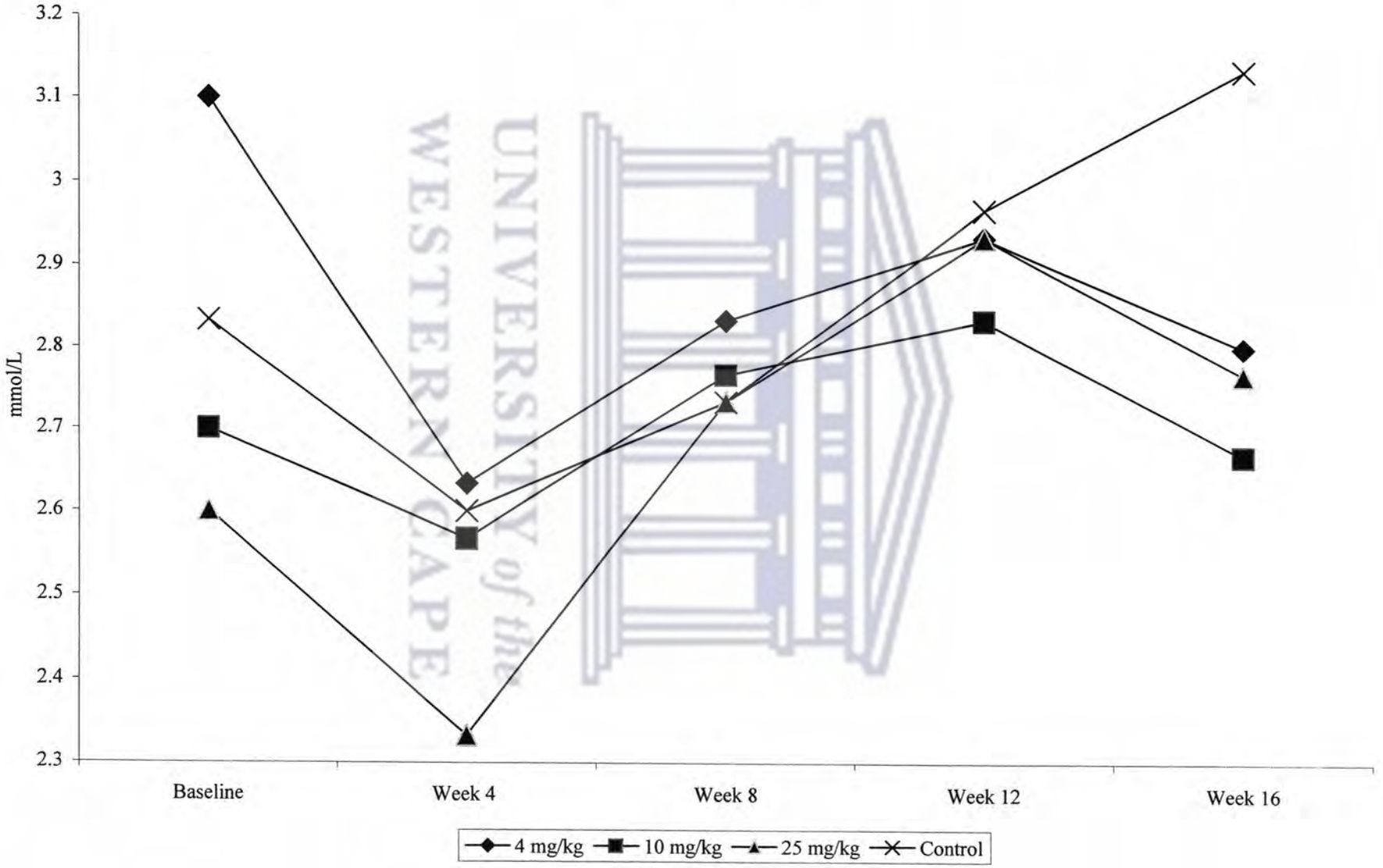


Figure 3.28. Total calcium

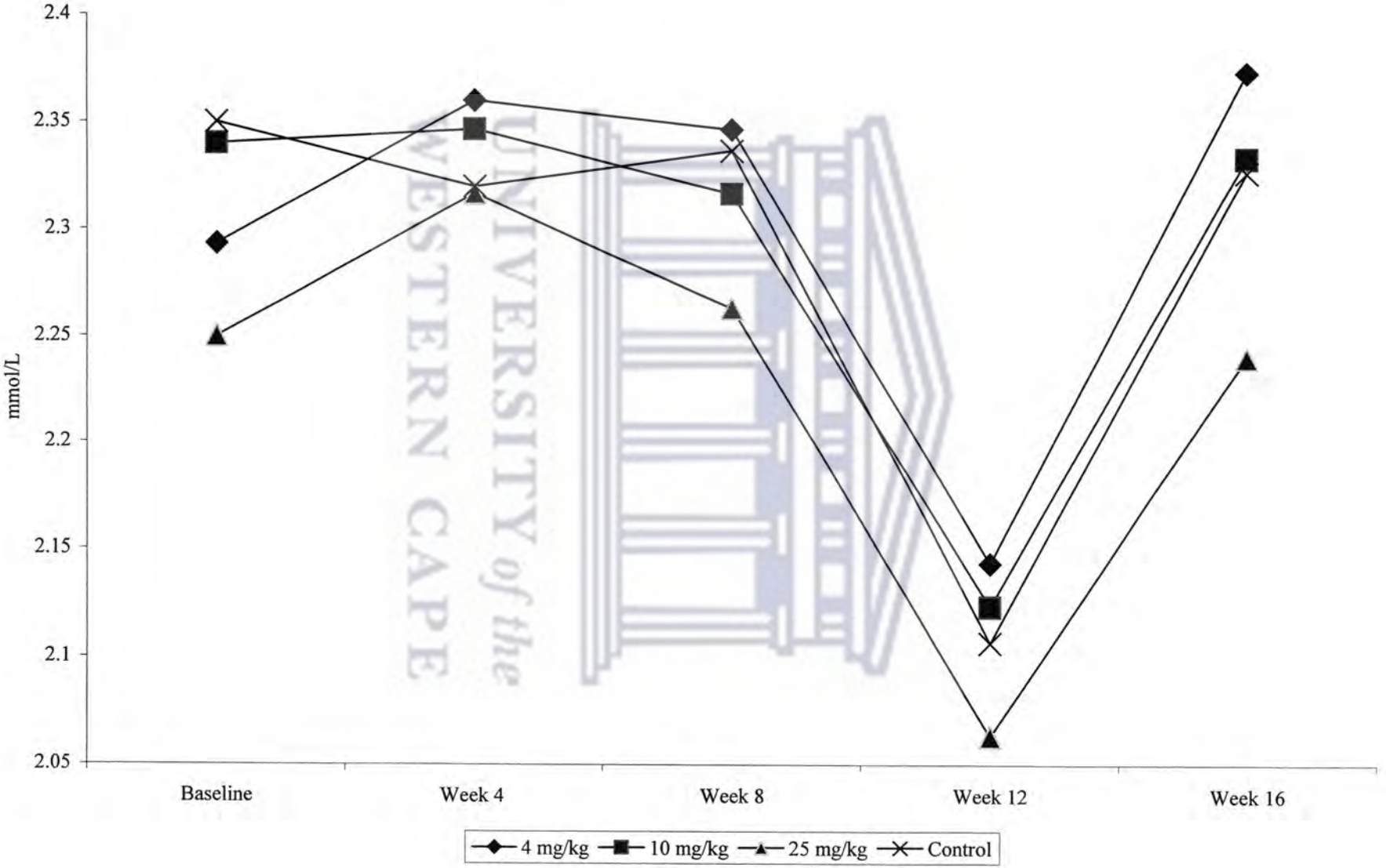


Figure 3.29. Magnesium

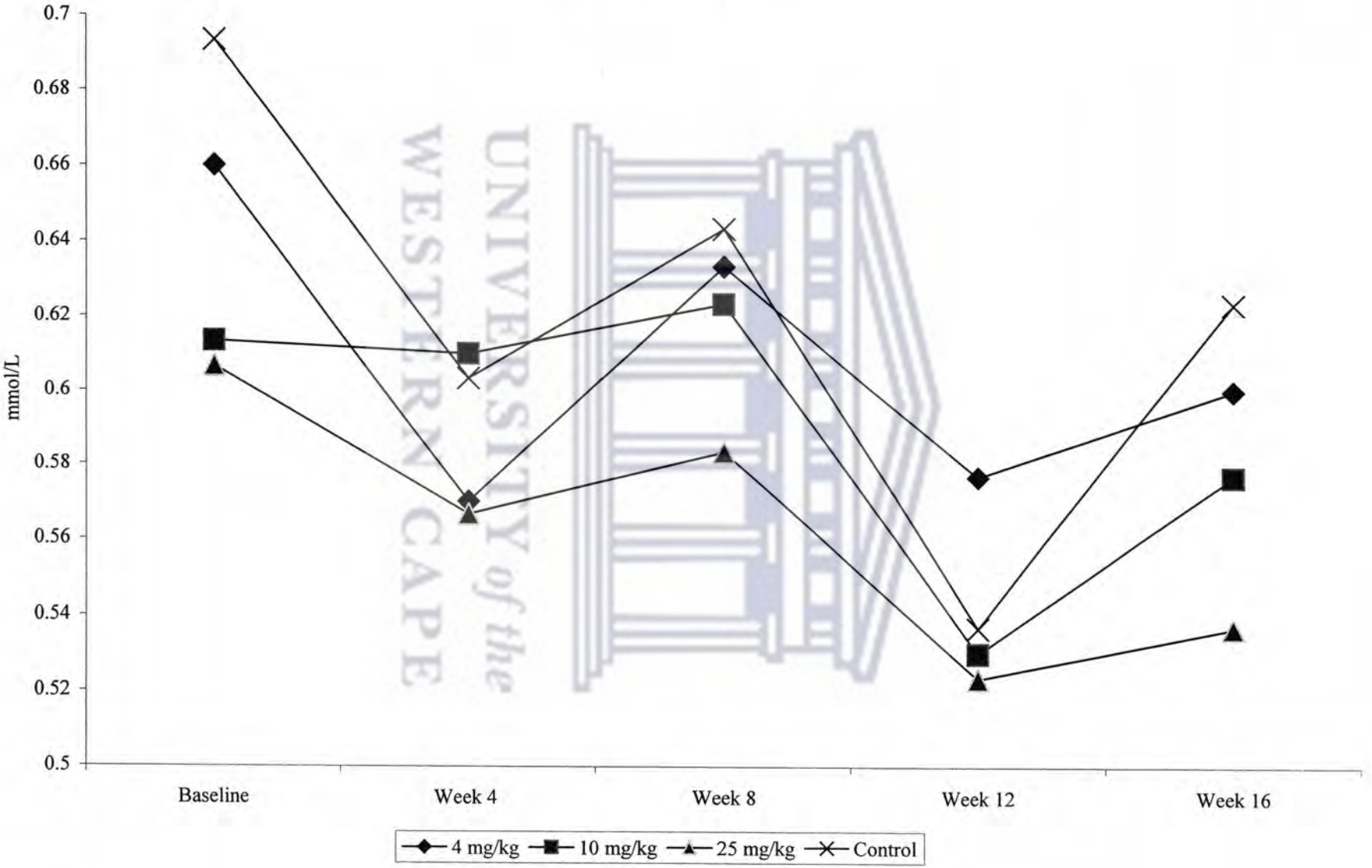
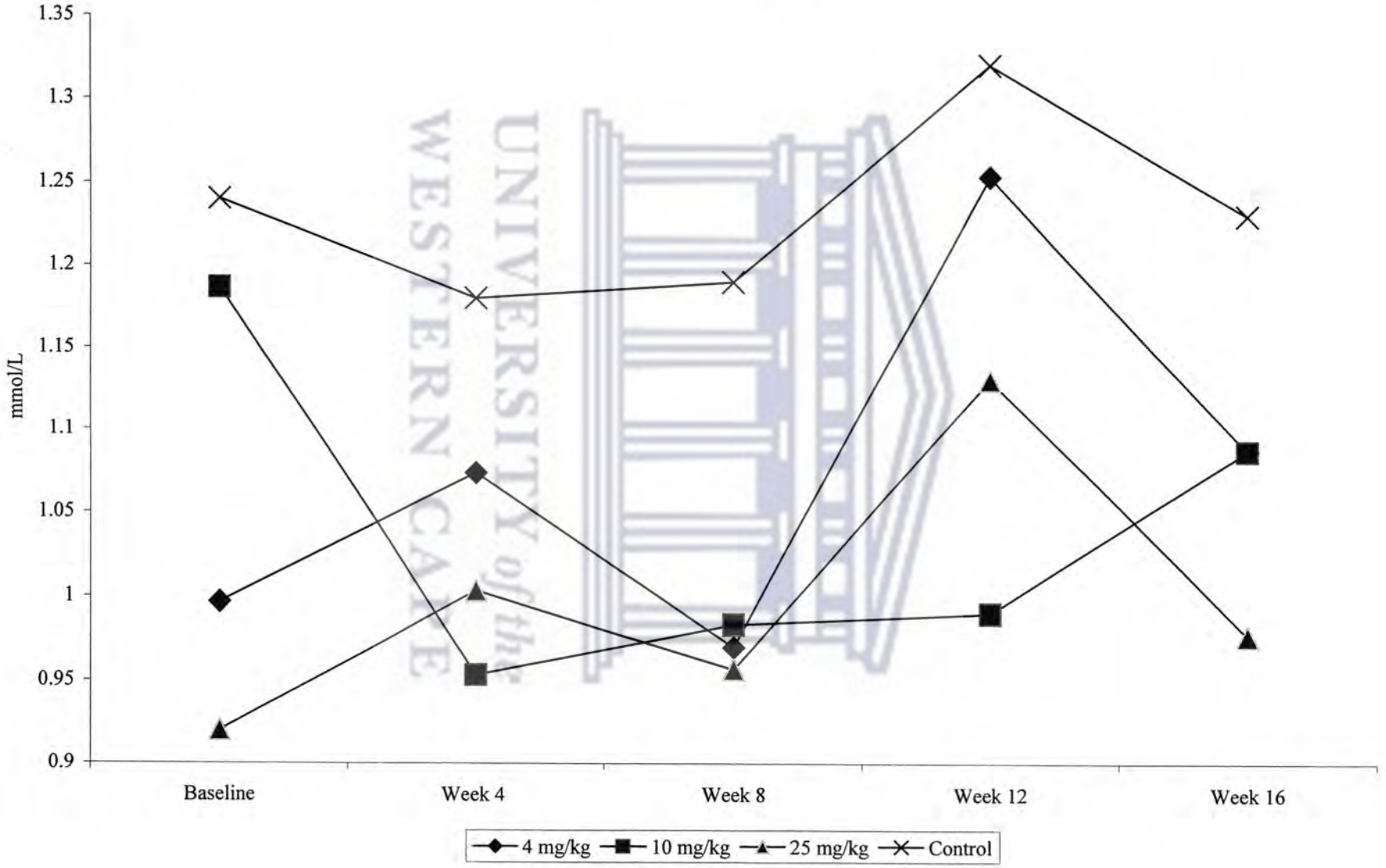


Figure 3.30. Phosphate



3.6. Hematological function

The numbers of circulating RBC (Figure 3.31) and Hb (Figure 3.32) decreased linearly in the controls and the group that received 25 mg/kg oleanolic acid. Consequently, no statistically significant differences were found between these groups. In addition, the RBC and Hct in the groups that received 4 and 10 mg/kg oleanolic acid were not statistically significantly different from the controls. Increased Hct concentrations in the latter groups at the end of the study (Figure 3.33), were not statistically significantly different from increases in the controls when compared to baseline. However, Hct concentrations were statistically significantly increased ($P = 0.0359$) in the group that received 25 mg/kg oleanolic acid when compared to the controls.

Increases that occurred during the study period in MCV concentrations of the treated groups were statistically significantly different when compared to that of the control group (Figure 3.34); $P = 0.0027$ (4 mg/kg), $P = 0.0010$ (10 mg/kg), and $P = 0.0022$ (25 mg/kg). In addition, there were statistically significant decreases in MCHC concentrations in the treated groups (Figure 3.35); $P = 0.0017$ (4 mg/kg), $P = 0.0004$ (10 mg/kg), $P = 0.0002$ (25 mg/kg). No significant statistical differences were found with changes observed in MCH values between controls and the treated groups (Figure 3.36).

The progressive reductions over time observed in WBC numbers of the groups that received 10 and 25 mg/kg oleanolic were not statistically significantly different from the numbers in the controls (Figure 3.37). The group that received 4 mg/kg oleanolic acid showed similar trend as the controls. Fluctuations over time in differential white cell numbers in the treated groups were not statistically significantly different from those in the controls (Figure 3.38 - 3.42).

Platelets

There were no statistically significant differences in the increases in baseline platelet numbers between controls and the treated groups (Figure 3.43).

