EFFECT OF FRESH AQUEOUS GARLIC EXRACT *(ALLIUM SATIVUM LONGICUPIS)* ON ERYTHROPOIESIS IN ADULT MALE RABBITS

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DECLARATION

I **MUNYIKOMBO M. WILLIAM**, confirm that this Thesis is my own original work and has not to my knowledge been presented for a degree in any other university. No part of this Thesis may be reproduced without the prior knowledge and permission of the author and/or the University of Nairobi.

All sources of information have been specifically acknowledged by means of references.

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DEDICATION

To my father and mother,

Lazarus and Rebecca Munyikombo,

For their self denial, love and overwhelming support!
A legacy of your incessant and fervent prayers
to God Almighty.

To my Lovely wife,

For her Love and Support!

Thank you for everything.

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TABLE OF CONTENT

Chapter 3

LIST OF FIGURES

LIST OF TABLES

PAGE

LIST OF PLATES

LIST OF SYMBOLS AND ACRONYMS/ABBREVIATIONS

ml	-	Milliliter(s).
ug	-	Microgram(s).
%	-	Percent.
°C	-	Degree(s) centigrade.
Mmol/L	-	Millimols per liter.
In"	-	Inch(s).
i.m	-	Intramuscular.
kJ	-	Kilo Joules.
kHz	-	Kilo Hertz.
RI	-	Reticulocyte index.
KNH	-	Kenyatta National Hospital.
RES	-	Reticuloendothelial system.
BM	-	Bone marrow.
Retics	-	Reticulocytes.
LACG	-	Laboratory Animal Committee Guidelines.
$NaH_2PO_4.H_2O$	-	Sodium dihydrogen phosphate, 1-hydrate.
Na_2HPO_4	-	di-Sodium hydrogen phosphate, anhydrous.
cm	-	centimeter.
g	-	gram(s).
mg	-	Milligram(s).
Kg	-	Kilogram(s).
mg/kg	-	Milligram per kilogram(s).
g/kg	-	gram per kilogram.

RNA	-	Ribonucleic acid.
mRNA	-	Messenger Ribonucleic acid.
DMSO	-	Dimethylsulfoxide
Ca	-	Calcium.
LSC	-	Lymphoid Stem Cell.
CFU-S	-	Colony-Forming Unit- Spleen.
IL	-	Interleukin
GM-CSF	-	Granulocyte-Macrophage Colony-Stimulating Factor.
G-CSF	-	Granulocyte Colony-Stimulating Factor.
CFU-GM	-	Colony-Forming Unit – Granulocyte Macrophage.
CFU-M	-	Colony-Forming Unit – Megakaryocytes.
CFU-E	-	Colony-Forming Unit – Erythrocytes.
M : E	-	Myeloid : Erythroid ratio.
WBC	-	White blood cells.
EDTA	-	Ethylene di-amine tetra-acetic acid.
AGE	-	Aqueous Garlic Extract.
AGS	-	Aqueous Garlic Stock.
CFU	-	Colony Forming Unit.
CG	-	Control Group.
CSC	-	Committed Stem Cell.
EG	-	Experimental Group.
EPO	-	Erythropoietin.
FDA	-	Food and Drug Administration.
GGC	-	German Government Commission
Hb	-	Hemoglobin.

ILRI	-	International Livestock and Research Institute.
PHSC	-	Pluripotent Hematopoietic Stem Cell.
PCV	-	Packed Cell Volume.
RBC	-	Red Blood Cell.
WHO	-	World Health Organization

ABSTRACT

Garlic *(Allium sativum Longicupis)* is a cultivated plant. Its wild progenor originated in the high planes of West-Central Asia. It has been widely used as food and medicine. Its effects have been demonstrated in both animals and humans. Garlic has been the subject of intensive scientific research; however, there is scanty information on garlic with regard to its action on specific blood cells. The study was conducted to investigate the effect of fresh Aqueous Garlic Extract (AGE) on the process of erythropoiesis (red blood cell formation) in adult male rabbits for a period of 30 days. The aim of the study was to determine whether garlic's active compounds had any erythropoietic effect in rabbits in vivo. It was also used to establish whether there is a dose-dependent relationship between garlic administered and erythropoiesis.