Figure 3.31. Red blood cell

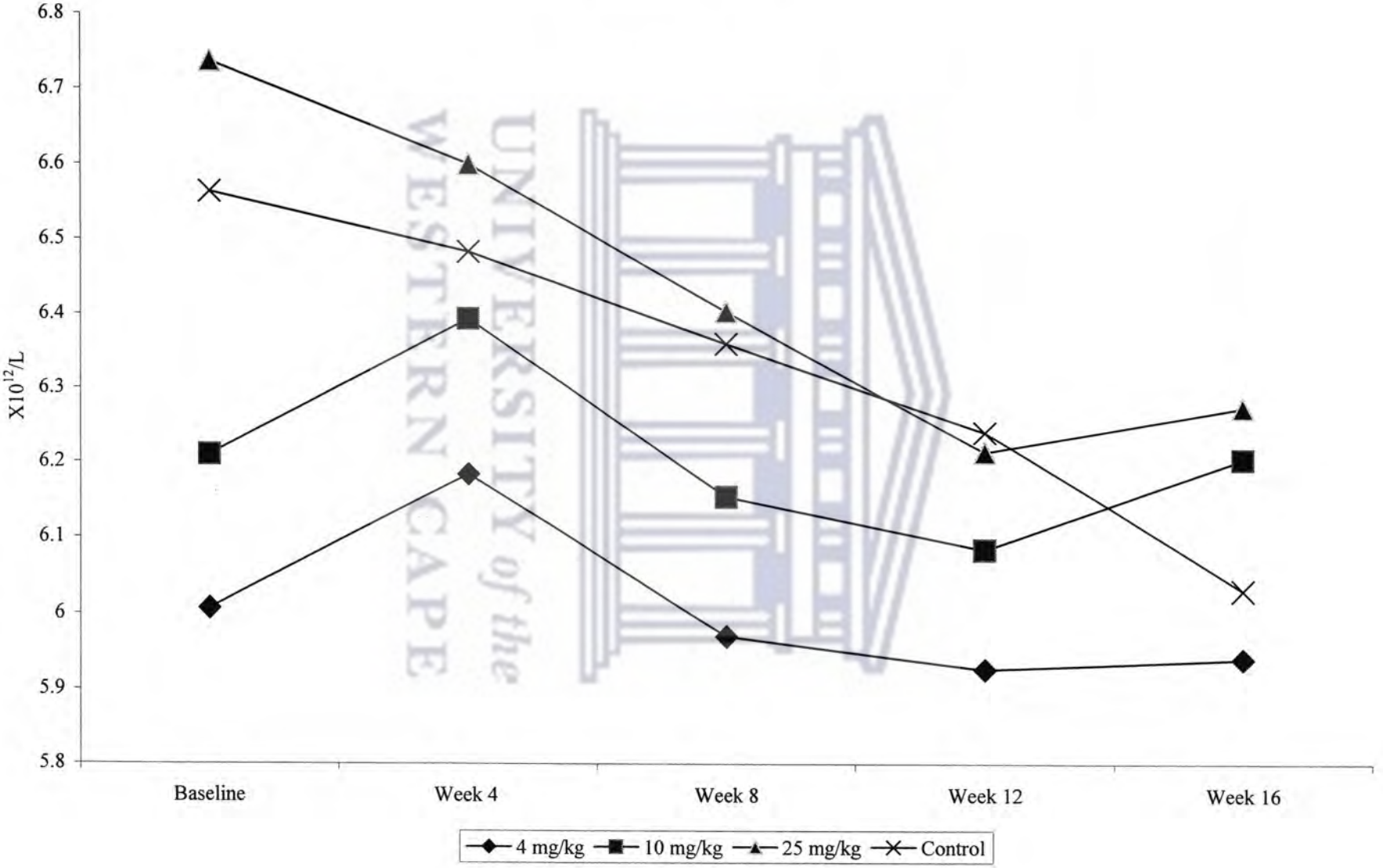


Figure 3.32. Hemoglobin

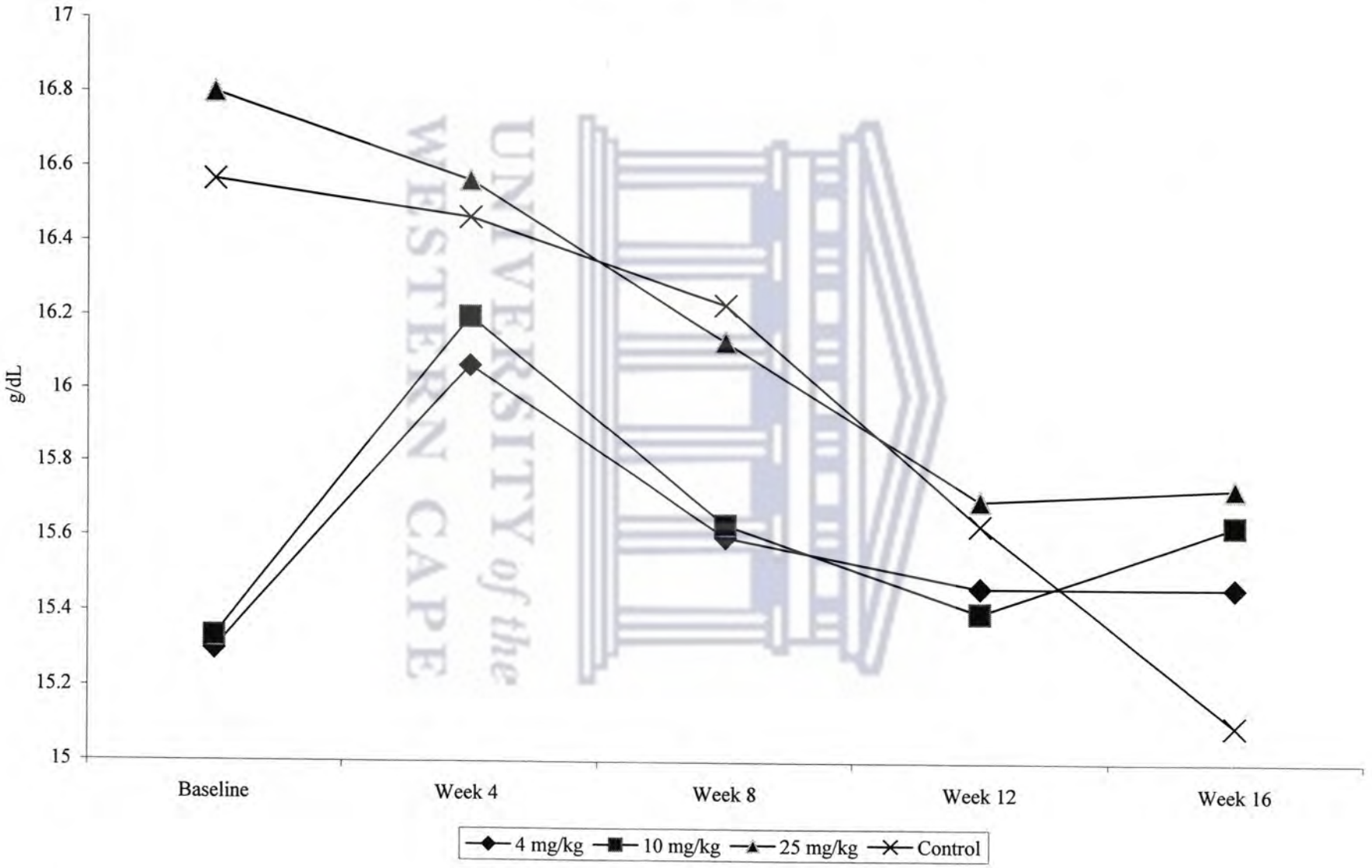


Figure 3.33. Hematocrit

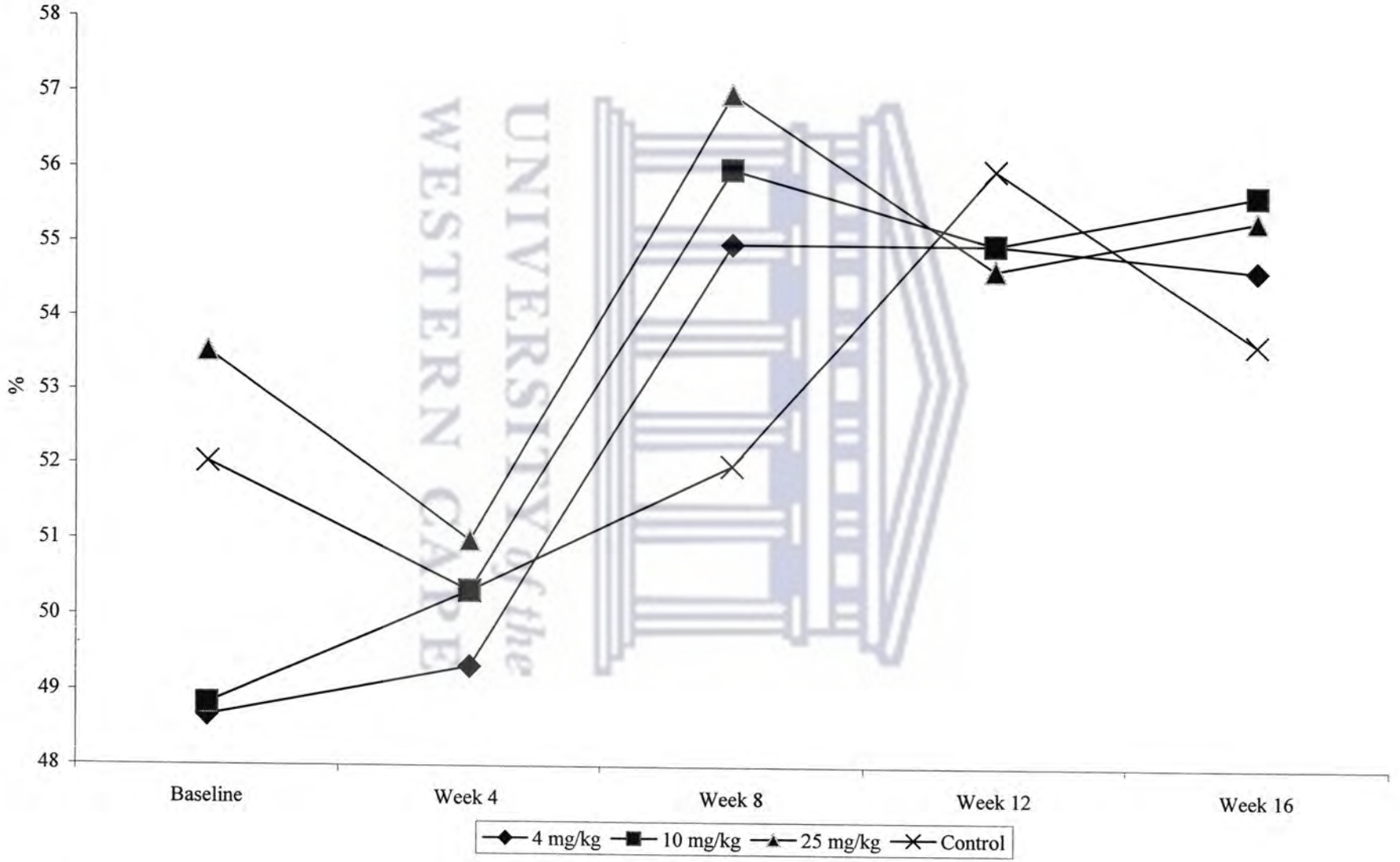


Figure 3.34. Mean cell volume

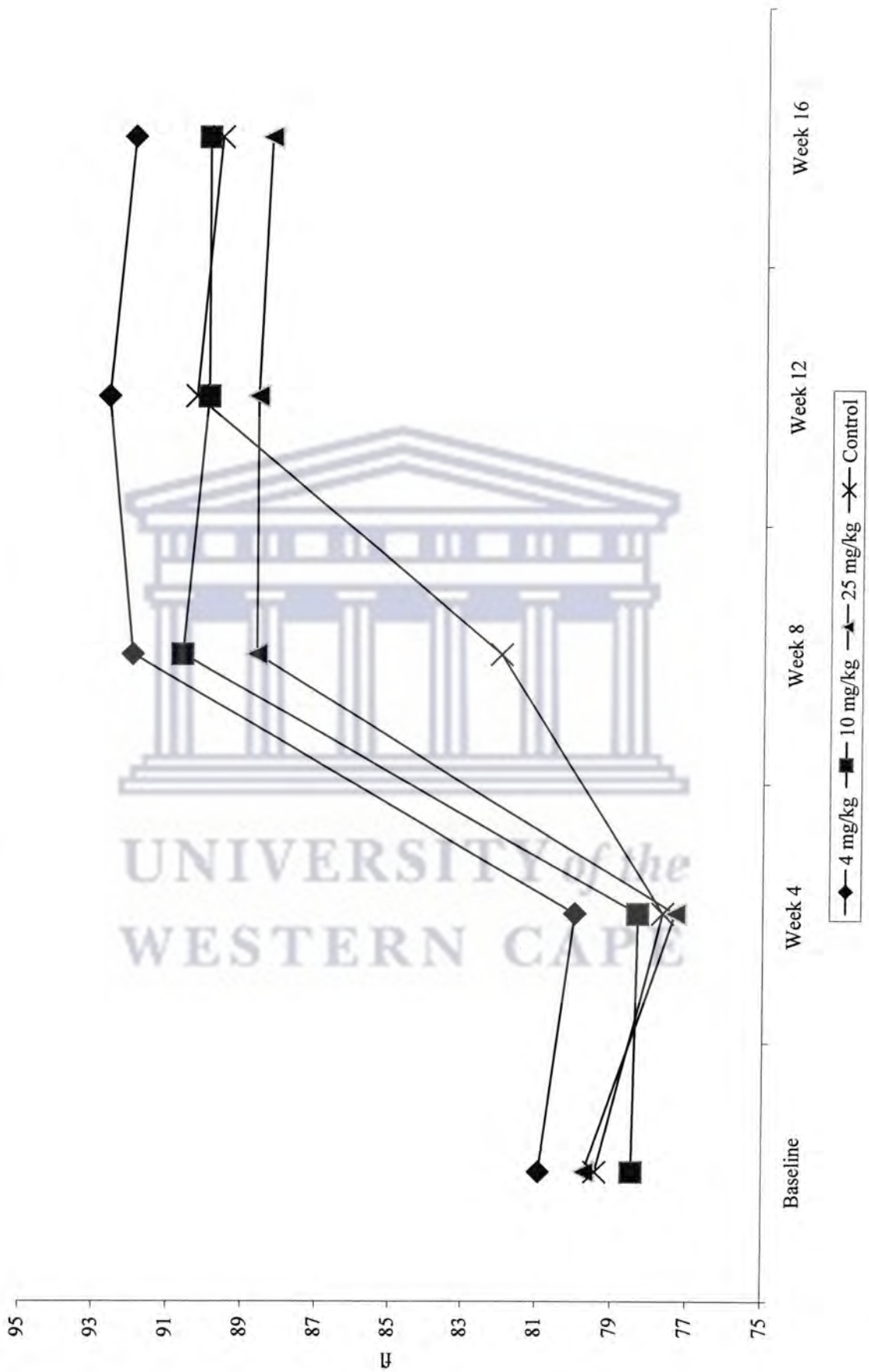


Figure 3.35. Mean cell hemoglobin concentration

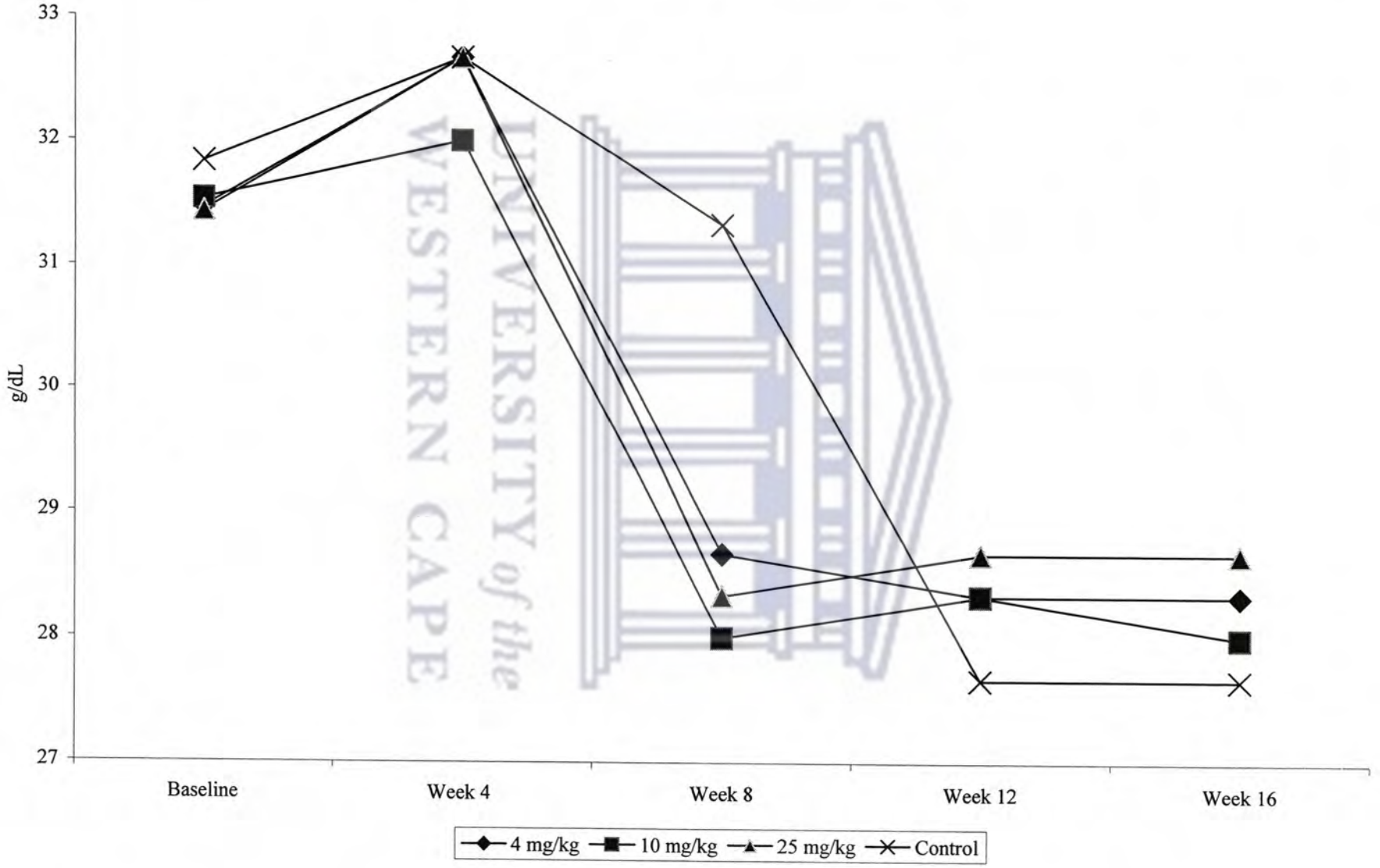


Figure 3.36. Mean cell hemoglobin

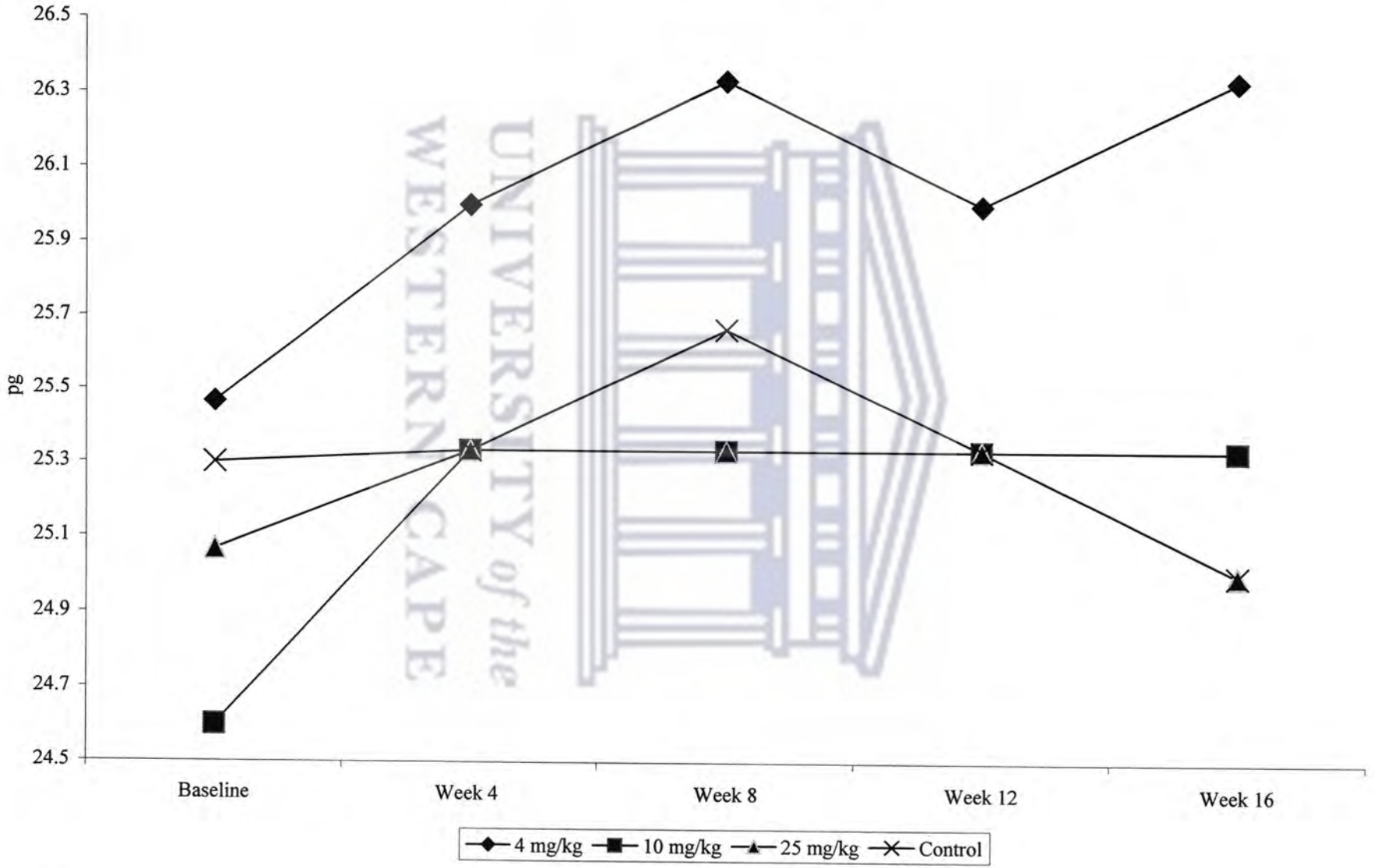


Figure 3.37. White cell count

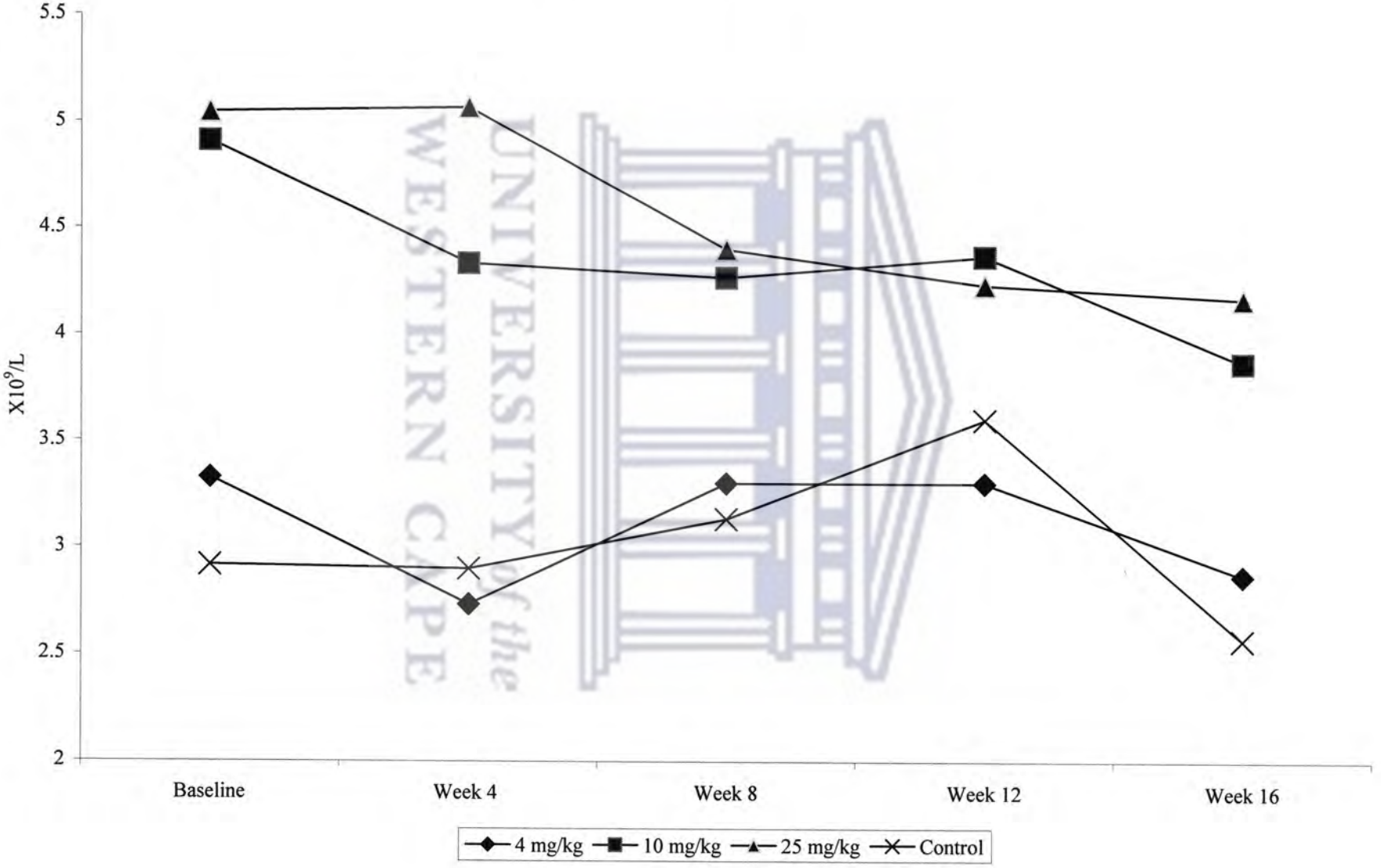


Figure 3.38. Neutrophils

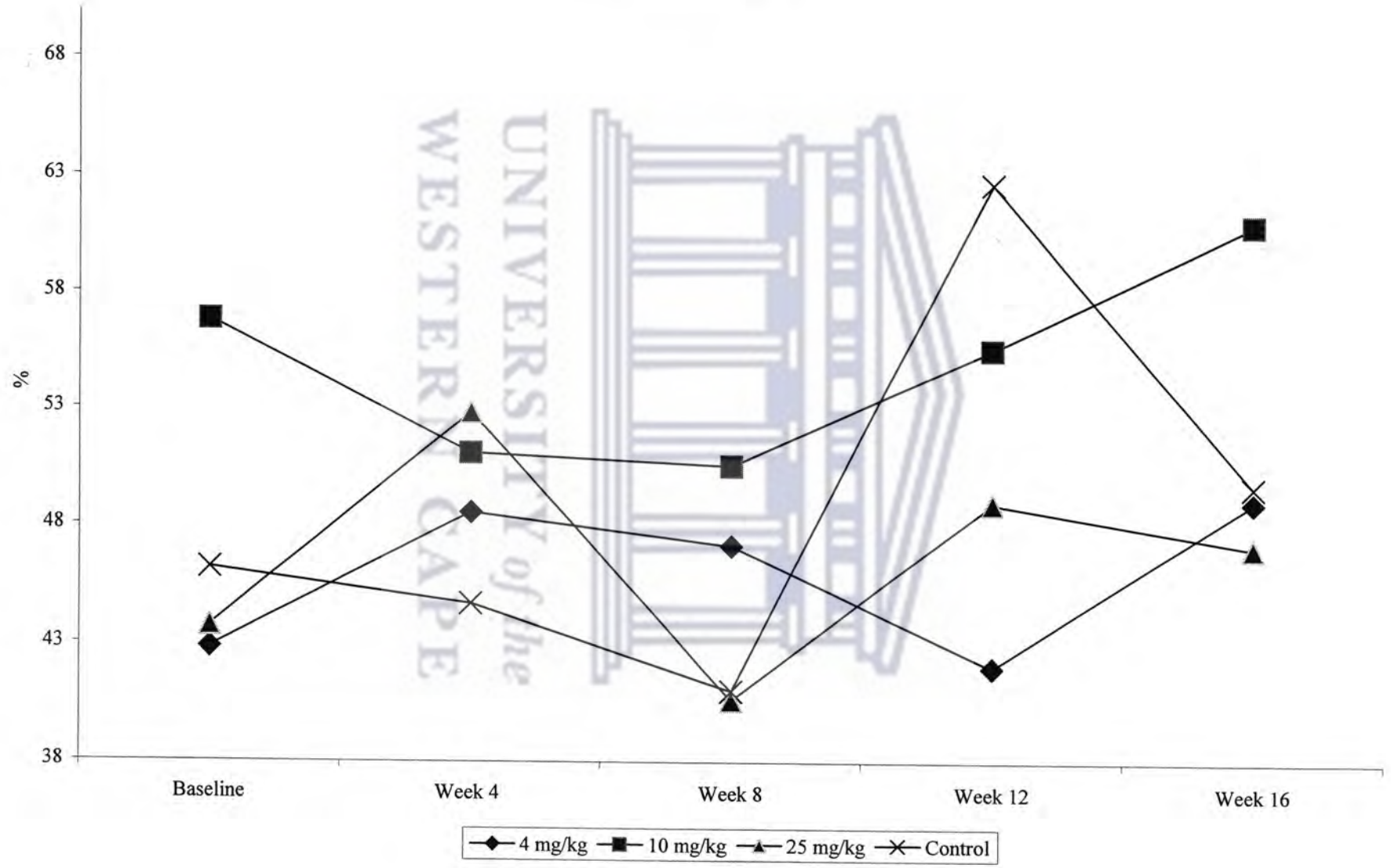


Figure 3.39. Eosinophils

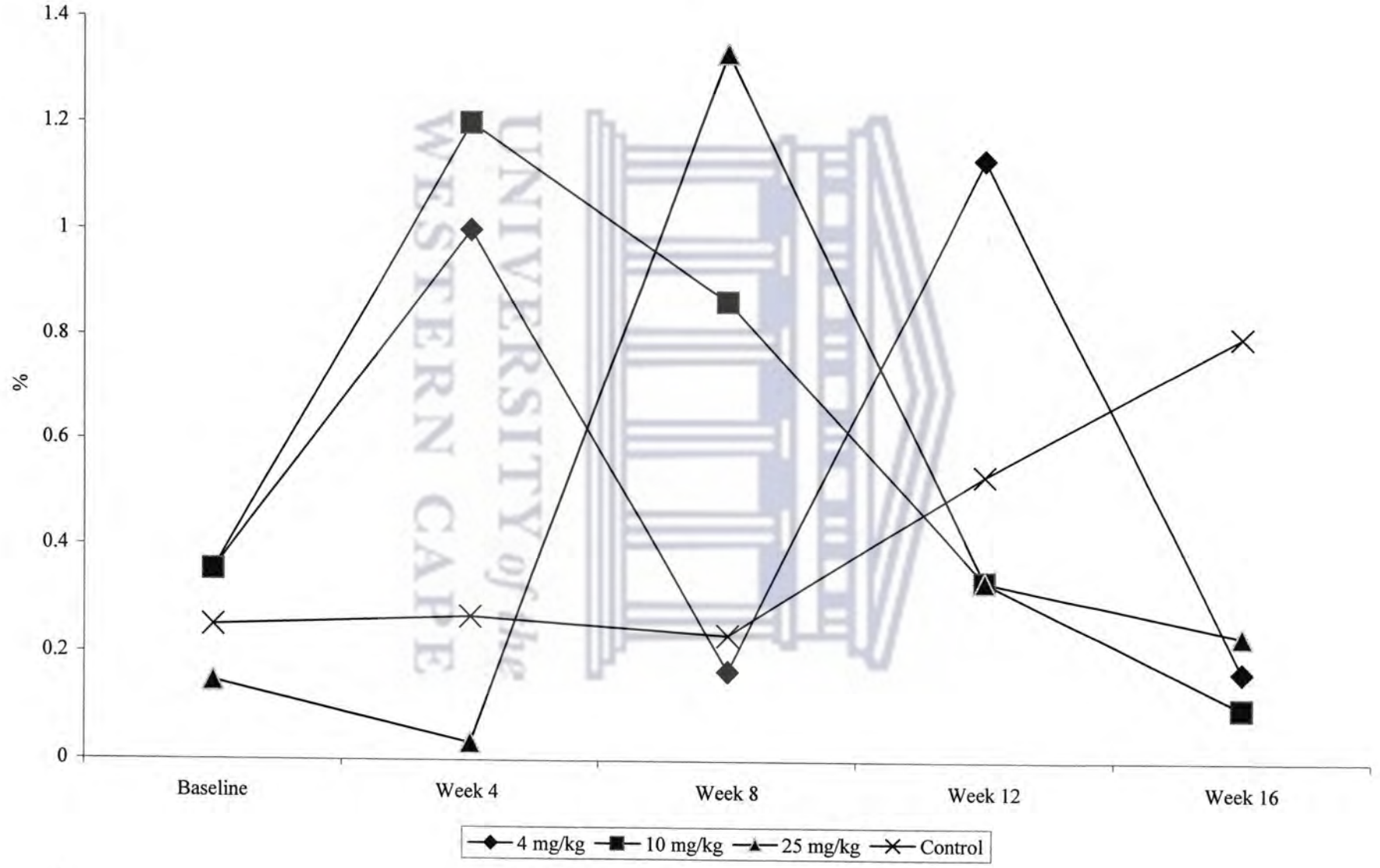


Figure 3.40. Basophils

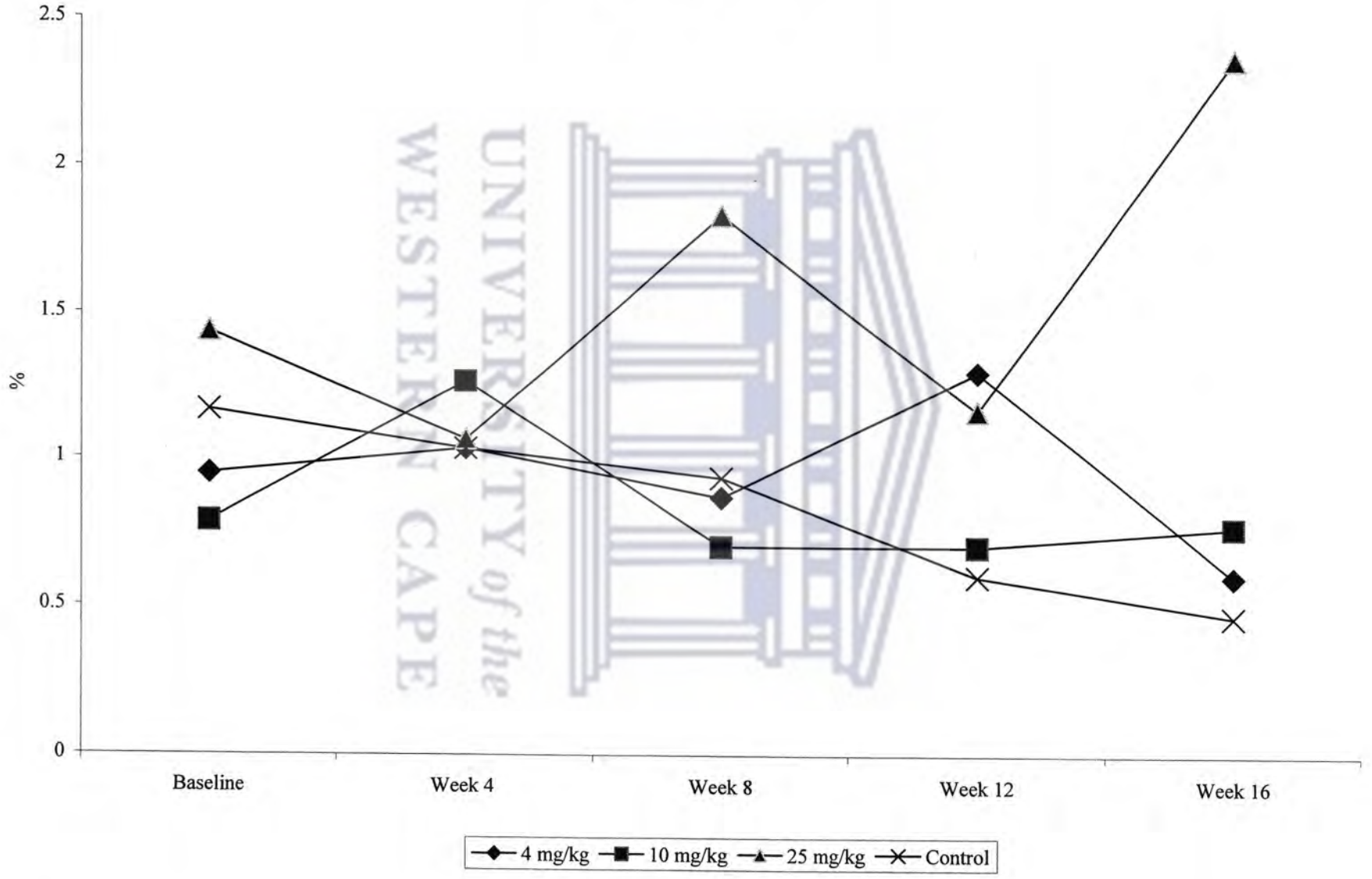


Figure 3.41. Monocytes

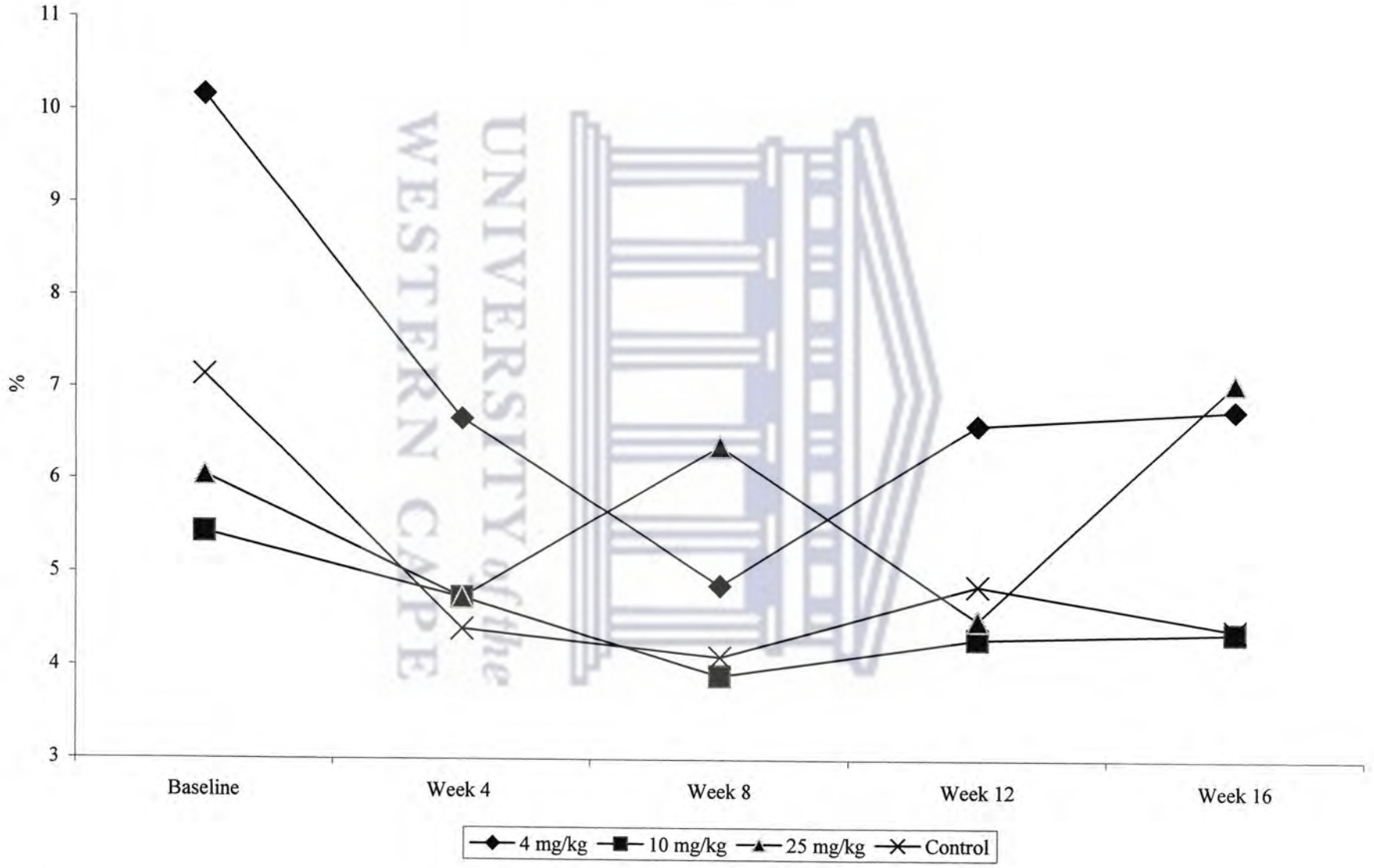


Figure 3.42. Lymphocytes

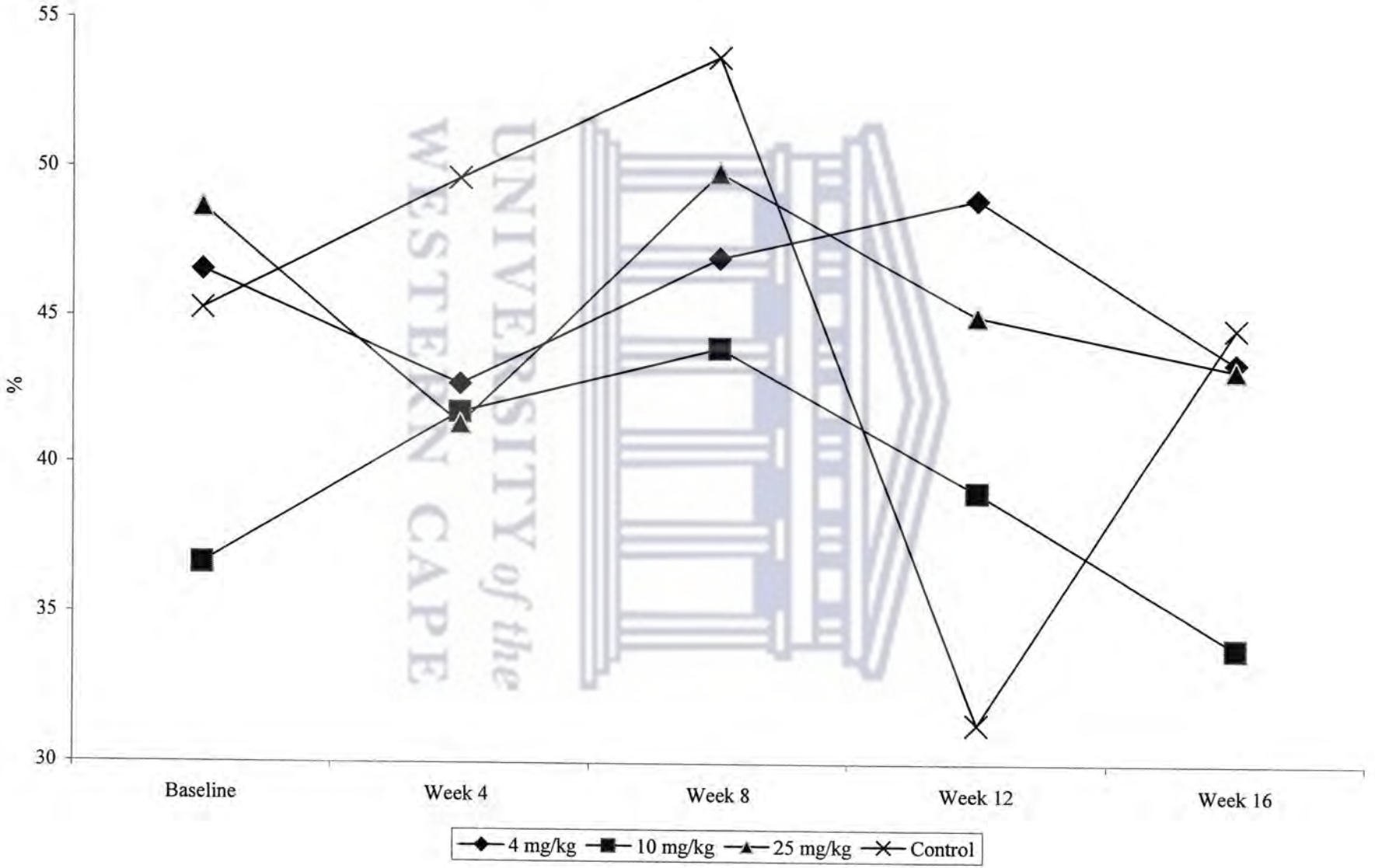
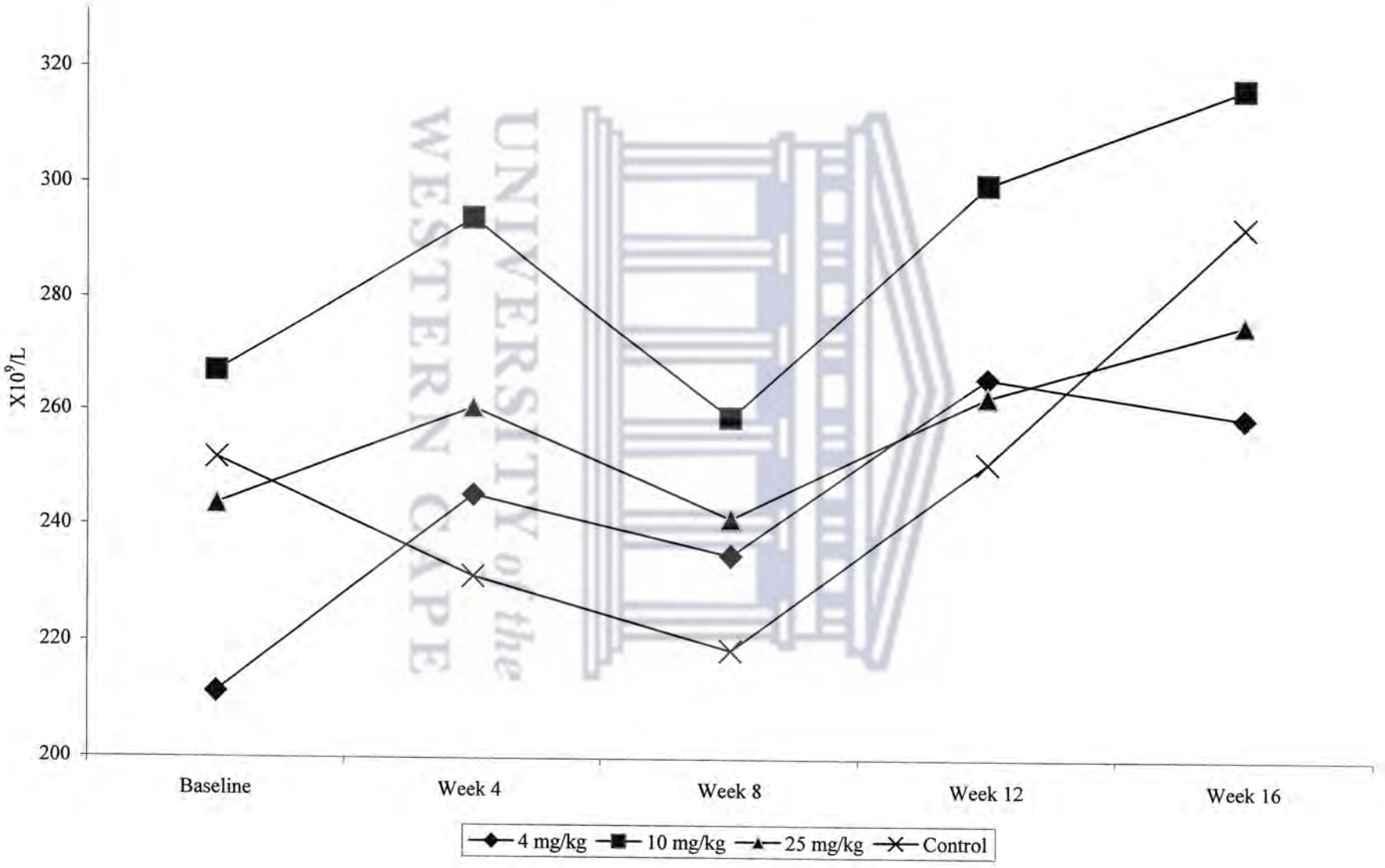


Figure 3.43. Platelets



Chapter 4

Discussion

Much research on the pharmacology of triterpenoids has been based on their therapeutic effects, and there is insufficient data documented on their toxicity to mammals. The most recently studied toxic effects of triterpenes are those from *Lantana camara var. aculeata* (Singh *et al.* 2001, Sharma *et al.* 1992, Sharma *et al.* 1989). A previous study carried out on ethanolic extracts of oleanolic acid from the button cactus plant revealed that this compound is toxic to mice (West and McLaughlin 1977). Although it is valuable, this data cannot be used exclusively to predict the biological effects of this compound in humans, since the model used lacks close similarity to humans compared to non human primates. The latter have drug metabolism similar to humans (Osman *et al.* 1998), hence their wide use in toxicological studies. To the knowledge of the author, this is the first toxicity study on oleanolic acid involving non human primates. Therefore, there are no comparative data for discussion.

4.1. Physical and physiological parameters

The results of this study show that oleanolic acid had no significant effect on bodyweights of the treated monkeys, which agrees with other indicators of muscle injury.

Consistent with the concept that most of the heat produced to maintain mammalian body temperature at normal range emanates from oxidation of consumed food, Lane *et al.* (1996) reported that calorie restriction without malnutrition, lead to reduction in body temperature of rhesus monkeys. Recent studies showed that the consumption of certain plant extracts caused a dose-dependent hypothermic response in mice (Pengsuparp *et al.* 2001). In this study, no significant or dose dependent changes were observed in the treated monkeys, indicating that oleanolic acid has

no effects on the mechanisms involved with the control of body temperature. This conclusion is supported by results of the respiratory rates observed in this study, since changes in the latter and body temperature are concomitant (Tortora and Anagnostakos 1990).

4.2. Cardiovascular function

This study has shown that the consumption of oleanolic acid has no hypertensive effects in vervet monkeys. In humans, renal disease is often the cause of hypertension (Hartley *et al.* 1984). Renal dysfunction played no role in the fluctuations of blood pressure values observed in this study. Guyton *et al.* (1975) reported that hypertension is also caused by hyperaldosteronism, which is characterized by a decrease in the serum potassium concentrations and excessive retention of sodium. However, none of these electrolytes in the treated groups was significantly different from the controls or was outside the normal limits reported for laboratory non human primates (de Neef *et al.* 1987), indicating normal adrenal functioning. In general, reductions observed in the systolic pressure of the monkeys receiving 25 mg/kg oleanolic acid needs further investigations.

As seen from the baseline data in Figure 3.7, the heart rate in vervet monkeys in comparison with humans is rather high. Increases in this parameter could be due to two uncontrollable factors. Firstly, the stress associated with the effects of physical immobilization for injection of the anesthetic. Secondly, the use of ketamine hydrochloride, since immobilization of animals with this compound has been shown to be highly stressful (Crockett *et al.* 2000), and thus stimulates the cardiovascular system (Jacobs *et al.* 1993). It is noteworthy that the heart rate did not only vary greatly in the treated groups during the period of the study, but also in the controls. These results indicate that a proper balance in the regulatory factors important for normal functioning of the heart was not adversely affected by oleanolic acid administration. Imbalances in these factors have an influence on the electrical properties of the cardiac muscle. For example, low potassium and high

calcium concentrations decrease the rate of impulse conduction in the papillary muscle (Hiraoka and Hirano 1986). The absence of extreme increases in heart rates of the treated monkeys, which are known to exert a direct atherogenic action on the arteries through increased wall stress (Pallatini 1999), is further evidence of the lack of oleanolic acid toxicity on the cardiovascular system of these monkeys.

Other determinants of cardiovascular function, total cholesterol and LDL were similarly increased between controls and the treated monkeys. Additionally, both parameters in the treated groups are within normal limits for male vervet monkeys (see Table 4.1), which indicate that these effects are not treatment related. On the contrary, oleanolic acid lead to increase in HDL concentrations of the monkeys in the treated groups, which probably indicate its antihyperlipidemic effects. Since antihyperlipidemic effects of this compound have been reported previously (Ma 1986), it therefore, appears that oleanolic acid will aid in clearing excess cholesterol from the tissues and could have therapeutic value. With this approach, it is thought that the risk of cardiovascular disease will be reduced. The results of this study suggest that the 10 and 25 mg/kg oleanolic acid doses are important in stabilizing physiological variation in triglycerides, considering the narrow range of fluctuations in these groups compared to the increase in the group that received 4 mg/kg oleanolic acid and the controls.

Serum activity of CK is a major biochemical marker used in the diagnosis of myocardial damage (Hierholzer and Finke 1997, Mullin and Eastern 1986). In this study, probable myocardial damage in the group that received 25 mg/kg oleanolic acid is indicated by simultaneous increases in CK and AST (another indicator of myocardial damage) during the first four weeks after oleanolic acid administration. While AST concentrations returned to normal limits by the end of the study, CK concentrations continued to rise beyond the normal limits listed in Table 4.1. However, AST

concentrations declined post week four, which suggests that the nonspecific increase in the CK activity could also be due to damage to other structures, particularly the skeletal muscle, where CK is highly conserved (Dahlstedt *et al.* 2000). A nonspecific increase in CK activity is mentioned because the activity of an isoenzyme (CK-MB) specific for myocardial injury (Navin and Hager 1979) was not determined in this study. In conclusion, findings of this study may indicate that serum CK levels, though helpful, should be interpreted carefully, since raised levels may be nonspecific, particularly in studies like this one that involves intramuscular injections.

Table 4.1. Clinical chemistry reference values for male vervet monkeys anaesthetized with ketamine hydrochloride (10 mg/kg bodyweight)

Parameters	Mean ± sd	Reference range
HDL (mmol/l)	2.16 ± 0.47	1.37 - 2.93*
LDL (mmol/l)	2.28 ± 0.47	1.71 - 3.37*
Total cholesterol (mmol/l)	4.44 ± 0.68	3.39 - 6.18*
Triglycerides (mmol/l)	0.35 ± 0.12	0.18 - 0.70 (Unpublished)
CK (u/l)	336.06 ± 130.23	141.00 - 675.00*
AST (u/l)	73.77 ± 21.27	24.70 - 108.10*
ALT (u/l)	48.89 ± 39.81	15.10 - 167.00*
ALP (u/l)	117.19 ± 53.63	60.00 - 259.00*
GGT (u/l)	65.13 ± 12.90	49.00 - 93.00*
Total bilirubin (µmol/l)	6.62 ± 2.77	3.30 - 12.80*
Conjugated bilirubin (µmol/l)	2.62 ± 0.96	1.40 - 5.20*
Glucose (mmol/l)	4.03 ± 0.43	3.30 - 4.70*
Albumin (g/l)	40.22 ± 3.08	29.00 - 50.00**
Globulin (g/l)	17.58 ± 4.53	9.00 - 25.20*
Total protein (g/l)	62.61 ± 4.27	53.00 - 75.00**
Creatinine (µmol/l)	81.09 ± 16.07	44.00 - 133.00**
Urea (mmol/l)	5.23 ± 1.55	1.92 - 11.94**
Sodium (mmol/l)	149.87 ± 2.79	141.00 - 166.00**
Chloride (mmol/l)	107.25 ± 2.85	99.00 - 117.00**
Potassium (mmol/l)	3.25 ± 0.34	2.60 - 4.20**
Total calcium (mmol/l)	2.17 ± 0.11	1.89 - 2.48**
Magnesium (mmol/l)	0.65 ± 0.06	0.54 - 0.77*
Phosphate (mmol/l)	1.27 ± 0.29	0.42 - 1.97**

*Seier *et al.* (2002)

**Seier *et al.* (1990)

4.3. Hepatic function

Many compounds are metabolized in the liver, but if too many demands are made on this organ's capacity, the continued function of its cells is no longer ensured. Different forms of stress promote leakage of cytoplasmic enzymes into the blood in man and laboratory animals by causing hepatic cell injury or increasing cell membrane permeability (Arakawa *et al.* 1997). Transaminases (AST and ALT) are some of the enzymes susceptible to oxidative stress; hence, they are useful as biomarkers for predicting possible toxicology (Rahman *et al.* 2001). Significant changes observed in the AST and ALT concentrations in the monkeys that received 25 mg/kg oleanolic acid are very interesting.

Firstly, since AST is of both mitochondrial and cytoplasmic origin, its raised serum activity seen immediately after treatment may indicate the first sign of cell damage by oleanolic acid, thus leading to outflow of the enzyme into the serum. However, the mitochondria contain at least half of the AST, which does not leave the cells immediately after damage (Brucker and Cohen 1976). Therefore, the augmented levels of AST during first few weeks might coincide with damage to the cell membrane and leakage of only cytoplasmic AST into the serum. Based on this assumption and the subsequent decrease in the enzyme's activity, it is possible that the mitochondria will be spared from the oleanolic acid effects. If this is correct, it appears that cells will not suffer from oxidative imbalances caused by oleanolic acid-induced defects in mechanisms such as pyruvate metabolism, mitochondrial fatty acids oxidation, citric acid cycle or electron transport by the mitochondrial respiratory chain.

Secondly, the presence of increased serum levels of ALT (which is solely cytoplasmic) during the first four weeks of the study, further supports the idea that cell membranes may have been damaged, with the result of leakage of this enzyme into the serum. Since the degenerating liver cells discharge predominantly ALT into the serum, sustained high levels of the latter enzyme between weeks four and 12 in the group that received 25 mg/kg oleanolic acid, could be interpreted as an

indication of oleanolic acid-induced hypersensitivity to hepatocellular alteration. Nonetheless, serum concentrations of AST were always within the normal limits (see Table 4.1).

In contrast, serum ALP and GGT activities in the treated monkeys appeared not to be negatively affected by oleanolic acid. These enzymes provide sensitive assays for variety of hepatobiliary disorders (Reichling and Kaplan 1988), and their increase is due to stimulation by cholestasis (retention of bile in intrahepatic ducts). The stable ALP levels together with the reduced GGT concentrations in the monkeys that received 25 mg/kg oleanolic acid may indicate that this dose reduces risks of hepatobiliary disorders associated with the increases in serum GGT concentrations. Liu *et al.* (1994) reported similar oleanolic acid's hepatoprotective effects against chemically induced liver injury. Since increased serum activity of GGT is a valuable biochemical marker for the assessment of inflammation *in vivo* (Singh *et al.* 1986), the decreased levels of this enzyme observed in this study further corroborate the anti-inflammatory properties of oleanolic acid observed by Kapil and Sharma (1995).

Elevated serum aminotransferases and ALP suggest injury of hepatocytes and any part of the biliary tree respectively (Mahl 1998). However, serum bilirubin levels as well as albumin levels are direct measures of liver function. Since albumin has the capacity to bind many substances (natural and synthetic), several drugs are known to compete with bilirubin for binding sites and so displace bilirubin. For example, flucloxacillin, a semi-synthetic penicillin, is 96% bound to albumin in the circulation (Bergan *et al.* 1986), whereas sulphafurazole (Friedman and Lewis 1980) and aspirin (Fung *et al.* 2000) displace bilirubin from albumin. In this study, the total bilirubin of all treated groups were within the normal range reported for laboratory primates (de Neef *et al.* 1987, Table 4.1) and were also not significantly different from the controls, which may suggest that oleanolic acid had minimal competition, if any, with bilirubin for binding sites on the albumin. The results obtained in this study further suggest that oleanolic acid, unlike other naturally occurring plant

compounds (Yin *et al.* 1991), has no effect on enzymes involved in bilirubin metabolism. This implies that oleanolic acid did not alter a balance between bilirubin production, on the one hand, and its elimination, on the other. This is interesting, since decreased bilirubin elimination, with the result of its increased serum concentrations, is suspected to be an essential factor in the pathogenesis of jaundice (Beutler 1994).

Glucose synthesis is controlled by the liver, which can generate free glucose from hepatic glycogen stores and *de novo* through gluconeogenesis (Tirone *et al.* 2001). Glucose metabolism by the liver is regulated by several hormones, including insulin, glucagon, growth hormone, cortisol, and catecholamines. These hormones, functioning as a single unit or in combination, may have mediated similarly reduced glucose concentrations in the treated groups and the controls monkeys during the study period. This suggests that oleanolic acid will not produce hypoglycemia, which is characterized by a decrease in plasma glucose levels to below 3.0 mmol/l in humans (Virally and Guillausseau 1999). Therefore, it is possible that oleanolic acid did not affect normal adrenal cortex function in the treated monkeys, since glucocorticoids required to maintain gluconeogenesis are not produced during adrenal cortex insufficiency (Bhattacharyya *et al.* 2001).

Albumin is the most common protein in the serum, and its two functions are to exert 80 % of the colloid osmotic pressure of blood with the result of maintaining blood volume, and transportation of numerous substances. The reduced amount of albumin relative to total protein can indicate either renal or liver disease. In renal disease, particularly nephrotic syndrome, kidneys are unable to retain smaller proteins such as albumin, because filtration is based mainly on macromolecular size (Castellino and Cataliotti 2002). In this study, serum albumin concentrations in the treated monkeys were constantly higher during treatment period as compared to baseline, which probably indicate normal functioning of both the hepatic and renal systems. According to these results, it appears that

oleanolic acid either stimulated increase in albumin synthesis by the parenchymal liver cells or decreased its catabolism. The assumed normal functioning of the liver is further supported by a corresponding decrease in the globulin levels of these monkeys. This is because in liver cirrhosis there is a considerable decrease in serum levels of albumin and an increase in globulin levels (Belpaire and Bogaert 1991).

On the other hand, Renoe *et al.* (1980) reported that increases in concentrations of serum albumin might be associated with venous stasis, which is caused by a prolonged use of tourniquet during blood collection by venepuncture. In this study, a tourniquet was not used to aid in obtaining blood, thus excluding any possible artefactual increase in albumin due to this technique.

4.4. Renal function

Kidneys are the main organs involved in drug elimination, and therefore, are particularly exposed to their toxic effects. In clinical investigations, the most frequently requested determinations of renal function are serum urea and creatinine. The serum creatinine level is less frequently influenced by extra-renal factors than is serum urea level, and is probably the most accurate test for both detecting and monitoring renal disease, but is not without its shortcomings (Lyman 1986, Ward 1981).

In this study, the parallel increases seen in urea concentrations of both control and the treated groups, may suggest that oleanolic acid did not accelerate hepatic urea synthesis in the treated monkeys. If this assumption is correct, it indirectly confirms that oleanolic acid has no stimulatory effects on protein catabolism. In humans, protein loss is seen in both myxedema and thyrotoxicosis (Grofte *et al.* 1997), which however, appeared not to be the case in this study. Creatinine results further confirm that there was no chronic failure of renal excretory function in the monkeys that received oleanolic acid. In general, serum urea and creatinine data of treated monkeys obtained in

this study, is in agreement with the reference data reported for macaques (de Neef *et al.* 1987), baboons (Harewood *et al.* 1999) and vervet monkeys (see Table 4.1).

Many pharmacological agents are implicated in a number of electrolyte disorders. Drugs may interfere with the normal intake, elimination, regulation and total body distribution of electrolytes (Brass and Thompson 1982). From the results presented in this study, it is clear that normal serum levels of electrolytes are not significantly altered from normal limits reported for vervet monkeys (see Table 4.1). Consequently, it may be tempting to speculate that oleanolic acid is devoid of drug-induced alterations in renal transport systems of the electrolytes investigated in this study. The particularly notable results are those of the serum sodium, potassium and calcium, since these cationic electrolytes are subject to active transport processes of the kidneys. The comparable data between controls and treated monkeys with regard to serum magnesium, most of which is reabsorbed in the loop of Henle after glomerular filtration (Heidenreich 1984), further represents normal kidney function in movement of this electrolyte between different compartments. This demonstrates that oleanolic acid has no loop blocking diuretic effects, which are known to cause major urinary wasting of magnesium (Meyer and Madias 1994, Quamme 1986, Ryan 1986). As for the anions, the major fraction of the filtered chloride is said to be reabsorbed passively or electrogenically in the proximal tubules in response to regulation of sodium reabsorption by aldosterone (Aronson and Giebisch 1997). Therefore, synchronized changes observed between sodium and chloride data in the treated and the control monkeys, supports this "cause and effect" mechanism. Results on this anion underscore the aforementioned normal functioning of the adrenals. Similarly, comparable changes in phosphate and glucose concentrations between control and treated monkeys may suggest that oleanolic acid has no effect on glomerular filtration of glucose, since the latter is reported to affect phosphate reabsorption (Westenfelder *et al.* 1998).

4.5. Hematological function

Experimental studies in rats and mice have shown that oleanolic acid has potent anti-inflammatory effects against dextran and carrageenan-induced oedema (Singh *et al.* 1992). Despite knowledge that the hematopoetic system is a sensitive target for toxicity, little or no information is available on the hematological effects of oleanolic acid in laboratory animals. In this study, these effects were determined by employing tests that detect anemia, damage on RBCs, and alterations in leukocytes and platelets.

The red blood cell abnormality most often associated with the toxic effects of drugs is premature destruction involving mechanisms such as denaturation of unstable hemoglobin, oxidation of sulfhydril groups in hemoglobin and the erythrocyte membrane in the presence of glucose-6-phosphate dehydrogenase deficiency, and direct effects on enzymes (Morse 1988). The results of this study suggest that oleanolic acid had no effect on production of the RBC. Since hemolysis of RBC's usually leads to an increase in unconjugated bilirubin, which is derived from hemoglobin of destroyed RBC, the data obtained in this study indicates that oleanolic acid is devoid of the drug-alteration toxic effects on the RBC. Usually, this kind of toxicity may lead to phagocytosis of the RBC by the cells of the reticulo-endothelial system, and thus shortening their normal life-span.

Although there were statistically significant increases in MCV and decreases in MCHC values of the treated monkeys over time, there are several indicators demonstrating that these changes do not necessarily indicate oleanolic acid-induced macrocytosis or microcytosis. Firstly, similar graphical patterns, except for week eight, seen between treated groups and the controls, suggest that the latter had a longer latent period to achieve a similar target as the treated groups. Secondly, the highest oleanolic acid dose had always the lowest effect when compared to the other treated groups and the controls. This suggests that oleanolic acid had neither a dose dependent nor a general treatment

effect in these monkeys. Thirdly, since an increase in MCH is indicative of macrocytosis (Kerr 1989), the relatively unchanged levels of this parameter in the treated groups during the study period, may suggest that oleanolic acid did affect haemoglobinization of these "supposedly macrocytic cells". Lastly, the Hct and Hb data of the treated monkeys were in contrast to the data reported for individuals with microcytic cells (Okuno and Chou 1975). In general, both MCV and MCHC values were within normal limits at the end of the study (see Table 4.1).

Table 4.2. Hematological reference values for male vervet monkeys anaesthetized with ketamine hydrochloride (10 mg/kg bodyweight)

Parameters	Mean ± sd	Reference range
RBC ($X10^{12}$/L)	6.60 ± 0.49	5.57 - 7.56*
Hb (g/dL)	16.09 ± 1.01	14.50 - 18.10*
Hct (%)	58.31 ± 3.82	50.00 - 64.00*
MCV (fl)	88.63 ± 3.93	81.00 - 96.00*
MCHC (g/dL)	27.69 ± 0.60	27.00 - 29.00*
MCH (pg)	24.50 ± 1.37	2.00 - 27.00*
WBC ($X10^9$/L)	5.49 ± 1.97	3.20 - 10.40*
Neutrophils (%)	49.60 ± 9.30	31.50 - 72.50#
Eosinophils (%)	1.10 ± 1.60	0.00 - 6.50#
Basophils (%)	0.20 ± 0.50	0.00 - 2.50#
Monocytes (%)	0.60 ± 0.80	0.00 - 3.00#
Lymphocytes (%)	48.50 ± 8.90	27.50 - 65.50#
Platelets ($X10^9$/L)	279.25 ± 46.16	180.00 - 350.00*

Wall *et al.* (1985)
*Seier *et al.* (2002)

Hct and MCV data of both control and the treated monkeys suggest that it is unlikely that oleanolic acid induces polycythemia. This is because the latter occurs where Hct increases without associated rise in the MCV (Andreasson *et al.* 1999). These findings emphasize the importance of simultaneous determination of both parameters during toxicity testing, because it is likely that in the initial stages of drug toxicity, an increase in MCV may balance a reduction in RBC numbers, with the result of not lowering Hct. In support of this concept, Paget (1979) reported that determinations

of MCV are important in order to avoid misinterpreting a relatively small decrease in the Hct. Again, Hct concentrations in the treated groups were within normal limits during the study period (see Table 4.1).

Changes in WBC are caused by exposure of animals to stress (Dominguez-Gerpe and Rey-Mendez 2001). In this study, the WBC counts of all monkeys were slightly reduced during the study period, which further indicates that stress had an insignificant impact on the biological systems of these monkeys. Loomis *et al.* (1980) and Wall *et al.* (1985) have documented the effects of ketamine hydrochloride on the hematological parameters of rhesus and vervet monkeys respectively. The authors found that in rhesus monkeys, ketamine reduces total WBC count. According to the WBC results of this study, it is possible that ketamine had slight influence on this parameter. This is consistent with the findings that ketamine has a relatively minor or insignificant biological effect on hematological parameters of vervet monkeys (Wall *et al.* 1985). Loomis *et al.* (1980) reported that the relative percentages of the neutrophils are increased and that of the lymphocytes are decreased because of ketamine use in rhesus monkeys. In contrast, results of this study do not reflect an effect of this compound on the differential count of the vervet monkeys. The concentrations of neutrophils, eosinophils and basophils in the controls and treated monkeys were within the normal range in the vervet monkeys (see Table 4.1). These findings also suggest that oleanolic acid had no effect on the proliferative pool of granulocytes in these monkeys. This is because destruction of this pool normally develops into a swift neutropenia (Weiss 1993). Kerr (1989) reported that it is common to find few or no monocytes at all in a blood film from a normal animal. Neither oleanolic acid nor ketamine had dramatic effects on monocyte and lymphocyte numbers of all monkeys in this study.

In conclusion, it appears that oleanolic acid is not associated with leucocytosis, and therefore, will not lead to significant changes in total WBC and differential counts.

Platelet production, like erythropoesis, occurs in the red bone marrow. Their function is to repair slightly damaged blood vessels and initiate a series of sequential reactions leading to blood clotting (Tortora and Anagnostakos 1990). Several compounds, including aspirin, interfere with platelet function/aggregation, thus leading to almost doubly increased bleeding time that may last for several days (Peter *et al.* 2002). In this study, it was observed that none of the treated monkeys had reduced platelet numbers or bleeding, which suggests that oleanolic acid did not suppress formation of platelets in adequate numbers or caused defect in platelet function in these monkeys. In addition, platelet numbers in the treated were within normal limits throughout the study period (see Table 4.1).

Conclusion

This study described effects of oleanolic acid on behavior, organs and systems functioning which, according to the author, were never reported for non human primate species before. The absence of any treatment effect on behavioral, physical or physiological parameters in any of the treated monkeys following administration of oleanolic acid, suggest that oleanolic acid is not toxic to vervet monkeys tested. Data obtained in this study suggest that oleanolic acid had no toxic effects on the cardiovascular system, renal function and electrolyte transport or on the biligenic function of the liver. The WBC results from the controls suggest that the vervet monkey colony environment in this study was stable, and thus reduced events of novel stimulation of the immune system.

References

Amagaya S, Iizuka A, Makino B, Kubo M, Komatsu Y, Cheng FC, Ruo TI, Itoh T, Terasawa K (2001) General pharmacological properties of Sho-seiryu-to (TJ-19) extracts. Phytomedicine **8**, 338-47

Andreasson B, Wahlstrom E, Jacobsson S, Bjorkholm M, Samuelsson J, Birgegard G, Wadenvik H, Kutti J (1999) The measurement of venous haematocrit in patients with polycythaemia vera. Journal of Internal Medicine **246**, 293-297

Ankier SI, Warrington SJ (1989) Research and development of new medicines. The Journal of International Medical Research **17**, 405-416

Arakawa H, Kodama H, Matsuoka N, Yamaguchi I (1997) Stress increases plasma enzyme activity in rats: differential effects of adrenergic and cholinergic blockades. The Journal of Pharmacology and Experimental Therapeutics **280**, 1296-1303

Aronson PS, Giebisch G (1997) Mechanisms of chloride transport in the proximal tubule. American Journal of Physiology **273**, 179-192

Artur Y, Wellman-Bednawska M, Jacquier A, Siest G (1984) Associations between serum gamma-glutamyltransferase and apolipoproteins: relationships with hepatobiliary diseases. Clinical Chemistry **30**, 1318-1321

Atroshi F, Ali-Vehmas T, Westermarck T, Rizzo A, Selga G, Baltais A, Linars J, Saulite V, Daukste A (2000) Evaluation of the antibacterial and hemolytic activities of Latvian herbal preparation. Veterinary and Human Toxicology **42**, 341-344

Auguscinska E, Kasprzyk Z (1982) Studies on the labelling of terpenoids in shoots and cells or protoplasts from *Calendula officinalis* leaves. Acta Biochimica Polonica **29**, 7-15

Bello R, Calatayud S, Beltran B, Primo-Yufera E, Esplugues J (2001) Cardiovascular effects of the methanol and dichloromethanol extracts from *Mentha suaveolens Ehrh.* Phytotherapy Research **15**, 447-448

Belpaire FM, Bogaert MG (1991) Binding of alfentanil to human alpha 1-acid glycoprotein, albumin and serum. International Journal of Clinical Pharmacology, Therapy and Toxicology **29**, 96-102

Bergan T, Engeset A, Olszewski W, Ostby N, Solberg R (1986) Extravascular penetration of highly protein-bound flucloxacillin. Antimicrobial Agents and Chemotherapy **30**, 729-732

Beutler E (1994) G6PD deficiency. Blood **84**, 3613-3636

Bhattacharyya A, Macdonald J, Lakhdar AA (2001) Acute adrenocortical crisis: three different presentations. International Journal of Clinical Practice **55**, 141-144

Bochner BS, Schleimer RP (2001) Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment. Immunological Reviews **179**, 5-15

Brass EP, Thompson WL (1982) Drug-induced electrolyte abnormalities. Drugs **24**, 207-28

Brierley CH, Burchell B (1993) Human UDP-glucuronosyl transferases: chemical defence, jaundice and gene therapy. Bioessays **15**, 749--754

Brommage R, Allison C, Stavisky R, Kaplan J (1999) Measurement of serum bone-specific alkaline phosphatase activity in cynomolgus macaques. Journal of Medical Primatology **28**, 329-333

Brucker RF, Cohen PP (1976) Alterations in enzyme and cytochrome profiles of Rana catesbeiana liver organelles during thyroxine-induced metamorphosis. Changes in membrane-localized phosphohydrolases, oxidoreductases, and cytochrome levels in response to *in vivo* thyroxine administration. The Journal of Biological Chemistry **251**, 6161-6169

Cai L, Wu CD (1996) Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. Journal of Natural Products **59**, 987-990

Cashman KD (2002) Calcium intake, calcium bioavailability and bone health. The British Journal of Nutrition **87**, 169-177

Castellino P, Cataliotti A (2002) Changes of protein kinetics in nephrotic patients. Current Opinion in Clinical Nutrition and Metabolic Care **5**, 51-54

Chae CU, Lloyd-Jones DM (2002) Isolated systolic hypertension in the elderly. Current Treatment Options in Cardiovascular Medicine **4**, 87-93

Cheng B, Hung CT, Chiu W (2002) Herbal medicine and anesthesia. Hong Kong Medical Journal **8**, 123-130

Cosyns JP, Dehoux JP, Guiot Y, Goebbels RM, Robert A, Bernard AM, van Ypersele de Strihou C (2001) Chronic aristolochic acid toxicity in rabbits: a model of Chinese herbs nephropathy? Kidney International **59**, 2164-2173

Crockett CM, Shimoji M, Bowden DM (2000) Behavior, appetite, and urinary cortisol responses by adult female pigtailed macaques to cage size, cage level, room change, and ketamine sedation. American Journal of Primatology **52**, 63-80

Dahlstedt AJ, Katz A, Wieringa B, Westerblad H (2000) Is creatine kinase responsible for fatigue? Studies of isolated skeletal muscle deficient in creatine kinase. The Federation of American Societies for Experimental Biology (FASEB) Journal **14**, 982-990

Dean JH, Olson HM (1993) The integration of investigative toxicology in the drug discovery process. Biology of the Cell **77**, 3-8

de Neef KJ, Nieuwenhuijsen K, Lammers AJ, Degen AJ, Verbon F (1987) Blood variables in adult stumptail macaques (*Macaca arctoides*) living in a captive group; annual variability. Journal of Medical Primatology **16**, 237-47

Dimo T, Rakotonirina A, Tan PV, Dongo E, Dongmo AB, Kamtchouing P, Azay J, Abegaz BM, Cros G, Ngadjui TB (2001) Antihypertensive effects of *Dorstenia psilurus* extract in fructose-fed hyperinsulinemic, hypertensive rats. Phytomedicine **8**, 101-106

Dominguez-Gerpe L, Rey-Mendez M (2001) Alterations induced by chronic stress in lymphocyte subsets of blood and primary and secondary immune organs of mice. BMC Immunology **2**, 7

Fahim M, Khan MS, Hameed HA (1995) Effect of Ajmaloon on the baroreceptor-heart rate reflex in anaesthetized rabbits and monkeys. Indian Journal of Physiology and Pharmacology **39**, 101-105

Farina C, Pinza M, Pifferi G (1998) Synthesis and anti-ulcer activity of new derivatives of glycyrrhetic, oleanolic and ursolic acids. Farmaco **53**, 22-32

Feldman BF, Ruehl WW (1984) Interpreting absolute WBC counts. Modern Veterinary Practice **65**, 446-449

Finsterer J, Stollberger C, Grossegger C, Kroiss A (1999) Hypothyroid myopathy with unusually high serum creatine kinase values. Hormone Research **52**, 205-208

Friedman LA, Lewis PJ (1980) The effect of semisynthetic penicillins on the binding of bilirubin by neonatal serum. British Journal of Clinical Pharmacology **9**, 61-65

Fung YS, Sun DX, Yeung CY (2000) Capillary electrophoresis for determination of free and albumin-bound bilirubin and the investigation of drug interaction with bilirubin-bound albumin. Electrophoresis **21**, 403-410

Ghio AJ, Piantadosi CA, Crumbliss AL (1997) Hypothesis: iron chelation plays a vital role in neutrophilic inflammation. Biometals **10**, 135-142

Goldenberg I, Jonas M, Thaler M, Grossman E (1997) Elevated levels of serum creatine kinase induced by hyponatraemia. Postgraduate Medical Journal **73**, 511-512

Greene DA (1986) A sodium-pump defect in diabetic peripheral nerve corrected by sorbinil administration: relationship to myo-inositol metabolism and nerve conduction slowing. Metabolism **35**, 60-65

Greger JL, Tseng E (1993) Longitudinal changes during the development of hypertension in rats fed excess chloride and sodium. Proceedings of the Society for Experimental Biology and Medicine **203**, 377-385

Grofte T, Wolthers T, Jensen DS, Moller N, Jorgensen JO, Orskov H, Vilstrup H (1997) Hepatic amino nitrogen conversion and organ N-contents in hypothyroidism, with thyroxine replacement, and in hyperthyroid rats. Journal of Hepatology **26**, 409-416

Guyton AC, Young DB, DeClue JW, Trippodo N, Hall JE (1975) Fluid balance and blood pressure. Clinical Nephrology **44**, 122-126

Haralampidis K, Trojanowska M, Osbourn AE (2002) Biosynthesis of triterpenoid saponins in plants. Advances in Biochemical Engineering/Biotechnology **75**, 31-49

Harewood WJ, Gillin A, Hennessy A, Armistead J, Horvath JS, Tiller DJ (1999) Biochemistry and haematology values for the baboon (*Papio hamadryas*): the effects of sex, growth, development and age. Journal of Medical Primatology **28**, 19-31

Harrison P (1998) Herbal medicine takes root in Germany. Canadian Medical Association Journal **158**, 637-639

Hartley LH, Rodger R, Nicolosi RJ, Hartley T (1984) Blood pressure values in *Macaca fascicularis*. Journal of Medical Primatology **13**, 183-189

Heidenreich O (1984) Mode of action of conventional and potassium-sparing diuretics--aspects with relevance to Mg-sparing effects. Review of the present state of the art and recent findings. Magnesium **3**, 248-256

Hierholzer K, Finke R (1997) Myxedema. Kidney International (Supplement) **59**, 82-89

Hiraoka M, Hirano Y (1986) Changes in passive electrical properties of guinea-pig ventricular muscle exposed to low K+ and high Ca2+ conditions. Journal of Molecular and Cellular Cardiology **18**, 1177-1186

Irwin JJ, Kirchner JT (1993) Anemia in children. American Family Physician **64**, 1379-1386

Ismaili H, Tortora S, Sosa S, Fkih-Tetouani S, Ilidrissi A, Della Loggia R, Tubaro A, Aquino R (2001) Topical anti-inflammatory activity of *Thymus willdenowii*. The Journal of Pharmacy and Pharmacology **53**, 1645-1652

Jacobs B, Harris GC, Allda V, Chugani HT, Pollack DB, Raleigh MJ (1993). Midazolam as an effective intravenous adjuvant to prolonged ketamine sedation in young rhesus (*Macaca mulatta*)

and vervet (*Cercopithecus aethiops sabaeus*) monkeys: A preliminary report. American Journal of Primatology **29**, 291-298

Jeon BH, Kim CS, Kim HS, Park JB, Nam KY, Chang SJ (2000) Effect of Korean red ginseng on blood pressure and nitric oxide production. Acta Pharmacologica Sinica **21**, 1095-1100

Jouad H, Lacaille-Dubois MA, Lyoussi B, Eddouks M (2001) Effects of the flavonoids extracted from Spergularia purpurea Pers. on arterial blood pressure and renal function in normal and hypertensive rats. Journal of Ethnopharmacology **76**, 159-163

Kannel WB (2000) Elevated systolic blood pressure as a cardiovascular risk factor. American Journal of Cardiology **85**, 251-255

Kapil A, Sharma S (1995) Effect of oleanolic acid on complement in adjuvant- and -induced inflammation in rats. The Journal of Pharmacy and Pharmacology **47**, 585-587

Kerr MG (1989) Veterinary Laboratory Medicine: Clinical Biochemistry and Haematology. Oxford Blackwell Scientific Publications

Kozai K, Suzuki J, Okada M, Nagasaka N (1999) Effect of oleanolic acid-cyclodextrin inclusion compounds on dental caries by in vitro experiment and rat-caries model. Microbios **97**, 179-188

Kromhout D (2001) Diet and cardiovascular diseases. The Journal of Nutrition Health and Aging **5**, 144-149

Kruth HS (2001) Lipoprotein cholesterol and atherosclerosis. Current Molecular Medicine **1**, 633-653

Kushner H, Kraft-Schreyer N, Angelakos ET, Wudarski EM (1982) Analysis of reproductive data in breeding colony of African Green Monkeys. Journal of Medical Primatology **11**, 77-84

Lane MA, Baer DJ, Rumpler WV, Weindruch R, Ingram DK, Tilmont EM, Cutler RG, Roth GS (1996) Calorie restriction lowers body temperature in rhesus monkeys, consistent with a postulated anti-aging mechanism in rodents. Proceedings of the National Academy of Sciences of the United States of America **93**, 4159--4164

Li J, Guo WJ, Yang QY (2002) Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. World Journal of Gastroenterology **8**, 493-495

Lin G, Nnane IP, Cheng TY (1999) The effects of pretreatment with glycyrrhizin and glycyrrhetinic acid on the retrorsine-induced hepatotoxicity in rats. Toxicon **37**, 1259-1270

Liu J, Liu Y, Madhu C, Klaassen CD (1993) Protective effects of oleanolic acid on acetaminophen-induced hepatotoxicity in mice. The Journal of Pharmacology and Experimental Therapeutics **266**, 1607-1613

Liu J, Liu YP, Klaassen (1994) The effect of Chinese hepatoprotective medicines on experimental liver injury in mice. Journal of Ethnopharmacology **42**, 181-193

Liu Y, Hartley DP, Liu J (1998) Protection against carbon tetrachloride hepatotoxicity by oleanolic acid is not mediated through metallothionein. Toxicology Letters **95**, 77-85

[a]Liu Y, Kreppel H, Liu J, Choudhuri S, Klaassen CD (1993) Oleanolic acid protects against cadmium hepatotoxicity by inducing metallothionein. The Journal of Pharmacology and Experimental Therapeutics **266**, 400-406

Loomis MR, Henrickson RV, Anderson JM (1980) Effects of ketamine hydrochloride on the hemogram of rhesus monkeys (*Macaca mulatta*). Laboratory Animal Science **30**, 851-853

Lyman JL (1986) Blood urea nitrogen and creatinine. Emergency Medicine Clinics of North America **4**, 223-233

Ma BL (1986) Hypolipidemic effects of oleanolic acid. Traditional Medicine and Pharmacology **2**, 28-29

Mahady GB (2001) Global harmonization of herbal health claims. The Journal of Nutrition **131**, 1120-1123

Mahl TC (1998) Approach to the patient with abnormal liver tests. Lippincotts Primary Care Practice **2**, 379-389

Matsuda H, Li Y, Murakami T, Yamahara J, Yoshikawa M (1998) Protective effects of oleanolic acid oligoglycosides on ethanol- or indomethacin-induced gastric mucosal lesions in rats. Life Sciences **63**, 245-250

Matthews HB, Lucier GW, Fisher KD (1999) Medicinal herbs in the United States: research needs. Environmental Health Perspectives **107**, 773-778

Mdhluli MC, van der Horst G (2002) The effect of oleanolic acid on sperm motion characteristics and fertility of male Wistar rats. Laboratory Animals **36**, 432-437

Meyer KB, Madias NE (1994) Cisplatin nephrotoxicity. Mineral and Electrolyte Metabolism **20**, 201-213

Miller LG (1998) Herbal medicinals: selected clinical considerations focusing on known or potential drug-herb interactions. Archives of Internal Medicine **158**, 2200-2211

Morse EE (1988) Toxic effects of drugs on erythrocytes. Annals of Clinical and Laboratory and Science **18**, 13-18

Moss DW (1997) Physicochemical and pathophysiological factors in the release of membrane-bound alkaline phosphatase from cells. Clinica Chimica Acta **257**, 133-40

Mullin GE, Eastern JS (1986) Cutaneous signs of thyroid disease. American Family Physician **4**, 93-98

Nadir A, Reddy D, Van Thiel DH (2000) Cascara sagrada-induced intrahepatic cholestasis causing portal hypertension: case report and review of herbal hepatotoxicity. The American Journal of Gastroenterology **95**, 3634-3637

Napier JR, Napier PH (1967) *Cercopithecus*. In: A handbook of living Primates (Nappier PH, ed). London: Academic Press, pp100-111

Navin TR, Hager WD (1979) Creatine kinase MB isoenzyme in the evaluation of myocardial infarction. Current Problems in Cardiology **3**, 1-32

Ohigashi H, Takamura H, Koshimizu K, Tokuda H, Ito Y (1986) Search for possible antitumor promoters by inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation; ursolic acid and oleanolic acid from an anti-inflammatory Chinese medicinal plant, *Glechoma hederaceae L.* Cancer Letters **30**, 143-151

Okuno T, Chou A (1975) The significance of small erythrocytes. American Journal of Clinical Pathology **64**, 48-52

Osman M, Chandrasekaran A, Chan K, Scatina J, Ermer J, Cevallos Wsisenwine SF (1998) Metabolic disposition of 14C-bromfenac in healthy male volunteers. Journal of Clinical Pharmacology 38, 744-752

Paget GE (1979) Methods in toxicology. Edinburgh: Blackwell Scientific Publications

Pallatini P (1999) Heart rate as a risk factor for atherosclerosis and cardiovascular mortality: the effect of antihypertensive drugs. Drugs **57**, 713-724

Pengsuparp T, Indra B, Nakagawasai O, Tadano T, Mimaki Y, Sashida Y, Ohizumi Y, Kisara K (2001) Pharmacological studies of geissoschizine methyl ether, isolated from *Uncaria sinensis Oliv.*, in the central nervous system. European Journal of Pharmacology **425**, 211-218

Peter FW, Benkovic C, Muehlberger T, Vogt PM, Homann HH, Kuhnen C, Wiebalck A, Steinau HU (2002) Effects of desmopressin on thrombogenesis in aspirin-induced platelet dysfunction. British Journal of Haematology **117**, 658-663

Pinn G. (2001) Herbal medicine in renal and genitourinary disease. Australian Family Physician **30**, 974-977

Pyatt DW, Yang Y, Mehos B, Le A, Stillman W, Irons RD (2000). Hematotoxicity of the chinese herbal medicine *Tripterygium wilfordii* hook F in CD34-positive human bone marrow cells. Molecular Pharmacology **57**, 512-518

Quamme GA (1986) Renal handling of magnesium: drug and hormone interactions. Magnesium **5**, 248-272

Quaschning T, Ruschitzka F, Shaw S, Luscher TF (2001) Aldosterone receptor antagonism normalizes vascular function in liquorice-induced hypertension. Hypertension **37**, 801-805

Rahman MF, Siddiqui MK, Jamil K (2001) Effects of Vepacide (*Azadirachta indica*) on aspartate and alanine aminotransferase profiles in a subchronic study with rats. Human and Experimental Toxicology **20**, 243-249

Rajasekaran M, Bapna JS, Lakshmanan S, Ramachandran NA, Veliath AJ and Panchanadam M (1988) Antifertility effect in male rats of oleanolic acid, a triterpene from *Eugenia jambolana* flowers. Journal of Ethnopharmacology **24,** 115- 121

Raleigh MJ, McGuire MT (1990) Social influences of endocrine function in male vervet monkeys. In: Socioendocrinology of primate reproduction, (Ziegler TE and Bercovitch FB, eds), New York, Wiley-Liss Inc, pp 95-111

Reichling JJ, Kaplan MM (1988) Clinical use of serum enzymes in liver disease. Digestive Diseases and Sciences **33**, 1601-1614

Renoe BW, McDonald JM, Ladenson JH (1980) The effects of stasis with and without exercise on free calcium, various cations, and related parameters. Clinica Chimica Acta **103**, 91-100

Rothschild MA, Oratz M, Schreiber SS (1988) Serum albumin. Hepatology **8**, 385-401

Russel FG, Masereeuw R, van Aubel RA (2002) Molecular aspects of renal anionic drug transport. Annual Review of Physiology **64**, 563-94

Ryan MP (1986) Magnesium and potassium-sparing diuretics. Magnesium **5**, 282-292

Savage RA (1993) The red cell indices. Yesterday, today, and tomorrow. Clinics in Laboratory Medicine **13**, 773-785

Seier JV (1986) Breeding vervet monkeys in closed environment. Journal of Medical Primatology **15**, 339-349

Seier JV, Fincham JE, Taljaard JJF (1990) Horseshoe kidney in vervet monkeys. Journal of Medical Primatology **19**, 595-599

Seier JV, Mdhluli M, Dhansay MA, Loza J, Laubscher R (2002) A toxicity study of *Sutherlandia* Leaf Powder (*Sutherlandia microphylla*) consumption. Scientific Report

Sharma OP, Dawra RK, Makkar HP (1989) Toxicity of isolated Lantana (*Lantana camara L*) constituents to male and female guinea pigs. Veterinary and Human Toxicology **31**, 10-13

Sharma OP, Vaid J, Pattabhi V, Bhutani KK (1992) Biological action of lantadene C, a new hepatotoxicant from *Lantana camara var. aculeata*. Journal of Biochemical Toxicology **7**, 73-79

Sheikh NM, Philen RM, Love LA (1997) Chaparral-associated hepatotoxicity. Archives of Internal Medicine **157**, 913-919

Sherman KE (1991) Alanine aminotransferase in clinical practice. A review. Archives of Internal Medicine **151**, 260-265

Sibley CG, Ahlquist JE (1987) DNA hybridization evidence of hominoid phylogeny: Results from an expanded data set. Journal of Molecular Evolution **26**, 99-121

Singh A, Sharma OP, Kurade NP, Ojha S. (2001) Detoxification of lantana hepatotoxin, lantadene A, using *Alcaligenes faecalis*. Journal of Applied Toxicology **21**, 225-228

Singh GB, Singh S, Bani S, Gupta BD, Banerjee SK (1992) Anti-inflammatory activity of oleanolic acid in rats and mice. The Journal of Pharmacy and Pharmacology **44**, 456- 458

Singh J, Chander J, Singh S, Singh G, Atal CK (1986) gamma-Glutamyl transpeptidase: a novel biochemical marker in inflammation. Biochemical Pharmacology **35**, 3753-3760

Smith CC (1969) Value of non human primates in predicting disposition of drugs in man. Annals of the New York Academy of Sciences **162**, 604-609

Smith RL, Williams RT (1974) Comparative metabolism of drugs in man and monkeys. Journal of Medical Primatology **3**, 138-152

Smith RL, Caldwell J (1976) Drug Metabolism from Microbe to Man, (Parke DV, Smith RL, eds). London: Taylor and Francis, 333-356

Stedman C (2002) Herbal hepatotoxicity. Seminars Liver Disease **22**, 195-206

Stonard MD (1990) Assessment of renal function and damage in animal species. A review of the current approach of the academic, governmental and industrial institutions represented by the Animal Clinical Chemistry Association. Journal of Applied Toxicology **10**, 267-274

Subat-Dezulovic M, Slavic I, Rozmanic V, Persic M, Medjimurec B, Scukanec-Spoljar M (2002) Drug-induced acute tubulointerstitial nephritis: a case with elevated urinary cadmium. Pediatric Nephrology **17**, 382-385

Takenaka O (1986) Blood characteristics of the crab-eating monkeys (*Macaca fascicularis*) in Bali Island, Indonesia: Implication of water deficiency in Bali. Journal of Medical Primatology **15**, 97-104

Teloh HA (1978) Serum proteins in hepatic disease. Annals of Clinical Laboratory Science **8**, 127-129

Tirone TA, Brunicardi FC (2001) Overview of glucose regulation. World Journal of Surgery **25**, 461-467

Tokuda H, Ohigashi H, Koshimizu K, Ito Y (1986) Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Cancer Letters **33**, 279-285

Tortora GR, Anagnostakos NP (1990) Principles of Anatomy and Physiology. New York, Harper & Row Publishers

Virally ML, Guillausseau PJ (1999) Hypoglycemia in adults. Diabetes and Metabolism **25**, 477-490

Wall HS, Wothman C, Else JG (1985) Effects of ketamine anaesthesia, stress and repeated bleeding on the haematology of vervet monkeys. Laboratory Animals **19**, 138-144

Ward PC (1981) Renal dysfunction. Urea and creatinine. Postgraduate Medicine **69**, 93-100

Weiss DJ (1993) Leukocyte response to injury. Toxicologic Pathology **21**, 135-140

West LG, McLaughlin JL (1977) Triterpenes from the button cactus, *Epithelantha micromeris*. Lloydia **40**, 499-504

Westenfelder C, Loghman-Adham M, Baranowski RL, Brownley R, Kablitz C (1998) Increased reabsorptive capacity after ureteral obstruction reduces the ability of glucose to inhibit phosphate reabsorption in rat kidney. Nephrology, Dialysis, Transplantation **13**, 1675-1681

Westerhof N, Elzinga G (1993) Why smaller animals have higher heart rates. Advances in Experimental Medicine and Biology **346**, 319-323

White LM, Gardner SF, Gurley BJ, Marx MA, Wang PL, Estes M (1997) Pharmacokinetics and cardiovascular effects of ma-huang (*Ephedra sinica*) in normotensive adults. Journal of Clinical Pharmacology **37**, 116-22

Wrzeciono U, Malecki I, Budzianowski J, Kierylowicz H, Zaprutko L, Beimcik E, Kostepska H (1985) Nitrogenous triterpene derivatives. 10. Hemisuccinates of some oleanolic acid derivatives and their antiulcer effect. Pharmazie **40**, 542-544

Yanagita T (1973) The use of monkeys in the evaluation of drug safety. In: The Laboratory Animal in Drug Testing, 5th ICLA Symposium (Spiegel A, ed). Stuttgart, Gustav Fischer Verlag, pp 177-184

Yim TK, Wu WK, Pak WF, Ko KM (2001) Hepatoprotective action of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruits is mediated through an enhancement on hepatic glutathione regeneration capacity in mice. Phytotherapy Research **15**, 589-592

Yin J, Miller M, Wennberg RP (1991) Induction of hepatic bilirubin-metabolizing enzymes by the traditional Chinese medicine yin zhi huang. Developmental Pharmacology and Therapeutics **16**, 176-184

Yoshikawa M, Matsuda H (2000) Antidiabetogenic activity of oleanolic acid glycosides from medicinal foodstuffs. Biofactors **13**, 231-237

Zhang CY, Tan BK (1996) Hypotensive activity of aqueous extract of *Andrographis paniculata* in rats. Clinical and Experimental Pharmacology and Physiology **23**, 675-678

Legends to appendix A

- For each group, data in Tables 1-43 represent means (written in bold) and standard deviations (below means), and relate to the graphs in Figures 3.1-3.43.
- Units for respective parameters are in brackets
- Data in Tables 1-7 were obtained every second week.
- Data in Tables 8-43 were obtained every fourth week.

APPENDIX A:

Means and standard deviations

Physical and physiological parameters

Table 1. Bodyweight (kg)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**5.32**	**5.31**	**5.29**	**5.22**	**5.22**	**5.14**	**5.30**	**5.20**	**5.17**
	0.53	0.51	0.49	0.47	0.46	0.47	0.44	0.44	0.43
10 mg/kg	**5.35**	**5.47**	**5.45**	**5.38**	**5.40**	**5.38**	**5.50**	**5.37**	**5.28**
	0.36	0.41	0.48	0.43	0.46	0.47	0.47	0.43	0.36
25 mg/kg	**5.30**	5.31	**5.22**	**5.18**	**5.10**	**4.99**	5.11	**4.98**	**5.00**
	0.56	0.64	0.61	0.56	0.58	0.48	0.45	0.46	0.53
Control	**5.22**	**5.33**	**5.20**	**5.16**	**5.11**	**5.18**	**5.13**	**5.09**	**4.98**
	0.29	0.49	0.26	0.32	0.35	0.28	0.41	0.362	0.40

Table 2. Body temperature (°C)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**39.06**	**39.06**	**39.16**	**38.90**	**38.83**	**39.46**	**39.03**	**38.86**	**38.86**
	0.05	0.20	0.28	0.17	0.28	0.305	0.15	0.28	0.15
10 mg/kg	**39.40**	**38.93**	**38.83**	**39.40**	**39.23**	**39.03**	**39.20**	**38.90**	**38.80**
	0.00	0.70	0.76	0.53	0.25	0.50	0.51	0.36	0.28
25 mg/kg	**39.26**	**38.83**	**38.93**	**39.23**	**39.10**	**38.83**	**39.40**	**39.10**	**39.03**
	0.23	0.65	0.46	0.25	0.50	0.28	0.34	0.45	0.28
Control	**39.06**	**38.76**	**39.13**	**39.36**	**38.86**	**38.93**	**38.63**	**38.90**	**38.66**
	0.40	0.20	0.11	0.15	0.61	0.46	0.37	0.17	0.28

Table 3. Respiratory rate (breaths/min)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**28.00**	**26.00**	**28.00**	**28.00**	**26.00**	**24.00**	**24.00**	**24.00**	**30.00**
	6.92	3.46	3.46	3.46	3.46	0.00	6.00	6.00	6.00
10 mg/kg	**22.00**	**26.00**	**28.00**	**28.00**	**28.00**	**22.00**	**26.00**	**24.00**	**26.00**
	3.46	3.46	9.16	3.46	6.92	3.46	9.16	0.00	3.46
25 mg/kg	**22.00**	**24.00**	**28.00**	**24.00**	**22.00**	**20.00**	**24.00**	**28.00**	**22.00**
	3.46	6.00	6.92	6.00	3.46	3.46	6.00	3.46	3.46
Control	**24.00**	**22.00**	**24.00**	**26.00**	**28.00**	**24.00**	**22.00**	**28.00**	**22.00**
	6.00	3.46	0.00	6.92	3.46	6.00	3.46	3.46	3.46

Cardiovascular function

Table 4. Systolic pressure (mmHg)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**101.66**	**104.33**	**108.33**	**106.33**	**103.33**	**103.33**	**95.00**	**108.33**	**105.33**
	12.01	16.77	9.45	6.80	12.85	10.59	9.00	6.80	12.89
10 mg/kg	**102.66**	**101.66**	**108.00**	**103.00**	**88.66**	**99.33**	**93.33**	**107.66**	**107.00**
	7.09	16.50	6.55	14.42	10.69	12.50	5.03	7.63	13.11
25 mg/kg	**109.66**	**117**	**98.33**	**103**	**97.33**	**91.33**	**92.66**	**101.00**	**93.66**
	7.76	2.64	9.71	9.84	16.74	7.57	7.50	10.81	11.23
Control	**102.66**	**102.66**	**100.00**	**99.66**	**97.33**	**93.00**	**91.00**	**92.33**	**90.00**
	18.00	14.57	15.52	12.66	16.16	19.31	9.53	5.68	14.10

Table 5. Diastolic pressure (mmHg)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**49.33**	**43.33**	**57.00**	**45.33**	**43.00**	**46.66**	**48.33**	**48.33**	**42.66**
	11.01	9.07	13.45	2.30	4.35	4.16	7.09	9.86	5.50
10 mg/kg	**42.66**	**41.33**	**46.33**	**44.66**	**42.66**	**46.3**	**48.33**	**53.00**	**47.66**
	5.68	10.11	5.68	9.29	6.42	7.37	6.65	2.64	7.76
25 mg/kg	**51.66**	**56.00**	**42.33**	**40.33**	**40.33**	**41.6**	**42.66**	**48.00**	**39.66**
	3.05	3.60	11.67	3.21	10.06	7.57	4.93	11.53	4.50
Control	**44.00**	**48.33**	**54.00**	**47.66**	**53.66**	**44.33**	**41.33**	**47.66**	**47.66**
	17.05	20.03	22.71	20.74	21.54	22.18	17.89	18.00	18.71

Table 6. Mean arterial pressure (mmHg)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**69.66**	**58.66**	**77.00**	**65.00**	**61.33**	**69.66**	**64.66**	**69.66**	**63.00**
	18.50	12.74	14.00	3.00	9.00	7.50	4.04	15.6	9.50
10 mg/kg	**65.00**	**64.33**	**67.66**	**64.66**	**60.00**	**67.66**	**66.66**	**78.00**	**66.66**
	12.76	15.69	10.50	13.79	5.56	10.11	1.52	1.73	10.40
25 mg/kg	**78.33**	**80.33**	**62.00**	**56.33**	**58.00**	**62.00**	**62.66**	**69.33**	**62.66**
	3.511	4.041	16.70	5.13	17.77	13.22	11.01	19.85	12.58
Control	**66.33**	**67.33**	**72.00**	**66.00**	**75.00**	**64.00**	**56.00**	**63.00**	**64.66**
	27.61	27.22	23.81	27.05	22.27	30.44	21.28	18.35	20.81

Table 7. Heart rate (beats/min)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**96.66**	**109.00**	**112.33**	**103.00**	**96.00**	**110.66**	**98.66**	**95.33**	**100.00**
	13.65	22.11	15.17	20.80	27.00	9.07	8.02	33.12	32.18
10 mg/kg	**91.33**	**125.66**	**125.33**	**122.00**	**120.66**	**107.00**	**104.33**	**109.33**	**111.33**
	20.84	29.50	31.13	30.00	29.77	23.25	23.79	20.03	9.60
25 mg/kg	**101.33**	**117.33**	**121.00**	**119.00**	**107.66**	**109.33**	**103.66**	**101.66**	**113.66**
	14.36	8.38	7.21	6.55	12.58	14.43	5.50	3.05	8.02
Control	**112.33**	**138.33**	**126.33**	**129.66**	**120.00**	**114.66**	**105.00**	**105.66**	**105.33**
	8.38	21.93	20.30	17.38	17.43	34.70	8.71	19.73	28.02

Table 8. High-density lipoproteins (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**3.06**	2.98	**3.10**	**3.30**	**3.45**
	0.25	0.15	0.07	0.06	0.11
10 mg/kg	**2.61**	**2.52**	**2.85**	**2.88**	**2.89**
	0.21	0.40	0.44	0.29	0.25
25 mg/kg	**2.06**	**2.19**	**2.34**	**2.43**	**2.40**
	0.43	0.42	0.27	0.41	0.96
Control	**2.89**	**2.77**	**2.88**	**2.78**	**2.84**
	0.19	0.67	0.80	0.97	0.75

Table 9. Low-density lipoproteins (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**1.21**	**1.72**	**2.23**	**1.63**	**1.62**
	0.28	0.17	0.46	0.33	0.47
10 mg/kg	**1.40**	**2.21**	**2.40**	**2.04**	**2.15**
	0.41	0.28	0.42	0.84	0.61
25 mg/kg	**1.71**	**1.97**	**2.31**	**1.75**	**1.82**
	0.84	0.98	0.68	0.52	0.78
Control	**1.60**	**2.47**	**2.73**	**2.11**	**2.28**
	0.72	0.72	0.83	0.72	0.62

Table 10. Total cholesterol (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**4.27**	**4.70**	**5.33**	**4.94**	**5.08**
	0.29	0.10	0.45	0.39	0.57
10 mg/kg	**4.02**	**4.73**	**5.25**	**4.93**	**5.05**
	0.20	0.67	0.86	1.03	0.56
25 mg/kg	**3.78**	**4.16**	**4.65**	**4.18**	**4.22**
	0.862	1.21	0.71	0.64	0.64
Control	**4.50**	**5.25**	**5.61**	**4.90**	**5.12**
	0.74	1.03	1.43	1.39	1.29

Table 11. Triglycerides (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**0.52**	**0.61**	**0.76**	**0.74**	**0.38**
	0.06	0.41	0.28	0.31	0.10
10 mg/kg	**0.43**	**0.53**	**0.56**	**0.47**	**0.26**
	0.02	0.13	0.04	0.05	0.12
25 mg/kg	**0.53**	**0.44**	**0.50**	**0.49**	**0.39**
	0.15	0.06	0.07	0.05	0.11
Control	**0.41**	**0.56**	**0.73**	**0.63**	**0.31**
	0.12	0.07	0.04	0.02	0.04

Table 12. Creatine kinase (U/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**241.66**	**525.33**	**479.33**	**1130.00**	**738.00**
	83.91	418.63	302.52	743.08	272.46
10 mg/kg	**744.33**	**529.66**	**322.00**	**718.66**	**769.33**
	315.76	330.58	50.74	588.35	355.51
25 mg/kg	**290.00**	**392.66**	**604.00**	**836.00**	**1033.33**
	129.42	340.70	235.61	125.79	372.35
Control	**319.66**	**318.33**	**416.00**	**489.33**	**596.33**
	230.79	26.57	193.03	160.50	120.26

Hepatic function

Table 13. Aspartate aminotransferase (U/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**56.13**	**109.10**	**92.70**	**115.33**	**80.83**
	20.33	13.94	4.85	36.70	7.52
10 mg/kg	**60.26**	**116.30**	**77.73**	**71.63**	**102.33**
	21.65	37.95	18.17	58.91	16.15
25 mg/kg	**69.46**	**176.86**	**121.36**	**87.10**	**82.56**
	13.53	36.21	6.49	18.57	19.67
Control	**75.13**	**97.46**	**74.56**	**63.40**	**70.53**
	9.71	20.75	17.91	17.36	9.20

Table 14. Alanine aminotransferase (U/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**29.26**	**59.76**	**88.33**	**69.06**	**58.76**
	3.32	27.38	29.92	29.90	33.74
10 mg/kg	**40.16**	**58.00**	**52.40**	**54.23**	**65.23**
	8.75	51.95	8.15	30.16	27.53
25 mg/kg	**44.56**	**100.70**	**89.70**	**90.23**	**45.26**
	13.69	17.31	38.74	20.05	18.08
Control	**27.80**	**48.93**	**53.30**	**51.33**	**47.20**
	13.68	38.74	24.64	28.67	29.54

Table 15. Alkaline phosphatase (U/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**146.66**	**147.33**	**149.00**	**139.66**	**154.66**
	47.25	64.82	49.42	47.72	24.58
10 mg/kg	**149.00**	**146.66**	**148.66**	**133.66**	**153.00**
	65.36	60.48	56.08	77.51	73.50
25 mg/kg	**106.00**	**111.33**	**117.66**	**109.00**	**106.33**
	31.74	31.50	28.30	42.03	37.64
Control	**149.00**	**154.66**	**171.66**	**137.00**	**157.33**
	53.01	47.68	57.97	40.14	64.93

Table 16. Gamma-glutamyl transferase (U/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	96.33	**65.33**	73.66	66.33	**70.33**
	42.77	23.45	26.27	23.71	20.55
10 mg/kg	**86.00**	**53.00**	**58.00**	**51.33**	**88.33**
	44.97	18.68	19.00	15.30	36.17
25 mg/kg	**91.66**	**82.33**	**87.66**	**67.00**	**60.00**
	51.18	33.47	45.35	23.89	11.35
Control	**61.00**	**62.33**	**57.00**	**55.66**	**63.00**
	7.54	17.03	5.29	16.77	12.16

Table 17. Total bilirubin (µmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**5.03**	**5.90**	**4.56**	**4.00**	**5.23**
	1.24	2.70	1.42	1.80	1.81
10 mg/kg	**5.26**	**5.3**	**3.96**	**3.96**	**4.66**
	3.06	1.57	0.72	0.92	1.15
25 mg/kg	**6.56**	**5.33**	**3.66**	**3.23**	**4.83**
	1.62	2.50	0.23	0.15	1.98
Control	**6.93**	**6.10**	**3.66**	**5.63**	**3.30**
	3.23	1.11	1.70	3.42	1.22

Table 18. Conjugated bilirubin (µmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**1.433**	**2.23**	**1.766**	**1.93**	**1.86**
	0.30	0.80	0.47	0.45	0.66
10 mg/kg	**1.90**	**2.066**	**1.60**	**1.73**	**1.33**
	0.62	0.60	0.52	0.51	0.87
25 mg/kg	**1.96**	**2.16**	**1.23**	**1.83**	**1.76**
	0.23	0.72	0.66	0.25	0.90
Control	**2.33**	**1.93**	**2.06**	**2.13**	**1.03**
	1.92	0.77	1.15	1.36	0.58

Table 19. Glucose (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**4.93**	**4.43**	**4.56**	**3.86**	**4.40**
	0.85	0.50	0.55	0.46	0.30
10 mg/kg	**5.03**	**5.16**	**4.66**	**4.33**	**4.23**
	1.66	1.65	1.67	1.13	0.60
25 mg/kg	**4.60**	**4.80**	**4.70**	**3.83**	**4.43**
	0.55	0.50	0.36	0.55	0.32
Control	**4.63**	**4.86**	**5.06**	**3.40**	**4.00**
	1.06	0.35	0.90	0.34	0.26

Table 20. Albumin (g/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**35.00**	**45.70**	**51.16**	43.06	**46.30**
	2.00	1.30	3.31	2.51	2.05
10 mg/kg	**37.53**	**43.66**	**47.66**	**39.33**	**42.86**
	2.33	3.09	4.85	3.35	4.44
25 mg/kg	**36.33**	**45.80**	**50.36**	40.66	**42.93**
	1.52	3.45	1.83	0.85	1.25
Control	**45.45**	**44.80**	**49.13**	**40.00**	**43.10**
	2.97	3.70	4.47	5.19	3.14

Table 21. Globulin (g/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**25.33**	**10.50**	**8.10**	13.86	**14.86**
	1.15	1.94	2.55	2.01	1.81
10 mg/kg	**22.93**	**13.50**	**11.30**	**16.03**	**16.86**
	2.10	4.85	4.69	3.78	4.10
25 mg/kg	**27.00**	**14.60**	**10.96**	**15.93**	**17.63**
	2.00	5.07	4.35	2.61	3.02
Control	**12.58**	**10.70**	**8.63**	**12.83**	**15.43**
	5.60	1.92	3.70	2.23	3.80

Table 22. Total proteins (g/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**60.33**	**56.00**	**59.26**	**56.93**	**61.16**
	2.30	3.37	5.75	4.50	3.85
10 mg/kg	**60.46**	**57.16**	**58.96**	**55.36**	**59.73**
	2.50	1.76	0.96	2.14	0.35
25 mg/kg	**63.33**	**60.40**	**61.33**	**56.60**	**60.56**
	0.57	1.65	2.59	2.52	2.13
Control	**58.03**	**55.50**	**57.76**	**52.83**	**58.53**
	4.40	2.55	3.72	4.80	1.00

Renal function

Table 23. Serum creatinine (µmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**110.66**	**103.00**	**104.33**	**129.33**	**97.66**
	4.93	3.46	3.21	50.81	9.81
10 mg/kg	**107.00**	**96.00**	**105.66**	**98.00**	**114.00**
	22.33	16.09	16.19	16.09	13.07
25 mg/kg	**111.33**	**100.00**	**105.00**	**94.66**	**91.66**
	12.01	13.45	12.76	7.50	11.93
Control	**95.66**	**102.33**	**103**	**100.66**	**99.00**
	26.02	17.95	24.24	21.50	24.75

Table 24. Urea (µmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**2.56**	**2.00**	**2.63**	**2.76**	**4.00**
	0.40	1.04	1.17	1.15	1.21
10 mg/kg	**3.33**	**2.40**	**3.03**	**3.36**	**3.33**
	0.40	1.22	1.55	1.04	1.32
25 mg/kg	**3.36**	**3.20**	**3.46**	**3.43**	**5.20**
	0.86	2.22	1.92	2.34	0.91
Control	**4.83**	**4.33**	**4.13**	**5.10**	**6.83**
	1.25	2.66	2.70	1.92	3.21

Table 25. Sodium (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**145.66**	**151.00**	**151.66**	**148.33**	**151.00**
	1.52	1.73	1.52	2.08	1.00
10 mg/kg	**147.67**	**151.33**	**151.66**	**147.66**	**151.66**
	5.85	2.51	2.081	2.08	1.15
25 mg/kg	**151.33**	**151.00**	**150.00**	**148.00**	**150.66**
	0.57	0.00	0.00	0.00	0.57
Control	**146.33**	**147.00**	**149.33**	**146.66**	**150.00**
	4.04	4.58	2.08	2.30	2.00

Table 26. Chloride (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**107.00**	**110.33**	**111.00**	**105.66**	**110.66**
	4.35	1.52	1.00	0.57	1.15
10 mg/kg	**104.00**	**110.00**	**109.66**	**104.33**	**110.00**
	2.00	2.64	0.57	2.51	0.00
25 mg/kg	**107.60**	**106.33**	**107.33**	**104.33**	**107.66**
	3.78	3.21	2.30	3.21	3.05
Control	**104.66**	**106.33**	**108.00**	**104.33**	**110.33**
	4.61	4.72	2.00	0.57	2.08

Table 27. Potassium (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**3.10**	**2.63**	**2.83**	**2.93**	**2.80**
	0.65	0.11	0.23	0.05	0.10
10 mg/kg	**2.70**	**2.56**	**2.76**	**2.83**	**2.66**
	0.30	0.15	0.28	0.23	0.11
25 mg/kg	**2.60**	**2.33**	**2.73**	**2.93**	**2.76**
	0.34	0.57	0.41	0.15	0.15
Control	**2.83**	**2.60**	**2.73**	**2.96**	**3.13**
	1.02	0.10	0.25	0.25	0.37

Table 28. Total calcium (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	2.29	**2.36**	**2.34**	**2.14**	**2.37**
	0.10	0.06	0.08	0.09	0.08
10 mg/kg	**2.34**	**2.34**	**2.31**	**2.12**	**2.33**
	0.10	0.11	0.17	0.11	0.10
25 mg/kg	**2.25**	**2.31**	**2.26**	**2.06**	**2.24**
	0.07	0.04	0.03	0.03	0.05
Control	**2.35**	**2.32**	**2.33**	**2.10**	**2.32**
	0.06	0.12	0.12	0.11	0.11

Table 29. Magnesium (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**0.66**	**0.57**	**0.63**	**0.57**	**0.60**
	0.11	0.07	0.09	0.07	0.06
10 mg/kg	**0.61**	**0.61**	**0.62**	**0.53**	**0.57**
	0.10	0.03	0.03	0.03	0.03
25 mg/kg	**0.60**	**0.56**	**0.58**	**0.52**	**0.53**
	0.03	0.01	0.01	0.02	0.03
Control	**0.69**	**0.60**	**0.64**	**0.53**	**0.62**
	0.18	0.04	0.10	0.02	0.04

Table 30. Phosphate (mmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**0.99**	**1.07**	**0.97**	**1.25**	**1.08**
	0.01	0.16	0.14	0.08	0.06
10 mg/kg	**1.18**	**0.95**	**0.98**	**0.99**	**1.08**
	0.08	0.30	0.18	0.12	0.06
25 mg/kg	**0.92**	**1.00**	**0.95**	**1.13**	**0.97**
	0.17	0.27	0.03	0.05	0.13
Control	**1.24**	**1.18**	**1.19**	**1.32**	**1.23**
	0.10	0.21	0.19	0.18	0.22

Hematological function

Table 31. Red cell count ($X10^{12}$/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**6.01**	**6.18**	**5.97**	**5.93**	**5.94**
	0.63	0.46	0.48	0.27	0.22
10 mg/kg	**6.21**	**6.39**	**6.15**	**6.08**	**6.20**
	0.28	0.13	0.13	0.17	0.13
25 mg/kg	**6.74**	**6.60**	**6.40**	**6.21**	**6.27**
	0.94	0.94	0.97	0.83	0.77
Control	**6.56**	**6.48**	**6.36**	**6.24**	**6.03**
	0.10	0.36	0.41	0.80	0.38

Table 32. Hemoglobin (g/dL)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**15.30**	16.07	**15.60**	**15.47**	**15.47**
	1.71	1.22	1.15	0.64	0.42
10 mg/kg	**15.33**	**16.20**	**15.63**	**15.40**	**15.63**
	1.25	1.28	0.93	0.56	0.45
25 mg/kg	**16.80**	**16.57**	**16.13**	**15.70**	**15.73**
	1.80	2.32	2.57	2.17	2.05
Control	**16.57**	**16.47**	**16.23**	**15.63**	**15.10**
	1.75	0.85	1.27	1.36	0.79

Table 33. Hematocrit (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**48.67**	**49.33**	**55.00**	**55.00**	**54.67**
	5.76	3.51	3.61	1.73	1.53
10 mg/kg	**48.83**	**50.33**	**56.00**	**55.00**	**55.67**
	5.70	3.06	2.65	1.00	1.53
25 mg/kg	**53.53**	**51.00**	**57.00**	**54.67**	**55.33**
	5.87	7.00	8.66	7.23	7.51
Control	**52.03**	**50.33**	**52.00**	**56.00**	**53.67**
	4.57	2.89	2.00	5.57	2.52

Table 34. Mean cell volume (fl)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**80.93**	**80.00**	**92.00**	**92.67**	**92.00**
	1.27	1.00	1.00	1.53	1.00
10 mg/kg	**78.47**	**78.33**	**90.67**	**90.00**	**90.00**
	5.82	3.79	3.51	3.00	4.36
25 mg/kg	**79.73**	**77.33**	**88.67**	**88.67**	**88.33**
	3.15	3.06	3.51	3.51	4.04
Control	**79.43**	**77.67**	**82.00**	**90.33**	**89.67**
	7.50	3.79	7.21	4.51	6.03

Table 35. Mean cell hemoglobin concentration (g/dL)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**31.47**	**32.67**	**28.67**	**28.33**	**28.33**
	0.29	0.58	0.58	0.58	0.58
10 mg/kg	**31.53**	**32.00**	**28.00**	**28.33**	**28.00**
	1.17	1.00	0.00	0.58	1.00
25 mg/kg	**31.43**	**32.67**	**28.33**	**28.67**	**28.67**
	0.57	0.58	0.58	0.58	0.58
Control	**31.83**	**32.67**	**31.33**	**27.67**	**27.67**
	0.29	0.58	2.08	0.58	0.58

Table 36. Mean cell hemoglobin (pg)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**25.47**	**26.00**	**26.33**	26.00	**26.33**
	0.31	0.00	0.58	0.00	0.58
10 mg/kg	**24.60**	**25.33**	**25.33**	**25.33**	**25.33**
	1.06	1.53	1.53	1.53	0.58
25 mg/kg	**25.07**	**25.33**	**25.33**	**25.33**	**25.00**
	1.33	0.58	1.15	1.15	1.00
Control	**25.30**	**25.33**	**25.67**	**25.33**	**25.00**
	2.52	1.53	1.53	1.53	2.00

Table 37. White cell count ($X10^9/L$)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**3.33**	**2.73**	**3.30**	**3.30**	**2.87**
	1.06	0.42	1.78	1.25	0.57
10 mg/kg	**4.91**	**4.33**	**4.27**	**4.37**	**3.87**
	1.73	2.83	2.12	2.31	2.14
25 mg/kg	**5.05**	**5.07**	**4.40**	**4.23**	**4.17**
	2.14	1.84	1.51	1.63	1.59
Control	**2.92**	**2.90**	**3.13**	**3.60**	**2.57**
	0.23	1.01	0.45	1.11	0.70

Table 38. Neutrophils (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**42.83**	**48.53**	**47.13**	**42.03**	**49.00**
	10.80	8.51	13.75	3.12	4.79
10 mg/kg	**56.83**	**51.07**	**50.53**	**55.57**	**60.93**
	6.53	9.92	13.67	7.71	7.74
25 mg/kg	**43.73**	**52.80**	**40.63**	**48.93**	**47.07**
	8.87	13.91	7.81	4.36	12.14
Control	**46.17**	**44.67**	**41.03**	**62.67**	**49.67**
	6.76	8.37	9.29	14.20	8.61

Table 39. Eosinophils (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**0.35**	**1.00**	**0.17**	**1.13**	**0.17**
	0.37	0.72	0.29	0.21	0.12
10 mg/kg	**0.35**	**1.20**	**0.87**	**0.33**	**0.10**
	0.54	0.75	0.55	0.21	0.00
25 mg/kg	**0.15**	**0.03**	**1.33**	**0.33**	**0.23**
	0.16	0.06	1.97	0.49	0.23
Control	**0.25**	**0.27**	**0.23**	**0.53**	**0.80**
	0.26	0.15	0.23	0.84	1.04

Table 40. Basophils (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**0.95**	**1.03**	**0.87**	**1.30**	**0.60**
	0.97	0.64	0.61	1.42	0.46
10 mg/kg	**0.78**	**1.27**	**0.70**	**0.70**	**0.77**
	0.44	1.42	0.52	0.36	0.60
25 mg/kg	**1.44**	**1.07**	**1.83**	**1.17**	**2.37**
	1.91	1.24	1.27	1.25	2.80
Control	**1.17**	**1.03**	**0.93**	**0.60**	**0.47**
	0.25	0.61	0.21	0.26	0.38

Table 41. Monocytes (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**10.16**	**6.67**	**4.87**	**6.60**	**6.77**
	1.05	2.61	2.96	3.69	2.19
10 mg/kg	**5.43**	**4.73**	**3.90**	**4.30**	**4.37**
	2.00	1.42	1.91	1.90	2.32
25 mg/kg	**6.04**	**4.73**	**6.37**	**4.50**	**7.07**
	0.38	2.06	1.50	1.73	1.66
Control	**7.14**	**4.40**	**4.10**	**4.87**	**4.40**
	1.69	0.78	2.48	2.56	1.13

Table 42. Lymphocytes (%)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**46.57**	**42.73**	**47.00**	**48.97**	**43.50**
	12.95	10.13	16.30	7.19	5.05
10 mg/kg	**36.63**	**41.77**	**43.97**	**39.07**	**33.87**
	8.06	10.09	14.76	9.21	9.83
25 mg/kg	**48.67**	**41.37**	**49.87**	**45.07**	**43.27**
	8.06	14.46	7.78	4.67	10.36
Control	**45.27**	**49.67**	**53.73**	**31.33**	**44.67**
	8.78	7.70	11.88	13.97	9.97

Table 43. Platelets ($X10^9/L$)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**211.33**	**245.33**	**235.00**	**266.00**	**259.00**
	33.61	35.35	55.67	27.62	35.00
10 mg/kg	**267.00**	**294.00**	**259.00**	**300.00**	**316.67**
	19.92	8.89	2.65	19.47	21.08
25 mg/kg	**243.67**	**260.67**	**241.33**	**262.67**	**275.67**
	54.78	49.07	76.06	61.60	62.01
Control	**251.67**	**231.33**	**218.67**	**251.00**	**292.67**
	88.44	61.71	54.98	78.08	54.59

Section II: Contraception

Abstract

Introduction: Except for the use of condoms and vasectomy, there are currently no established chemical methods of male contraception. Investigations on some promising compounds indicate that their antifertility effects are based on the interruption of spermatogenesis at different stages, and thus produce prolonged or even permanent sterility. Generally, the major drawback in the development of chemical contraceptives for men is the fear of genetic damage and permanent infertility. Therefore new and ongoing research into chemical compounds that have contraceptive effects on later stages of spermatogenesis (spermiogenesis or spermiation) or maturation of sperm in the epididymis is urgently needed. Oleanolic acid had promising contraceptive effects in rats. However, this compound has not been tested in non human primates, which are biologically similar to man. Thus, the aim of this study is to determine antifertility effects of this compound in vervet monkeys.

Materials and methods: This study took place at the same time as the toxicological trial, using the same monkeys. Sample collection schedules and durations of both investigations were the same. Testicular lengths and widths were measured at baseline and every second week, for 16 weeks. Semen samples were collected at these time points by rectal electrostimulation. Motility was determined by subjective methods based on estimation of total progressive motility and speed of forward progression. A hemocytometric method was used for the determination of sperm concentrations. Visual analyses of sperm morphology were carried out using bright field microscopy after Spermac staining. Percentage vitalities were determined by staining spermatozoa with vital stain, Hoechst 33258. Peanut agglutinin (PNA) was used to assess the sperm acrosomal integrity. Blood samples were collected at baseline and every fourth week for serum testosterone analysis. At the end of 16 weeks, treatment continued on each treated male, while they were mated

with a female for two months. Successful matings were determined by presence of spermatozoa on vaginal smear. After four weeks of mating, females were examined by ultrasound for pregnancy. Oleanolic acid administration was stopped after this mating period, and monkeys in the group that received 4 mg/kg oleanolic acid were remated for further four months to determine reversibility of the drug.

Results: Right and left testicular volumes were not statistically significantly different between controls and the treated groups. In all groups, sperm concentrations varied considerably between and within individuals with each successive semen collection. However, there were no statistically significant differences between controls and the treated groups.

Sperm forward progressive motility was statistically significantly reduced ($P = 0.0184$) in the group that received 4 mg/kg oleanolic acid, while reductions in the other treated groups were not statistically significantly different to the control group. No statistically significant differences were observed in the speed of forward progression between controls and the treated groups.

Although not statistically significantly different from the controls, the tapered sperm heads and nipple acrosomes were the most common head abnormalities in the treated groups. Statistically significant fluctuations were observed in the percentages of microcephalic ($P = 0.0007$) and acrosomal cyst ($P = 0.0019$) forms in the group that received 10 mg/kg oleanolic acid. Fluctuations in the amorphous bulging form were statistically significantly ($P = 0.0473$) different from the controls in the group that received 25 mg/kg oleanolic acid. Compared to head abnormalities, midpiece defects were most common in both controls and the treated groups. The majority of these imperfections, on which oleanolic acid had no significant effect, consisted of broken, bent and abaxially implanted midpieces. Tail defects were the second most common anomalies, with coiled and hairpin forms predominating. Changes in the hairpin forms in the group that received 25 mg/kg oleanolic acid were statistically significantly different ($P = 0.0249$) from the controls.

Although not statistically significantly different from the controls, the numbers of spermatozoa with intact outer acrosomal membranes were reduced in all treated groups, with a particularly pronounced reduction in the group that received 4 mg/kg oleanolic acid. A statistically significant increase ($P = 0.0276$) in the spermatozoa with patchy acrosomes was observed in the group that received 4 mg/kg oleanolic acid. No significant changes in spermatozoa with equatorial staining and absent acrosomes were observed. Reductions of live spermatozoa in the treated groups, particularly more pronounced in the group that received 4 mg/kg oleanolic acid, were not statistically significantly different from fluctuations in the controls. Although not statistically significantly different from the controls, serum testosterone concentrations were considerably reduced in the treated monkeys. All males copulated successfully with their female partners. The conception rate in the group that received 4 mg/kg oleanolic acid was zero, while this rate was 33 and 66% in the groups that received 10 and 25 mg/kg oleanolic acid respectively. During two months of drug withdrawal, there was an apparent return to normal fertility for the monkeys in the group that received 4 mg/kg oleanolic acid.

Discussions: Effects of oleanolic acid administration on the reproductive function of vervet monkeys were not dose-dependent. The finding that testicular volumes of monkeys in the treated groups were not affected, suggests that oleanolic acid is not associated with varicocele induction. Varying concentrations of spermatozoa in semen samples of the treated and control monkeys signal that oleanolic acid did not produce spermatogenic arrest. Compared to the controls, sperm from the oleanolic acid-treated monkeys showed considerable reduction of forward progressive motility, whereas the speeds of forward progression were relatively unchanged. This compound did not induce any specific morphological defects, since all categories of morphological abnormalities observed in spermatozoa from the treated monkeys were also present in the controls. However, increased occurrence of microcephalic and nipple acrosomal defects in the treated groups is an

indication of oleanolic acid effect on spermiogenesis. The changes in peanut agglutinin labeling of the spermatozoa from treated monkeys suggest that oleanolic acid interfered with acrosomal modification taking place during spermatozoa maturation in the epididymis. However, most of these spermatozoa were still viable at ejaculation. The marked decrease in testosterone levels associated with oleanolic acid treatment may render this drug suitable for reducing this androgen during sperm's epididymal maturation. The results of this study suggest that the mating ability of all males was not affected. The 4 mg/kg oleanolic acid dose was apparently effective in rendering males infertile, and these effects were reversible. According to the results obtained in this study, it is speculated that the effects of oleanolic acid are at the epididymal level.

Conclusion: From results of this investigation, it is evident that oleanolic acid has biphasic dose responses. However, it is concluded that oleanolic acid represents another approach toward development of a chemical male contraceptive.

Chapter 1

Introduction

Note: References for section II (contraception) are in pages 218 – 241

1. General introduction

During the 1950's, the world witnessed progress in contraceptive technology that can only be described as a revolution. Like other powerful revolutions, e.g. information, globalization, environmental, reproductive health, and gender equity, the contraceptive revolution has changed people's perception (Diczfalusy 1999*). This development broadened contraceptive choices, and has since offered protection with short or long term acting reversible methods. The products of this revolution have been used by millions of people all around the world, i.e. people with different socio-economic status, cultures, religious beliefs and value systems.

Since the first contraceptive revolution in the sixties, birth control has been practiced by about 900 million couples by the year 1990 (Diczfalusy 1999). In all developing countries, where it is thought that birth control is practiced less than in developed nations, the number of contraceptive users increased from an estimated 31 to 381 million between the years 1960-65 and 1985-90 (Fathalla 1994). However, when this revolution lost some impetus due to unmet needs of women, it encouraged the Rockefeller Foundation to adopt the Contraception-21 strategy, aimed at developing novel contraceptive approaches for the current century (Fathalla 1999). Since its adoption, this strategy has led to new developments in female contraceptive technology, which includes introduction of hormone-releasing intrauterine devices, hormone implants and femidoms (Andrews 2000). This provided women with reliable methods, independent of male co-operation, to regulate and control their fertility.

The appropriate use of modern contraceptive methods is effective and safe for most women, and has had considerable positive impact on maternal and infant health, and on population growth (Diaz 1998). However, this dramatic success in the currently available contraceptive methods has been predominantly targeted towards the female clientele, with few significant developments in male fertility regulation for over a century (Hair and Wu 2000). Among other factors that hampered research into new male contraceptive methods, is the fear of litigation should the method have deleterious effects, such as genetic damage and impotence (Greep 1998), and being irreversible. Ultraconservatism or hostile attitude of men, towards taking part in fertility control is not a major concern anymore, since surveys showed that men from both developed and developing countries present with the same degree of family planning and child spacing acceptance (Chipfakacha 1993, Duarte 1998, Ozvaris *et al.* 1998). Additionally, a survey shows that women in these countries show high level of trust (70 - 90%) that their partners will use the 'male pill' reliably (Glasier *et al.* 2000).

Despite their widespread use, current male contraceptive practices (premature withdrawal, condom and vasectomy), do not fully meet the requirements of an ideal male contraceptive method, which includes high efficacy, little or almost no side effects and easy reversibility when withdrawn (Frick and Aulitzky 1988). For example, the surgical nature of vasectomy, together with the reversibility problems and suspected link with subsequent prostate cancer (Comhaire 1994), render this method far from ideal. The past few decades have witnessed an enormous increase in research on the development of an ideal chemical male contraceptive method. Of the currently pursued five strategies of male fertility control (Puri *et al.* 2000), inhibition of spermatogenesis and interference with sperm maturation in the epididymis, are the two potential sites in the male reproductive processes that can be used as targets for fertility regulation (Prasad and Rajalakshmi 1976). However, before discussing the potential of these sites in male contraception, it is important to review testicular function and hormonal control of spermatogenesis.

1.1. Testicular function

The testis is composed of two compartments, i.e. seminiferous tubules and interstitial cells, with functions which seem to be interrelated. The identification of two gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary, which are released under the influence of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Schally *et al.* 1971), indicates the independent control of these compartments. Receptors for FSH are located on the Sertoli cells in the seminiferous tubular compartment, and those for LH are on the Leydig cells in the interstitial cell compartment (Simoni *et al.* 1999).

1.1.1. Spermatogenesis

Investigations into spermatogenesis have established that it involves complete series of the successive cellular associations appearing in sequence with time in any particular area of the seminiferous tubule (Barr 1973). It involves series of mitotic divisions of spermatogonial (stem) cells and followed by meiotic divisions, which involves both primary and secondary spermatocytes to form spermatids (Barr 1973). The latter then undergo extensive metamorphic remodeling during spermiogenesis. Finally, free cells are released into lumen of the seminiferous tubules during a process called spermiation. In the rat and humans, this process takes 48 - 52 and 64 days respectively (Clermont 1972).

Specialized seminiferous tubular environment for successfully completion of spermatogenesis is achieved by organization of the Sertoli cells to form blood-testis barrier, which prevents free exchange of fluids between the seminiferous tubules and blood (Li *et al.* 2001). However, some substances pass at different rates through this barrier. For example, proteins enter the tubules slowly, whereas gonadotropins and testosterone (Cooper and Waites 1975), and α-chlorohydrin

(Edwards *et al.* 1975) penetrate the barrier very rapidly. A precursor of testosterone, cholesterol, is excluded from entering the barrier.

1.1.2. Hormonal control of spermatogenesis

Testosterone is a necessary prerequisite for the maintenance of spermatogenesis in the adult testes, as well as for the restoration of spermatogenesis in testes induced experimentally to become azoospermic (Zirkin 1998). The requirement of testosterone for normal spermatogenesis is demonstrated by positive correlation between testosterone synthesis and sperm output. For example, blockade of the LH receptor with the LH-receptor peptide antibody, results in a significant reduction of serum testosterone and inhibition of spermatogenesis (Moudgal *et al.* 2001). Although precise functions of FSH and LH remain to be defined, considerable evidence exist that they are able to stimulate all phases of mammalian spermatogenesis. FSH appears to stimulate spermatogenesis by decreasing the number of germ cells that normally degenerate (Billig *et al.* 1995). Absence of LH receptors in the seminiferous tubules indicates that Leydig cells mediate effects of LH on spermatogenesis through production of testosterone (Simoni *et al.* 1999). In turn, testosterone acts on germ cells indirectly by stimulating the Sertoli cells (de Kretser *et al.*1998). This mediation of hormonal signal through the somatic cells is due to the apparent presence of the testosterone receptors on the Sertoli cells, and not on the germ cells (Verhoeven 1992).

The main effects of testosterone are directed towards successful completion of spermiogenesis (O'Donnell *et al.* 1996), suggesting that maintenance of the pre-meiotic cells (spermatogonia and spermatocytes) may be testosterone independent. Testosterone is highly involved in the conversion of round spermatids, between stages VII and VIII of the spermatogenic cycle, to elongated spermatids (O'Donnell 1994). However, without a major involvement of FSH, testosterone alone is less effective in maintaining the conversion of spermatocytes to round spermatids (Sun *et al.* 1990).

Reduction in testosterone serum levels between stages VIII to IX result in the unstable attachment of spermatids to Sertoli cells (Cameron *et al.* 1993), suggesting that proper attachment of spermatids to the Sertoli cells is important, thus preventing their immature detachment from the epithelium.

1.2. Epididymal function

Although the exact process of epididymal sperm maturation is inconclusive, it is accepted that spermatozoa are directly bathed in the fluid or microenvironment provided by epithelial secretions of the seminiferous tubules and the epididymal ducts (Hinton 1980). Wilson and French (1976) showed that cytoplasm and nuclei of mammalian epididymis possess androgen receptors, which are similar in physico-chemical characteristics to those in the testes and prostate. This concept is supported by findings that expression of messenger ribonucleic acid (mRNA), which in turn regulates protein synthesis in the epididymis, is highly dependent on the presence of androgens and testicular factors (Cornwall *et al.* 2001). Several maturational changes on the independent living spermatozoa in the epididymal milieu have been documented. However, sperm motility, surface changes and the segregation of certain proteins and lipids to specialized domains of the sperm plasma membrane, are among the most frequently studied phenomena (Jones 1999). Gatti *et al.* (2000) reported that specific regions of the epididymal epithelium secrete and integrate into the sperm membrane the glycoproteins, which modify surface characteristics of sperm in preparation for the events of fertilization. In short, these findings indicate that any biochemical changes to the specialized microenvironment will have direct effect on sperm maturation. Against this background, it seems possible to develop compounds that act directly on the spermatozoa or that exert their action on the epididymis and thus disrupts formation of a suitable microenvironment around the spermatozoa.

1.2.1. Changes in motility patterns

The concept that mammalian spermatozoa released from the seminiferous tubules are highly differentiated but still lack the ability to move forward, partly due to immaturity of the plasma membrane, is widely accepted. In support of this theory, White and Volglmayr (1986) observed that testicular spermatozoa can move almost as actively as their cauda epididymal counterparts, only after membrane removal and exposure to adenosine triphosphate, cyclic adenosine monophosphate and magnesium ions. Similarly, caput epididymal sperm of the rat show different patterns of flagellation, i.e. vibrating, motile in place and motile with a static curvature of the midpiece resulting in a spinning motion or a circular path (Jeulin *et al.* 1996). However, those from the cauda epididymis usually swim with a vigorous motion that results in forward rapid movement. This indicates that there are physiological and biochemical changes that spermatozoa undergo during their maturation in the epididymial duct. Despite increases in sperm motility and the ability to move progressively (Soler *et al.* 1994), it has been reported that cauda epididymal sperm are held in a quiescent state by factors of the epididymal fluid, and it is not until they are ejaculated or diluted into an appropriate medium that they rapidly display active motility and the greatly enhanced metabolic rate (Wong 1990).

In bovine, the quiescence of spermatozoa produced by cauda epididymal fluid was found to be strongly dependent upon the extracellular pH, i.e., motility is inhibited at pH 5.5 but not at pH 7.6 (Carr and Acott 1984). In rats, immobilization of the cauda epididymal spermatozoa is caused by "immobilin", a protein factor which creates a highly viscoelastic environment (Turner and Reich 1987, Usselman and Cone 1983). In search of the site of origin of this protein factor, Turner and Giles (1982) suggested that it could be of testicular origin. However, Ruiz-Bravo (1988) conclusively found that the principal cells in the caput epididymis synthesize and secrete this protein into the lumen of the tubules to travel with the sperm into the cauda epididymis.

1.2.2. Changes in sperm membrane lectin-binding properties

Despite being immature, plasma membranes of mammalian spermatozoa already have both the membrane-integrated and the surface-adsorbed protein. It is during epididymal maturation when some of these intrinsic proteins are located elsewhere in or on the plasma membrane, altered, masked or replaced by tight-binding proteins of epididymal origin (Kohane *et al.* 1980, Vreeburg *et al.* 1992, Retamal *et al.* 2000). In addition, functional changes in plasma membrane composition and organization during epididymal transit are reflected by changes in the lectin binding properties (Sarkar *et al.* 1991, Fourie *et al.* 1996). These changes are particularly important for spermatozoa to specifically recognize the complimentary binding sites on the zona pellucida during sperm-oocyte interaction.

The epididymal glycoprotein modifying enzymes, glycosyltransferases (synthetic enzymes) and glycosidases (hydrolytic enzymes), are responsible for this modification of sperm surface glycoproteins (Tulsiani *et al.* 1998). With regard to the findings that only plasma membranes of mature epididymal sperm possesses PNA specific lectin-binding glycoprotein (GP-83), it is presumed that the principal cells of the corpus and cauda epididymides secrete and conjugate GP-83 to the spermatozoa during epididymal transit (Liu *et al.* 2000). According to Hamilton *et al.* (1986), these dramatic changes in the lectin-binding ability of the sperm surface, are indications of active glycosylation of sperm surface components. Srivastav (2000) reported that due to the high degree of differences in glycosylation between immature and mature epididymal sperm surfaces, these differences in modifications of the glycoproteins could be used as markers of sperm maturation during the screening of antifertility agents acting at the epididymal level.

1.3. Sperm morphology

1.3.1. The normal sperm

Normal mammalian spermatozoa have similar cytoskeletal features, which are species-specific in terms of the size and shape of the head, and the length and relative size of the components of the tail. However, it is these components of the head and features of the tail that reflect the specialized structural organization and unique functional activities of the mammalian spermatozoa. The next sub-sections summarize cytoskeletal characteristics of spermatozoa.

1.3.1.1. The head

The acrosome and nucleus of most mammalian spermatozoa that have spatulate heads are usually symmetrical. The unique features of the sperm nucleus are the organization and amount of its deoxyribonucleic acid (DNA), and the arrangement and composition of its nucleoproteins (Ward and Coffey 1991). At the acrosomal region, plasma and outer acrosomal membranes of the acrosomal cap fuse and vesiculate during acrosomal reaction, leading to releases of hydrolytic enzymes involved in fertilization. The equatorial region of the spatulate head spermatozoon forms a narrow band that nearly overlies the sperm head. It is this region and the anterior portion of the post acrosomal region that initiate fusion with the oocyte membrane during fertilization.

1.3.1.2. The tail

The tail is composed of the neck, the midpiece, the principal piece and the end piece. The neck forms the basal plate, which continues with nine coarse fibers that project posteriorly throughout most of the tail. The midpiece has nine radially and two centrally arranged microtubules, surrounded by nine coarse fibers, which possess both elastic and contractile properties. Additionally, the entire midpiece is covered by helically arranged mitochondrial sheath, which is believed to generate the energy needed for sperm motility. A fibrous sheath surrounding the

axoneme and its associated coarse fibers of the principal piece, provide stability for the contractile elements of the tail. The end piece lacks any components other than the microtubules of the axoneme covered by the plasma membrane.

1.3.2. The abnormal sperm

1.3.2.1. The head

Table 1.1 summarizes common mammalian sperm head abnormalities, some of which are mostly as a result of genetic defects of primary spermatocytes and spermatogonia.

Table 1.1. Common mammalian sperm head abnormalities

Abnormality	Characteristics or causes of the abnormalities
Microcephalic	Possibly could be a familial trait producing sperm with small nucleus and no acrosome (Nistal *et al.* 1978). Age is also a contributory factor (Bujan *et al.* 1988).
Macrocephalic	Probably of familial genetic origin, it indicates meiotic division deficiency, where spermatozoa are tetraploid (Escalier 2002). Spermatozoa undergo a threefold increase in nuclear volume and acrosomal hyper-development and malformation (Escalier 1983).
Amorphous	Numbers increases in vasectomized men (Wen *et al.* 1999) and in patients with varicocele (Rodriguez-Rigau *et al.* 1981).
Duplication	Caused by inflammatory processes either of microorganisms or immunologic origin (Hofmann *et al.* 1992). Spermatozoa are binucleated, with both nuclei separately packaged within the sperm head (Burkhart and Malling 1989).
Tapering	Environmental factors or stress, acting via the Sertoli cells on the latter stages of spermatogenesis, play a role in this defect (MacLeod 1974).
Round heads	Associated with incomplete second meiotic division (Kullander and Rausing 1975), round head sperm lack acrosome (Tyler *et al.* 1985). Sperm with this defect are unable to interact with and penetrate the oolemma (Syms *et al.* 1984).
Acrosomal cysts	Although not clear whether it is hereditary, cyst formation occurs during spermiogenesis (Blom and Birch-Andersen 1975).
Vacuolated	In an oligoasthenoteratozoospermia patient, this condition was believed to have resulted from a genetically induced spermiation failure (Benzacken *et al.* 2001).
Pyriform	In bulls, spermatozoa have a reduced capability to bind to and penetrate the zona pellucida (Thundathil *et al.* 1999). In cases where fertilization occurs, zygotes appear to have a reduced ability to initiate cleavage.

1.3.2.2. The midpiece, principal and end piece

Tables 1.2 and 1.3 summarize common sperm midpiece and principal as well as end piece respectively.

Table 1.2. Sperm midpiece abnormalities

Abnormality	Characteristics or causes of the abnormalities
Bent	This defect is linked with high zinc content in the outer dense tail fibers (Blom and Wolstrup 1976). Zinc is negatively correlated with sperm motility and velocity (Henkel *et al.* 1999).
Asymmetrical insertion	It was observed that Maltese poodle with this defect are unable to impregnate fertile bitches (Oettlé and Soley 1985).
Detached	This defect is associated with ultrastructural abnormality in the neck or implantation region of the spermatozoon (Blom 1977).
Stripped mitochondria	The deficit in mitochondria has been seen in asthenozoospermic men (Mundy *et al.* 1995). Since energy for sperm movement is provided by this organelle, its absence explains poor sperm motility seen in semen samples from these men.
Corkscrew	Defects occur due to irregular distribution or spiraling of the midpiece mitochondria, compared to the regular mitochondrial helices in normal mammalian spermatozoa (Blom 1978).
Thickened	As seen in asthenozoospermia men, thickened midpieces contain supernumerary mitochondria (Piasecka *et al.* 1998).

Table 1.3. Sperm principal and end piece abnormalities

Abnormality	Characteristics or causes of the abnormalities
Coiled	Elevated zinc levels in sperm and seminal plasma cause coiling of the principal piece (Blom and Wolstrup 1976). Defective migration of the cytoplasmic droplet is also a cause of this defect (Holt 1982, Pelfrey *et al.* 1982).
Hairpin	This defect, causing the spermatozoa to progress backward, has been seen in bull spermatozoa after cryopreservation (Phillips and Kalay 1984) and in other species.
Duplicate	Nistal *et al.* (1977) observed that the axonemes, which were arranged in parallel and enclosed by same plasma membrane throughout the middle piece, separate at the beginning of the principal piece to become individual tails.

1.4. Sperm motility

Sperm motility is one of the essential functional properties of spermatozoa that can be correlated with fertilizing capacity. It is comprehensively described in the previous section that spermatozoa from mammalian species acquire the potential to move in a forward direction during their transit through different regions of the epididymis. Acquisition of this type of motility is important for the spermatozoa to reach the site of fertilization. In support of this notion, Gaddum-Rosse (1981) observed that after uterine insemination in rats, only forward progressing spermatozoa could negotiate the uterotubal junction.

Despite changes during epididymal transit, sperm also undergo additional changes in the pattern of motility after residing in the female reproductive tract. As capacitation proceeds, sperm become hyperactivated, and display a movement with an increased thrust and whiplashlike beating of the flagella, which is believed to aid spermatozoa in swimming through the viscous fluid of the oviduct (Demott and Suarez 1992), as well as in the penetration of the zona-intact eggs. According to this physiological role, it appears that those spermatozoa that become hyperactivated too soon at a wrong site will eventually be exhausted and thus fail to fertilize the ovum.

The introduction of Computer Aided Sperm Analysis (CASA) systems to obtain objective measurements of the trajectories of human spermatozoa has revolutionized the techniques used to assess the quality of sperm motility. Despite the diagnostic value of such systems, it has been observed that the expense and expertise required to run such functional systems has narrowed their use by many infertility clinicians (Irvine and Aitken 1994). It is suggested that this problem is mostly encountered in the developing or third world countries compared to the developed world. Therefore, these practitioners turn to the conventional or subjective methods of sperm motility analysis. It will be noted in the next chapter that the latter method was also used in this study.

1.5. Acrosomal integrity

1.5.1. Lectins as molecular probes in fluorescence microscopy

In modern biology, fluorescence microscopy has become an indispensable tool for probing the mechanism of cell function and localization of cellular components, i.e. molecules and organelles, using target-specific fluorescent probes/lectins (Pedley 1997). The latter allow deep understanding of cell and organelle biology and cellular visualization of the general cyto-architecture as close to the native organization as possible (Opas 1999). For example, spermatozoa are stained with lectin-labeled fluorescent dyes for the visualization/ assessment of their acrosomal integrity (Zeginiadou *et al.* 2000).

What are lectins, and why are they so important in surface and membrane biology? They are sugar binding proteins or glycoproteins of non-immune origin, which are isolated from a wide variety of natural sources (Sigma catalogue 2000-2001). Due to their wide abundance in the plant kingdom, lectins serve as, (1) a recognition factor between symbiotic nitrogen fixing bacteria and host plants, (2) a deterrent to phytopathogens like fungi, insects, and animals, (3) a storage protein, and (4) an aid in sexual reproduction in *Chlamydomonas* (Sengupta *et al.* 1997). Lectins are important in surface and membrane biology because they bind mono- and oligosaccharides with great specificity, and can interact with sugar moieties of polysaccharides, glycoproteins, and glycolipids on cell walls and surface membranes of both prokaryotic and eukaryotic cells (Lotan 1979). They are potential mediators in recognitive processes and cell adhesion by interaction with glycoconjugates. In cancer studies, structural transformation of cells from normal to malignant stage is accompanied by changes in cell membrane, and hence lectin binding patterns are used to indicate the invasive potential of tumors (Haseenabeevi *et al.* 1991).

In moribund or dead spermatozoa, the outer acrosomal membrane, together with its overlying plasma membrane, may either be partially or totally destroyed or detached from the head of the spermatozoa. It is for these reasons that assessment of the acrosomal status is increasingly used in evaluating the effects of novel contraceptive drugs on the sperm acrosome. Advances in modern light microscopy, bright field or fluorescence, have largely facilitated quantitative studies and improved imaging of cells, including spermatozoa. Notwithstanding the considerably lower spatial resolution of light microscopy compared to electron microscopy, its essentially non-invasive nature provides a unique tool for analyzing behavior of cells, even at a single stage, and thus bringing new insight into both cellular diversity and cell to cell interactions (Pedley 1997).

1.5.2. Lectin binding specificity on spermatozoa

The binding sites of lectins, even of the same group of compounds, i.e. with similar structural features, are diverse. It is the exact shape of the binding sites and the nature of the amino acid residues to which the carbohydrate is linked that determines the specificity of each lectin (Sharon and Lis 1995). Therefore, any changes to these features, such as substitution of one or two amino acids, will markedly change binding specificity of the lectin. Although some lectin binding sites on spermatozoa are generally comparable to those of many somatic cells, the distribution of most lectins is highly restricted to certain domains of the cell surface. For example, domains that lectins interact with are often related to specific underlying morphological entities such as the acrosome, postacrosomal region, and mitochondria associated with the middle piece (Koehler 1981). Ahluwalia *et al.* (1990) discovered that lectins with specificity for mannosyl, glucosyl, and sialic acid predominantly stain the sperm acrosomal region, whereas those specific for fucosyl residues predominantly stain the postacrosomal regions. In addition, there is a higher density of lectin-binding sites on sperm plasma membrane of the head than of the tail (Lee and Ahuja 1987). The locations of these binding sites on the sperm head from different species, as demonstrated with the

use of different fluorescent-conjugated lectins, are provided in Table 1.4. It is important to note that lectin specificities are not altered by conjugation to these fluorescent markers. Fluorescein isothiocyanate (FITC) is one of the most widely used fluorescent labeling reagents due to conjugate stability and the fluorophore's high quantum efficiency. FITC has a yellow-orange color with absorption maximum at 495nm, and emits a yellow-green color at 520nm (Sigma catalogue 2000-2001). FITC conjugates of lectins, antibodies, hormones and growth factors have been used in variety of immunohistochemical and immunocytochemical, using both visible and fluorescence microscopy, and in flow cytometric applications (Sigma catalogue 2000-2001[a]).

The pioneer work on labeling permeable sperm plasma membrane with internally directed lectin, *Ricinus communis* agglutinin (RCA), should be ascribed to Talbot and Chacon (1980). Like other lectins, RCA has a greater binding affinity on sperm head than the tail (Nicolson and Yanagimachi 1974), with intense binding to the anterior and weaker to the equatorial acrosomal regions (Marti *et al.* 2000). However, the use of RCA in monitoring sperm acrosomal status and/or acrosome reaction was never popular because of the high toxicity of ricin from castor oil plant seeds. This toxin consists of two-disulfide bonded polypeptide chains, i.e. an enzymatically active A chain and a B chain with lectin properties (Olsnes and Pihl 1973). Internalization of ricin into the cell is mediated through binding of its B chain to the cell surface glycoconjugate with non-reducing terminal galactose (ricin receptors), followed by slow endocytosis and release of the free A-chain into the cytosol (Morino *et al.* 1995). The translocation of bound toxin from the cell surface to the cytosol is under retrograde transport through the Golgi apparatus to the endoplasmic reticulum (Olsnes and Kozlov 2001). Once inside the cell, the toxin inactivates ribosomes by depurinating single adenosine residue in 28S ribosomal RNA, and thus inhibiting protein synthesis (Endo and Tsurugi 1987).

Table 1.4. Lectin specificity and regional heterogeneity of the sperm plasma membrane from different species

Lectin	Sugar specificity	Binding site
SBA	α-D-GalNAc,D-Gal	Principal segment of the anterior acrosome of guinea pig sperm (Koehler 1981).
Con A	α-D-Glc,D-Man	Anterior acrosome of mouse sperm (Edelman and Millette 1971), and inner acrosomal membrane of human sperm (Holden *et al.* 1990).
WGA	$[\beta(1\text{-}4)$D-GalNAc$]_2$	Anterior acrosome of guinea pig and goat sperm (Schwarz and Koehler 1976, Sarkar *et al.* 1991).
PSA	α-D-Glc, α-D –Man	Acrosomal contents of human sperm (Cross *et al.* 1986).
RCA	β-D-Gal, D-GalNAc	Anterior acrosome of ram spermatozoa (Marti *et al.* 2000).
PNA	D-Gal,β[1-3]GalNAc	Anterior acrosome of mouse sperm and outer acrosomal membrane of human sperm (Mortimer *et al.* 1987, Hennigar *et al.*1987).

SBA= Soybean agglutinin, Con A= Concanavalin A, WGA= Wheat-germ agglutinin, PSA= *Pisum sativum* agglutinin, PNA= Peanut agglutinin.

Subsequent to the toxicity of RCA, FITC-PSA and FITC-PNA were identified as suitable substitutes for acrosomal staining. However, Cross *et al.* (1986) found that PSA bind to acrosome reacted human spermatozoa, and that its labeling (which is inhibited by α-methyl mannoside and not by β-D-galactose) was localized within the acrosomal contents. However, these investigators found that PNA, which has very similar sugar specificity as RCA, bind to spermatozoa which still have an outer acrosomal membrane present. Therefore, with the use PNA, acrosomally stained cells will indicate intact acrosomes, while unstained cells indicate acrosomal loss. Mortimer *et al.* (1987) and Cheng *et al.* (1996) reported specific binding of PNA to the outer acrosomal membrane of human and stallion spermatozoa respectively. Binding of PNA to the outer acrosomal membrane of macaques is regardless of their epididymal origin (Yeung *et al.* 1996). It is for these reasons, that

PNA was selected for use in assessing acrosomal integrity in this study. Structurally, PNA is a tetramer composed of four identical subunits and has a molecular weight of 110,000 (Lotan *et al.* 1975). It agglutinates lymphocytes from mouse, rat, guinea pig, and man, but only after their treatment with neuraminidase (Novogrodsky *et al.* 1975). However, agglutination of neuraminidase-treated human erythrocytes by PNA is inhibited by galactose (Shanker and Das 2001).

Although the use of FITC-PNA in detailed evaluation of sperm acrosomal status is well established, when compared to the triple-stain technique (Talbot and Chacon 1981), it does not provide a simultaneous assessment of sperm vitality. Therefore, Mortimer *et al.* (1990) suggested the inclusion of fluorescent dye, Hoechst 33258, as a supravital stain in the FITC-PNA labeling procedure. Hoechst 33258 is very often used to detect deoxyribonucleic acid (DNA), and like DAPI preferentially binds to A-T rich sequences of DNA (Holmquist 1975, Schnedl *et al.* 1977). This dye has limited permeability to the plasma membrane, and therefore only nuclei of dead spermatozoa, with permeable or damaged plasma membrane, will pick up the dye and stain blue, whereas living spermatozoa with intact plasma membranes will remain unstained.

1.6. Possible strategies for male fertility control

1.6.1. Inhibition of spermatogenesis

Although the chance of pregnancy is significantly reduced at very low sperm density, it is still practically possible if even only a few motile sperm are present. Administration of progestins and androgens, either alone or in combination with other gonadotropin-suppressing agents, are able to suppress the secretion of LH and FSH (Reddy 2000). Amory and Bremmer (1998) showed that reduction in the concentration of the gonadotropins leads to a decrease in endogenous testosterone

secretion from the testis, and thus deprives the developing sperm of the signals required for normal maturation.

It is well documented that administration of testosterone suppresses production of gonadotropins and, hence, inhibits spermatogenesis. In normospermic men, intramuscular and oral administration of active testosterone or testosterone in combination with progesterone leads to a reversible azoospermia (Reddy 2000). A weekly injection of 200 mg-testosterone enanthate, the longer-acting ester, suppresses LH and FSH to undetectable levels in men (Anawalt and Amory 2001). However, common side effects associated with this regimen include acne, fatigue, weight gain, and reductions in the plasma levels of HDL (Wu *et al.* 1996, Bhasin *et al.* 2001). Suppression of gonadotropins with the result of azoospermia and severe oligozoospermia has been achieved with a six weekly interval injection of 1000 mg-testosterone undecanoate, either alone or in combination with levonorgestrel (Kamischke *et al.* 2000). Administration of this regimen also leads to a decrease in HDL cholesterol, besides being better in terms of having longer half-life compared to testosterone enanthate. Results of a study using subdermal implants of four 200 mg-testosterone pellets in combination with single intramuscular injection of 300 mg-depot medroxyprogesterone acetate, showed an enhanced extent of spermatogenic suppression, which lasted for six months, without any serious androgenic side effects (Handelsman *et al.* 1996). These results are in contrast to the findings that administration of testosterone promotes the development of prostate cancer (Morales 2002).

Although male fertility control at the testicular level appears to be achievable, there is a vital factor to be considered before any chemical can be safely regarded as an ideal male contraceptive agent. LH deprivation that in turn leads to decrease in endogenous testosterone production could pose a

possible loss in *libido* and potency. This is not the case with the female steroidal pills, since the LH surge necessary to stimulate ovulation is readily suppressed by progesterone.

1.6.2. Interference with sperm maturation

Although inhibition of spermatogenesis proves to be a successful method of male contraception, its use may be limited due to possibilities of undesirable side effects. Therefore, aspects of epididymal physiology have received considerable attention in research with the aim of finding new approaches to male contraception at a post testicular level.

The antifertility effects of low doses of α-chlorohydrin, a monochloro-derivative of glycerol, are directed towards mature sperm. In rats, α-chlorohydrin accumulates in the lumen of the terminal segment of the epididymis and acts directly on the spermatozoa (Back *et al.* 1975). This mechanism, which is based on inhibiting the activity of the glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase), hinders glycolytic metabolism of glucose and other sugars by spermatozoa (Brown-Woodman *et al.* 1975). As a result, inability of spermatozoa to synthesize adenosine triphosphate by the time they are ejaculated, they will appear morphologically normal, but will also be deprived of potential to generate energy, which is necessary for the maintenance of motility. This mode of action has the advantage over spermatogenic inhibitors, because of the fast onset of infertility after administration, and almost immediate restoration of fertility after withdrawal of the contraceptive agent. However, α-chlorohydrin also shows some species-specificity, i.e. it is effective in rat, ram, boar, guinea pig, hamster, rhesus monkey and upon ejaculated human sperm, but ineffective against epididymal spermatozoa from a mouse and rabbit (Jones 1983). The contraceptive failure in these species could be due to the presence of a barrier that restricts the entrance of the drug into the lumen of the epididymal duct (Back *et al.* 1975). Despite an apparent toxicity when administered at high doses, the simplicity of use and the site of

antifertility activity of α-chlorohydrin justifies more research on this compound. The chlorinated sugar, 6-chloro-6-deoxyglucose, impairs sperm motility and produce a metabolic profile similar to spermatozoa recovered from rats treated with α-chlorohydrin (Ford *et al.* 1981). However, this inhibitory activity on the metabolic process of mature rat sperm is only feasible *in vivo* but not *in vitro*. Therefore, it is possible that this compound undergoes conversion *in vitro* to an active inhibitory metabolite.

Triptolide, a diterpene isolated from a Chinese medicinal plant (*Tripterygium wilfordii Hook F*), has been suggested as a possible candidate for post-testicular contraception. Hikim *et al.* (2000) reported that daily dose, for 70 days, of triptolide to male rats, causes changes of the cauda epididymal sperm. The most conspicuous changes observed by the authors were complete absence of plasma membrane over the entire middle and principal piece, premature chromatin decondensation of the nuclei and the disorganization of the mitochondrial sheath with many vacuolated mitochondria. Huynh *et al.* (2000) showed that, in addition to these effects, longer treatment with triptolide also leads to sperm head-tail separation as well as to aggregation of tails.

With the understanding of involvement of androgens in sperm maturation, several studies investigated the potential of anti-androgens as post-testicular antifertility agents. Daily administration of Cetrolix (a GnRH antagonist) to cynomolgus monkeys lead to 80 and 50 percentage reduction in serum and epididymal androgen levels respectively (Yeung *et al.*1999). Except for velocities, other sperm motion parameters are not affected, indicating insufficiency of this compound if it was to be used as a male contraceptive. Since it was found that cyproterone acetate has an antifertility action in rats without loss of *libido* (Whalen and Luttge 1969), this antiandrogen has been a subject of several investigations in this field. In rhesus monkeys, administration of cypoterone acetate reverses the androgen-dependent maturational changes

occurring between corpus and cauda epididymides, and prohibits migration of cytoplasmic droplet from proximal to the distal end of the midpiece (Kaur *et al.* 1990). In Black Bengal goats (a dwarf breed), cyproterone acetate produced a significant reduction in epididymal sperm concentration and progressive motility, lead to retention of the cytoplasmic droplet by the cauda epididymal sperm, and altered the length and width of the sperm head (Panda and Jindal 1982). It also produces moderate reduction in height of the epithelium, and the number of narrow and apical cells (Kumar and Panda 1983).

Even though there is currently no available chemical drug for immediate application in the extragonadal control of sperm fertilizing capacity, the theoretical advantages of this method of fertility control are useful in the quest for a safe and reversible male contraceptive.

1.7. Use of non-human primates and suitability of vervet monkeys in reproductive research

Non human primates remain essential models for human reproductive studies. Similarities in the endocrine control of spermatogenesis in the male and ovarian function in the female, which exhibit menstrual cycle, exist between human and non human primate species (Moudgal and Sairam 1998, Hatasaka *et al.* 1997). The physiological response to endocrine stimuli, which is common to both humans and primates, make them suitable for the testing and development of novel methods of fertility control. It has been suggested that studies involving molecular changes associated with the acquisition of fertilizing capacity in primate spermatozoa are vital for the development of specific methods for male fertility control at the cellular level (Young *et al.* 1985).

The vervet monkey has been suggested as an alternative experimental model in reproduction to the traditionally used Old World primate species such as rhesus monkeys and baboons (Gombe *et al.* 1980). The moderately small size of the vervet monkey makes it less expensive than many other Old World primates, and yet it is large enough for most biomedical procedures (Else 1985). Vervet monkeys adapt and breed successfully all year round in captivity (Seier 1986, Fairbanks and McGuire 1984), and are not affected by seasonal variations. Both in captivity and in the wild, mating is not only restricted to the peri-ovulatory phase, but rather occurs throughout the entire menstrual cycle, during pregnancy and lactation (Rowel 1971). This is in contrast to other male primate species, which are subjected to seasonal variations in testicular volume/size, spermatogenic arrest, semen quality, sperm number, spontaneous ejaculation, sexual behavior, frequency of birth rate, and concentrations of reproductive hormones (Gupta *et al.* 2000). For example, testicular volume and circulating testosterone levels increase significantly during the mating season in adult male mandrills (Setchell and Dixson 2001) and in rhesus monkeys (Herndon *et al.* 1996). Formation of spermatozoa in the male vervet monkey follows similar stages to other mammals. The total duration of spermatogenesis, timed from the first appearance of the B spermatogonia, is estimated to be between 39 and 48 days (Barr 1973). The ultrastructure of Leydig cells, even though its endoplasmic reticulum is more extensive and polymorphic than in man, is similar to those described in other mammals (Camatini *et al.* 1981).

1.8. Objective of the study

To determine the effect of oleanolic acid on the reproductive physiology of male vervet monkeys, with special reference to the following parameters:

(a) Testicular volume.

(b) Sperm concentration.

(c) Sperm morphology.

(d) Sperm motility.

(e) Sperm acrosomal integrity and vitality.

(f) Testosterone concentration.

(g) Fertility.

Chapter 2

Materials and methods

2.1. Ethical approval, subjects, housing conditions, diets, treatments and anaesthesia

See toxicology section

2.2. Testicular volume

Widths (w) and lengths (ℓ) of right and left testicles were measured using a Vernier caliper (Mitotuyo, Japan) every two weeks throughout the study period. Volume of each testicle was calculated by using an elipsoid formula:

$$\text{Volume} = \pi w^2 \ell/6$$

where π= 22/7, length and width measured in centimeters (cm), and volume in cubic centimeters (cm^3).

2.3. Semen collection

Ejaculates were obtained from each of the twelve individuals by peri-prostatic electrostimulation, as previously described by Seier *et al* (1989). Briefly, monkeys were placed in dorsal recumbency and a probe, lubricated with a gel (Kryojel, Kyron Laboratories, Benrose, South Africa), was inserted gently into the *rectum*. One operator controlled the probe position and another the transformer and semen collection. The same pair of operators was used throughout the study to ensure consistency. A stimulating current was applied continuously by means of a transformer, operated manually at the lowest current (19.7 milli-amperes) necessary to obtain a necessary response and ejaculation, without urine contamination. The semen was collected directly into pre-warmed 5-ml screw top polycarbonate tubes and placed in a water bath at 37°C.

2.4. Sperm motility

Immediately after collection, semen was aspirated from the polycarbonate tube with a Pasteur pipette, and a drop was placed on a 75 X 25 mm glass microscope slide (Superior, Germany). A 22 X 22 mm coverslip (Superior, Germany) was placed on the drop, spreading it over the surface between coverslip and the glass microscope slide. Subjective rating of motility was immediately determined under bright field illumination of the Nikon SE light microscope (Nikon, Japan) at 400X magnification. The overall motility was rated in 10% units of motile spermatozoa and the speed of forward progression on a scale of 0 – 4, zero being no progression and 4 maximum speed (Seier *et al.* 1989).

2.5. Sperm concentration

Sperm concentration was determined by the hemocytometry method. In this study, semen was diluted with the use of either white or red cell pipettes (Superior, Germany), depending on the initial estimated concentrations which was determined under bright field microscopy at 100X magnification. The diluting fluid consisted of 50g sodium bicarbonate and 0.25g tryptan blue dissolved in 1000ml of double distilled water. An aliquot of liquefied semen was aspirated to either first or second mark of the white or red cell pipettes, depending on the desired dilution. The diluting fluid was then aspirated up to the third mark of pipette, which is above the bulb containing the mixing bead, this represents dilution factors ranging from 1:100 to 1:200 (for the red cell pipette and 1:10 to 1:20 (for the white cell pipette).

After thorough mixing of semen and the diluent for 1 minute, a quarter of the fluid in the pipette was discarded, followed by placing a drop to spread under the coverslip on the Improved Neubauer hemocytometer BS748 (Weber, England). The hemocytometer was then left in a humid chamber for five minutes to prevent drying, and let the spermatozoa settle onto the counting grid. The four large

outside squares of the hemocytometer, each with sixteen smaller squares were counted under bright-field illumination at 400X magnification. The concentration of spermatozoa per milliliter (ml) of semen was calculated according to the formula below.

$$*\text{Spermatozoa concentration/ml} = \frac{\text{number of cells counted x dilution factor x 10 000}}{\text{number of blocks counted}}$$

(*Seier 1995)

2.6. Sperm morphology

Spermac stain was bought from Stain Enterprises (Wellington, South Africa). The stains are supplied in four bottles labeled fixative and stains A, B, and C respectively. Thin smears of sperm were made by either placing a small or big drop, depending on whether sperm concentration was high or low respectively. The drops were spread with another slide at an angle, and the smears were air-dried for approximately 5 minutes, and stained according to the following manufacture's instructions.

(a) Fixation by immersion in formaldehyde based fixative for 30 minutes.

(b) Rinsing by dipping twice in tap water.

(c) Staining for 1 minute in stain A and rinsed in tap water.

(d) Staining for 45 seconds in stain B and rinsed in tap water.

(e) Stained for 1 minute in stain C and rinsed in tap water.

(f) Air drying.

Interpretation of results

Slides were viewed under bright field illumination at 100X (oil immersion) objective of the Nikon SE light microscope (Nikon, Japan). Two hundred spermatozoa, including normal shaped (Figure 2.1.), were counted from each slide.

Legend to figures

Figures 2.1-2.14 (Seier 1995) are representations of some of the abnormal morphological forms observed in vervet monkey spermatozoa which were considered in this study. The bar at the bottom left hand side of Figure 2.1 represents 6.7 μm, whereas one on Figure 2.2 represents 3.3 μm and applies to all subsequent figures.

Figure 2.1. Normal morphological characteristics of vervet monkey spermatozoa
The sperm in the centre has bilaterally flattened head, and the acrosomal region occupies approximately 66 percent of the head region. The midpiece is slender and long in relation to the head. The tail is more slender than the midpiece, and has no coils, kinks or folds.

In illustrating the multiple defects of vervet monkey spermatozoa used in this study, a multi-parametric, rather than a single entry scoring, differential morphological assessment was used. Each defect was tallied independently. Based on the abnormal morphology of spermatozoa, the following list of common categories, according to World Health Organization (1992), were considered (Figure 2.2-2.14):

Note: Analysis of sperm morphology was not only restricted to these categories

2.6.1. Head abnormalities

Figure 2.2. **Multiple head abnormalities**.
The duplicate heads observed in this figure are also microcephalic, round and have no acrosome. Additionally, the spermatozoon also has a short, thickened and triangular-shaped midpiece, which is probably as a result of two fused midpieces.

Figure 2.3. **Macrocephalic**
The head is bigger than normal (see Figure 2.1). Note a coiled tail forms a figure eight at the distal end of the midpiece.

Figure 2.4. **Elongation**
Elongation of the sperm head, with a tapering at the post acrosomal region. The midpiece is thickened and the tail is coiled.

Figure 2.5. **Nipple acrosome**
Apical aggregation of the acrosomal material forms a nipple acrosome, which is a large apical cyst. The sperm tail is coiled.

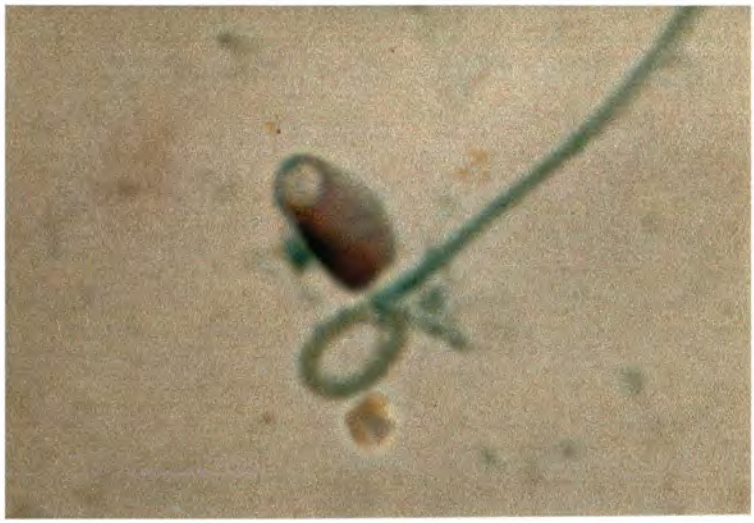


Figure 2.6. **Acrosomal cyst**
It is anterior and occupies almost a third of the head size. The cyst has a lighter center and a darker perimeter. The midpiece is bent and detached from the asymmetrically shaped head.

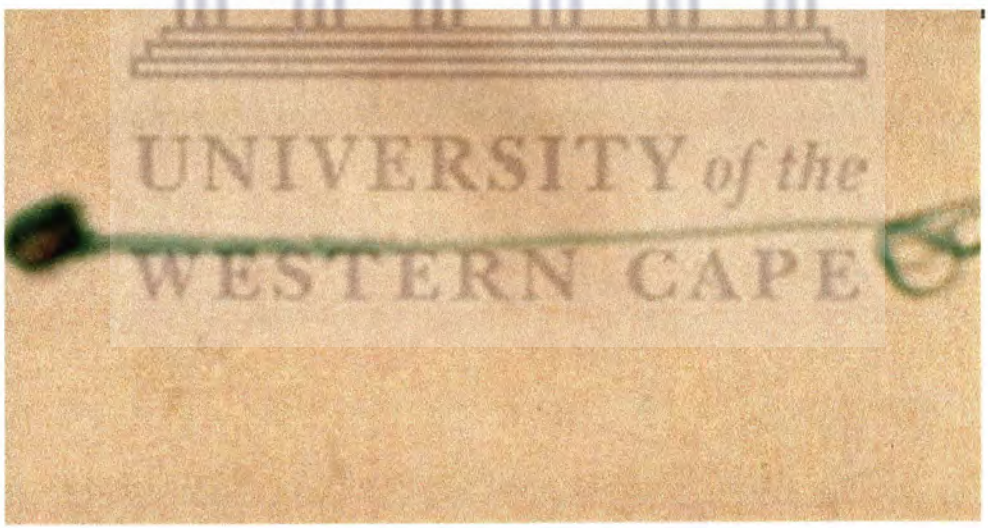


Figure 2.7. **Amorphous and microcephalic**
Note the midpiece is straight, whereas the tail is coiled terminally.

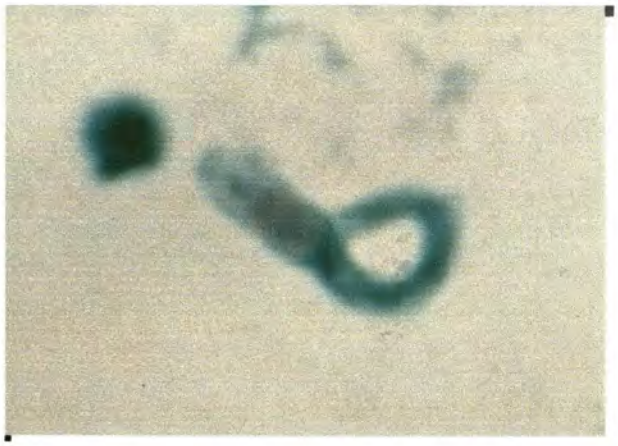


Figure 2.8. Constriction
Constriction over the equatorial region. The acrosome is patchy and appears to have lost most of its contents. The midpiece is thickened, probably due to two fused midpieces.

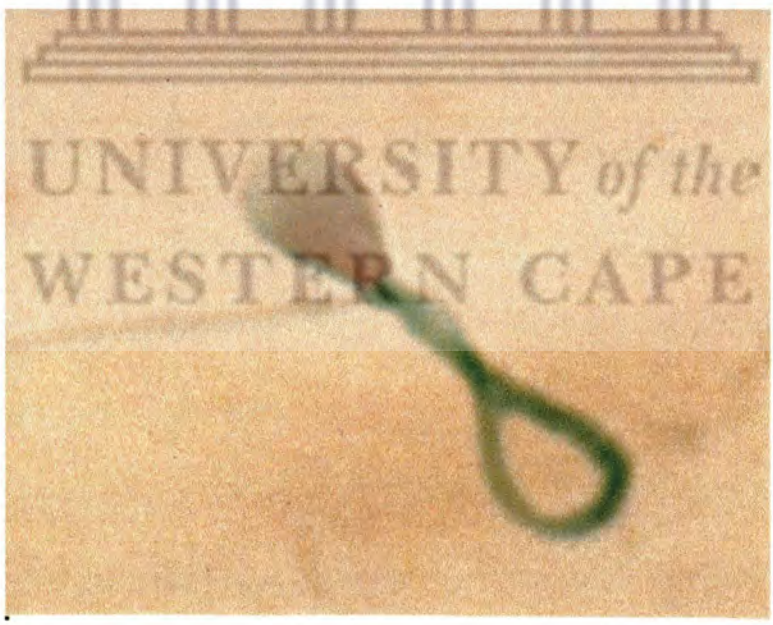


Figure 2.9. Pyriform
Narrowing at the postacrosomal region produces the pyriform defect. There is a complete loss of the acrosome. The midpiece is bent at the distal end, with the tail wrapped around and projects at the proximal region of the midpiece.

2.6.2. Midpiece abnormalities

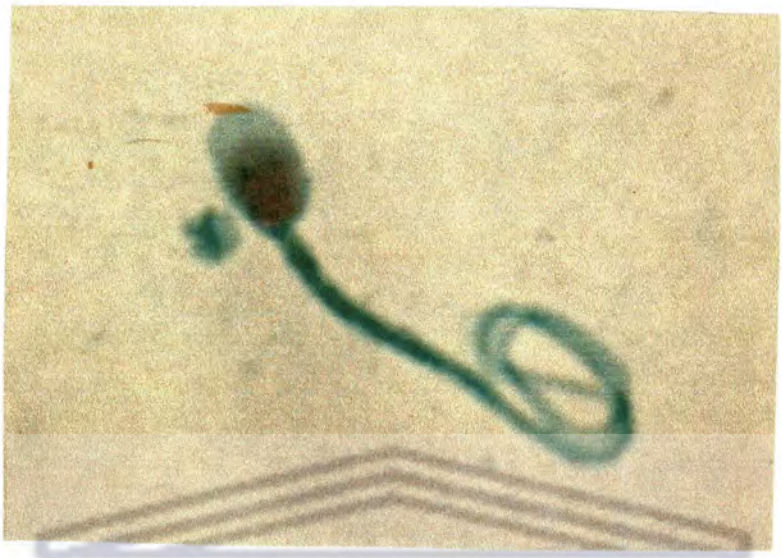


Figure 2.10. Abaxial implantation
Abaxially implanted midpieces on the left side of the basal plate. There is coiling of the tail

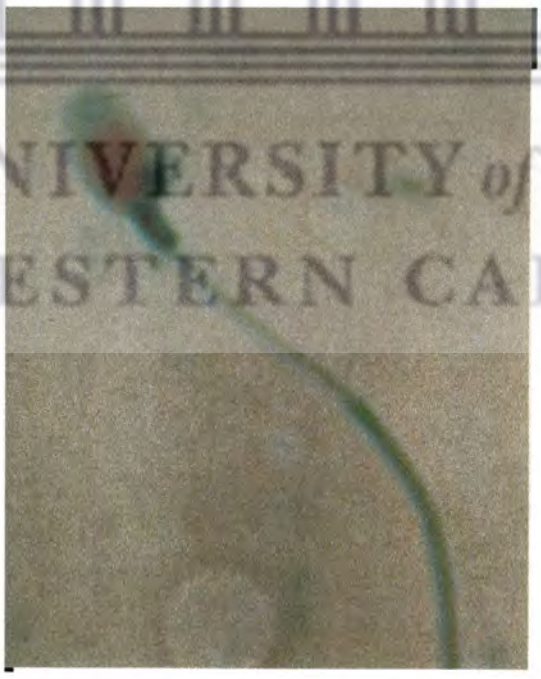


Figure 2.11. Stripped mitochondria
The mitochondria are stripped from most of the midpiece region, leaving an uncovered axoneme.

2.6.3. Principal and endpiece abnormalities

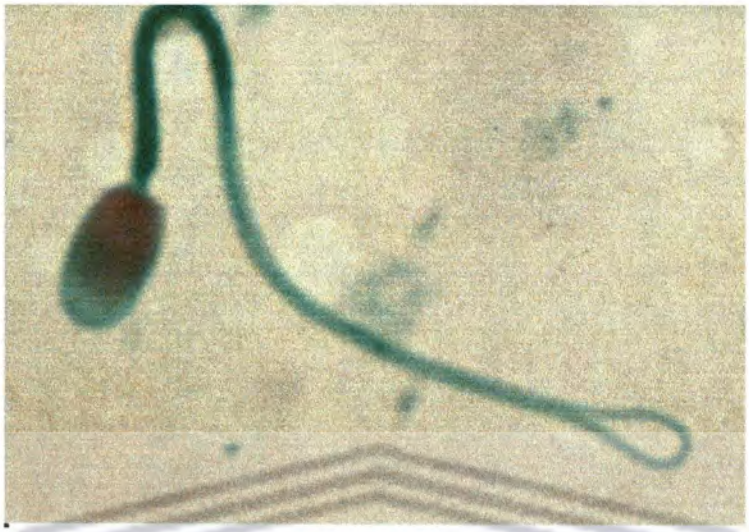


Figure 2.12. Hairpin
Folding back of the principal portion of the tail into a hairpin shape. The midpiece is also bent.

Figure 2.13. Tail duplication
The midpiece is thickened and appears to be detached from the basal plate.

Figure 2.14. Detached tail
There is also nipple acrosome, and a slight abaxial implantation of the midpiece.

2.7. Sperm vitality and acrosomal integrity: Staining sperm with fluorescent probes

Samples of spermatozoa were washed twice in Hams F-10 by centrifugation at 1800 revolutions per minute (rpm) for five minutes in Eppendorf tubes. After washing, supernatants were discarded and pellets were re-suspended to about 15X 10^6 cells/ml in Phosphate buffered saline (PBS). Hoechst 33258 was added to the suspension at 1µl/ml, contained in a foil covered eppendorf tube, and left to stain for five minutes at room temperature. Spermatozoa were then centrifuged and re-suspended in PBS, spread over the edge of the microscope slide and left to air-dry in the dark. Air-dried slides were fixed in 95% ethanol for seven minutes in a foil covered Coplin jar and allowed to air dry. Dried ethanol-fixed slides were then labeled by immersion in the FITC-PNA for 15 minutes in another foil covered Coplin jar and allowed to air dry. One drop of mounting medium (glycerol in PBS + n-propyl gallate) was placed on a smear and covered with a coverslip.

Quantitative analysis

Slides were viewed the next day on an Olympus microscope (BX 50) using 100X objective (Uplan FI Oil Ph3). The rotatory optical cube in the microscope, allowed quick and easy interchange of fluorescence filter sets for FITC- PNA and Hoechst 33258 respectively. Photomicrographs were taken with an Olympus camera (C35DX) connected to the exposure control unit (Olympus PM-20).

For the Hoechst 33258 (supravitally) stained smears, the fluorescent types were classified according to the intensity of the blue fluorescence displayed on the sperm head. Spermatozoa that had not taken up Hoechst 33258, i.e. those fluorescing pale blue (Type PB), possessed intact plasma membrane and were vital. On the other hand, the heads (nuclei) of spermatozoa that had taken up Hoechst 33258, with the result of bright blue fluorescence (Type BB), indicated that their plasma membranes were disrupted or damaged. Regarding FITC-PNA labeling, the proportions of spermatozoa showing categories of fluorescence described in Figure 2.15 were determined for each smear. Each slide was examined in duplicate and 200 spermatozoa were counted. The average of two counts was determined and results were expressed as percentages.

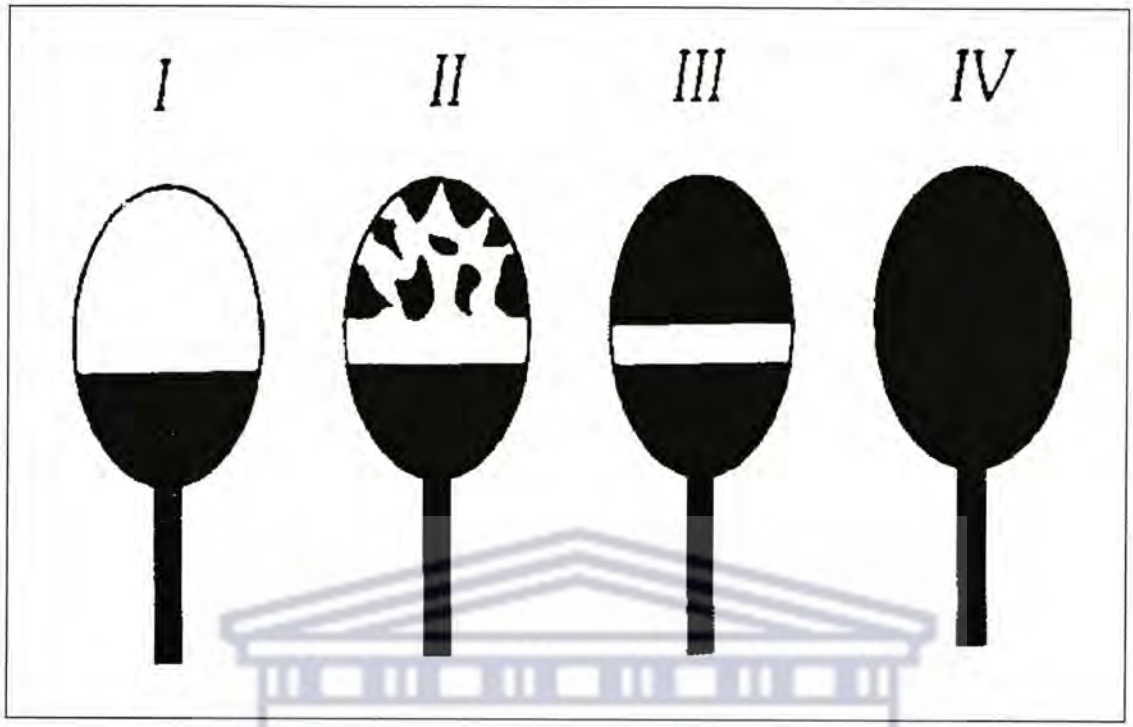


Figure 2.15. Representation of the fluorescence patterns of human spermatozoa stained with FITC-PNA lectin (According to Mortimer *et al.* 1990**).**

Fluorescence pattern I showing intensely fluorescent region of acrosome intact sperm and the dark postacrosomal region. Fluorescence pattern II displaying irregular fluorescence of the acrosomal region. Note this type of fluorescence is also limited to the acrosomal region of the head. Fluorescence pattern III showing bright fluorescence at the equatorial region of the sperm. Note the lack of fluorescence at both the acrosomal and post-acrosomal regions. Fluorescence pattern IV, showing lack of fluorescence on the sperm head due to absence of the acrosome.

2.8. Testosterone concentration

Blood samples were collected at baseline and every fourth week for serum testosterone analyses, which were performed at the commercial laboratory (PathCare, Cape Town, South Africa). The analyses were done by competitive immunoassay using chemoluninescent technology (Chiron Diagnostics), detection limit 0.35 nmol/L, intrassay variation 6.5% and interassay variation 9.7%.

2.9. Mating and reversibility of oleanolic acid

After four months of oleanolic acid administration, each male was mated, in the two combined single cages (size: 60X120X80 cm), with one multiparous female for a period of two months. The oleanolic acid administration continued during this period, but no semen or blood samples were collected. During the morning feeding time, females were prevented from eating oleanolic acid-mixed-food by separating them from the males with a partition between the cages. Matings were confirmed by the presence of spermatozoa on vaginal smears, which were prepared a day after pairing. Pregnancy was diagnosed by unltrasonography using an Aloka real time electronic scanner (SSD-210DXII), equipped with a 7.5 MHz scanhead after four and eight weeks of mating.

Reversal of antifertility effects was assessed by extending the mating period by additional two months, for a group that did not produce pregnancy during the initial two-month mating period. During this period, monkeys in this group received no oleanolic acid, but were maintained under conditions identical to the treatment period. Fertility of these monkeys were again assessed by the conception rate, as outlined above.

2.10. Statistical analysis

All parameters were subjected to Repeated Measures Analysis of variance using statistical package SAS (Version 8). $P < 0.05$ was regarded as significant.

Chapter 3

Results

Note:

- Since inclusion of standard deviations in the graphs would have made visualization impossible, they are tabulated in the Appendix B (pages 243 – 252).
- Statistical data (*P*-values) provided in this study represent changes observed between and within groups during the entire period of the study.

3.1. Testicular volume

The right and left testicular volumes are presented in Figures 3.1 and 3.2 respectively. During the study period, there were no statistically significant differences in testicular volumes of either testis within the same group, and between controls and the treated groups.

3.2. Semen collection

The electrostimulation technique was effective in consistently producing ejaculates from all male monkeys. Stimulation of somatic musculature resulting in strong contractions of the thigh muscles, and testicular and tail movements, per selected voltage were observed on all subjects. Weakening of these responses and erection cessation indicated the necessity of the next higher current. Accompanying these responses was erection with an enlargement of the glans penis and ejaculation. There were inter-individual differences in the stimulation period required to achieve ejaculation.

Legends to figures

- Data in Figures 3.1 - 3.29 were obtained every second week, in Figure 3.30 every fourth week.
- Figures 3.1 and 3.2 are testicular volumes from the controls and the oleanolic acid-treated male vervet monkeys used in this study.
- Figures 3.4 - 3.6 are representations of sperm motility and concentrations from the controls and the oleanolic acid-treated male vervet monkeys used in this study.
- Figures 3.7 - 3.22 are representations abnormal sperm morphological forms found in the semen of the controls and oleanolic acid-treated male vervet monkeys used in this study.
- Figure 3.23 is a micrograph of four acrosomal integrity patterns observed in the sperm from the treated and control monkeys, and percentages of each pattern are shown in Figures 3.24 - 3.27.
- Figure 3.30 is a representation of testosterone concentrations from the controls and the oleanolic acid-treated male vervet monkeys used in this study.

Explanations for unit abbreviations in Figures 3.1, 3.2 and 3.30

Unit	Explanation
cm^3	cubic centimetre
nmol/L	nanomol per litre

Figure 3.1. Right testicular volume

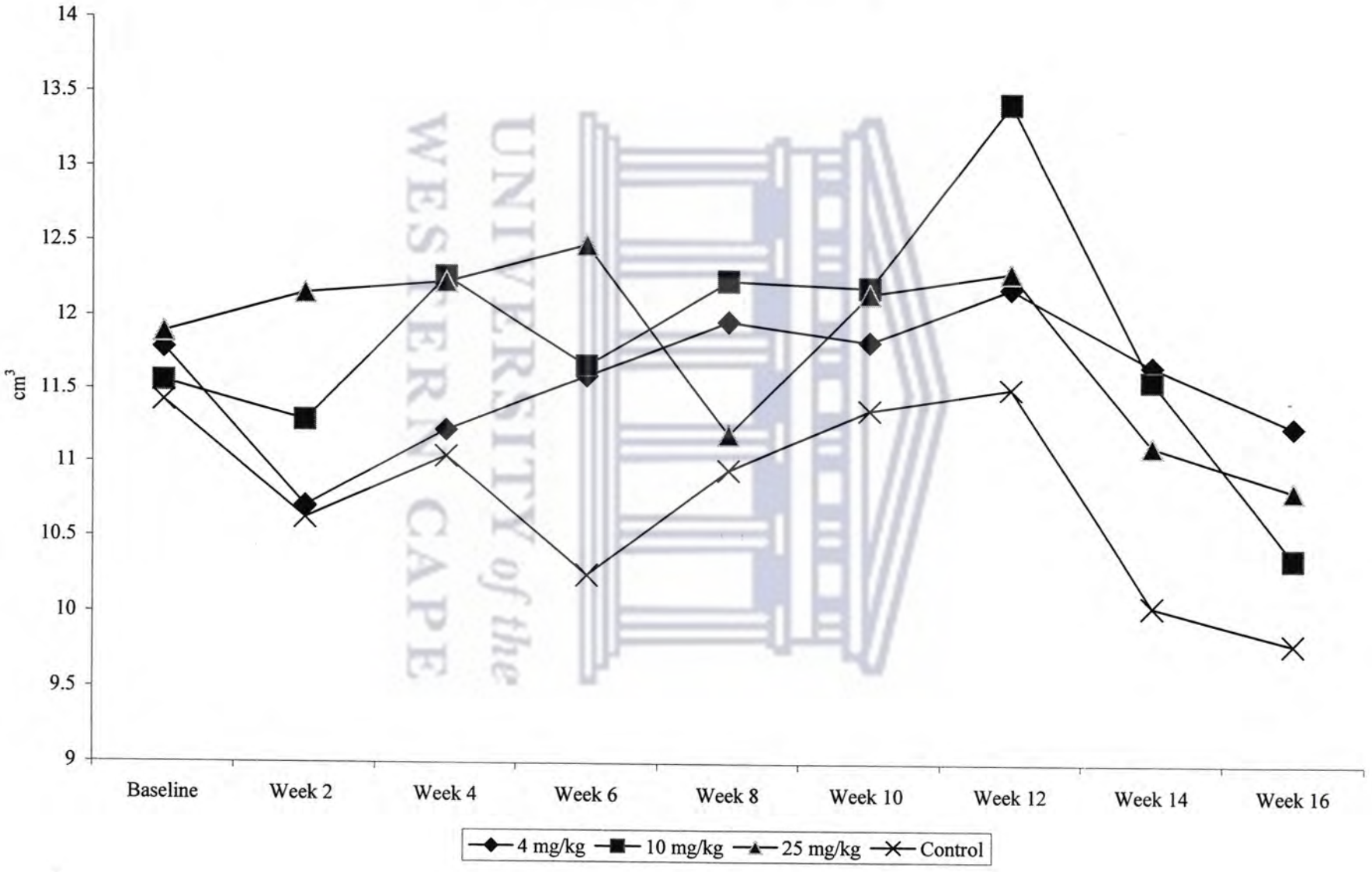
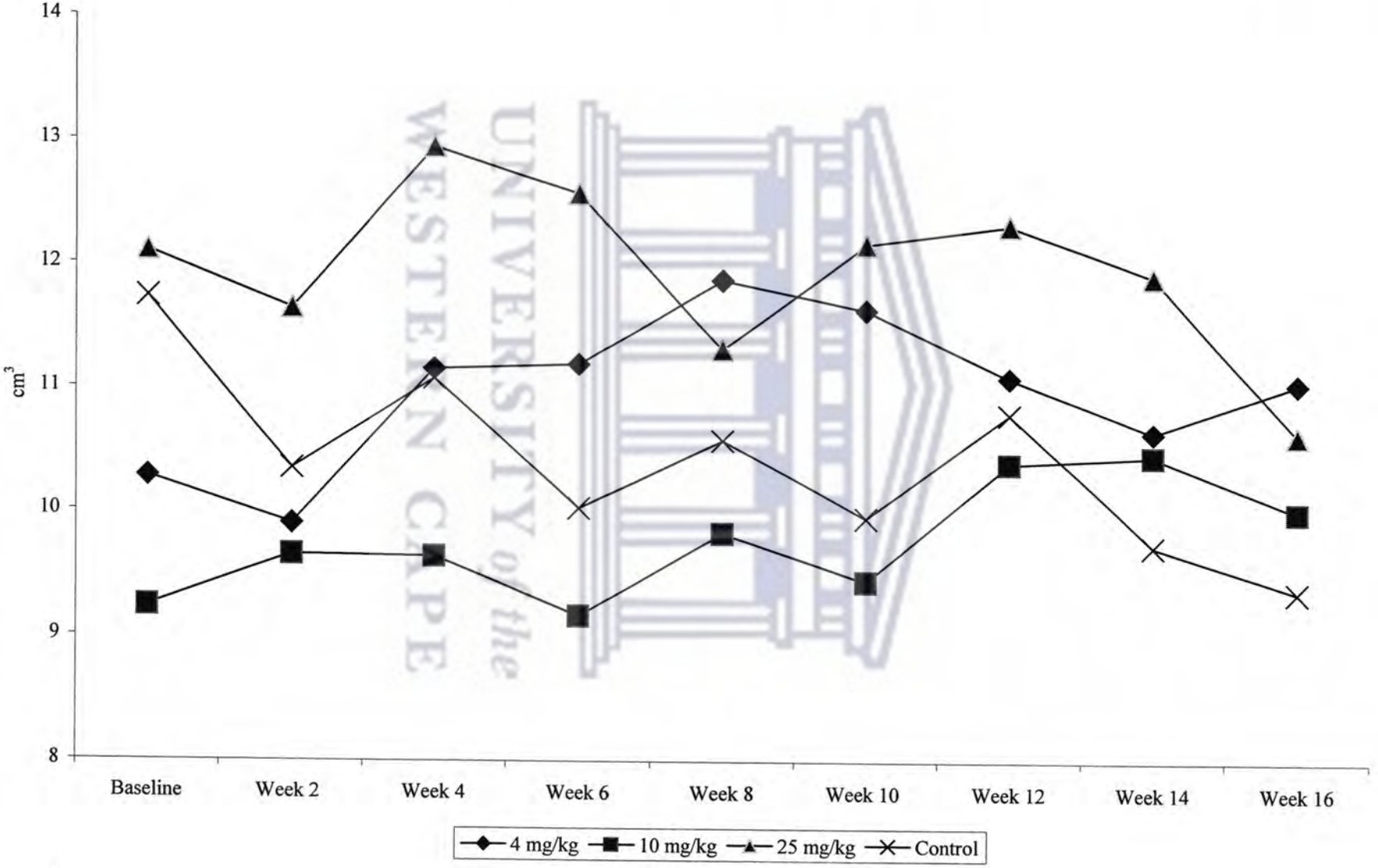


Figure 3.2. Left testicular volume



3.3. Sperm motility and concentration

The average sperm progressive motility, speed of forward progression and concentration are presented in Figures 3.3, 3.4 and 3.5 respectively. Although there were reductions in progressive motility in the two higher dose groups, a statistically significant reduction (P = 0.0184) in this parameter was only observed in the group that received 4 mg/kg oleanolic acid when compared to the controls.

No statistical significant differences were observed with the speed of forward progression between controls and the treated groups.

No statistically significant differences or treatment-related effects on sperm concentration were observed between treated groups and the controls. Sperm concentrations varied considerably within individuals in any group, as reflected by large standard deviations with each sample (Table 3.1).

Table 3.1. Sperm concentration X 10^6/ml (means ± standard deviations) for successive semen collection

Time points	4 mg/kg	10 mg/kg	25 mg/kg	Control
Baseline	80.33 ± 43.68	176.67 ± 118.02	544.00 ± 467.90	321.00 ± 200.33
Week 2	202.00 ± 163.33	897.33 ± 1307.43*	815.33 ± 263.90	457.33 ± 125.48
Week 4	431.33 ± 335.48	782.00 ± 1068.97*	254.67 ± 154.04	1125.33 ± 1189.72*
Week 6	534.67 ± 399.16	608.00 ± 872.96*	364.00 ± 323.38	320.67 ± 91.48
Week 8	305.33 ± 198.56	412.00 ± 380.88	421.33 ± 255.17	332.00 ± 195.10
Week 10	298.67 ± 173.79	482.00 ± 468.49	362.00 ± 158.38	300.00 ± 40.15
Week 12	324.00 ± 209.23	402.67 ± 465.55*	392.67 ± 277.00	284.00 ± 19.08
Week 14	442.00 ± 321.93	824.00 ± 979.83*	363.33 ± 83.58	508.00 ± 57.65
Week 16	286.67 ± 202.32	415.67 ± 562.53*	632.67 ± 567.79	454.00 ± 101.73

*standard deviations greater than mean values

Figure 3.3. Progressive motility

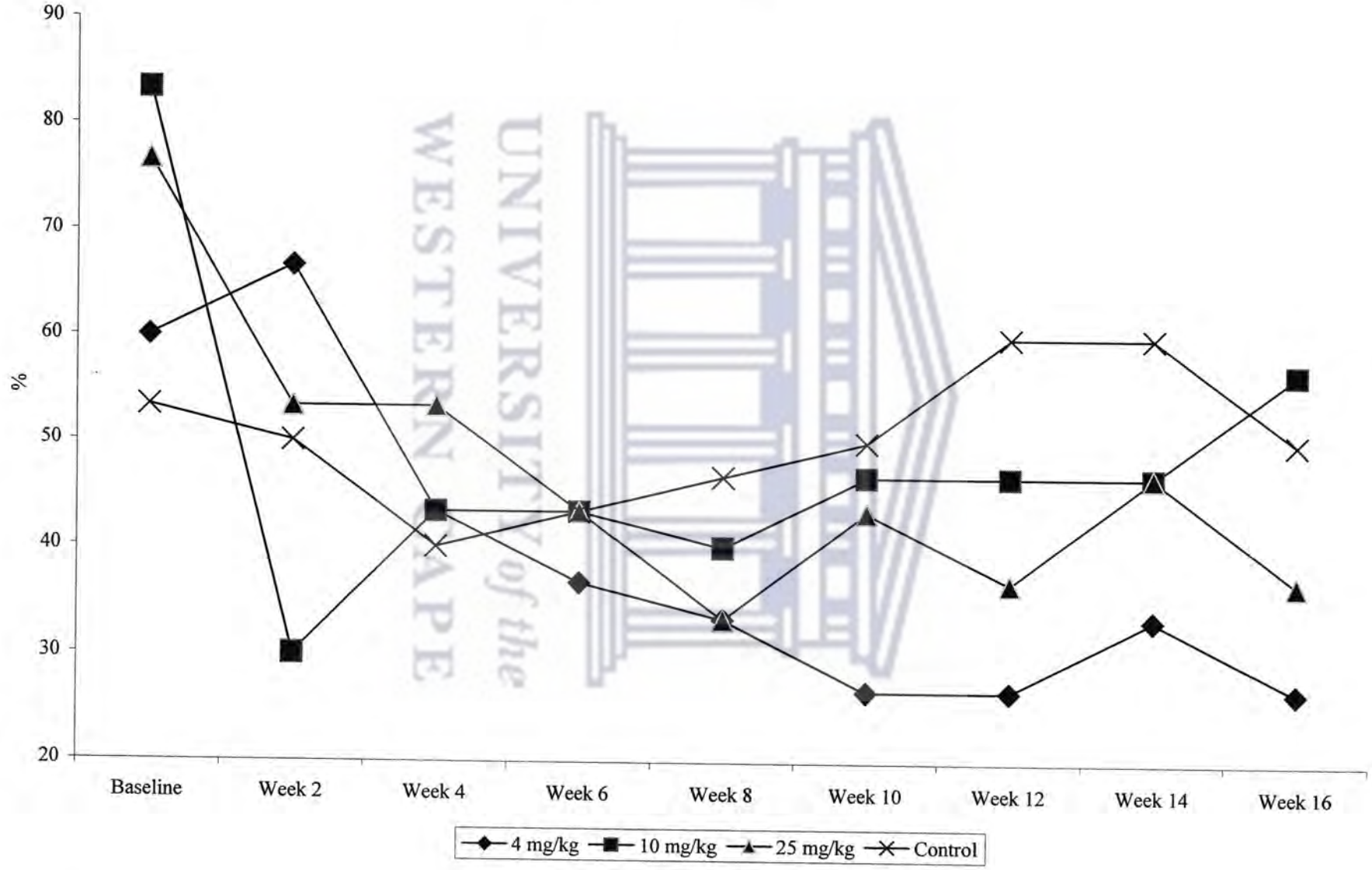


Figure 3.4. Speed of forward progression

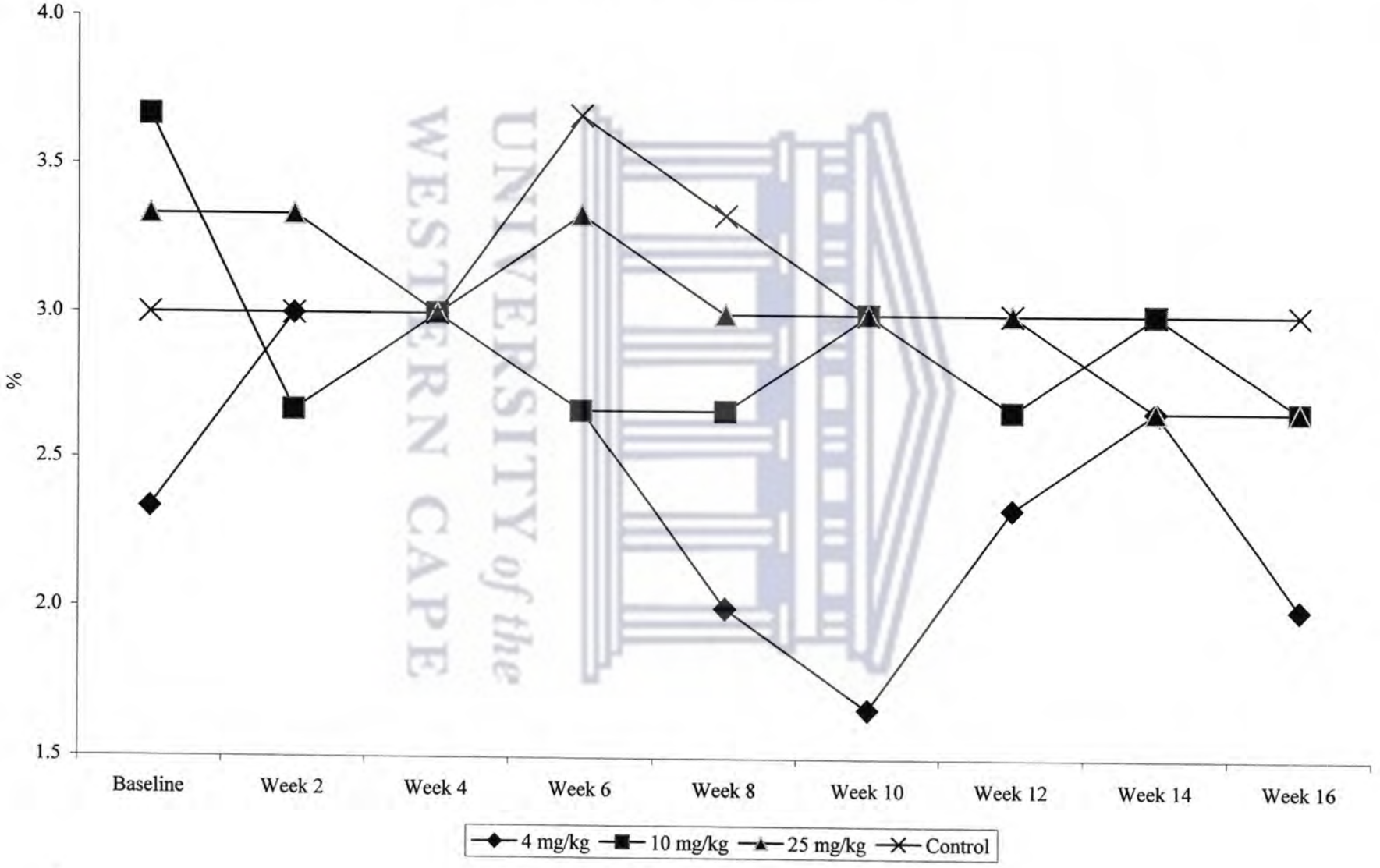
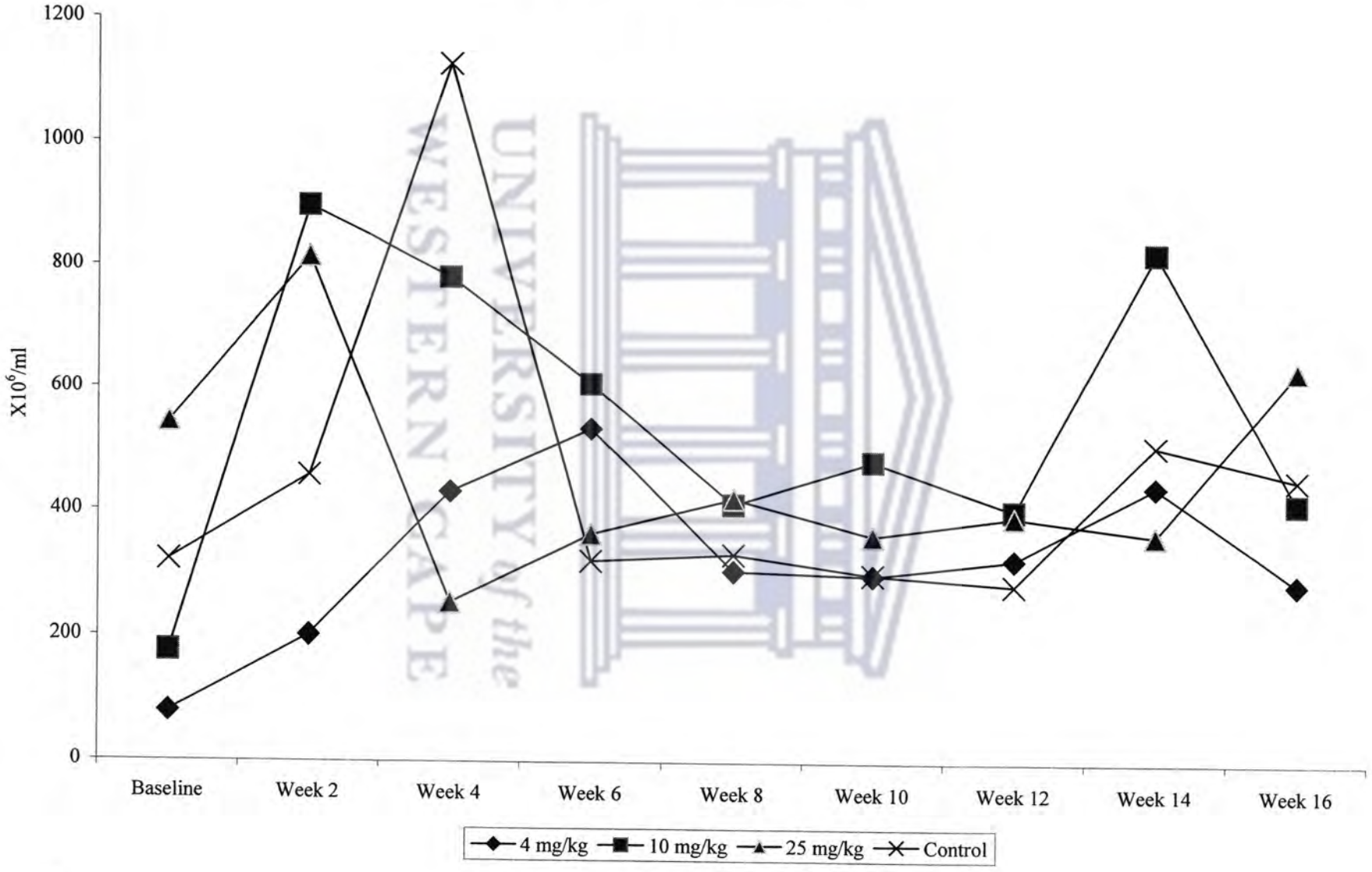


Figure 3.5. Sperm concentration



3.4. Sperm morphology

A total of 20 different sperm abnormalities were found amongst ejaculates of the treated and control groups.

3.4.1. Head abnormalities

The percentages of spermatozoa with microcephalic defects were not statistically significantly different between controls and the groups that received 4 and 25 mg/kg oleanolic acid (Figure 3.6). However, a highly statistically significant increase ($P = 0.0007$) over time in this defect was observed in the group that received 10 mg/kg oleanolic acid. None of the treated groups were statistically significantly different from the controls in respect to the pyriform (Figure 3.7), macrocephalic (Figure 3.8) and tapering (Figure 3.9) defects. Fluctuations in the numbers of spermatozoa with acrosomal cysts (Figure 3.10) in the group that received 10 mg/kg oleanolic acid were statistically significantly different ($P = 0.0019$) from the controls. No spermatozoa with acrosomal cysts were present in the groups that received 4 and 25 mg/kg oleanolic acid throughout the study period. Fluctuations in amorphous head defects (Figure 3.11) in the group that received 25 mg/kg oleanolic acid were statistically significantly different ($P = 0.0473$) from the controls. No amorphous head defects were observed in the other treated groups throughout the study period.

Fluctuations in the numbers of spermatozoa with round heads in the groups that received 10 and 25 mg/kg oleanolic (Figure 3.12) were not statistically significantly different from the controls. No amorphous head defects were observed in the group that received 4 mg/kg oleanolic acid throughout the study period. Although the percentages of nipple acrosomal defects were higher in the group that received 25 mg/kg oleanolic acid compared to other treated groups and the controls (Figure 3.13), no statistically significant differences were observed between controls and all treated groups.

The combined results show a gradual increase in total head abnormalities of the treated groups as the study progresses (Figure 3.14). Although the controls had consistently fewer head abnormalities, they however, equaled those of the treated groups at the end of the study. Nonetheless, no statistically significant differences were found between treated groups and the control.

Figure 3.6. Microcephalic

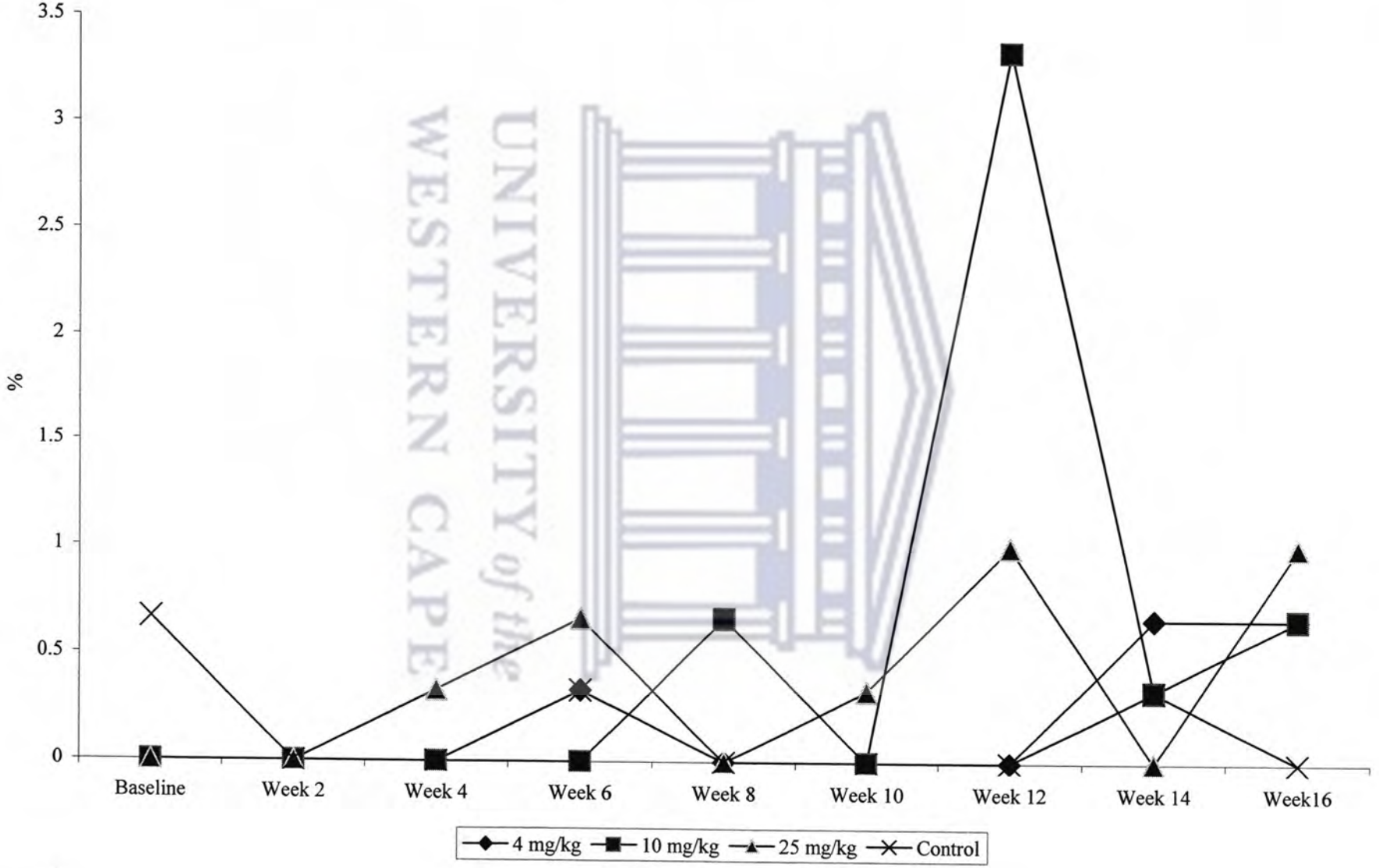


Figure 3.7. Pyriform

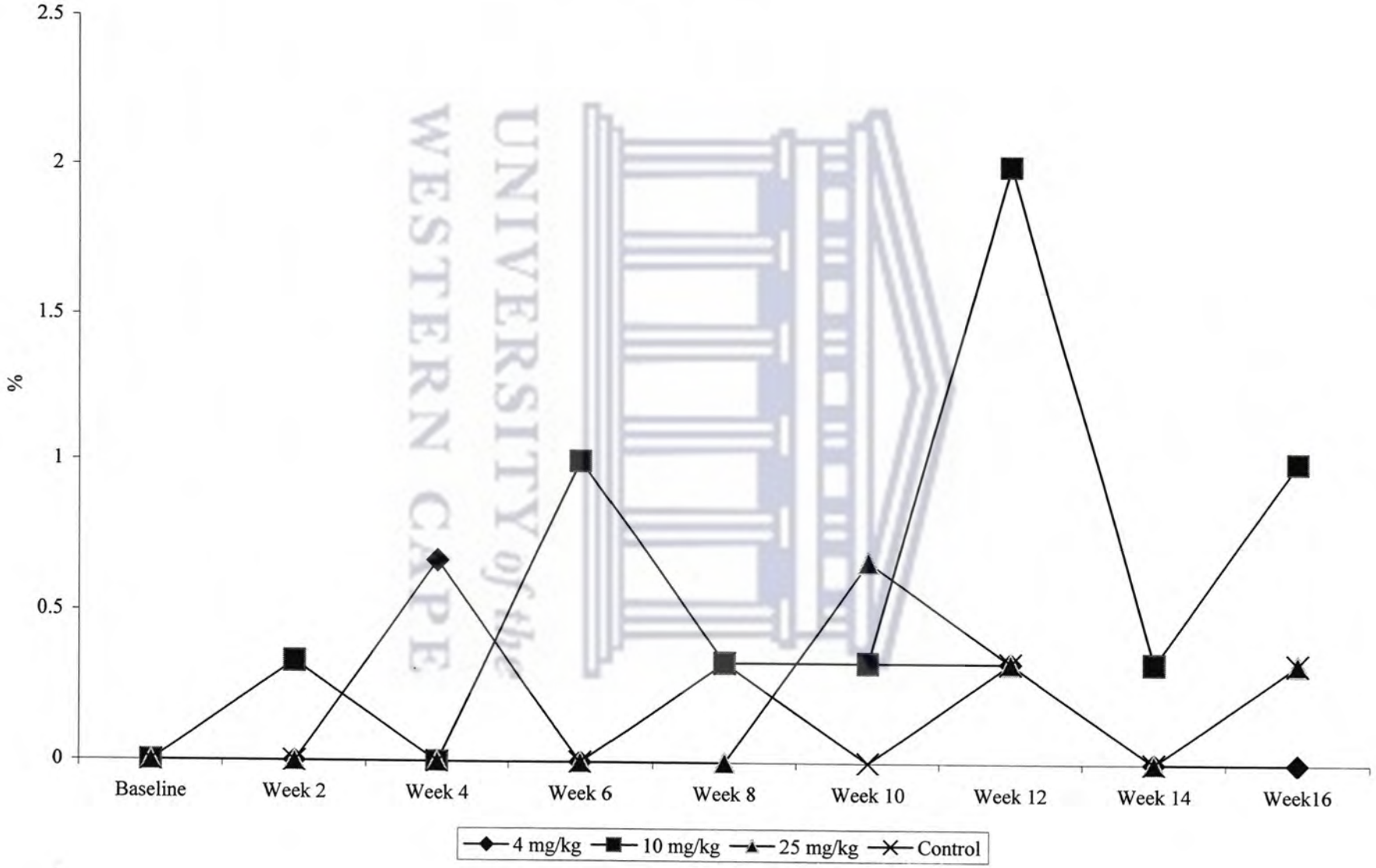


Figure 3.8. Macrocephalic

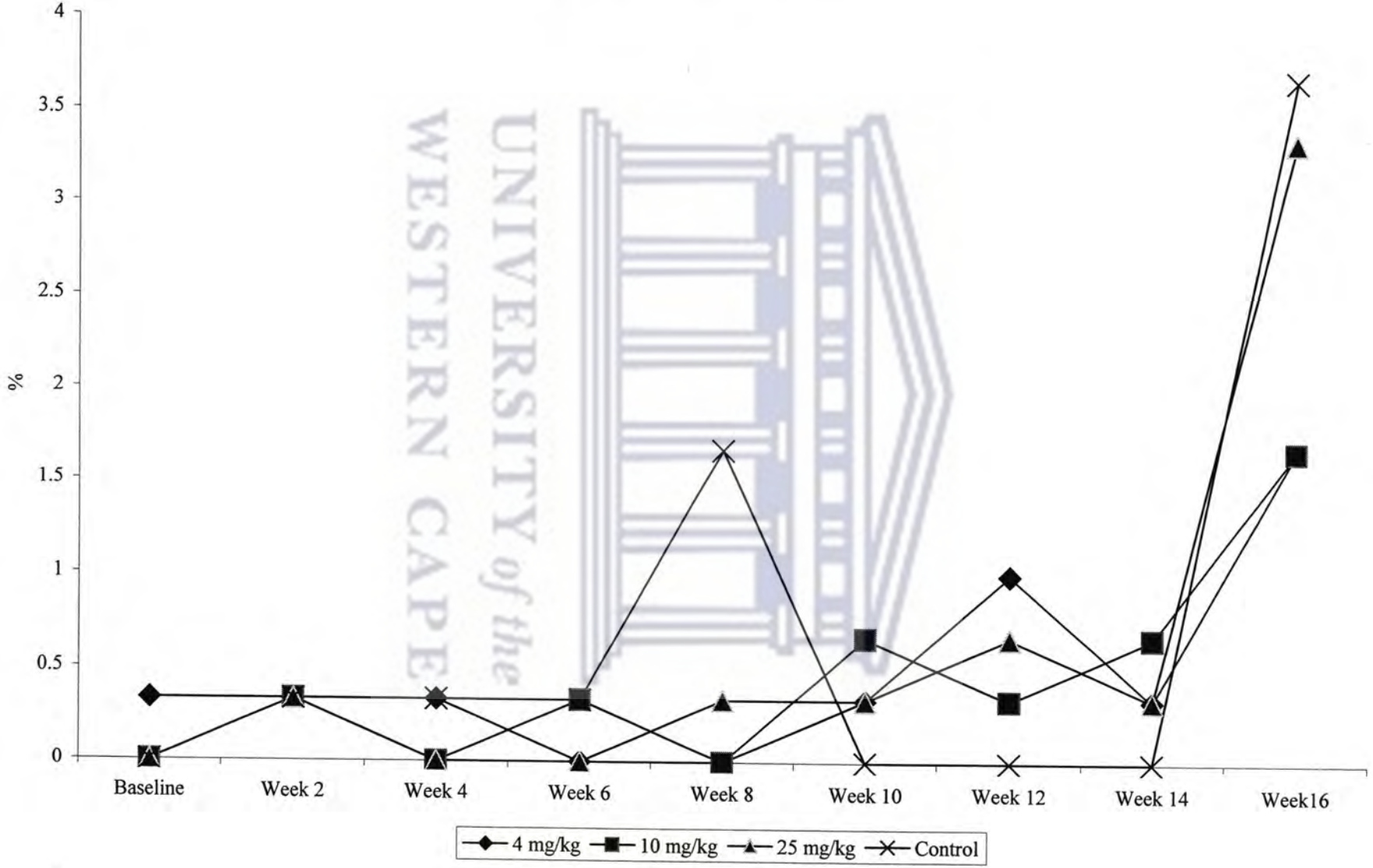


Figure 3.9. Tapering

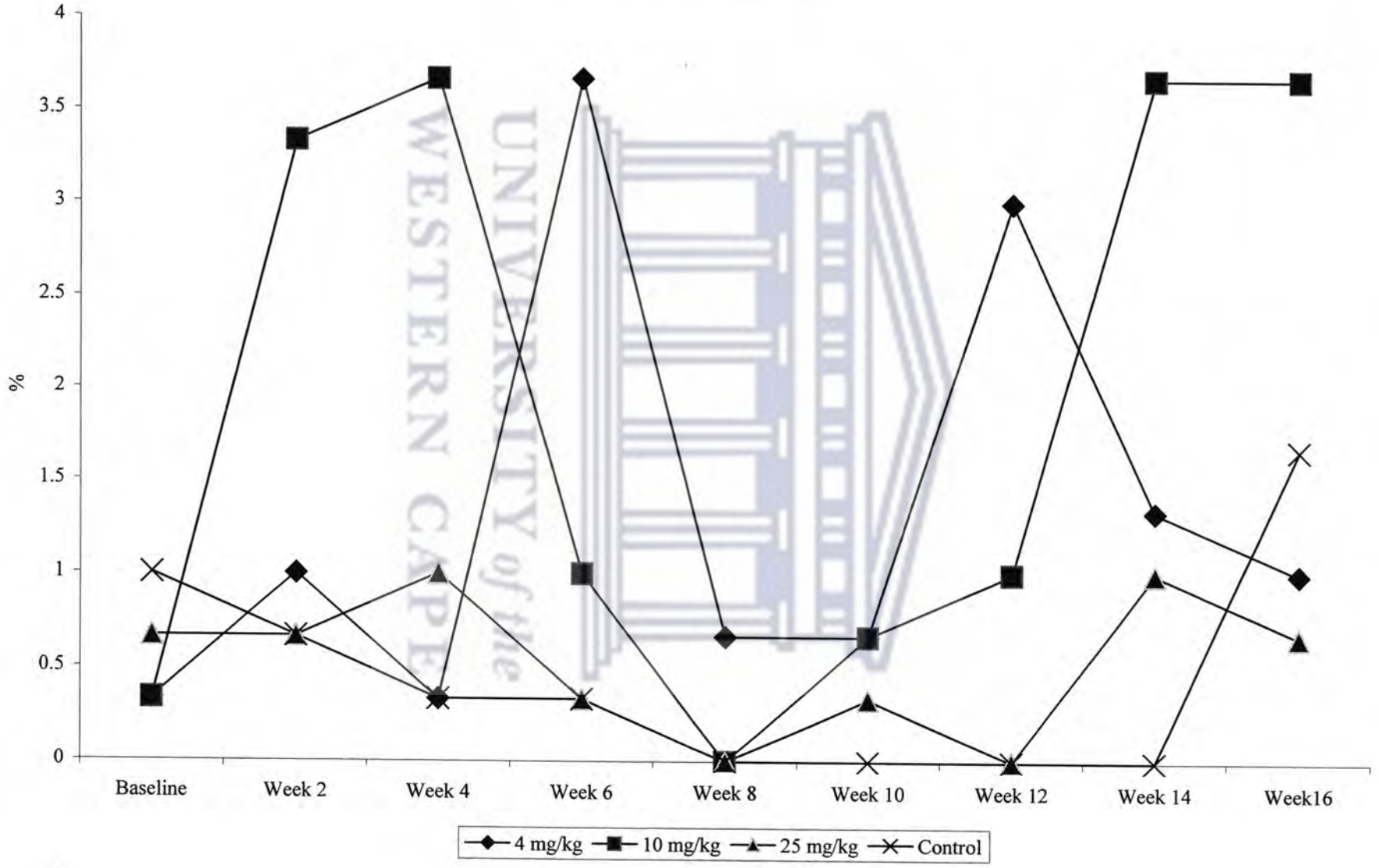


Figure 3.10. Acrosomal cyst

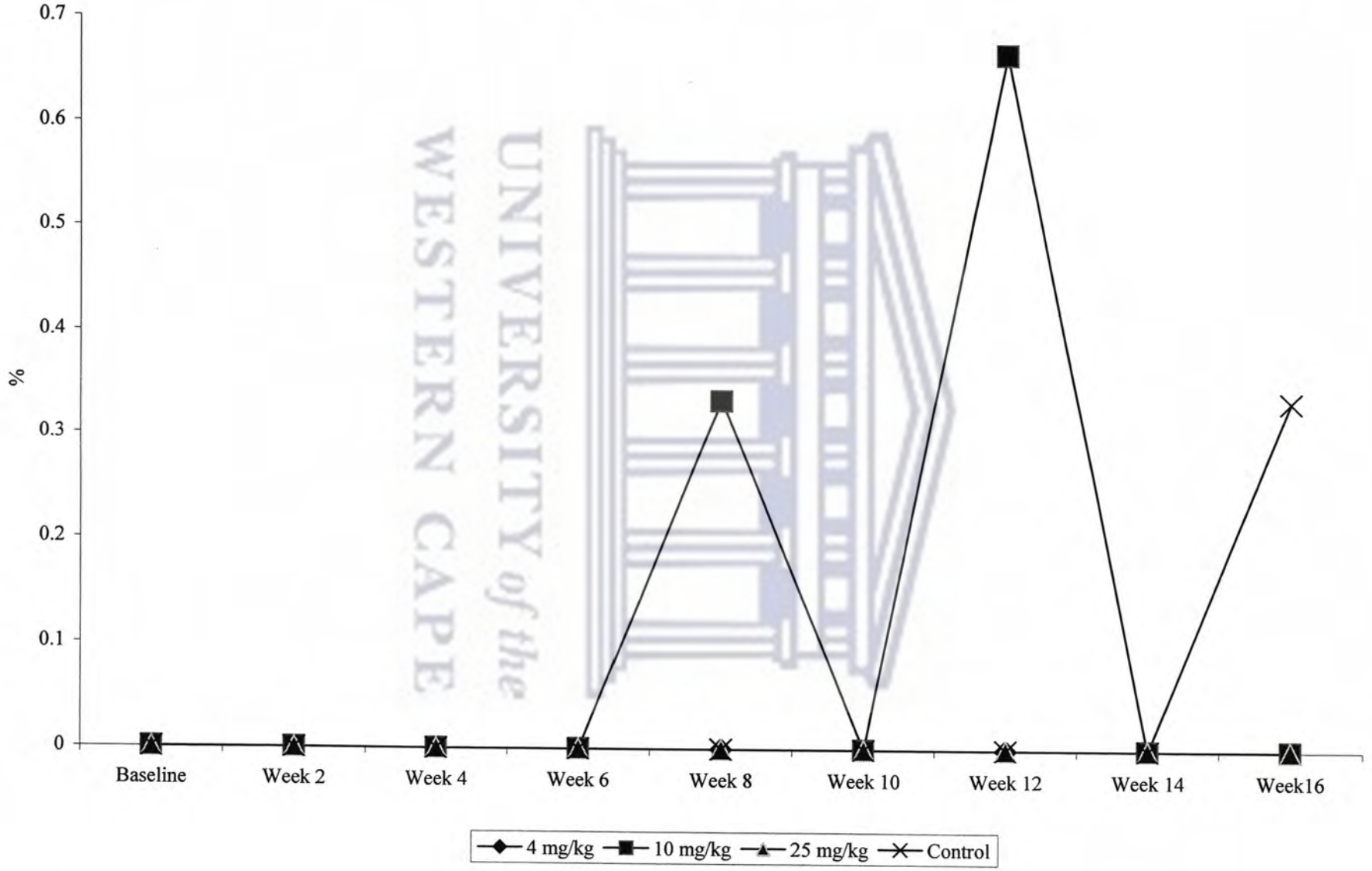


Figure 3.11. Amorphous

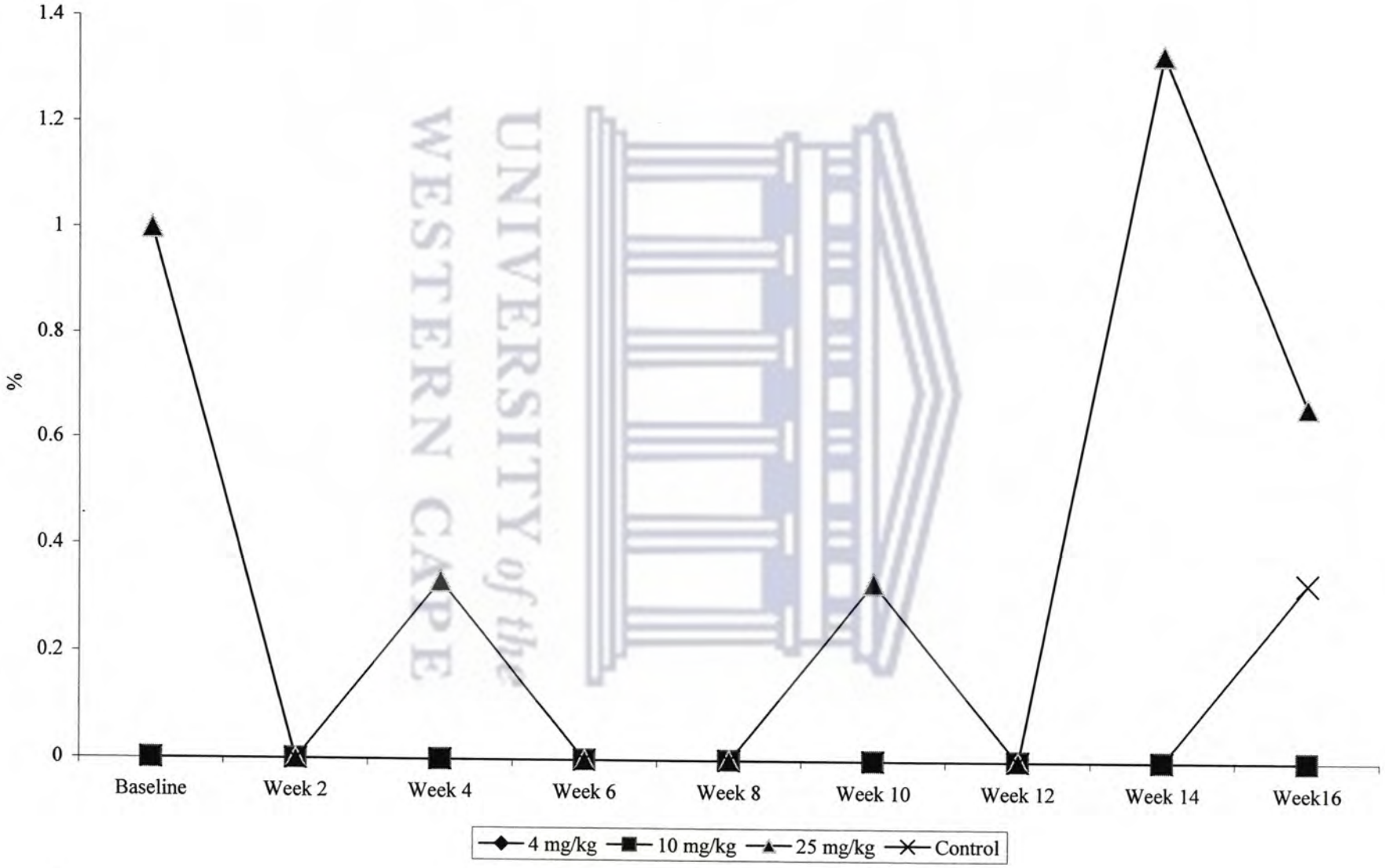


Figure 3.12. Round heads

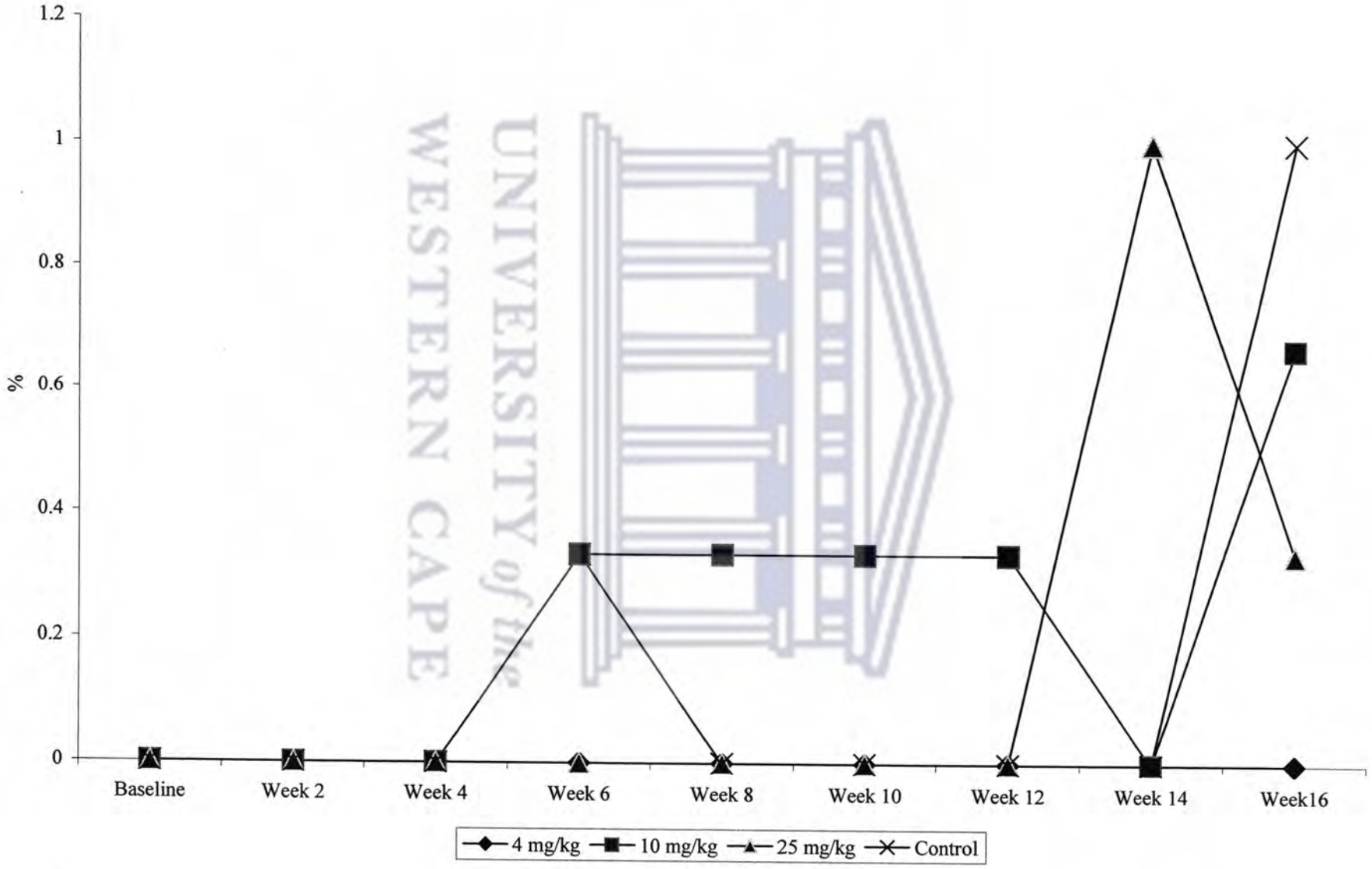


Figure 3.13. Nipple acrosome

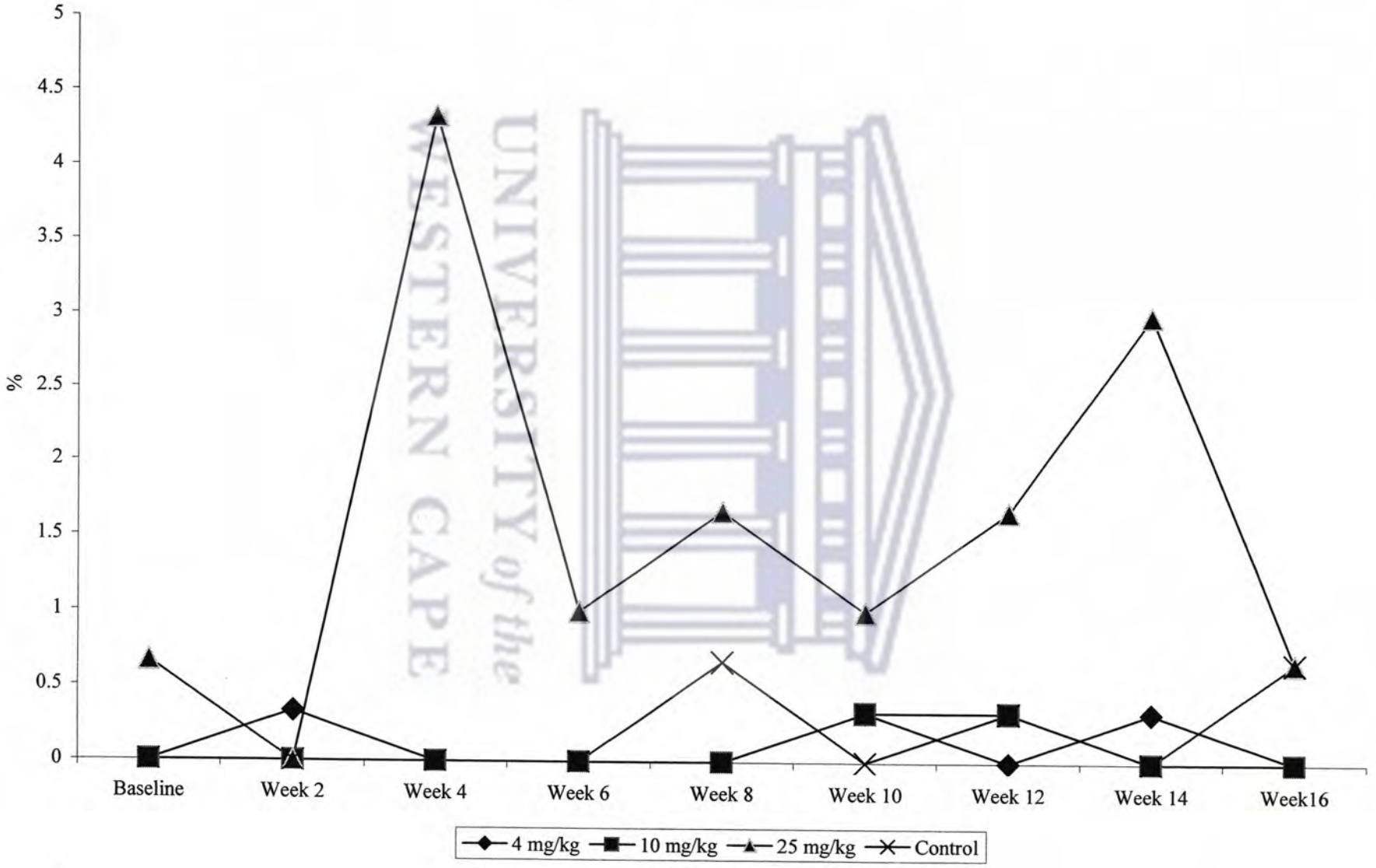
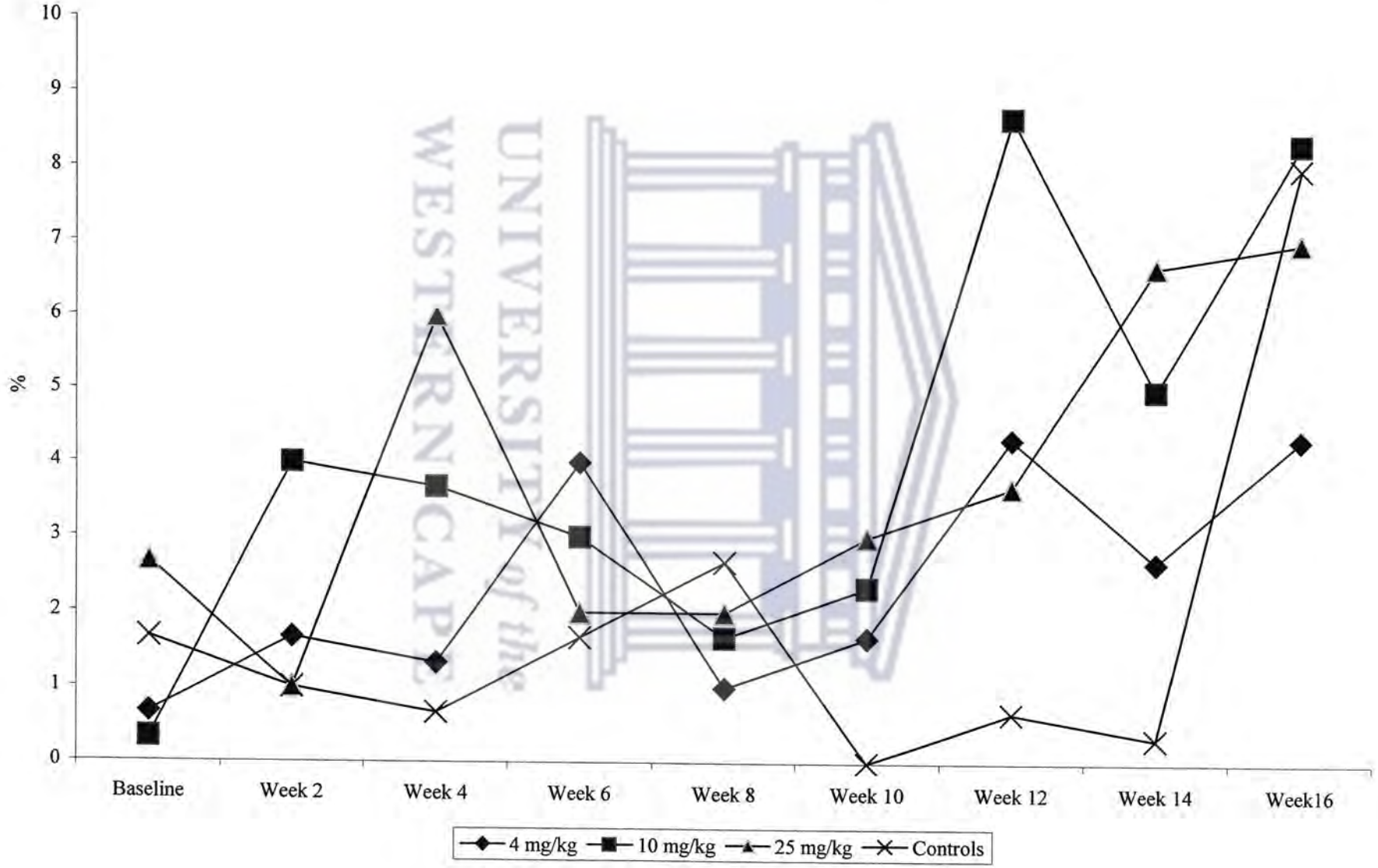


Figure 3.14. Combined head abnormalities



3.4.2. Midpiece abnormalities

The predominant defect in the ejaculates of all monkeys was bent midpiece (Figure 3.15). Fluctuations in the numbers of spermatozoa with this defect in the group that received 25 mg/kg oleanolic acid were statistically significantly different (P = 0.0018) from the controls, and not in other treated groups. The second most common midpiece defects were broken (Figure 3.16) and abaxial implantation (Figure 3.17). Nonetheless, no statistically significant differences were found in these defects between treated groups and the controls.

The combined midpiece abnormalities are presented in Figure 3.18. Although this parameter showed an upward trend in all groups as the study progressed, no statistically significant differences were evident between treated groups and the controls.

Figure 3.15. Bent midpiece

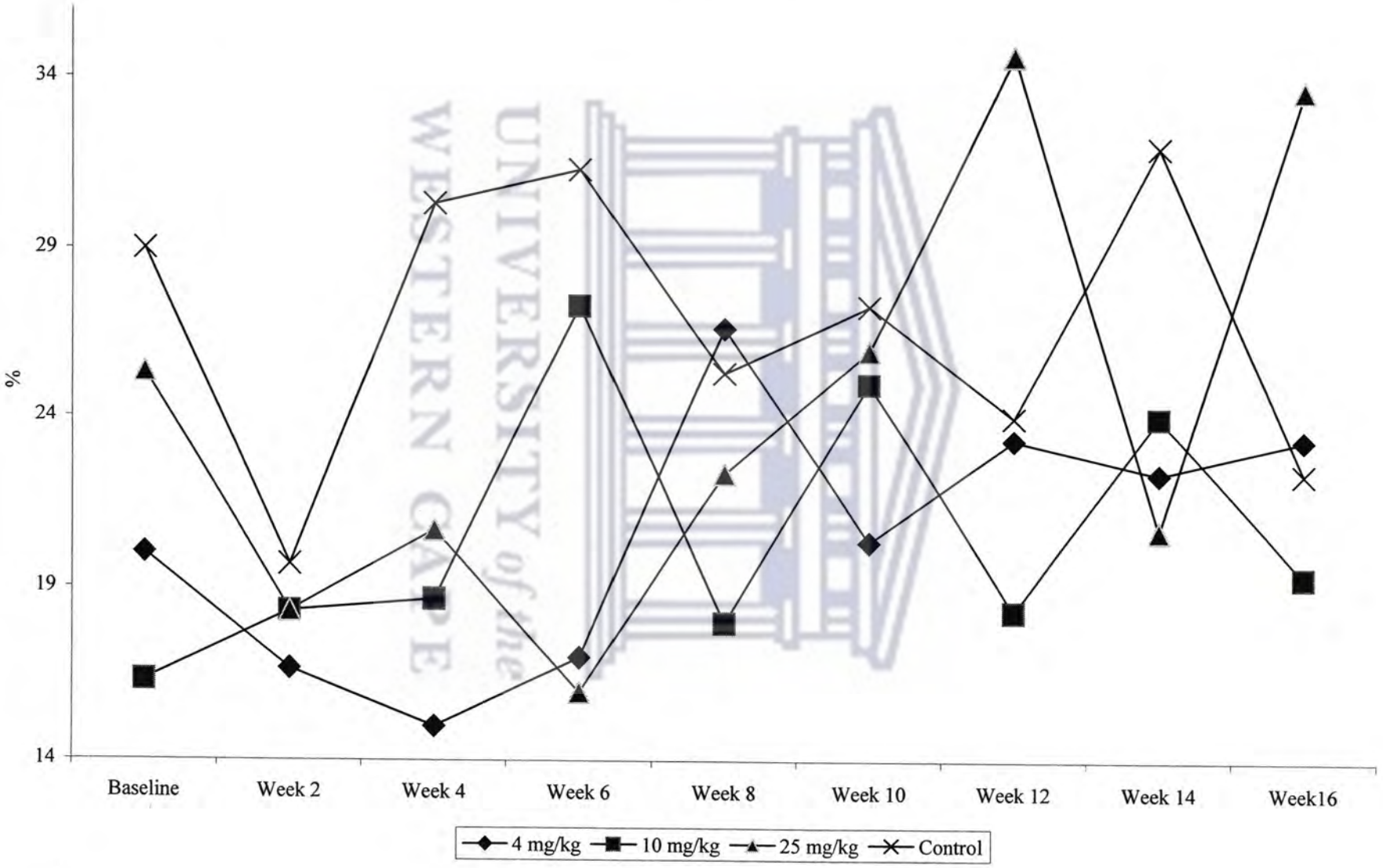


Figure 3.16. Broken midpiece

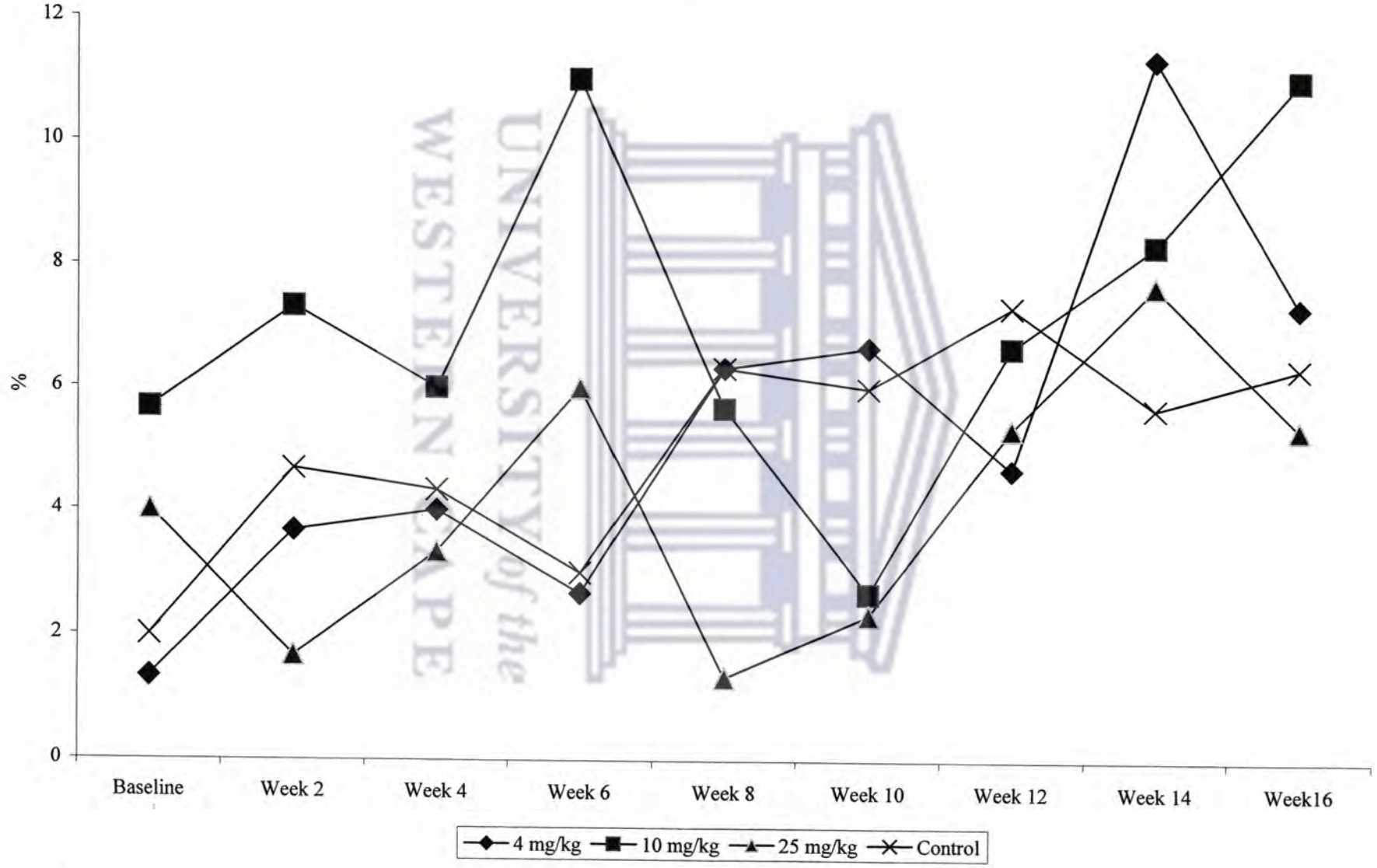


Figure 3.17. Abaxially implanted midpiece

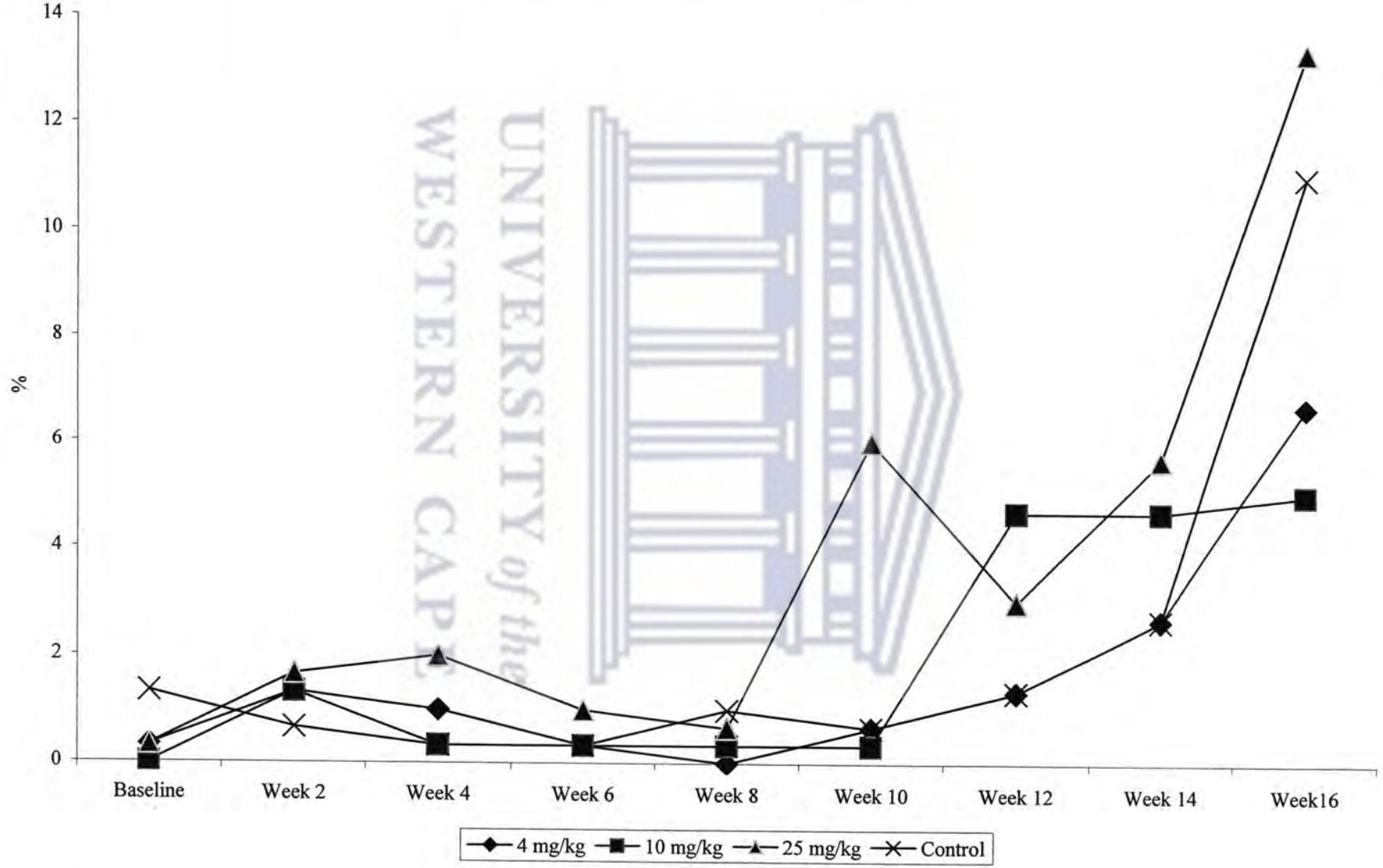
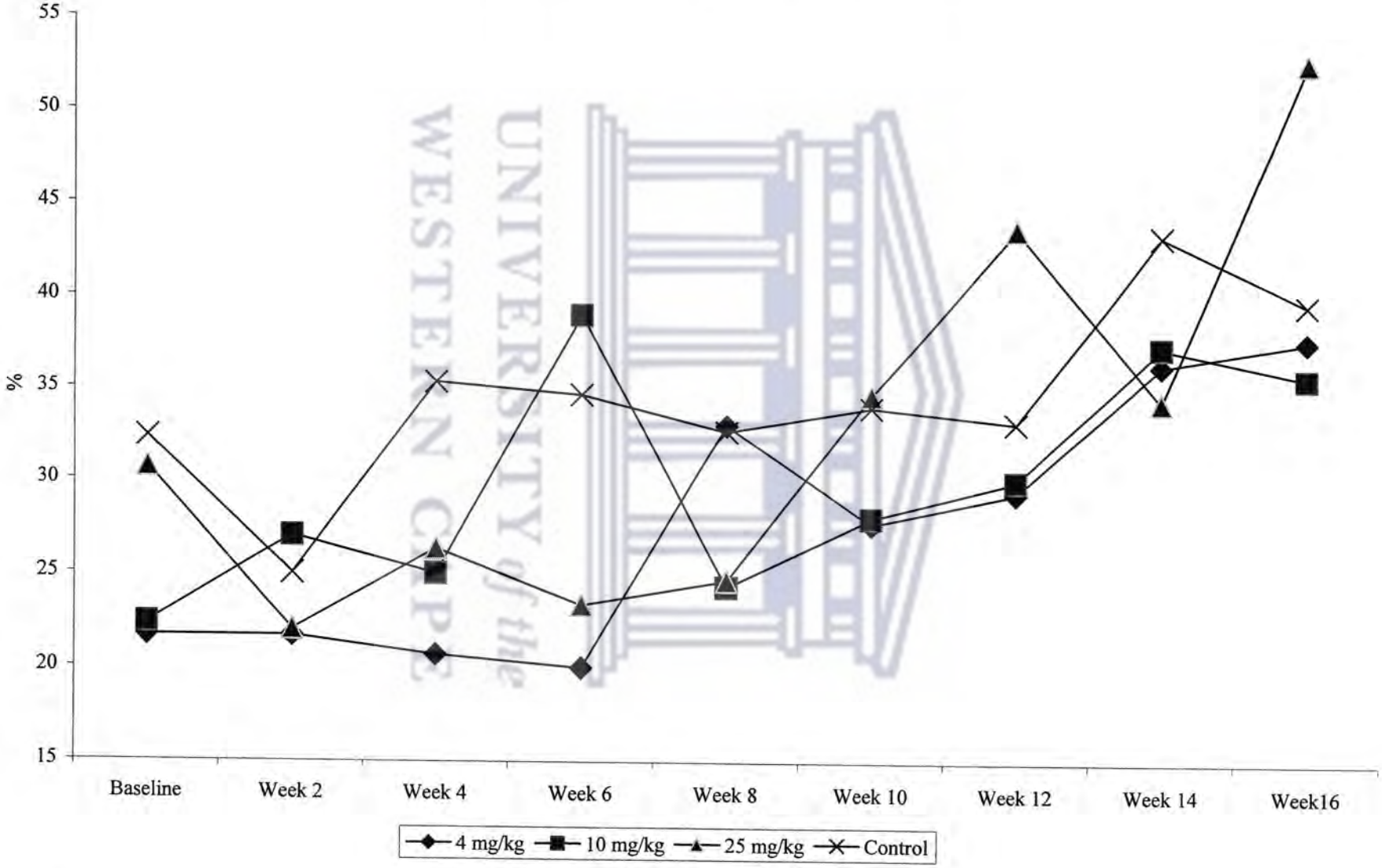


Figure 3.18. Combined midpiece abnormalities



3.4.3. Principal and end piece abnormalities

The percentages of sperm with coiled tails for each group are presented in Figure 3.19. Although there were increases in the coiled tail defects between baseline and the end of the study in the group that received 25 mg/kg oleanolic acid, no statistically significant differences were found between all treated groups and the controls.

Fluctuations in the number of sperm with hairpin tails in the treated groups (Figure 3.20) were only statistically significantly different (P = 0.0249) from the controls in the group that received 25 mg/kg oleanolic acid. Microscopic evaluation of spermatozoa with hairpin tail defects showed that these spermatozoa swim backwards. Detached tails were the least occurring defects (Figure 3.21), and no statistically significant differences were observed between treated groups and the controls.

Figure 3.22 illustrates combined tail abnormalities. These were more common than head abnormalities, but slightly less frequent than the defects of the midpiece. The results observed in this parameter were mostly caused by changes in the coiled and hairpin tails. Nonetheless, no statistically significant differences were found between combined tail abnormalities of the treated groups and the controls.

Figure 3.19. Coiled tail

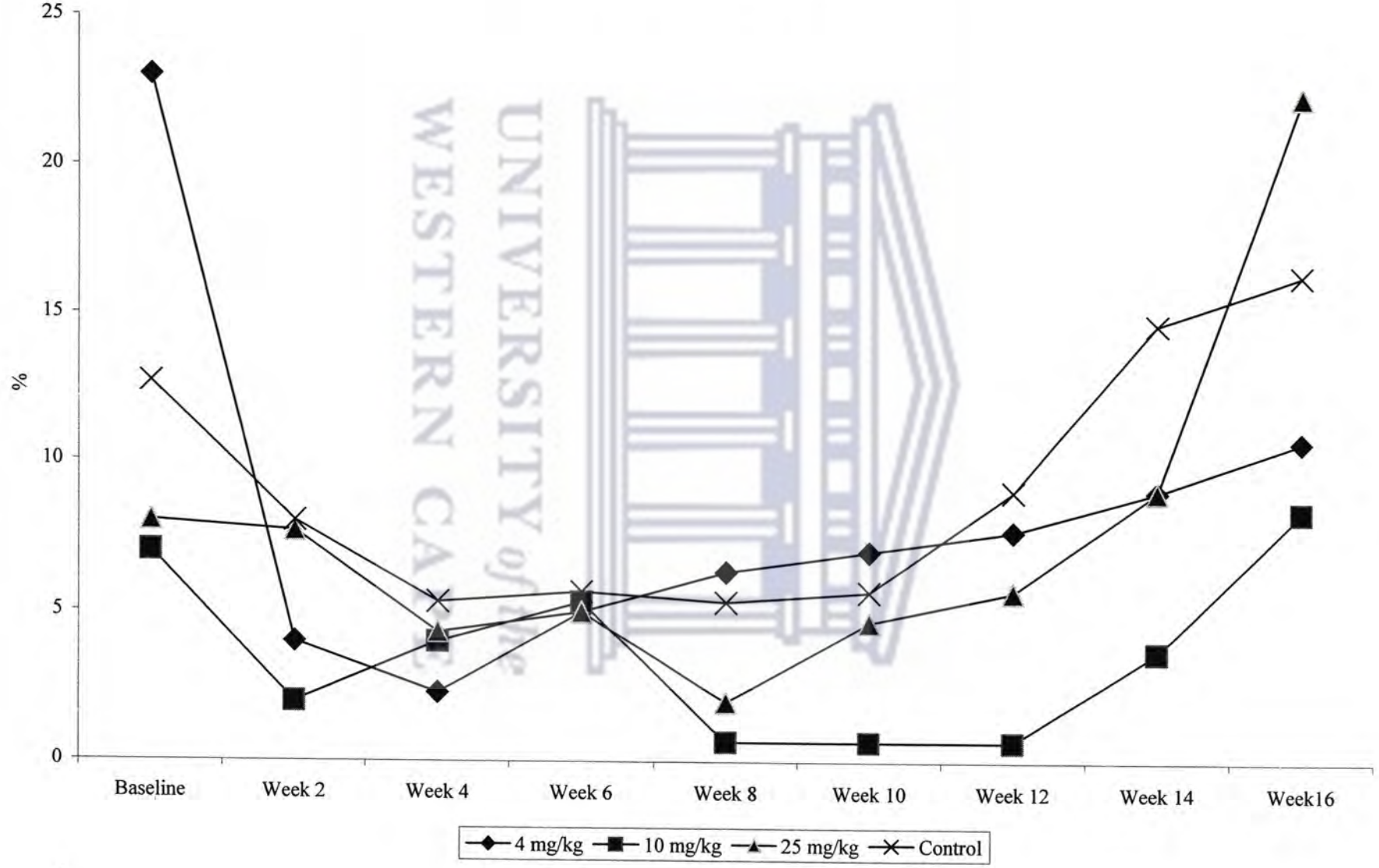


Figure 3.20. Hairpin tail

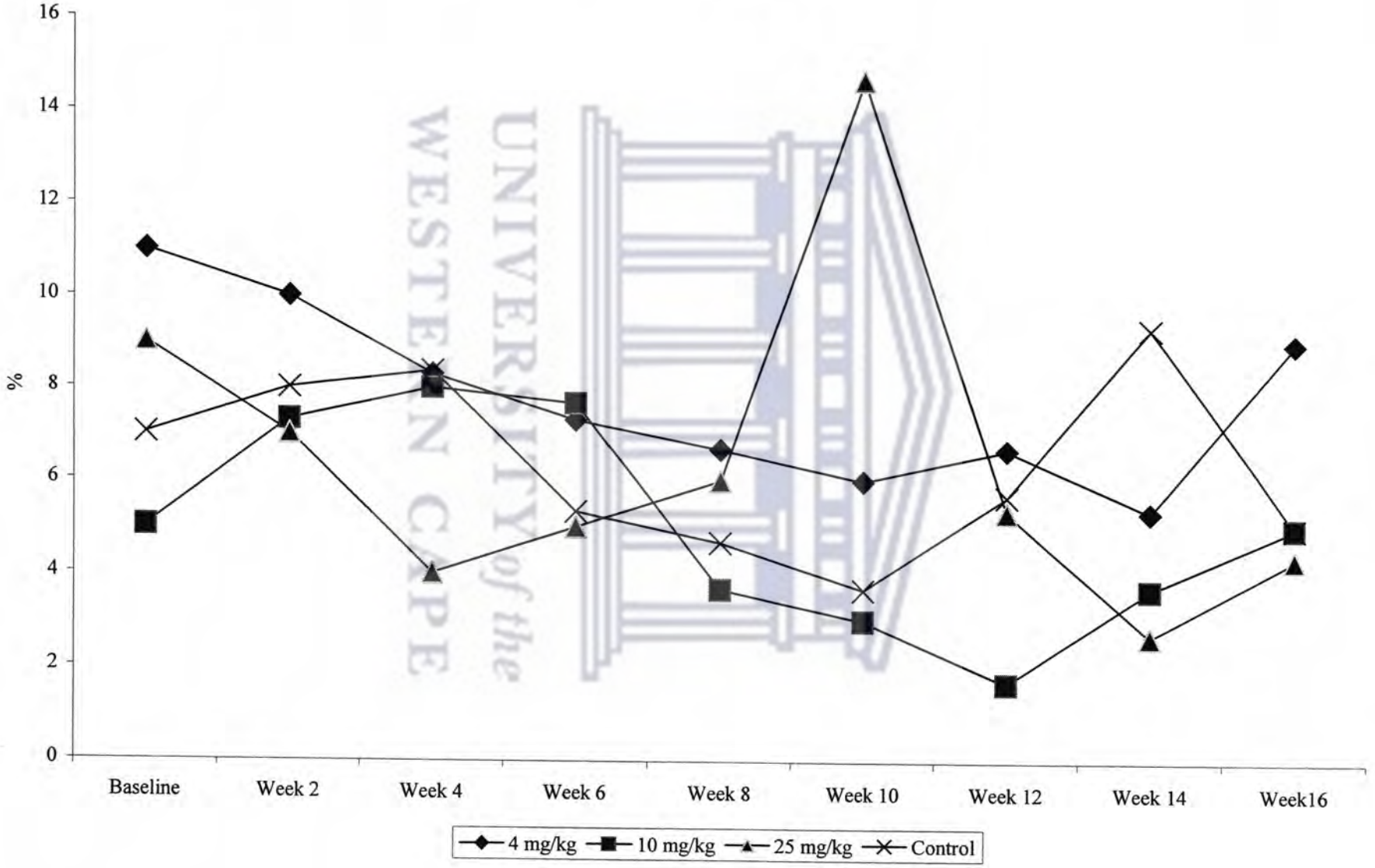


Figure 3.21. Detached tail

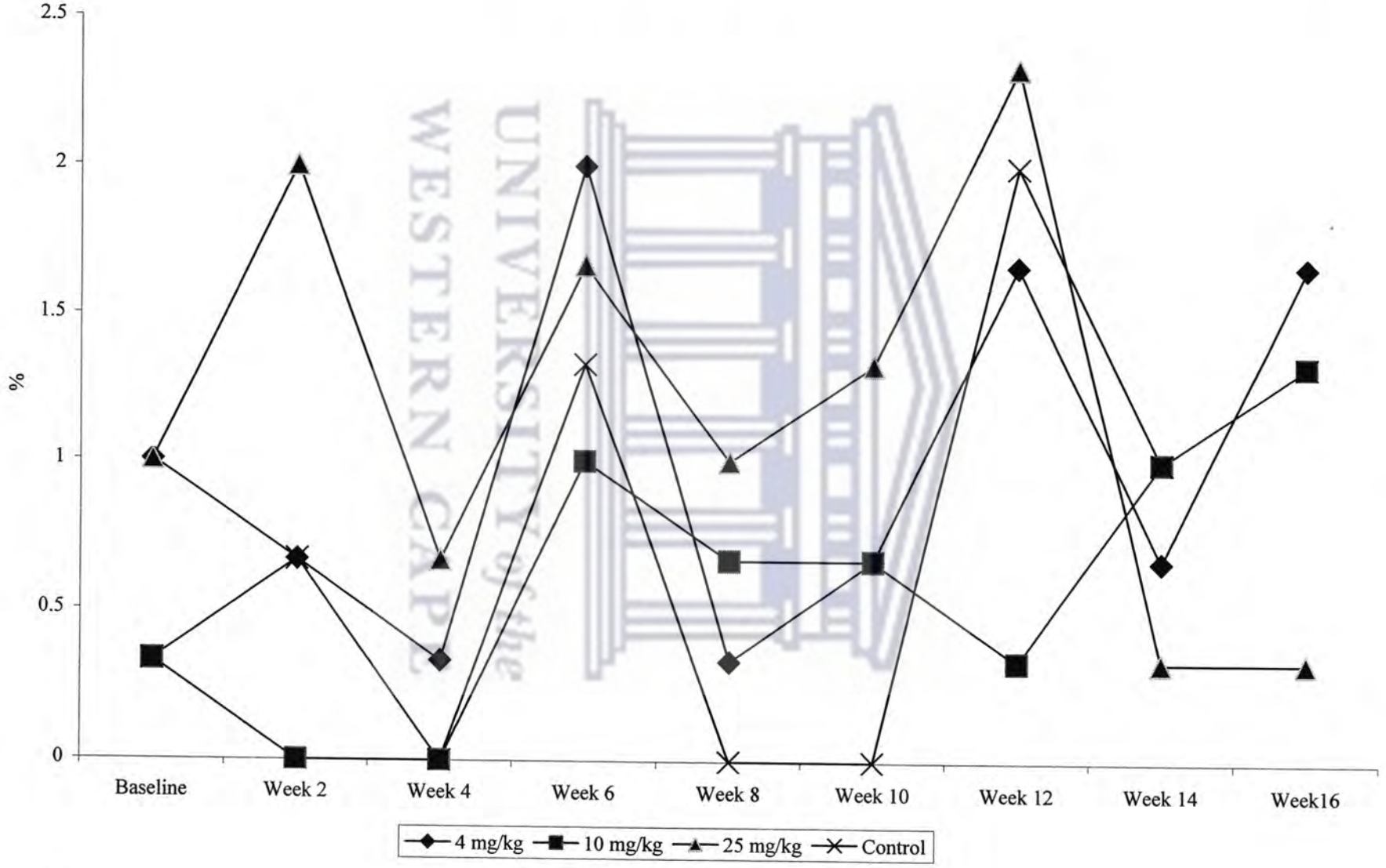
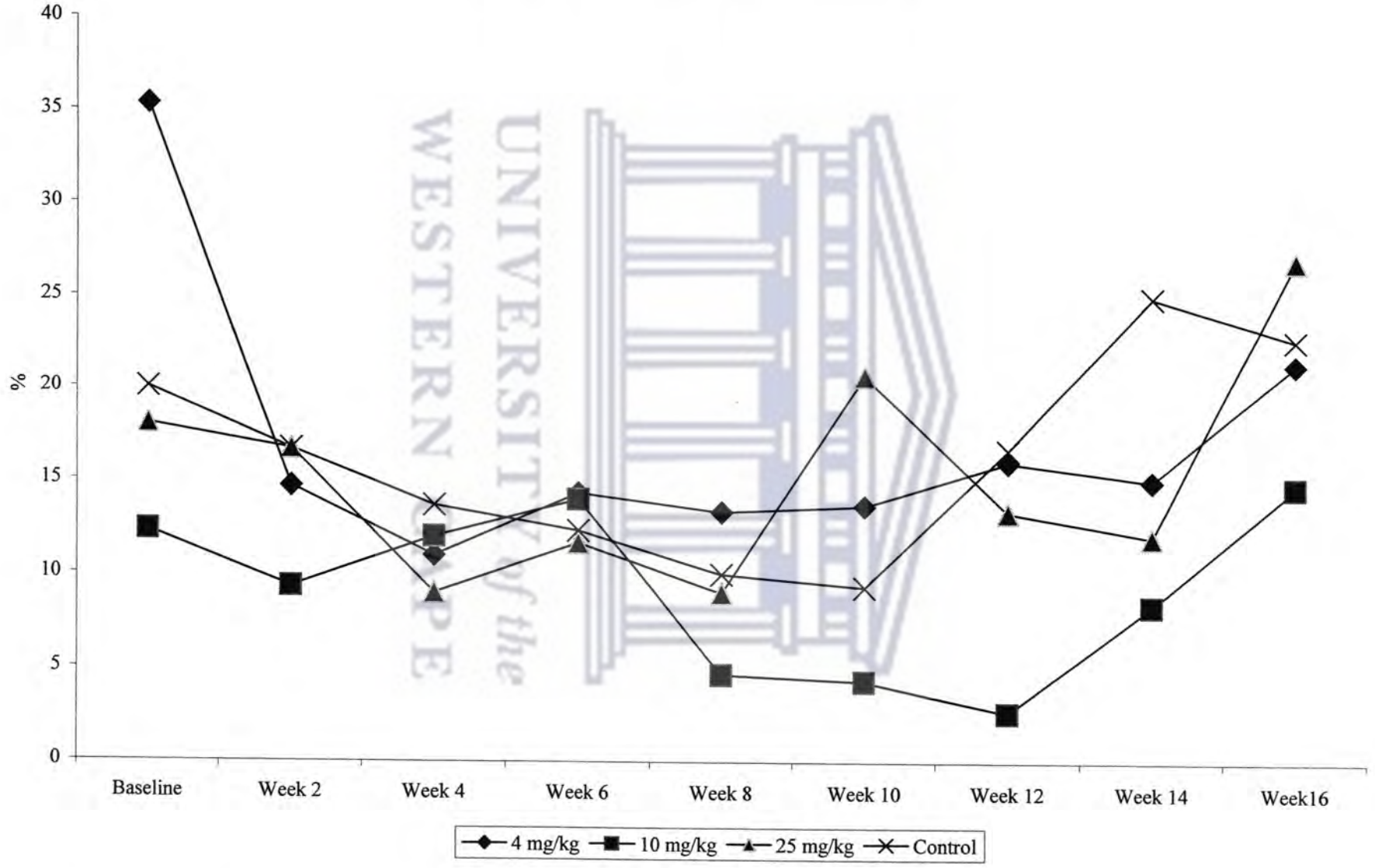


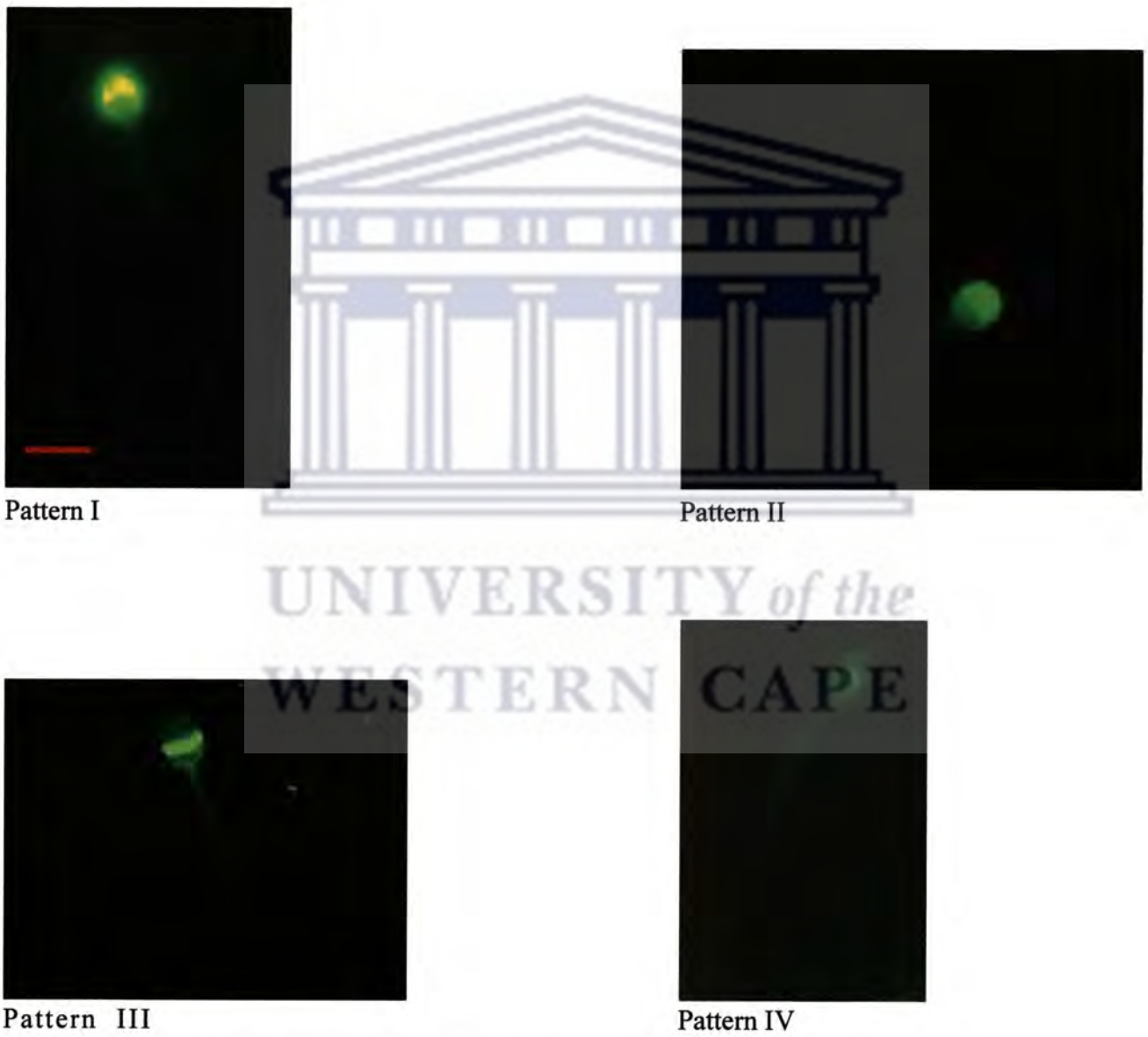
Figure 3.22. Combined tail abnormalities



3.5. Acrosomal integrity and sperm vitality

3.5.1. Acrosomal integrity

There were four distinct staining patterns of FITC-PNA to the acrosomal and equatorial regions of the vervet monkey spermatozoa (Figure 3.23). The bar at the bottom of pattern I photomicrograph (Figure 3.27) represents 6.3 μm, and also applies to Figure 3.28.



Pattern I Pattern II

Pattern III Pattern IV

Figure 3.23. Staining of vervet monkey spermatozoa after labeling with FITC-PNA

Although not statistically significantly different from the controls, decreases observed in the pattern I fluorescence in the treated groups were considerable (Figure 3.24). Associated with these changes were increases in the proportion of spermatozoa displaying pattern II fluorescence in all treated groups (Figure 3.25). Increases in this pattern were statistically significantly different ($P = 0.0276$) from the controls in the group that received 4 mg/kg oleanolic acid and not in the others. No statistically significant differences were observed for patterns III (Figure 3.26) and IV (Figure 3.27) between controls and the treated groups.

Figure 3.24. Intact acrosome (Pattern I)

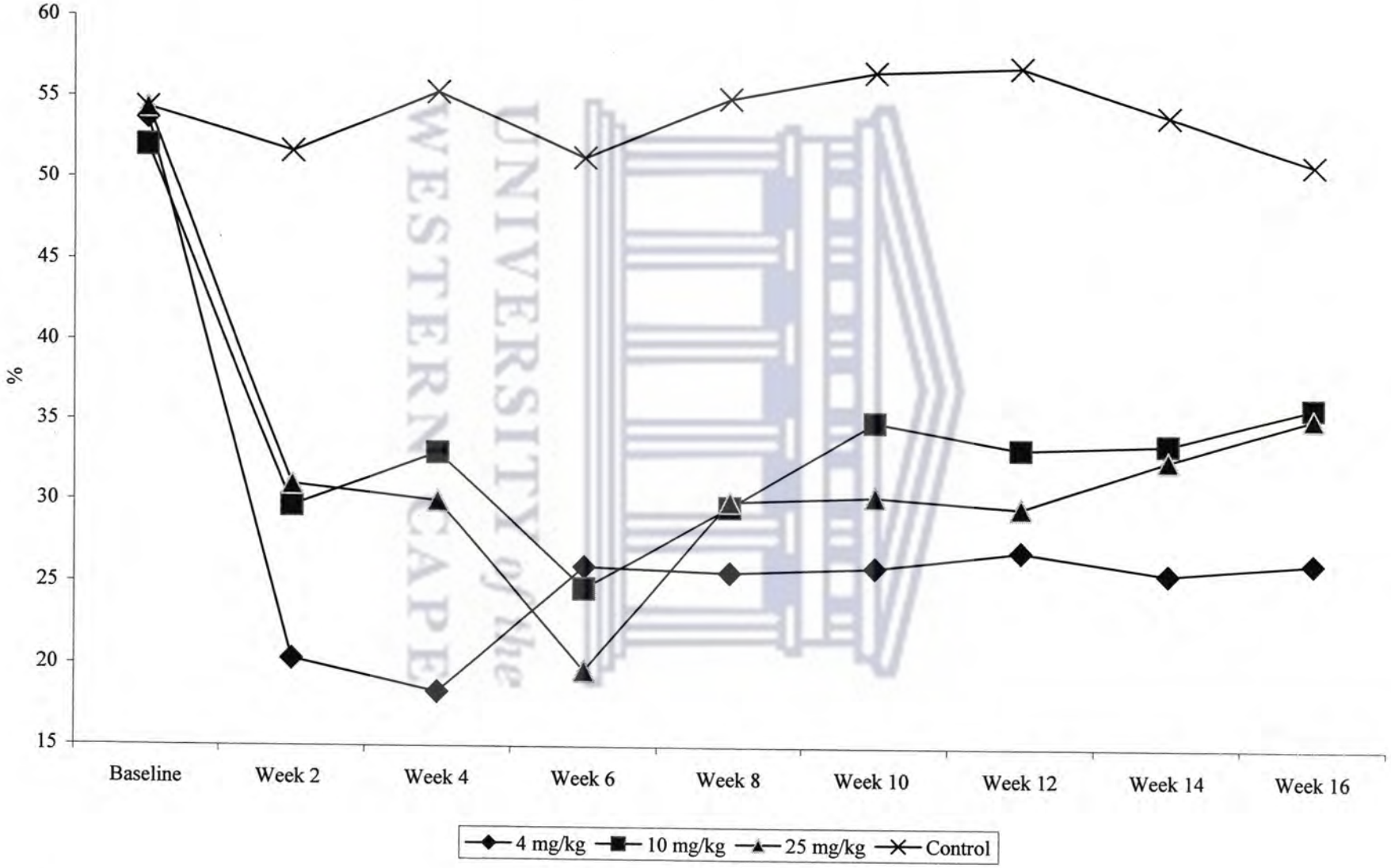


Figure 3.25. Patchy acrosome (Pattern II)

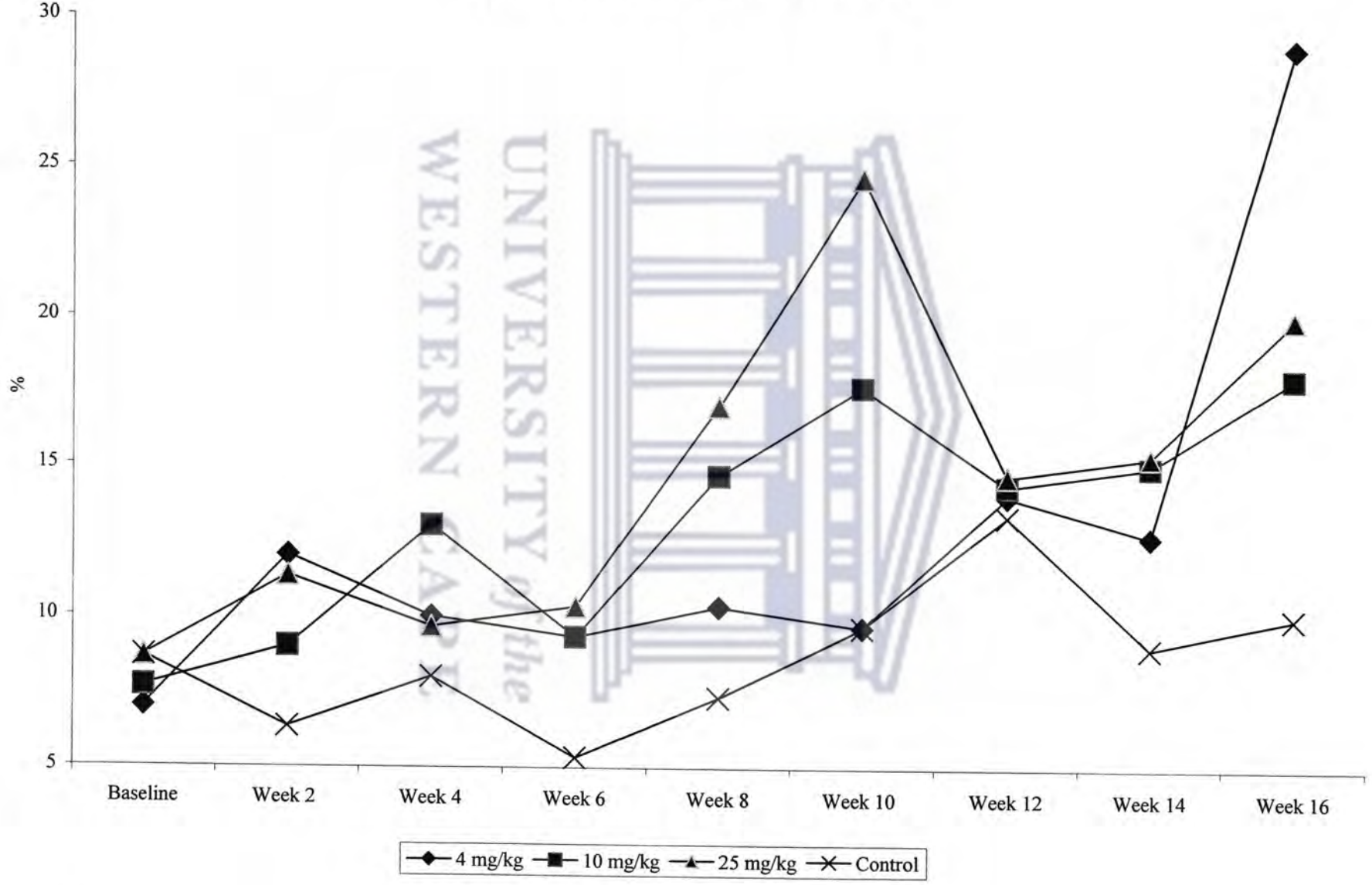


Figure 3.26. Equatorial staining (pattern III)

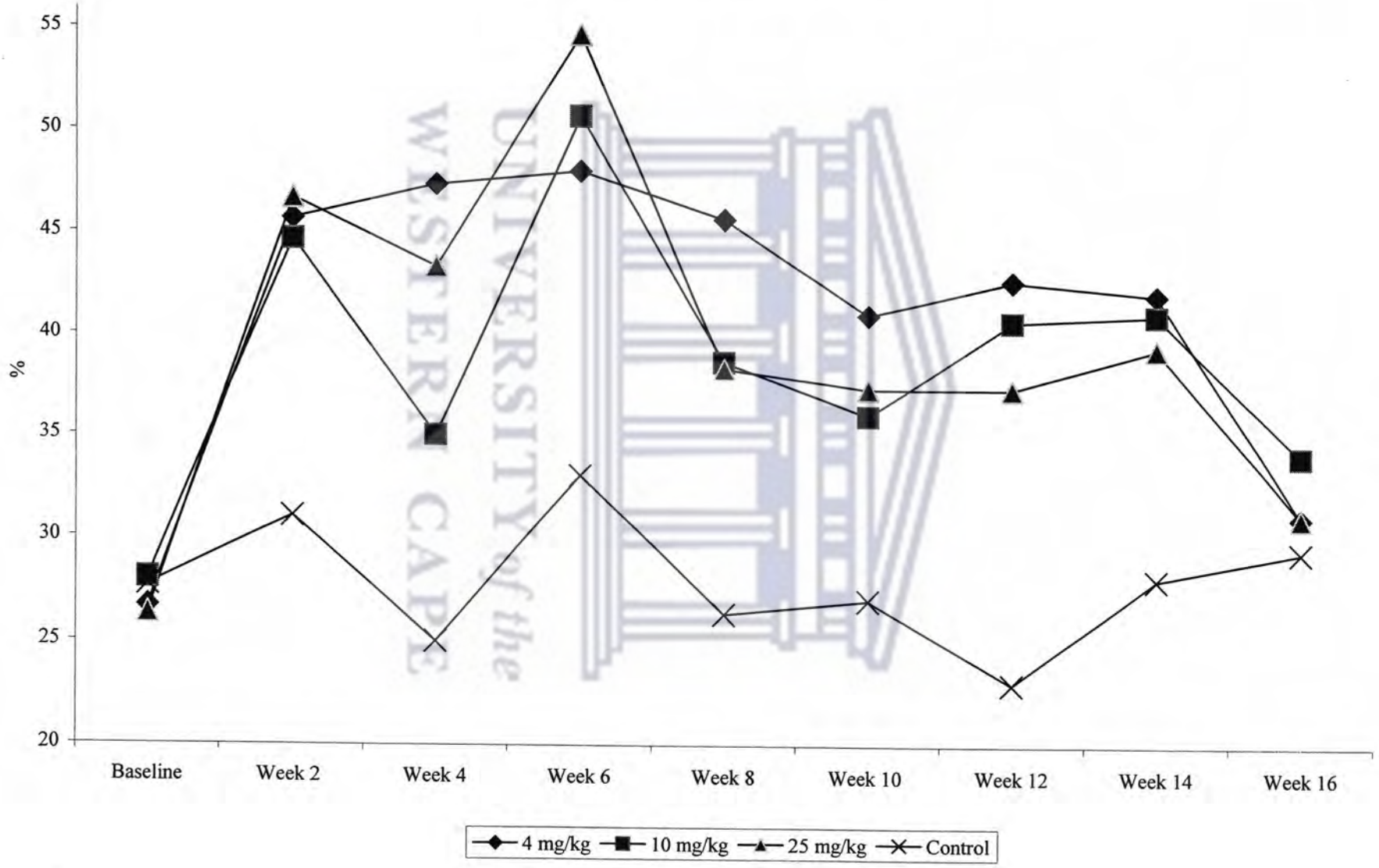
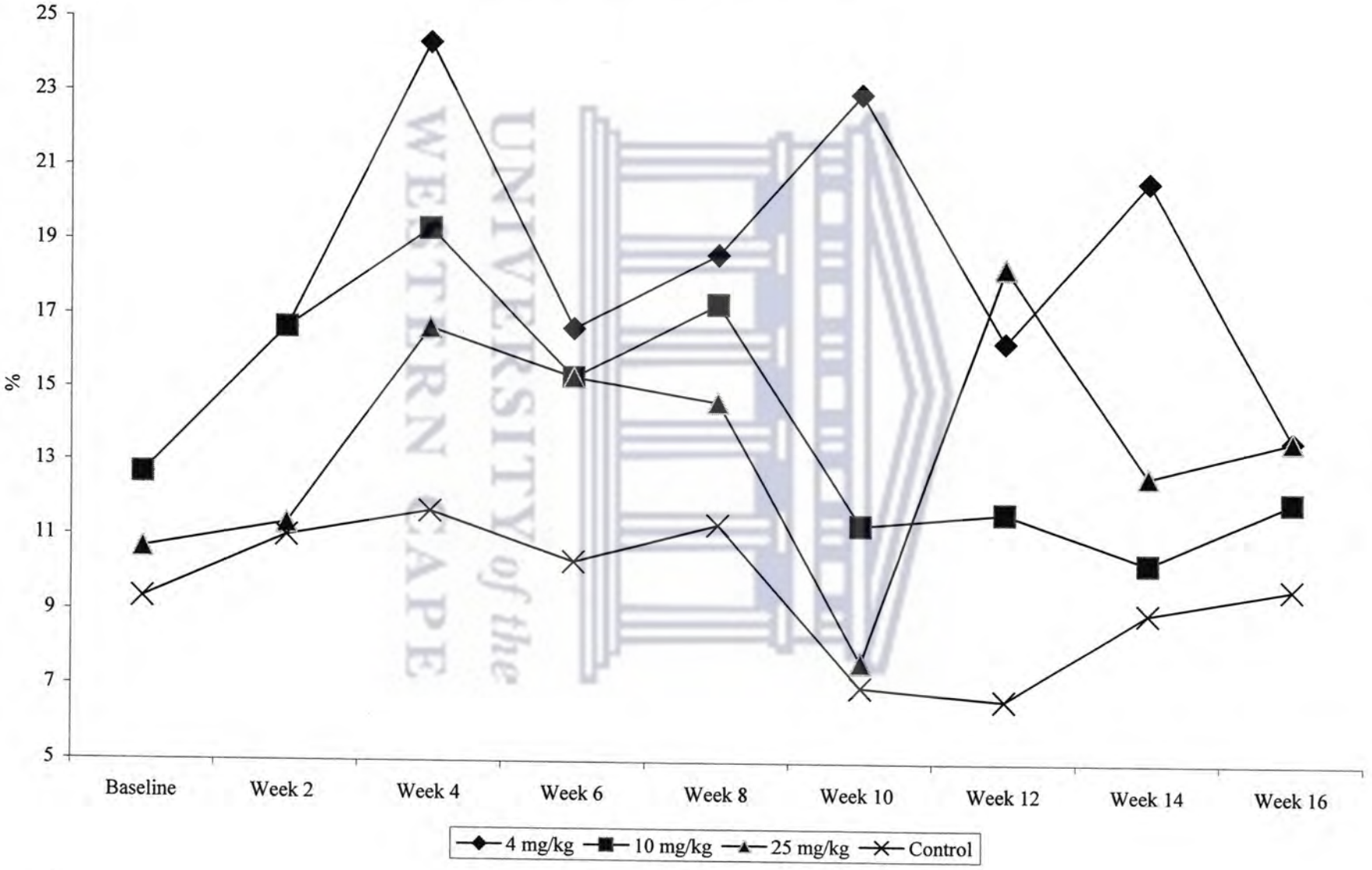


Figure 3.27. No acrosome (Pattern IV)



3.5.2. Sperm vitality

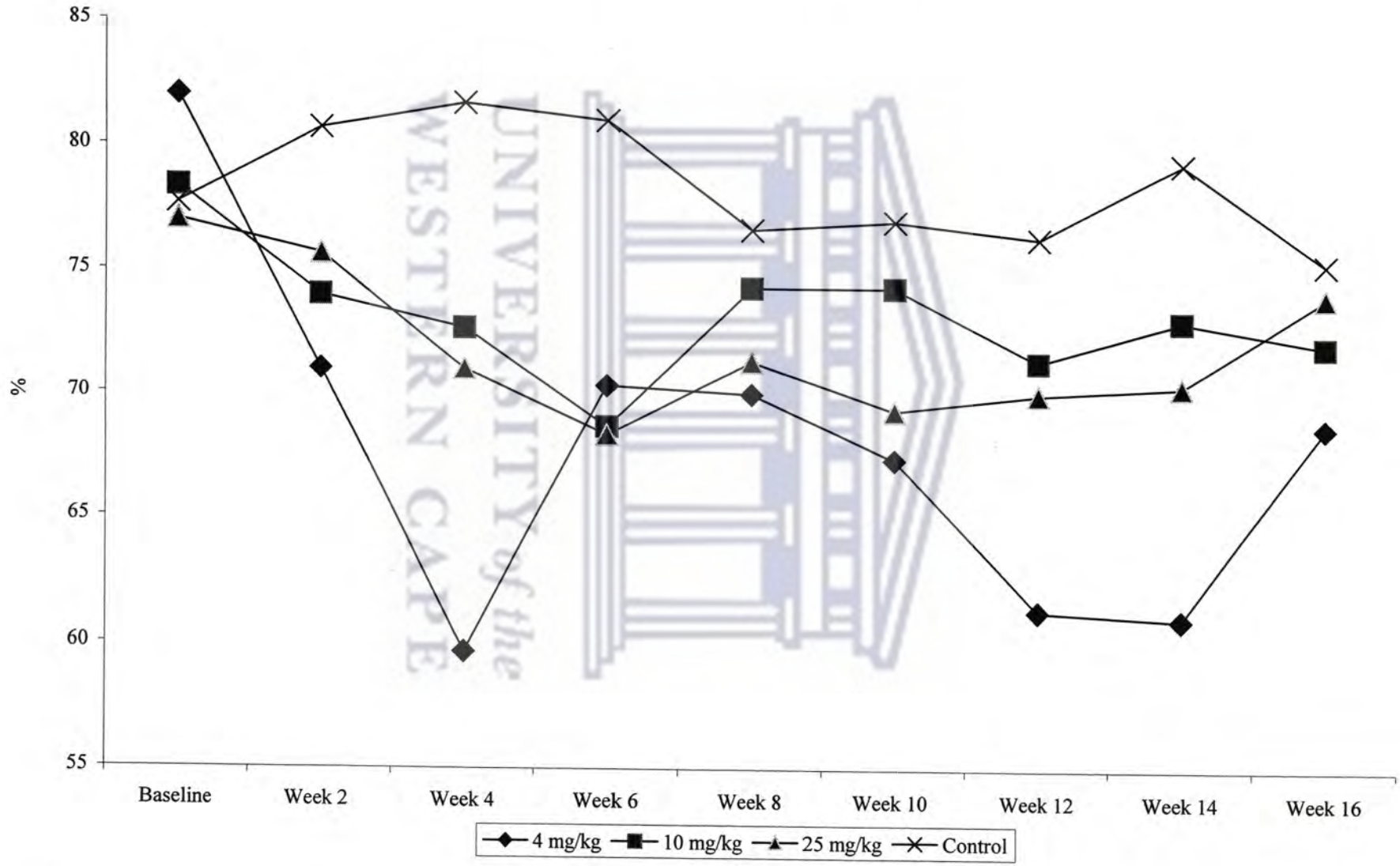
Two types of fluorescence observed after staining vervet monkey spermatozoa with a supravital stain, Hoechst 33258, are presented in Figure 3.28.

Figure 3.28. Fluorescent types of vervet monkey spermatozoa after staining with Hoechst 33258

Spermatozoon on the left has pale blue (type PB) fluorescence and is alive, whereas the one on the right displays bright blue (type BB) fluorescence and is dead.

Decline in the numbers of spermatozoa showing type PB fluorescence in the treated groups (Figure 3.29) were not statistically significantly different from the controls.

Figure 3.29. Live spermatozoa (Type PB)



3.6. Testosterone

Circulating concentrations of testosterone in the treated groups and the controls are shown in Figure 3.30. Although reductions observed in the serum testosterone concentrations of the treated groups, particularly in the group that received 4 mg/kg oleanolic acid, were not statistically significantly different from the controls, a subtle decrease may be associated with a treatment effect.

3.7. Fertility during treatment and reversibility

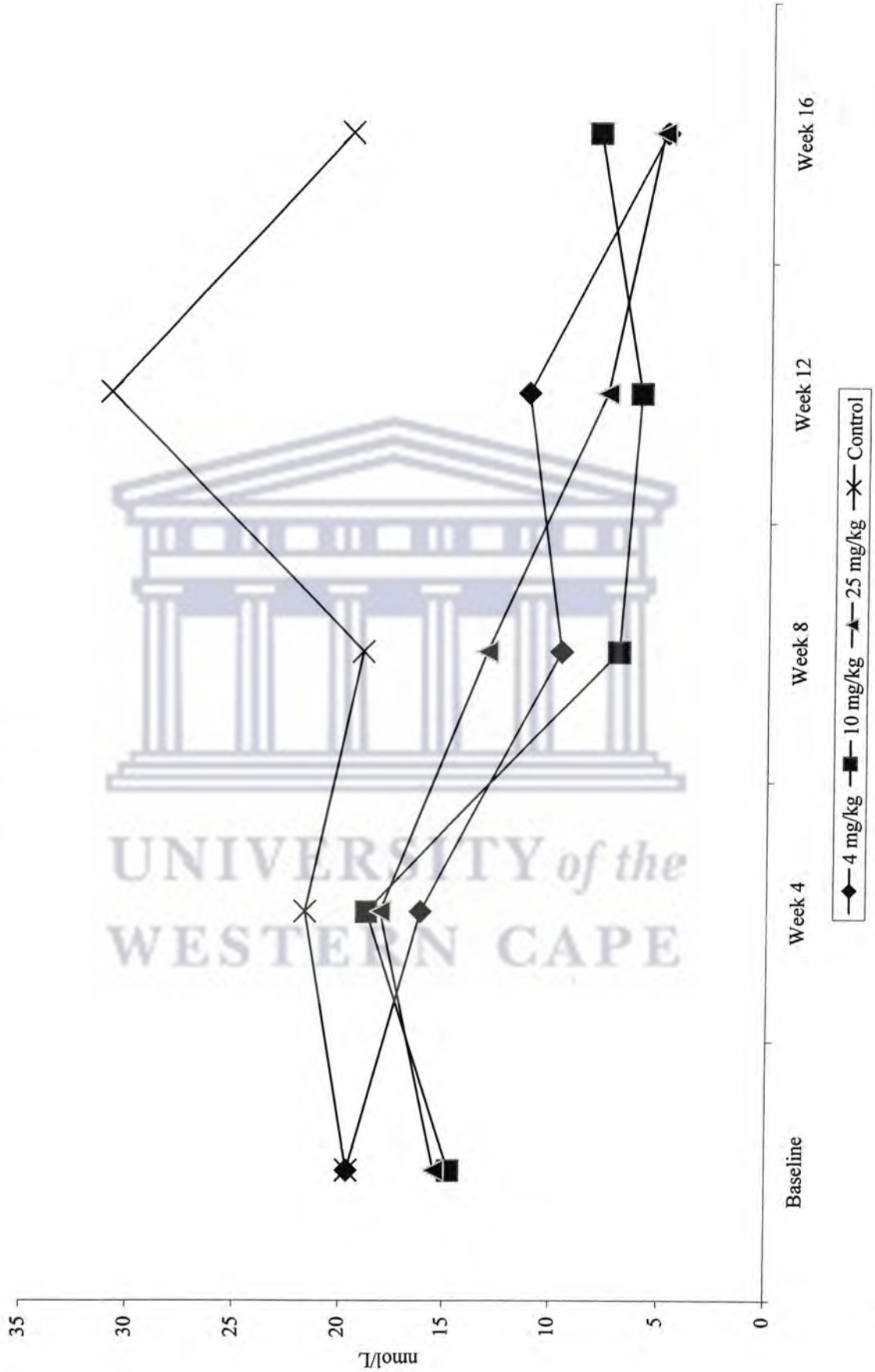
Vaginal smears prepared from females used in this study indicate that all males were able to copulate. Table 3.3 shows that the conception rates were not reduced in the groups that received 10 and 25 mg/kg oleanolic acid during the treatment period. No conception was observed in the group that received 4 mg/kg oleanolic acid, which returned to control levels two months after treatment withdrawal.

Table 3.2. Conception rates (percentages)

Group	During first 2 months of mating*	During additional 2 months of mating#
4 mg/kg	0	33.33
10 mg/kg	33.33	Not mated
25 mg/kg	66.66	Not mated
Control	33.33	Not mated

*During oleanolic acid treatment.
After oleanolic acid withdrawal.

Figure 3.30. Testosterone



Chapter 4

Discussion

Although the effects of oleanolic acid have been widely investigated in a variety of studies, there has been little emphasis on its potential antifertility effects. The only available data in this regard are those reported by Mdhluli and van der Horst (2002) and Rajasekaran *et al.* (1988) who showed that this compound has promising antifertility effects in rats, which might have applications in humans. However, it would be difficult to predict the effects this compound will have in humans using results from the rat studies alone, since this species is not as closely related to humans, as compared to non human primates. Data from the rat indicate that there are differences in the control of the male reproductive processes between this species, humans and non human primates. For example, rats are highly sensitive to the anti-gonadal and anti-pituitary effects of GnRH agonists (Thau *et al.* 1985). These peptides induced permanent testicular damage in rats, but not in humans and non human primates (Weinbauer and Nieschlag 1989).

To the knowledge of the author, there is no data in the literature on male antifertility effects of oleanolic acid in non human primates, despite the significant use of this order as a model for human reproduction. Therefore, this study presents for the first time, data about the effects of oleanolic acid on the different reproductive parameters of the adult male non human primate. For the above mentioned reason, the results presented in this study can only be compared with data from rodents.

4.1. Testicular volume

Testicular volume is regarded to be a reliable indicator of testicular function (Bujan *et al.*1989). In this study, the two-dimensional caliper measurement of length and width proved to be more accurate predictor of testicular volume than the one-dimensional measurement of scrotal circumference. Previous investigators reported that testicular measurements done with a caliper are in agreement with those of the ultrasound method (Love *et al.* 1991).

Reduced testicular volume, which is associated with the presence of varicocele, is often cited as the most common cause of male factor fertility (Gentile and Cockett 1992). Consistent with this theory, Coutinho *et al.* (2000) observed reduction in testicular volume and suppression of spermatogenesis in men after administration of gossypol. It was also noticed that where varicocele occurs there was a failure to reverse azoospermia, even after discontinuation of treatment (Coutinho and Melo 1988, Coutinho *et al.* 1984). In the current study, the changes observed in both testicular volumes of monkeys in the treated groups were negligible and of the same magnitude as in the controls. Therefore, it appears that administration of oleanolic acid has no risk of causing varicose swelling of the testicular veins, and thus may not affect defined threshold of testicular function.

4.2. Sperm motility and concentration

Among various maturational processes in the epididymis, spermatozoa develop the potential for sustained and progressive motility. This alteration of spermatozoa motility is not only associated with changes in their physiological, but also with their fertilizing capacity by enabling them to reach the egg and penetrate its investments. Gossypol is reported to cause proportional and complete abolishment of forward progressive motility in guinea pig spermatozoa (Shi and Friend 1983). In this study, forward progressive motility was reduced in the treated groups, which was particularly significant in the group that received 4 mg/kg oleanolic acid. This indicates that these

spermatozoa had motility similar to those displayed by immature testicular sperm (Perez-Sanchez *et al.* 1996). Since forward progressive motility of vervet monkey spermatozoa starts in the corpus epididymis and becomes more predominant in the cauda epididymis (van der Horst *et al.* 1999), results of this study suggest that oleanolic acid effects on sperm motility may have been directed towards these epididymal regions. It is further speculated that the mechanism of oleanolic acid effects on sperm motility could involve inhibition of epididymal forward motility protein action on spermatozoa, since this protein is important for acquisition of forward motility (Raynaud and Kann 1986). Considerable reductions in progressive forward motility and not speed of forward progression of the treated monkeys indicate that oleanolic acid treatment only affected the ability of sperm to move in a forward but not the vigor of motile spermatozoa. The finding that oleanolic acid has no strong influence on sperm swimming speeds is in contrast to the results of the previous study (Mdhluli and van der Horst 2002), in which several parameters of sperm velocities were significantly affected. However, it is probable that the discrepancy in the result of this study with those observed previously might be due to three factors. Firstly, inter-species differences could play a major role. Since it is known that there are differences in the size, structure and beat frequency of sperm between species (Phillips 1972). This explains variation in the hyperactivated sperm motility between species (Ho and Suarez 2001). Secondly, the sperm used in these studies were from different sources, i.e. ejaculate against cauda epididymal sperm. Although cauda epididymal and ejaculated sperm are regarded to be equally mature, evidence exist that they do not necessarily behave in the same way *in vitro* (Shalgi *et al.* 1981). Thirdly, the differences in the methods used to assess sperm motility, i.e. subjective versus computerized analysis, might have had some influence on the results of this study. It has been observed that there is variation between motility values generated by Computer Aided Sperm Analysis and subjective analysis (Kolibianakis *et al.* 1992).

Despite considerable fluctuations in sperm concentrations of the treated groups, results of this study do not demonstrate a tendency toward depressed spermatogenesis in any one of these groups. However, a definite variation within groups is reflected by large standard deviations of the means. For example, six out of nine sperm concentration measurements in the group that received 10 mg/kg oleanolic acid had larger standard deviations than the means. Since similar results were observed in the controls, it is speculated that several uncontrolled factors, rather than treatment effect, could be responsible for these varying sperm concentrations. Firstly, results from all groups suggest that there is a high level of intra- and inter-individual variability in sperm concentration. Similar intra-individual discrepancy in this parameter has also been observed in human males (Tyler *et al.* 1982) and vervet monkeys (Seier 1995). Secondly, variations in sperm concentrations may be associated with the frequent tendency among the non human primates to masturbate (Mohamed *et al.* 1987). This may have affected the abstinence period between semen sampling, and consequently sperm concentration. According to the results obtained in this parameter it is tempting to presume that sperm concentration is of little importance to the overall diagnosis of male fertility status in non human primates, unless azoospermia is achieved.

4.3. Sperm morphology

Sperm morphology correlates positively with fertility (Colenbrander and Kemp 1990). Therefore, recognition of specific forms of imperfect spermatozoa in stained smears under the microscope will provide useful information about the fertility status of a male. Generally, a high proportion teratospermic forms (ejaculation of less that 40% of morphologically normal spermatozoa) is rare in most mammals except in certain large wild felid species such as cheetah and clouded leopard (Pukazhenthi *et al.* 1998). In addition, teratospermia is also common in human (Pukazhenthi *et al.* 2001) and gorilla males and less encountered in other primate species (Cui *et al.* 1991). In view of

this finding, one would expect to find few morphologically defects in the normal semen sample of the vervet monkey.

4.3.1. Head abnormalities

The results of this study show that nipple acrosomes and the macrocephalic were the more prevalent head abnormalities in the group that received 25 mg/kg oleanolic acid, followed by tapering in the groups that received 4 and 10 mg/kg oleanolic acid. Despite the lack of statistically significant differences, increased fluctuations in the nipple acrosomal defects in the group that received 25 mg/kg oleanolic acid compared to the controls, may be associated with a treatment effect. This effect could be at one of two levels: testicular or epididymal. At the testicular level, oleanolic acid may have disturbed acrosomal development during spermiogenesis through prevention of spreading of the Golgi complex (GC) over the nuclear surface of the sperm to form an acrosomal cap. At the epididymal level, oleanolic acid may have interfered with the epididymal processes involved in the complete morphological changes of the acrosomes. In view of the decreased testosterone concentrations in the treated groups, it appears that this oleanolic acid-induced androgen deficiency lead to general disturbances in sperm surface carbohydrates. This is because alteration of the sperm surface carbohydrates in the epididymis by glycosylation involves androgen-dependent glycosyltransferases, i.e. glucosyl-and mannosyl-transferases (Iusem *et al.* 1984).

One of the interesting findings of this study was that oleanolic acid increased occurrence of the tapered head defects without alterations of the testicular sizes or formation of the varicocele in the treated monkeys. This is because increases in the tapered head defects mostly emanate from these conditions (Ayodeji and Baker 1986, Ali *et al.* 1990). The question to be answered would be what causes these head defects? It is tempting to speculate again that they may have resulted, in part, from the lowered testosterone concentrations by oleanolic acid. This could have led to androgen depletion during later stages of spermatogenesis, which are critical for shaping of the sperm heads.

The lack of statistically significant differences in macrocephalic defects between controls and the group that received 25 mg/kg oleanolic acid further suggest that oleanolic acid has no effects at early to middle stages of spermatogenesis. These defects indicate deficiency of the meiotic division, where spermatogenesis is arrested at the round spermatid stage (Escalier 2002).

Despite significant fluctuations in the numbers of sperm with acrosomal cyst in the group that received 10 mg/kg oleanolic acid, the results also show that both this defect and the microcephalic defects followed an almost similar trend throughout the period of the study in this group. These results are presumably in agreement with the finding that spermatozoa with the latter condition usually have no acrosomes (Nistal *et al.* 1978).

Another interesting finding is that the above-mentioned defects, as well as the pyriform and constricted defects were correspondingly very high in the 10 mg/kg oleanolic acid group at week 12 of the study. The potentiating action of oleanolic acid on the appearance of these defects at this period cannot be explained at present, and necessitates further investigations.

Although amorphous were the least (0.33%) occurring head abnormalities, the statistically significant fluctuations in the numbers of sperm with this defect in the group that received 25 mg/kg oleanolic acid might be treatment related. On the other hand, the round headed spermatozoa occurred at almost the same rate in treated and control groups, which indicates a lack of treatment effect.

4.3.2. Midpiece abnormalities

In this study, the broken, bent and abaxially implanted midpieces were the principal types of midpiece abnormalities. The highly significant fluctuations in the bent midpieces of the group that received 25 mg/kg oleanolic acid may be associated with a treatment effect.

Since no statistically significant differences were observed in the percentages of broken and abaxially implanted midpieces between controls and the treated groups, this suggests a lack of oleanolic acid effect. From the results, it is observed that the combined midpiece defects in the controls and treated groups were much higher compared to the combined head defects of the same groups. This is in agreement with the report by Seier *et al.* (1996), indicating that non human primates have a low rate of sperm head defects compared to human males.

4.3.3. Principal and end piece abnormalities

The second most common abnormalities observed in this study were tail defects, in which hairpin and coiled tails being the primary defects. Smikle and Turek (1997) reported that coiled tails may be induced *in vitro* by exposing spermatozoa to hypo-osmotic solution with a different ionic strength to that of the seminal plasma. Therefore, despite lack of statistical significance, the considerably higher number of spermatozoa with this defect at the end of the study compared to baseline in the group that received 25 mg/kg oleanolic acid may be associated with a treatment effect. It is possible that oleanolic acid accumulates in the epididymis and uncouples the disulfide bonds, which are necessary to stabilize sperm tail and reduce coiling during epididymal maturation (Sutovsky *et al.* 1997).

Regarding the hairpin defect, this condition results from folding back of the sperm tail on itself at a certain point behind the neck to form a kink (Phillips and Kalay 1984). The backward movement observed on spermatozoa with this defect in this study is reported to be caused by the base-to-tip wave propagation at the distal portion of the tail, and not at the region between the kink and the sperm head (Phillips and Kalay 1984). The significantly high fluctuations in the spermatozoa with this defect in the group that received 25 mg/kg oleanolic acid may be associated with oleanolic acid

treatment. It is suggested that oleanolic acid could be acting on the spermatozoa through alterations of the epididymal milieu responsible for ultrastructural modification of the sperm tail.

4.4. Acrosomal integrity and sperm vitality

The data from this study demonstrate that at baseline, fluorescence pattern I was a dominant characteristic for all three treated groups, as well as the controls. However, following administration of oleanolic acid, proportions of spermatozoa displaying pattern I fluorescence were lower, whereas those of patterns II to IV were increased in the treated groups compared to the controls. Despite a lack of statistically significant difference between controls and the groups that received 10 and 25 mg/kg oleanolic acid, reductions in pattern I fluorescence in these treated groups may be treatment related. In addition, a statistically significant increase in pattern II fluorescence in the group that received 4 mg/kg oleanolic acid, suggests that oleanolic acid may have biphasic dose effects. This phenomenon is also observed in the vitality data, where there was a higher decline in vital pattern PB fluorescence in the group that received 4 mg/kg oleanolic acid as compared to other treated groups.

In conclusion, the huge reduction in pattern I fluorescence in the treated groups suggests that oleanolic acid may have effects on the functional changes in sperm plasma membrane composition and organization of the PNA specific lectin-binding glycoproteins, which takes place during epididymal transit.

4.5. Testosterone

The average baseline testosterone levels in the males from the control and treated groups were similar to the concentrations reported previously in this species (Beattie and Bullock 1978). Another member in the triterpene family, Quassin, is reported to inhibit basal and LH-stimulated

testosterone secretion by Leydig cells in rats (Raji and Bolarinwa 1997). Although no statistically significant differences were found, the tendency of reduction in serum testosterone concentrations in all treated groups, and not in the controls, suggests that oleanolic acid interfers with testosterone secretion/synthesis. However, Rajasekaran *et al.* (1988) suggested that oleanolic acid does not alter synthesis of testosterone. The discrepancy in our results from that of Rajasekaran *et al.* (1988) seems to be due to two factors. Firstly, they estimated alteration of testosterone synthesis based on changes in the weight of androgen-dependent organs, compared to direct serum testosterone analysis in this study. Secondly, the use of different species, i.e. monkeys in this study and rats in their study. It is known that there is a considerable interspecies variation in response to compound absorption, binding to plasma proteins, pattern of metabolism and mode of excretion (Smith and Williams 1974). Therefore, it is possible that there were differences in routes and rates of metabolism between these species.

In conclusion, further investigations are necessary to elucidate whether oleanolic acid affects testosterone release through the hypothalamo-hypophyseal-gonadal axis or by a local testicular mechanism.

4.6. Fertility during treatment and reversibility

The results of this study show that the ability to mate with females by male monkeys treated with oleanolic acid was unaffected. These observations provide evidence that the treated males had normal sexual activity and possibly no erectile dysfunction.

The previous data suggest that oleanolic acid has a potential for the development as a reversible human male contraceptive compound (Mdhluli and van der Horst 2002, Rajasekaran *et al.* 1988). As in rat studies, two questions had to be answered, i.e. whether oleanolic acid affects fertility of male vervet monkeys, and if yes, whether these antifertility effects are reversible? To answer the

first question, the conception rates show that only the group that received 4 mg/kg oleanolic acid did not produce pregnancies during the first two months of mating. This is interesting, since the effects of oleanolic acid on sperm progressive motility, acrosomal integrity, vitality and serum testosterone concentrations were also pronounced in this group and not in the groups that received higher doses. Therefore, an agreement between sperm status, androgen concentrations and mating results in the group that received 4 mg/kg oleanolic acid further confirms the existence of biphasic dose responses observed in this study.

With respect to the second question, the results of this study reveal that cessation of treatment resulted in an apparent return to normal fertility, suggesting that the effects of oleanolic acid are reversible. The reversibility of antifertility effects of oleanolic acid have been reported before (Mdhluli and van der Horst 2002). Based on these findings, it can be speculated that oleanolic acid is devoid of permanent sterility effects.

Conclusion

This study has shown that oleanolic acid has no effect on the testicular volumes of the treated monkeys. The swift onset at which changes were observed in sperm characteristics, particularly sperm with intact acrosomes in the treated groups, suggest that oleanolic acid could be acting on the late stages of spermatogenesis (i.e. spermiogenesis) and/or during sperm epididymal transit, thus rendering it a potential post-testicular male antifertility compound. Since most changes observed in the sperm characteristics of the treated monkeys stabilized at a certain point, it appears that there was an inherent/acquired cellular drug uptake threshold, or enhanced but limited drug detoxification process in these monkeys.

According to the low rate at which sperm head defects occurred in the treated groups, it is reasonable to speculate that oleanolic acid has no major effects on spermatogenesis in male vervet monkeys. In contrast, the apparent post-testicular effects of oleanolic acid are demonstrated by reductions in the treated groups' sperm progressive motility and lectin binding ability, which are acquired during epididymal transit.

The results of this study demonstrate the presence of biphasic dose responses. The influence of biphasic action on the sperm parameters, androgens and fertility are not understood. Further studies are required to investigate the mechanism of these biphasic responses.

In conclusion, oleanolic acid represents an alternative approach towards the development of a chemical male contraceptive.

References

Ahluwalia B, Farshori P, Jamuar M, Baccetti B, Anderson WA (1990) Specific localization of lectins in boar and bull spermatozoa. Journal of Submicroscopic Cytology and Pathology **22**,53-62

Ali JI, Weaver DJ, Weinstein SH, Grimes EM (1990) Scrotal temperature and semen quality in men with and without varicocele. Archives of Andrology **24**, 215-219

Amory JK, Bremner WJ (1998) The use of testosterone as a male contraceptive. Bailliere's Clinical Endocrinology Metabolism **12**, 471-484

Anawalt BD, Amory JK (2001) Male hormonal contraceptives. Expert Opinion on Pharmacotherapy **2**, 1389-1398

Andrews G (2000) Contraception: what has changed over the past decade? British Journal of Nursing **9**, 326-333

Ayodeji O, Baker HW (1986) Is there a specific abnormality of sperm morphology in men with varicoceles? Fertility and Sterility **45**, 839-842

Back DJ, Glover TD, Shenton JC, Boyd GP (1975) The effects of alpha-chlorohydrin on the composition of rat and rabbit epididymal plasma: a possible explanation of species difference. Journal of Reproduction and Fertility **45**, 117-128

Barr AB (1973) Timing of spermatogenesis in four non-human primate species. Fertility and Sterility **24**, 381-389

Beattie CW, Bullock BC (1978) Diurnal variation of serum androgens and oestradiol-17ß in the adult male green monkey (*Cercopithecus aethiops*). Biology of Reproduction **19**, 36-39

Benzacken B, Gavelle FM, Martin-Pont B, Dupuy O, Lievre N, Hugues JN, Wolf JP (2001) Familial sperm polyploidy induced by genetic spermatogenesis failure: case report. Human Reproduction **16**, 2646-2651

Bhasin S, Woodhouse L, Casaburi R, Singh, AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW (2001) Testosterone dose-response relationships in healthy young men. American Journal of Physiology Endocrinology and Metabolism **281**, 1172-1181

Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M, Hsueh AJ (1995) Apoptosis in testis germ cells: Developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology **136**, 5-12

Blom E (1977) A decapitated sperm defect in two sterile Hereford bulls. Nordic Veterinary Medicine **29**, 119-123

Blom E (1978) The corkscrew sperm defect in Danish bulls--a possible indicator of nuclear fallout? Nordic Veterinary Medicine **30**, 1-8

Blom E, Birch-Andersen A (1975) The ultrastructure of a characteristic sperm head-defect in the boar: the SME-defect. Andrologia **7**, 199-209

Blom E, Wolstrup C (1976) Zinc as a possible causal factor in the sterilizing sperm tail defect, the 'Dag-defect', in Jersey bulls. Nordic Veterinary Medicine **28**, 515-518

Brown-Woodman PD, Mohri H, Mohri T, Suter D, White IG (1978) Mode of action of alpha-chlorohydrin as a male anti-fertility agent. Inhibition of the metabolism of ram spermatozoa by alpha-chlorohydrin and location of block in glycolysis. Biochemical Journal **170**, 23-37

Bujan L, Mieusset R, Mansat A, Moatti JP, Mondinat C, Pontonnier F (1989) Testicular sizes in infertile men: Relationship to semen characteristics and hormonal blood levels. British Journal of Urology **64**, 632-637

Bujan L, Mieusset R, Mondinat C, Mansat A, Pontonnier F (1988) Sperm morphology in fertile men and its age related variation. Andrologia **20**, 121-128

Burkhart JG, Malling HV (1989) Differentiation of binuclear spermatozoa in mice. Gamete Research **22**, 399-410

Camatini M, Franchi E, De Curtis I (1981) Ultrastructure of Leydig cells in the African green monkey. Journal of Ultrastructure Research **76**, 224-234

Cameron DF, Muffly KE, Nazian SJ (1993) Reduced testosterone during puberty results in a midspermiogenic lesion. Proceedings of the Society for Experimental Biology and Medicine **202**, 457-464

Carr DW, Acott TS (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: Studies of a sperm motility quiescence factor. Biology of Reproduction **30**, 913-925

Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B (1996) Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. Journal of Andrology **17**, 674-682

Chipfakacha VG (1993) Attitudes of males on contraception: a KAPE survey. East African Medical Journal **70**, 82-84

Clermont Y (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiological Review **52**, 198-236

Colenbrander B, Kemp B (1990) Factors influencing semen quality in pigs. Journal of Reproduction and Fertility **40**, 105-115

Comhaire FH (1994) Male contraception: hormonal, mechanical and other. Human Reproduction **9**, 586-590

Cooper TG, Waites GM (1975) Steroid entry into rete testis fluid and the blood-testis barrier. Journal of Endocrinology **65**, 195-205

Cornwall GA, Collis R, Xiao Q, Hsia N, Hann SR. (2001) B-Myc, a proximal caput epididymal protein, is dependent on androgens and testicular factors for expression. Biology of Reproduction **64**, 1600-1607

Coutinho EM, Athayde C, Atta G, Gu ZP, Chen ZW, Sang GW, Emuveyan E, Adekunle AO, Mati J, Otubu J, Reidenberg MM, Segal SJ (2000) Gossypol blood levels and inhibition of spermatogenesis in men taking gossypol as a contraceptive. A multicenter, international, dose-finding study. Contraception **61**, 61-67

Coutinho EM, Melo JF (1988) Clinical experience with gossypol in non-Chinese men: a follow-up. Contraception **37**, 137-151

Coutinho EM, Melo JF, Barbosa I, Segal SJ (1984) Antispermatogenic action of gossypol in men. Fertility and Sterility **42**, 424-430

Cross NL, Morales P, Overstreet JW, Hanson FW (1986) Two simple methods for detecting acrosome-reacted human sperm. Gamete Research **15**, 213-226

Cui KH, Flaherty SP, Newble CD, Guerin MV, Napier AJ, Matthews CD (1991) Collection and analysis of semen from the common marmoset (*Callithrix jacchus*). Journal of Andrology **12**, 214-220

de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N (1998) Spermatogenesis. Human Reproduction **13**, 1-8

Demott RP, Suarez SS (1992) Hyperactivated sperm progress in the mouse oviduct. Biology of Reproduction **9**, 328-337

Diaz S (1998) Contraceptive technology and family planning services. International Journal of Gynaecology and Obstetrics **63**, 85-90

Diczfalusy E (1999) The past, present and future. International Journal of Gynaecology and Obstetrics **67**, 153-157

Diczfalusy E (1999*) From the contraceptive to the anthropocentric revolution (Gregory Pincus in memoriam). The European Journal of Contraception and Reproductive Health Care **4**, 187-201

Duarte GA (1998) Male perspectives on contraceptive methods. Cadernos de Saude Publica **14**, 125-130

Edelman GM, Millette CF (1971) Molecular probes of spermatozoon structure. Proceedings of the National Academy of Sciences of the United States of America **68**, 2436-2440

Edwards EM, Jones AR, Waites GM (1975) The entry of alpha-chlorohydrin into body fluids of male rats and its effect upon the incorporation of glycerol into lipids. Journal of Reproduction and Fertility **43**, 225-232

Else JG (1985) Captive propagation of vervet monkeys (*Cercopithecus aethiops*) in harems. Laboratory Animal Sciences **35**, 373-375

Endo Y, Tsurugi K (1987) RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. The Journal of Biological Chemistry **262**, 8128-8130

Escalier D (1983) Human spermatozoa with large heads and multiple flagella: a quantitative ultrastructural study of 6 cases. Biology of the Cell **48**, 65-74

Escalier D (2002) Genetic approach to male meiotic division deficiency: the human macronuclear spermatozoa. Molecular Human Reproduction **8**, 1-7

Fairbanks LA, McGuire MT (1984). Determinants of fecundity and reproductive success in captive vervet monkeys **7**, 27-38

Fathalla MF (1994) The global view of reproductive health. The Australian and New Zealand Journal of Obstetrics and Gynaecology **34**, 295-298

Fathalla MF (1999) Contraception. International Journal of Gynaecology and Obstetrics 67, 5-12

Ford WC, Harrison A, Waites GM (1981) Effects of 6-chloro-6-deoxysugars on glucose oxidation in rat spermatozoa. Journal of Reproduction and Fertility **63**, 67-73

Fourie MH, Du Toit D, Bornman MS, Viljoen E (1996) Changes in WGA-lectin binding sites on sperm during epididymal transit in the Chacma baboon (*Papio ursinus*) and the vervet monkey (*Cercopithecus aethiops*). Archives of Andrology **36**, 89-100

Frick J, Aulitzky W (1988) Male contraception. Human Reproduction **3**, 147-151

Gaddum-Rosse P (1981) Some observations on sperm transport through the uterotubal junction of the rat. The American Journal of Anatomy **160**, 333-341

Gatti JL, Druart X, Syntin P, Guerin Y, Dacheux JL, Dacheux F (2000) Biochemical characterization of two ram cauda epididymal maturation-dependent sperm glycoproteins. Biology of Reproduction **62**, 950-958

Gentile DP, Cockett AT (1992) The effect of varicocelectomy on testicular volume in 89 infertile adult males with varicoceles. Fertility and Sterility **58**, 209-211

Glasier AF, Anakwe R, Everington D, Martin CW, van der Spuy Z, Cheng L, Ho PC, Anderson RA (2000) Would women trust their partners to use a male pill? Human Reproduction **15**, 646-649

Gombe S, Oduor-Okelo D, Else J (1980) The potential of African mammals as new models for research in human reproduction. In: Animal Models in Human Reproduction (Serio M, Martini L, eds). New York: Raven Press, pp 345-358

Greep RO (1998) Whither global population problem. Biochemical Pharmacology **55**, 383-386

Gupta G, Maikhuri JP, Setty BS, Dhar JD (2000). Seasonal variations in daily sperm production rate of rhesus and bonnet monkeys. Journal of Medical Primatology **29,** 411-414

Hair WM, Wu FC (2000) Male contraception: prospects for the new millennium. Asian Journal of Andrology **2**, 3-12

Hamilton DW, Wenstrom JC, Baker JB (1986) Membrane glycoproteins from spermatozoa: partial characterization of an integral Mr = ca. 24,000 molecule from rat spermatozoa that is glycosylated during epididymal maturation. Biology of Reproduction **34**, 925-936

Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey MA (1996) Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. The Journal of Clinical Endocrinology and Metabolism **81**, 4113-4121

Haseenabeevi VM, Remani P, Anil S, Vijayakumar T (1991) Plant lectins-histochemical and cytochemical applications in oncology. Indian Journal of Dental Research **2**, 45-53

Hatasaka HH, Schaffer NE, Chenette PE, Kowalski W, Hecht BR, Meehan TP, Wentz AC, Valle RF, Chatterton RT, Jeyendran RS (1997) Strategies for ovulation induction and oocyte retrieval in the lowland gorilla. Journal of Assisted Reproduction and Genetics **14**, 102-110

Henkel R, Bittner J, Weber R, Huther F, Miska W (1999) Relevance of zinc in human sperm flagella and its relation to motility. Fertility and Sterility **71**, 1138-1143

Hennigar LM, Hennigar RA, Schulte BA. (1987) Histochemical specificity of beta-galactose binding lectins from *Arachis hypogaea* (peanut) and *Ricinus communis* (castor bean). Stain Technology **62**, 317-325

Herndon JG, Bein ML, Nordmeyer DL, Turner JJ (1996) Seasonal testicular function in male rhesus monkeys. Hormones and Behavior **30**, 266-271

Hikim AP, Lue YH, Wang C, Reutrakul V, Sangsuwan R, Swerdloff RS (2000) Posttesticular antifertility action of triptolide in the male rat: evidence for severe impairment of cauda epididymal sperm ultrastructure. Journal of Andrology **21**, 431-437

Hinton (1980) The epididymal microenvironment: a site of attack for a male contraceptive? Investigative Urology **18**, 1-10

Ho HC, Suarez SS (2001) Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction **122**, 519-526

Hofmann N, Fervers B, Hilscher B (1992) Correlation between multi-headed spermatozoa in the ejaculate and histological findings in the testis. Andrologia **24**, 227-230

Holden CA, Hyne RV, Sathananthan AH, Trounson AO (1990) Assessment of the human sperm acrosome reaction using concanavalin A lectin. Molecular Reproduction and Development **25**, 247-257

Holmquist G (1975) Hoechst 33258 fluorescent staining of Drosophila chromosomes. Chromosoma **49**, 333-356

Holt WV (1982) Epididymal origin of a coiled-tail sperm defect in a boar. Journal of Reproduction and Fertility **64**, 485-489

Huynh PN, Hikim AP, Wang C, Stefonovic K, Lue YH, Leung A, Atienza V, Baravarian S, Reutrakul V, Swerdloff RS (2000) Long term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. Journal of Andrology **21**, 689-699

Irvine DS, Aitken RJ (1994) Seminal fluid analysis and sperm function testing. Endocrinology and Metabolism Clinics of North America **23**, 725-748

Iusem ND, De Larminat MA, Tezon JG, Blaquier JA, Belocopitow E (1984) Androgen dependence of protein N-glycosylation in rat epididymis. Endocrinology **114**, 1448-1453

Jeulin C, Lewin LM, Chevrier C, Schoevaert-Brossault D (1996) Changes in flagella movement of rat spermatozoa along the length of the epididymis: manual and computer-aided image analysis. Cell Motility and Cytoskeleton **35**, 147-161

Jones AR (1983) Antifertility actions of alpha-chlorohydrin in the male. Australian Journal of Biological Sciences **36**, 333-350

Jones R (1999) Plasma membrane structure and remodeling during sperm maturation in the epididymis. Journal of Reproduction and Fertility **53**,73-84

Kamischke A, Ploger D, Venherm S, von Eckardstein S, von Eckardstein A, Nieschlag E (2000) Intramuscular testosterone undecanoate with or without oral levonorgestel: a randomized placebo-controlled feasibility study for male contraception. Clinical Endocrinology **53**, 43-52

Kaur J, Ramakrishnan PR, Rajalakshmi M (1990) Inhibition of spermatozoa maturation in rhesus monkey by cyproterone acetate. Contraception **42**, 349-359

Koehler JK (1981) Lectins as molecular probes of the spermatozoon surface. Archives of Andrology **6**, 197-217

Kohane AC, Gonzalez-Echeverria FMC, Piniero L, Blanquier JA (1980) Interaction of proteins of epididymal origin with spermatozoa. Biology of Reproduction **23**, 737-742

Kolibianakis EM, Tarlatzis BC, Bontis J, Papadimas J, Spanos E, Mantalenakis S (1992) Evaluation of Hamilton-Thorn automated semen analysis system. Archives of Andrology **28**, 213-222

Kullander S, Rausing A (1975) On round-headed human spermatozoa. International Journal of Fertility **20**, 33-40

Kumar MV, Panda JN (1983) Epididymal histoarchitecture of goats under chronic cyproterone acetate. Andrologia **15**, 193-195

Lee SH, Ahuja KK (1987) An investigation using lectins of glyco-components of mouse spermatozoa during capacitation and spermatozoa binding. Journal of Reproduction and Fertility **80**, 65-74

Li JC, Mruk D, Cheng CY (2001) The inter-Sertoli tight junction permeability barrier is regulated by the interplay of protein phosphatases and kinases: an *in vitro* study. Journal of Andrology **22**, 847-856

Liu HW, Lin YC, Chao CF, Chang SY, Sun GH (2000) GP-83 and GP-39, two glycoproteins secreted by human epididymis are conjugated to spermatozoa during maturation. Molecular Human Reproduction **6**, 422-428

Lotan R (1979) Qualitative and quantitative aspects of labeling cell surface carbohydrates using lectins as probes. Scanning Electron Microscope **3**, 549-564

Lotan R, Skutelsky E, Danon D, Sharon N (1975) The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*) The Journal of Biological Chemistry **250**, 8518-8523

Love CC, Garcia MC, Riera FR, Kenney RM (1991) Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume and predict daily sperm output in the stallion. Journal of Reproduction and Fertility **44**, 99-105

MacLeod J (1974) Effects of environmental factors and of antispermatogenic compounds on the human testis as reflected in seminal cytology. In: Male Fertility and Sterility (Mancini RE, Martini L, eds). New York: Academic Press, pp 123-148

Marti JI, Cebrian-Perez JA, Muino-Blanco T (2000) Assessment of the acrosomal status of ram spermatozoa by RCA lectin-binding and partition in aqueous two-phase system. Journal of Andrology **21**, 541-548

Mdhluli MC, van der Horst G (2002) The effect of oleanolic acid on sperm motion characteristics and fertility of male Wistar rats. Laboratory Animals **36**, 432-437

Mohamed MK, Burbacher TM, Mottet NK (1987) Effects of methyl mercury on testicular functions in *Macaca fascicularis* monkeys. Pharmacology and Toxicology **60**, 29-36

Morales A (2002) Androgen replacement therapy and prostate safety. European Urology **41**, 113-120

Morino H, Sakakibara R, Ishiguro M (1995) The binding of ricin to its receptors is not required for the expression of its toxicity. Biological and Pharmaceutical Bulletin **18**, 1770-1772

Mortimer D, Curtis EF, Camenzind AR (1990) Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. Human Reproduction **5**, 99-103

Mortimer D, Curtis EF, Miller RG (1987) Specific labeling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoa. Journal of Reproduction and Fertility **81**, 127-135

Moudgal NR, Krishnamurthy HN, Surekha S, Krishnamurthy H, Dhople VM, Nagaraj R, Sairam MR (2001) Immunobiology of a synthetic luteinizing hormone receptor peptide 21-41. Journal of Andrology **22**, 992-998

Moudgal NR, Sairam MR. (1998) Is there a true requirement for follicle stimulating hormone in promoting spermatogenesis and fertility in primates? Human Reproduction 13, 916-919

Mundy AJ, Ryder TA, Edmonds DK (1995) Asthenozoospermia and the human sperm mid-piece. Human Reproduction **10**, 116-119

Nicolson G, Yanagimachi R (1974) Mobility and restriction of mobility of plasma membrane lectin-binding components. Science **184**, 1294 -1296

Nistal M, Herruzo A, Sanchez-Corral F (1978) Absolute teratozoospermia in a family. Irregular microcephalic spermatozoa without acrosome. Andrologia **10**, 234-240

Nistal M, Paniagua R, Herruzo A (1977) Multi-tailed spermatozoa in a case with asthenospermia and teratospermia. Virchows Archiv. B: Cell Pathology **26**, 111-118

Novogrodsky A, Lotan R, Ravid A, Sharon N (1975) Peanut agglutinin, a new mitogen that binds to galactosyl sites exposed after neuraminidase treatment. Journal of Immunology **115**, 1243-1248

O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM (1996) Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. Biology of Reproduction **55**, 895-901

O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM (1994) Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. Endocrinology **135**, 2608-2614

Oettlé EE, Soley JT (1985) Infertility in a Maltese poodle as a result of a sperm midpiece defect. Journal of South African Veterinary Association **56**, 103-106

Olsnes S, Kozlov JV (2001) Ricin. Toxicon **39**, 1723-1728

Olsnes S, Pihl A (1973) Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. Biochemistry 12, 3121-3126

Opas M (1999) Fluorescence tracing of intracellular proteins. Biotechnic and Histochemistry **74**, 294-310

Ozvaris SB, Dogan BG, Akin A (1998) Male involvement in family planning in Turkey. World Health Forum **19**, 76-78

Panda JN, Jindal SK (1982) Effect of chronic use of cyproterone acetate on the epididymis of goat. Andrologia **14**, 397-402

Pedley KC (1997) Applications of confocal and fluorescence microscopy. Digestion **58**, 62-68

Pelfrey RJ, Overstreet JW, Lewis EL (1982) Abnormalities of sperm morphology in cases of persistent infertility after vasectomy reversal. Fertility and Sterility **38**, 112 -114

Perez-Sanchez F, Tablado L, Yeung CH, Cooper TG, Soler C (1996) Changes in the motility patterns of spermatozoa from the rabbit epididymis as assessed by computer-aided sperm motion analysis. Molecular Reproduction and Development **45**, 364-371

Phillips DM (1972) Comparative analysis of mammalian sperm motility. Journal of Cell Biology **53**, 561-573

Phillips DM, Kalay D (1984) Mechanisms of flagella motility deduced from backward swimming bull sperm. Journal of Experimental Zoology **231**, 109-116

Piasecka M, Wenda-Rozewicka L, Kozanecka A, Rozewicki S, Laszczynska M, Marchlewicz M (1998) Changes in sperm mitochondria in patients with asthenozoospermia. Ginekologia Polska **69**, 479-484

Prasad MR, Rajalakshmi M (1976) Target sites for suppressing fertility in the male. Advances in Sex Hormone Research **2**, 263-287

Pukazhenthi BS, Long JA, Wildt DE, Ottinger MA, Armstrong DL, Howard J (1998) Regulation of sperm function by protein tyrosine phosphorylation in diverse wild felid species. Journal of Andrology **19**, 675-685

Pukazhenthi BS, Wildt DE, Howard JG (2001) The phenomenon and significance of teratospermia in felids. Journal of Reproduction and Fertility **57**, 423-433

Puri CP, Gopalkrishnan K, Iyer KS (2000) Constraints in the development of contraceptives for men. Asian Journal of Andrology **2**, 179-190

Rajasekaran M, Bapna JS, Lakshmanan S, Ramachandran NA, Veliath AJ and Panchanadam M (1988) Antifertility effect in male rats of oleanolic acid, a triterpene from *Eugenia jambolana* flowers. Journal of Ethnopharmacology **24,** 115- 121

Raji Y, Bolarinwa AF (1997) Antifertility activity of *Quassia amara* in male rats - *in vivo* study. Life Sciences **61**, 1067-1074

Raynaud F, Kann ML (1986) Forward motility protein (FMP): localization in hamster epididymal spermatozoa. International Journal of Andrology **9**, 371-380

Reddy PR (2000) Hormonal contraception for human males: prospects. Asian Journal of Andrology **2**, 46-50

Retamal C, Urzua J, Lorca C, Lopez ML, Alves EW (2000) Changes in the plasma membrane proteins of stallion spermatozoa during maturation in the epididymis. Journal of Submicroscopic Cytology and Pathology **32**, 229-239

Rodriguez-Rigau LJ, Smith KD, Steinberger E (1981) Varicocele and the morphology of spermatozoa. Fertility and Sterility **35**, 54-57

Rowel TE (1971) Organization of caged groups of *Cercopithecus* monkeys. Animal Behaviour **19**, 625-645

Ruiz-Bravo N (1988) Tissue and cell specificity of immobilin biosynthesis. Biology of Reproduction **39**, 901-911

Sarkar M, Majumder GC, Chatterjee T (1991) Goat sperm membrane: lectin-binding sites of sperm surface and lectin affinity chromatography of the mature sperm membrane antigens. Biochimica *et* Biophysica Acta **1070**, 198-204

Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF (1971) Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle stimulating hormones. Science **10,** 1036-1038

Schnedl W, Breitenbach M, Stranzinger G (1977) Mithramycin and DIPI: a pair of fluorochromes specific for GC-and AT-rich DNA respectively. Human Genetics **36**, 299-305

Schwarz MA, Kohler JK (1976) Alterations in lectin binding to guinea pig spermatozoa accompanying *in vitro* capacitation and the acrosome reaction. Biology of Reproduction **21**, 1295-1307

Seier JV (1986) Breeding vervet monkeys in closed environment. Journal of Medical Primatology **15**, 339-349

Seier JV, Fincham JE, Menkveld R, Venter FS (1989) Semen characteristics of vervet monkeys. Laboratory Animals **23**, 43-47

Seier JV (1995) Aspects of the reproductive physiology of male vervet monkeys maintained in a laboratory. MSc Thesis

Seier JV, van der Horst G, Laubscher R (1996) Abnormal morphology of vervet monkey sperm. Journal of Medical Primatology **25**, 397-403

Sengupta S, Singh, Sengupta LK, Bisen PS (1997) Phytolectins: natural molecules with immense biotechnological potential. Indian Journal of Experimental Biology **35**, 103-110

Setchell JM, Dixson AF (2001) Circannual changes in the secondary sexual adornments of semifree-ranging male and female mandrills (*Mandrillus sphinx*). American Journal of Primatology **53**, 109-121

Shalgi R, Kaplan R, Nebel L, Kraicer PF (1981) The male factor in fertilization of rat eggs *in vitro*. Journal of Experimental Zoology **217**, 399-402

Shanker S, Das RH (2001) Identification of a cDNA clone encoding for a galactose-binding lectin from peanut (*Arachis hypogaea*) seedling roots. Biochimica *et.* Biophysica Acta **1568**, 105-110

Sharon N, Lis H (1995) Lectins-proteins with sweet tooth: functions in cell recognition. Essays in Biochemistry **30**, 59-75

Shi QX, Friend DS (1983) Gossypol-induced inhibition of guinea pig sperm capacitation *in vitro*. Biology of Reproduction **29**, 1027-1032

Sigma (2000-2001) Biochemicals and reagents for life science research. Atlasville: Sigma-Aldrich Co

Sigma (2000-2001[a]) Products for Life Science Research. Atlasville: Sigma-Aldrich Co

Simoni M, Weinbauer GF, Grommoll J, Nieschlag E (1999) Roles of FSH in male gonadal function. Annales D'endocrinologie **60**, 102-106

Smikle CB, Turek PJ (1997) Hypo-osmotic swelling can accurately assess the viability of nonmotile sperm. Molecular Reproduction and Development **47**, 200-203

Smith RL, Williams RT (1974) Comparative metabolism of drugs in man and monkeys. Journal of Medical Primatology **3**, 138-152

Soler C, Yeung CH, Cooper TG (1994) Development of sperm motility patterns in the murine epididymis. International Journal of Andrology **17**, 271-278

Srivastav A (2000) Maturation-dependent glycoproteins containing both N- and O-linked oligosaccharides in epididymal sperm plasma membrane of rhesus monkeys (*Macaca mulatta*). Journal of Reproduction and Fertility **119**, 241-252

Sun YT, Wreford NG, Robertson DM, de Kretser DM (1990) Quantitative cytological studies of spermatogenesis in intact and hypophysectomized rats: identification of androgen-dependent stages. Endocrinology **127**, 1215-1223

Sutovsky P, Tengowski MW, Navara CS, Zoran SS, Schatten G (1997) Mitochondrial sheath movement and detachment in mammalian, but not nonmammalian, sperm induced by disulfide bond reduction. Molecular Reproduction and Development **47**, 79-86

Syms AJ, Johnson AR, Lipshultz LI, Smith RG (1984) Studies on human spermatozoa with round head syndrome. Fertility and Sterility **42**, 431-435

Talbot P, Chacon R (1980) A new procedure for rapidly scoring acrosome reactions of human sperm. Gamete Research **3**, 211-216

Talbot P, Chacon RS (1981) A triple-stain technique for evaluating normal acrosome reactions of human sperm. Journal of Experimental Zoology **215**, 201-208

Thau RB, Limonta P, Schmidt F, Sundaram K (1985) Species differences in the sensitivity to GnRH analogs. Journal of Steroid Biochemistry **23**, 811-817

Thundathil J, Palasz AT, Mapletoft RJ, Barth AD (1999) An investigation of the fertilizing characteristics of pyriform-shaped bovine spermatozoa. Animal Reproduction Science **57**, 35-50

Tulsiani DR, Orgebin-Crist MC, Skudlarek MD (1998) Role of luminal fluid glycosyltransferases and glycosidases in the modification of rat sperm plasma membrane glycoproteins during epididymal maturation. Journal of Reproduction and Fertility **53**, 85-97

Turner TT, Giles RD (1982) Sperm motility-inhibiting factor in rat epididymis. American Journal Physiology **242**, 199-203

Turner TT, Reich GW (1987) Influence of proteins in rat cauda epididymal lumen fluid on cauda sperm motility. Gamete Research **18**, 267-278

Tyler JP, Boadle RA, Stevens SM (1985) Round-headed spermatozoa: a case report. Pathology **17**, 67-70

Tyler JP, Crockett NG, Driscoll GL (1982) Studies of human seminal parameters with frequent ejaculation. I. Clinical characteristics. Clinical Reproduction and Fertility **1**, 273-285

Usselman MC, Cone RA (1983) Rat sperm are mechanically immobilized in the caudal epididymis by "immobilin," a high molecular weight glycoprotein. Biology of Reproduction **29**, 1241-1253

van der Horst G, Seier JV, Spinks AC, Hendricks S (1999). The maturation of sperm motility in the epididymis and vas deferens of the vervet monkey, *Cercopithecus aethiops*. International Journal of Andrology **22**, 197-207

Verhoeven G (1992) Androgens and the testis. Verhandelingen-Koninkijke Academie voor Geneeskunde van Beljie **54**, 299-326

Vreeburg JTM, Hollqind MK, Orgebin-Crist MC (1992) Binding of the epididymal proteins to rat spermatozoa *in vivo*. Biology of Reproduction **47**, 588-597

Ward WS, Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. Biology of Reproduction **44**, 569-574

Weinbauer GF, Nieschlag E (1989) Reversibility of GnRH agonist-induced inhibition of testicular function: differences between rats and primates. Progress in Clinical and Biological Research **303**, 75-87

Wen RQ, Li SQ, Wang CX, Wang QH, Liu MY. (1999) Morphological changes of spermatozoa in proximal vas deferens after vasectomy. Asian Journal of Andrology **1**, 61-65

Whalen RE, Luttge (1969) Contraceptive properties of the antiandrogen cyproterone acetate. Nature **223**, 633-634

White IG, Volglmayr JK (1986) ATP induced reactivation of ram testicular, caudal epididymal, and ejaculated spermatozoa extracts with Triton X-100. Biology of Reproduction **34**, 183-193

Wilson EM, French FS (1976) Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis and prostate. The Journal of Biological Chemistry **251**, 5620-5629

Wong PY (1990) Abnormal fluid transport by the epididymis as a cause of obstructive azoospermia. Reproduction Fertility and Development **2**, 115-127

World Health Organization (1992) Laboratory manual for the examination of human semen and sperm-cervical mucus interaction 3rd ed., pp 6-18

Wu FC, Farley TM, Peregoudov A, Waites GM (1996) Effects of testosterone enanthate in normal men: experience from multicenter contraceptive efficacy study. World Health Organization Task Force on Methods for the Regulation of Male Fertility. Fertility and Sterility **65**, 626-636

Yeung CH, Wienbauer GF, Cooper TG (1999) Effect of acute androgen withdrawal by GnRH antagonist on epididymal sperm motility and morphology in the cynomolgus monkey. Journal of Andrology **20**, 72-79

Yeung, Cooper TG, Weinbauer GF (1996) Maturation of monkey spermatozoa in the epididymis with respect to their ability to undergo the acrosome reaction. Journal of Andrology **17**, 427-432

Young LG, Hinton BT, Gould KG (1985) Surface changes in chimpanzee sperm during epididymal transit. Biology of Reproduction **32**, 399-412

Zeginiadou T, Papadimas J, Mantalenakis S (2000) Acrosome reaction: methods for detection and clinical significance. Andrologia **32**, 335-343

Zirkin BR (1998) Spermatogenesis: its regulation by testosterone and FSH. Seminars in Cell and Developmental Biology **9**, 379-391

Legends to appendix B

- For each group, data in Tables 1-30 represent means (written in bold) and standard deviations (below means), and relate to the graphs in Figures 3.1-3.22, 3.24-3.27, 3.29-3.30.
- Units for respective parameters are in brackets.
- Data in Tables 1–29 were obtained every second week.
- Data in Table 30 were obtained every fourth week.

APPENDIX B:

Means and standard deviations

Testicular volumes

Table 1. Right testicular volume (cm^3)

Groups	Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**11.78**	**10.70**	**11.22**	**11.59**	**11.97**	**11.83**	**12.19**	**11.67**	**11.25**
	3.32	2.56	1.67	1.85	1.67	0.67	1.70	1.00	0.92
10 mg/kg	**11.55**	**11.29**	**12.27**	**11.67**	**12.24**	**12.20**	**13.43**	**11.57**	**10.36**
	2.74	3.04	3.43	3.40	3.81	4.11	4.38	3.94	3.92
25 mg/kg	**11.89**	**12.15**	**12.2**	**12.48**	**11.19**	**12.16**	**12.30**	**11.12**	**10.82**
	1.29	1.90	2.16	2.20	2.48	0.80	0.817	0.61	1.08
Control	**11.42**	**10.62**	**11.04**	**10.25**	**10.95**	**11.36**	**11.51**	**10.04**	**9.80**
	0.59	0.15	1.33	1.26	1.25	1.24	1.71	1.97	1.27

Table 2. Left testicular volume (cm^3)

Groups	Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**10.27**	**9.89**	**11.15**	**11.19**	**11.87**	**11.63**	**11.08**	**10.63**	**11.04**
	3.45	1.66	1.51	1.97	1.64	0.50	0.84	1.07	0.20
10 mg/kg	**9.23**	**9.64**	**9.63**	**9.16**	**9.82**	**9.43**	**10.38**	**10.44**	**9.99**
	4.92	4.41	4.57	2.89	3.68	4.01	4.18	3.84	3.91
25 mg/kg	**12.10**	**11.64**	**12.94**	**12.56**	**11.31**	**12.16**	**12.31**	**11.90**	**10.61**
	0.86	0.788	0.16	1.41	0.76	0.80	0.95	1.03	0.57
Control	**11.73**	**10.34**	**11.07**	**10.01**	**10.57**	**9.94**	**10.78**	**9.72**	**9.35**
	0.35	0.79	1.33	1.81	1.58	1.01	1.49	1.50	1.36

Sperm motility and concentration

Table 3. Progressive motility (%)

Groups	Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**60.00**	**66.67**	**43.33**	**36.67**	**33.33**	**26.67**	**26.67**	**33.33**	**26.67**
	17.32	15.28	20.82	11.55	15.28	5.77	15.28	20.82	11.55
10 mg/kg	**83.33**	**30.00**	**43.33**	**43.33**	**40.00**	**46.67**	**46.67**	**46.67**	**56.67**
	11.55	26.46	25.17	11.55	10.00	15.28	15.28	15.28	5.77
25 mg/kg	**76.67**	**53.33**	**53.33**	**43.33**	**33.33**	**43.33**	**36.67**	**46.67**	**36.67**
	15.28	25.17	23.09	28.87	11.55	15.28	30.55	20.82	5.77
Control	**53.33**	**50.00**	**40.00**	**43.33**	**46.67**	**50.00**	**60.00**	**60.00**	**50.00**
	20.82	17.32	17.32	15.28	25.17	17.32	20.00	10.00	10.00

Table 4. Speed of forward progression (%)

Groups	Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**2.33**	**3.00**	**3.00**	**2.67**	**2.00**	**1.67**	**2.33**	**2.67**	**2.00**
	0.58	1.00	0.00	0.58	1.00	0.58	0.58	1.53	1.00
10 mg/kg	**3.67**	**2.67**	**3.00**	**2.67**	**2.67**	**3.00**	**2.67**	**3.00**	**2.67**
	0.58	0.58	0.00	0.58	0.58	0.00	0.58	0.00	0.58
25 mg/kg	**3.33**	**3.33**	**3.00**	**3.33**	**3.00**	**3.00**	**3.00**	**2.67**	**2.67**
	0.58	0.58	0.00	0.58	0.00	0.00	0.00	0.58	1.15
Control	**3.00**	**3.00**	**3.00**	**3.67**	**3.33**	**3.00**	**3.00**	**3.00**	**3.00**
	0.00	0.00	1.00	0.58	0.58	0.00	0.00	0.00	0.00

Table 5. Sperm concentration ($X10^6$/ml)

Groups	Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**80.33**	**202.00**	**431.33**	**534.67**	**305.33**	**298.67**	**324.00**	**442.00**	**286.67**
	43.68	163.33	335.48	399.16	198.56	173.79	209.23	321.93	202.32
10 mg/kg	**176.67**	**897.33**	**782.00**	**608.00**	**412.00**	**482.00**	**402.67**	**824.00**	**415.67**
	118.02	1307.43	1068.97	872.96	380.88	468.49	465.55	979.83	562.53
25 mg/kg	**544.00**	**815.33**	**254.67**	**364.00**	**421.33**	**362.00**	**392.67**	**363.33**	**632.67**
	467.90	263.90	154.04	323.38	255.17	158.38	277.00	83.58	567.79
Control	**321.00**	**457.33**	**1125.33**	**320.67**	**332.00**	**300.00**	**284.00**	**508.00**	**454.00**
	200.33	125.48	1189.72	91.48	195.10	40.15	19.08	57.65	101.73

Sperm morphology

Head abnormalities

Table 6. Microcephalic (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.00**	**0.00**	**0.66**	**0.66**
	0.00	0.00	0.00	0.57	0.00	0.00	0.00	0.57	1.15
10 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.66**	**0.00**	**3.33**	**0.33**	**0.66**
	0.00	0.00	0.00	0.00	1.15	0.00	0.57	0.57	1.15
25 mg/kg	**0.00**	**0.00**	**0.33**	**0.66**	**0.00**	**0.33**	**1.00**	**0.00**	**1.00**
	0.00	0.00	0.57	0.57	0.00	0.57	1.00	0.00	1.73
Control	**0.66**	**0.00**	**0.00**	**0.33**	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**
	1.15	0.00	0.00	0.57	0.00	0.00	0.00	0.57	0.00

Table 7. Pyriform (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.00**	**0.66**	**0.00**	**0.33**	**0.33**	**0.33**	**0.00**	**0.00**
	0.00	0.00	1.15	0.00	0.57	0.57	0.57	0.00	0.00
10 mg/kg	**0.00**	**0.33**	**0.00**	**1.00**	**0.33**	**0.33**	**2.00**	**0.33**	**1.00**
	0.00	0.57	0.00	1.00	0.57	0.57	1.00	0.57	1.73
25 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.66**	**0.33**	**0.00**	**0.33**
	0.57	0.00	0.00	0.00	0.00	0.57	0.57	0.00	0.57
Control	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.33**	**0.00**	**0.33**
	0.00	0.00	0.00	0.00	0.57	0.00	0.57	0.00	0.57

Table 8. Macrocephalic (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.33**	**0.33**	**0.33**	**0.00**	**0.00**	**0.33**	**1.00**	**0.33**	**1.66**
	0.57	0.57	0.57	0.00	0.00	0.57	1.00	0.57	1.15
10 mg/kg	**0.00**	**0.33**	**0.00**	**0.33**	**0.00**	**0.66**	**0.33**	**0.66**	**1.66**
	0.00	0.57	0.00	0.57	0.00	1.15	0.57	1.15	0.57
25 mg/kg	**0.00**	**0.33**	**0.00**	**0.00**	**0.33**	**0.33**	**0.66**	**0.33**	**3.33**
	0.00	0.57	0.00	0.00	0.57	0.57	1.15	0.57	2.51
Control	**0.00**	**0.33**	**0.33**	**0.33**	**1.66**	**0.00**	**0.00**	**0.00**	**3.667**
	0.00	0.57	0.57	0.57	1.15	0.00	0.00	0.00	3.78

Table 9. Tapered (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.33**	**1.00**	**0.33**	**3.66**	**0.66**	**0.66**	**3.00**	**1.33**	**1.00**
	0.57	1.00	0.57	5.50	1.15	0.57	3.64	1.15	1.73
10 mg/kg	**0.33**	**3.33**	**3.66**	**1.00**	**0.00**	**0.66**	**1.00**	**3.66**	**3.66**
	0.57	4.90	5.50	1.73	0.00	1.15	1.00	4.04	3.05
25 mg/kg	**0.66**	**0.66**	**1.00**	**0.33**	**0.00**	**0.33**	**0.00**	**1.00**	**0.66**
	0.57	0.57	0.00	0.57	0.00	0.57	0.00	1.00	0.57
Control	**1.00**	**0.66**	**0.33**	**0.33**	**0.00**	**0.00**	**0.00**	**0.00**	**1.66**
	1.00	1.15	0.57	0.57	0.00	0.00	0.00	0.00	1.52

Table 10. Acrosomal cyst (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.66**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.57	0.00	0.57	0.00	0.00
25 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57

Table 11. Amorphous (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.00
10 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25 mg/kg	**1.00**	**0.00**	**0.33**	**0.00**	**0.00**	**0.33**	**0.00**	**1.33**	**0.66**
	1.73	0.00	0.57	0.00	0.00	0.57	0.00	1.52	0.57
Control	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57

Table 12. Round (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10 mg/kg	**0.00**	**0.00**	**0.00**	**0.33**	**0.33**	**0.33**	**0.33**	**0.00**	**0.66**
	0.00	0.00	0.00	0.57	0.57	0.57	0.57	0.00	1.15
25 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.00**	**0.33**
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.57
Control	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.00**	**0.00**	**0.00**	**1.00**
	0.00	0.00	0.00	0.57	0.00	0.00	0.00	0.00	1.00

Table 13. Nipple acrosomes (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.00**	**0.33**	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.33**	**0.00**
	0.00	0.57	0.00	0.00	0.00	0.57	0.00	0.57	0.00
10 mg/kg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**	**0.33**	**0.00**	**0.00**
	0.00	0.00	0.00	0.00	0.00	0.57	0.57	0.00	0.00
25 mg/kg	**0.66**	**0.00**	**4.33**	**1.00**	**1.66**	**1.00**	**1.66**	**3.00**	**0.66**
	1.15	0.00	7.50	1.73	7.88	1.73	2.88	5.19	1.15
Control	**0.00**	**0.00**	**0.00**	**0.00**	**0.66**	**0.00**	**0.33**	**0.00**	**0.66**
	0.00	0.00	0.00	0.00	1.15	0.00	0.57	0.00	0.57

Table 14. Combined head abnormalities (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.67**	**1.67**	**1.33**	**4.00**	**1.00**	**1.67**	**4.33**	**2.67**	**4.33**
	0.58	1.53	1.53	6.08	1.00	1.15	2.52	2.31	4.93
10 mg/kg	**0.33**	**4.00**	**3.67**	**3.00**	**1.67**	**2.33**	**8.67**	**5.00**	**8.33**
	0.58	5.20	5.51	1.00	2.08	1.53	2.08	4.36	5.13
25 mg/kg	**2.67**	**1.00**	**6.00**	**2.00**	**2.00**	**3.00**	**3.67**	**6.67**	**7.00**
	3.79	1.00	8.66	1.73	2.65	2.65	3.06	8.14	4.00
Control	**1.67**	**1.00**	**0.67**	**1.67**	**2.67**	**0.00**	**0.67**	**0.33**	**8.00**
	2.08	1.00	0.58	0.58	2.08	0.00	1.15	0.58	7.00

Midpiece abnormalities

Table 15. Bent midpiece (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**20.00**	**16.67**	**15.00**	**17.00**	**26.67**	**20.33**	**23.33**	**22.33**	**23.33**
	17.43	2.08	1.73	4.58	5.50	5.50	4.16	10.59	3.21
10 mg/kg	**16.33**	**18.33**	**18.67**	**27.33**	**18.00**	**25.00**	**18.33**	**24.00**	**19.33**
	9.45	8.50	10.69	3.51	6.08	11.13	6.50	12.49	1.52
25 mg/kg	**25.33**	**18.33**	**20.67**	**16.00**	**22.33**	**26.00**	**34.67**	**20.67**	**33.67**
	15.27	4.04	5.50	5.00	4.50	6.00	6.65	7.50	5.13
Control	**29.00**	**19.67**	**30.33**	**31.33**	**25.33**	**27.33**	**24.00**	**32.00**	**22.33**
	3.46	4.50	15.04	4.93	6.02	7.63	11.13	0.00	8.32

Table 16. Broken midpieces (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**1.33**	**3.67**	**4.00**	**2.67**	**6.33**	**6.67**	**4.67**	**11.33**	**7.33**
	1.52	0.57	3.00	1.15	3.78	1.15	2.88	4.50	6.80
10 mg/kg	**5.67**	**7.33**	**6.00**	**11.00**	**5.67**	**2.67**	**6.67**	**8.33**	**11.00**
	1.52	3.78	4.58	2.64	3.05	0.57	3.51	3.78	7.93
25 mg/kg	**4.00**	**1.67**	**3.33**	**6.00**	**1.33**	**2.33**	**5.33**	**7.67**	**5.33**
	2.00	0.57	3.51	3.00	1.52	2.08	5.50	5.68	2.51
Control	**2.00**	**4.67**	**4.33**	**3.00**	**6.33**	**6.00**	**7.33**	**5.67**	**6.33**
	1.73	1.52	3.05	2.64	6.65	1.73	4.61	4.72	0.57

Table 17. Abaxially implanted midpieces (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**0.33**	**1.33**	**1.00**	**0.33**	**0.00**	**0.66**	**1.33**	**2.66**	**6.66**
	0.57	1.52	1.73	0.57	0.00	0.57	1.52	3.78	8.32
10 mg/kg	**0.00**	**1.33**	**0.33**	**0.33**	**0.33**	**0.33**	**4.66**	**4.66**	**5.00**
	0.00	1.15	0.57	0.57	0.57	0.57	3.51	2.08	4.00
25 mg/kg	**0.33**	**1.66**	**2.00**	**1.00**	**0.66**	**6.00**	**3.00**	**5.66**	**13.33**
	0.57	1.52	1.00	1.00	1.15	9.53	2.64	3.51	6.80
Control	**1.33**	**0.66**	**0.33**	**0.33**	**1.00**	**0.66**	**1.33**	**2.66**	**11.00**
	0.57	1.15	0.57	0.57	0.00	1.15	1.52	2.88	2.64

Table 18. Combined midpiece abnormalities (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**21.67**	21.67	20.67	20.00	33.00	27.67	29.33	**36.33**	**37.67**
	19.40	2.52	3.21	4.36	1.73	4.16	5.51	15.31	11.15
10 mg/kg	**22.33**	**27.00**	**25.00**	**39.00**	**24.33**	**28.00**	**30.00**	**37.33**	**35.67**
	11.02	5.20	6.56	6.56	6.03	10.54	2.65	13.05	11.93
25 mg/kg	**30.67**	**22.00**	**26.33**	**23.33**	**24.67**	**34.67**	**43.67**	**34.33**	**52.67**
	14.36	4.36	6.66	4.62	7.64	12.90	9.71	13.05	10.26
Control	**32.33**	**25.00**	**35.33**	**34.67**	**32.67**	**34**	**33.17**	**43.33**	**39.67**
	1.53	5.29	17.16	5.13	2.31	5.29	11.36	8.50	7.57

Principal and end piece abnormalities

Table 19. Coiled (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**23.00**	**4.00**	**2.33**	**5.00**	**6.33**	**7.00**	**7.66**	**9.00**	**10.66**
	20.95	3.00	2.08	3.46	3.21	2.64	4.5	1.73	6.65
10 mg/kg	**7.00**	**2.00**	**4.00**	**5.33**	**0.66**	**0.66**	**0.66**	**3.66**	**8.33**
	3.60	1.00	2.65	3.05	0.57	0.57	0.57	3.51	7.09
25 mg/kg	**8.00**	**7.66**	**4.33**	**5.00**	**2.00**	**4.66**	**5.66**	**9.00**	**22.33**
	9.53	3.51	2.51	3.00	1.00	5.03	3.52	6.24	16.28
Control	**12.66**	**8.00**	**5.33**	**5.66**	**5.33**	**5.66**	**9.00**	**14.66**	**16.33**
	11.93	1.00	4.04	4.93	7.57	5.03	7.00	0.57	1.52

Table 20. Hairpin (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**11.00**	**10.00**	**8.30**	**7.3**	**6.67**	**6.00**	**6.67**	**5.33**	**9.00**
	7.02	7.00	4.16	4.04	7.63	2.64	0.57	2.08	5.29
10 mg/kg	**5.00**	**7.3**	**8.00**	**7.67**	**3.67**	**3.00**	**1.67**	**3.67**	**5.00**
	2.00	1.52	5.19	1.52	1.15	2.00	1.15	2.08	1.00
25 mg/kg	**9.00**	**7.00**	**4.00**	**5.00**	**6.00**	**14.67**	**5.33**	**2.67**	**4.33**
	2.00	1.73	1.00	2.64	2.64	10.69	2.51	2.08	1.52
Control	**7.00**	**8.00**	**8.37**	**5.33**	**4.67**	**3.67**	**5.67**	**9.33**	**5.00**
	6.08	3.60	1.52	2.51	1.15	1.15	2.51	4.50	2.00

Table 21. Detached (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**1.00**	0.66	0.33	2.00	0.33	0.66	1.66	**0.66**	**1.66**
	1.00	**0.57**	**0.57**	**1.73**	**0.57**	0.57	1.52	1.15	1.52
10 mg/kg	**0.33**	**0.00**	**0.00**	**1.00**	**0.66**	**0.66**	**0.33**	**1.00**	**1.33**
	0.57	0.00	0.00	1.00	0.57	0.57	0.57	1.73	0.57
25 mg/kg	**1.00**	**2.00**	**0.66**	**1.66**	**1.00**	**1.33**	**2.33**	**0.33**	**0.33**
	1.73	2.64	0.57	0.57	1.00	1.52	2.30	0.57	0.57
Control	**0.33**	**0.66**	**0.00**	**1.33**	**0.00**	**0.00**	**2.00**	**1.00**	**1.33**
	0.57	0.57	0.00	1.52	0.00	0.00	1.00	1.73	1.52

Table 22. Combined tail abnormalities (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**35.33**	**14.67**	**11.00**	**14.33**	**13.33**	**13.67**	**16.00**	**15.00**	**21.33**
	23.59	7.51	2.65	4.16	10.21	3.21	3.00	4.00	10.69
10 mg/kg	**12.33**	**9.33**	**12.00**	**14.00**	**4.67**	**4.33**	**2.67**	**8.33**	**14.67**
	5.51	2.52	7.81	2.00	0.00	2.31	1.73	5.03	7.02
25 mg/kg	**18.00**	**16.67**	**9.00**	**11.67**	**9.00**	**20.67**	**13.33**	**12.00**	**27.00**
	7.55	3.79	3.00	2.65	3.46	8.33	5.03	7.02	16.09
Control	**20.00**	**16.67**	**13.67**	**12.33**	**10.00**	**9.33**	**16.67**	**25.00**	**22.67**
	11.79	3.79	4.93	5.69	6.93	6.11	6.81	3.46	1.53

Acrosomal integrity and sperm vitality

Table 23. Intact acrosome (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**53.66**	**20.33**	**18.33**	**26.00**	**25.66**	**26.00**	**27.00**	**25.66**	**26.33**
	11.06	0.57	5.68	8.71	1.154	4.58	4.35	9.45	9.07
10 mg/kg	**52.00**	**29.66**	**33.00**	**24.66**	**29.66**	**35.00**	**33.33**	**33.66**	**36.00**
	3.60	12.50	18.52	6.11	4.50	4.35	8.02	9.29	14.79
25 mg/kg	**54.33**	**31.00**	**30.00**	**19.66**	**30.00**	**30.33**	**29.66**	**32.66**	**35.33**
	13.79	4.35	6.00	4.16	3.00	9.07	8.14	7.37	6.80
Control	**54.33**	**51.66**	**55.33**	**51.33**	**55.00**	**56.66**	**57.00**	**54.00**	**51.00**
	6.80	8.14	11.71	6.42	7.00	7.63	12.52	13.11	11.53

Table 24. Patchy acrosome (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**7.00**	**12**	**10.00**	**9.33**	**10.33**	**9.66**	**14.00**	**12.66**	**29**
	1.00	2.64	4.00	6.11	0.57	2.30	7.00	6.65	15.62
10 mg/kg	**7.66**	**9.00**	**13.00**	**9.333**	**14.66**	**17.66**	**14.33**	**15.00**	**18.00**
	2.51	2.00	4.58	5.77	4.04	10.01	9.01	7.81	4.00
25 mg/kg	**8.66**	**11.33**	**9.66**	**10.33**	**17.00**	**24.66**	**14.66**	**15.33**	**20.00**
	4.16	3.05	1.527	0.57	2.00	5.68	4.16	4.04	3.46
Control	**8.66**	**6.33**	**8.00**	**5.33**	**7.33**	**9.66**	**13.33**	**9.00**	**10.00**
	2.08	1.52	2.00	1.52	2.08	3.05	4.93	3.60	5.00

Table 25. Equatorial staining (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**26.66**	**45.66**	**47.33**	**48.00**	**45.66**	**41.00**	**42.66**	**42.00**	**31.00**
	9.86	5.85	3.21	14.10	5.03	2.00	10.96	7.00	11.35
10 mg/kg	**28.00**	**44.66**	**35.00**	**50.66**	**38.66**	**36.00**	**40.66**	**41.00**	**34.00**
	2.64	6.50	7.00	11.67	8.50	5.00	5.68	12.28	9.64
25 mg/kg	**26.33**	**46.66**	**43.33**	**54.66**	**38.33**	**37.33**	**37.33**	**39.33**	**31.00**
	4.72	8.32	6.11	11.93	6.50	6.50	5.50	3.51	7.81
Control	**27.66**	**31.00**	**25.00**	**33.00**	**26.33**	**27.00**	**23.00**	**28.00**	**29.33**
	5.13	6.08	10.44	6.08	3.21	5.19	7.54	9.53	3.78

Table 26. No acrosome (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**12.66**	**16.66**	**24.33**	**16.66**	**18.66**	**23**	**16.33**	**20.66**	**13.66**
	4.50	3.05	3.21	3.78	5.85	8.66	10.21	13.61	4.16
10 mg/kg	**12.66**	**16.66**	**19.33**	**15.33**	**17.33**	**11.33**	**11.66**	**10.33**	**12.00**
	2.30	5.50	9.86	8.02	2.88	1.15	4.16	2.51	2.00
25 mg/kg	**10.66**	**11.33**	**16.66**	**15.33**	**14.66**	**7.666**	**18.33**	**12.66**	**13.66**
	5.50	0.57	6.11	8.50	6.11	3.78	6.65	4.61	5.68
Control	**9.33**	**11.00**	**11.66**	**10.33**	**11.33**	**7.00**	**6.66**	**9.00**	**9.66**
	2.30	1.73	2.88	0.57	3.21	1.00	0.57	2.00	4.72

Table 27. Sperm vitality (%)

Groups	Baseline	Week2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
4 mg/kg	**82.00**	**71.00**	**59.66**	**70.33**	**70.00**	**67.33**	**61.33**	**61.00**	**68.66**
	5.56	7.81	15.14	10.11	11.35	11.50	13.27	11.53	6.80
10 mg/kg	**78.33**	**74.00**	**72.66**	**68.66**	**74.33**	**74.33**	**71.33**	**73.00**	**72.00**
	1.52	2.00	13.31	7.63	4.04	6.02	6.027	2.64	5.56
25 mg/kg	**77.00**	**75.66**	**71.00**	**68.33**	**71.33**	**69.33**	**70.00**	**70.33**	**74.00**
	9.64	4.50	3.60	2.51	7.57	3.78	4.35	3.78	2.64
Control	**77.66**	**80.66**	**81.66**	**81.00**	**76.66**	**77.00**	**76.33**	**79.33**	**75.33**
	5.13	5.85	1.52	2.00	3.05	1.00	4.04	3.21	2.30

Steroid

Table 28. Testosterone (nmol/L)

Groups	Baseline	Week 4	Week 8	Week 12	Week 16
4 mg/kg	**19.66**	**16.20**	**9.63**	**11.23**	**4.93**
	10.02	8.29	2.72	7.43	2.51
10 mg/kg	**14.76**	**18.8**	**6.96**	**6.03**	**8.00**
	14.26	15.06	3.46	2.05	4.13
25 mg/kg	**15.46**	**18.13**	**13.10**	**7.60**	**5.06**
	8.29	13.34	8.02	3.83	3.66
Control	**19.66**	**21.73**	**19.03**	**30.96**	**19.73**
	16.47	28.35	12.45	23.79	28.03

Acknowledgements

I would like to thank the following persons and institutions for making this thesis possible:

My promoter Professor Gerhard van der Horst

My co-promoter Dr. Jürgen Seier

Mr. Timothy Collop and Abraham Davids for excellent animal care and technical assistance

Mrs. Ria Laubscher for statistical analyses

The Primate Unit for supply of animals

The Medical Research Council for financial assistance

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3 September 1999

Dr S Wolfe-Coote
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Dear Dr Wolfe-Coote

ETHICAL APPROVAL

The Ethics Committee for Research on Animals (ECRA) discussed Mr C Mdhluli's application entitled *To determine the effect of oleanolic acid on the reproductive physiology of male Vervet monkeys*, on 23 August 1999.

ECRA approved the application, but requested that the veterinarian sign on page 9 of the application form, as he is legally responsible for the supervision of the use of the drug.

As the duration of the experiment is longer than one year, Mr Mdhluli is requested to submit a short progress report at the end of each year. ECRA must also be informed when the experiment has been completed.

Yours sincerely

R. Klaas

AP DR AJS BENADÉ
CONVENOR : ECRA