Thirty out of fifty adult male New Zealand white rabbits were **keenly** selected (with the best characteristics) and used for the study. They were left to acclimatize to their new environment for 3 weeks. The 30 rabbits were then subdivided into 6 groups of 5 animals each, making a control group, experimental groups 1, 2, 3, 4, and 5. The control group was orally fed on 2ml of a placebo (distilled water). Animals in the 5 experimental groups were orally fed (using a syringe connected to a metal cannula) on 5 different absolute doses of freshly prepared AGE as 26, 52, 104, 208 and 416 mg/kg body weight. Using Paget and Barnes Conversion Table (Appendix G), the doses were extrapolated to the following volumes: 0.5, 1.0, 2.1, 4.78 and 8.4 ml respectively. 2ml distilled water was used **only** as a **vehicle** to ensure the content (**garlic fiber and its active ingredients**) was swallowed in solution.

In ***phase 1***, 1ml of blood was withdrawn from the lateral ear vein of each animal every 7th day. Red blood cell count (RBC), Packed cell volume (PCV), Hemoglobin levels (Hb) and Reticulocyte counts (Retics) were done to assess the effect of garlic on peripheral

blood. In ***phase 2***, two animals from each group were irreversibly anaesthetized and from their bone marrow aspirates, histological analysis of their cellular components were carried out on 5 bones; i.e. femur, ulna, sternum, humerous and tibia. This was to assess the effect of AGE on the morphology and rate of erythropoietic activity on the precursor cells in the bone marrow.

The results suggest that the placebo did not affect the erythropoietic activity in the peripheral blood of animals in the control group as they therefore remained erythropoietically normal. From the descriptive statistics tabulated, there was a statistically significant increase in the mean **RBC** production between the experimental groups and this was compared with the control at 0.01 level with F (5) =11.419, p=0.000. There was a significant increase in the erythropoietic activity in experimental groups 4 and 5 (71.4%, $p<0.05$) as compared to groups 1 and 2 (33.3%, $p< 0.01$), and consequently the control group, as microscopically seen from the peripheral blood parameters (RBC and Retics), and in the bone marrow. Though there was a steady rise in the mean **PCV**, it was not statistically significant at p=0.00. However, when compared with the control, experimental group 5 that was given the largest dose, the mean PCV was statistically significant at 0.01 level, F (1, 5) = 4.108, p=0.002. The ANOVA results for **Hb** level revealed a highly significant effect with increasing dosage with F (1, 5) = 6.192 at p=0.000, especially group 5 and the control at 0.05 level. Similarly, there were highly statistically significant increase in the mean rate of **reticulocyte** production within experimental groups, and as compared to the control, where F (1, 5) = 19.529, p=0.000 was highly significant at 0.01 level. An increase in AGE dose consequently led to an increase in the mean rate of reticulocyte production.

Finally, dependent on the dosage, AGE had an influence in each experimental group, thereby eliciting sigmoid (S-shaped) dose-response curves for PCV and Hb levels, and an exponential growth curves in RBC and reticulocyte counts between the groups.

Based on the study, these results indicate that freshly prepared AGE, or as used in its naturally occurring form (fresh garlic bulbs), has a positively significant influence on the process of erythropoiesis, for it accelerates the rate of reticulocyte production, which was used as an **index** for the increased rate of red blood cell production. It also showed that garlic had no adverse effects on cell morphology and function as was observed from the bone marrow analysis.

CHAPTER 1

1.0 INTRODUCTION

Now known as a variable cultigen (found only in cultivations), garlic's wild progenor is thought to have originated in the high planes of West-Central Asia. Garlic may have derived from the Central Asian species *A. longicupis* (Mabberly, 1987). The plant spread east and west with nomadic tribes, and was known to be cultivated in the Middle East more than 5000 years ago. The word `**garlic**' originates from the Anglo-Saxon `gar-leac` or `spear-plant' (Pickering, 1879).

From the time of the Egyptian pharaohs and the earliest Chinese dynasties, garlic *(Allium sativum)* has been widely consumed as both food and medicine. However, according to Pliny, there are some drawbacks, which include dulling the sight, causing flatulence, injuring the stomach and causing thirst, if taken in excess (Jones, 1964).

Garlic used as food and medicine throughout the world, has a history of use predating the written word. Garlic supplies are abundant, the herb is inexpensive, and is generally recognized as safe (Murray, 1996). Consequently, garlic has been the subject of intensive scientific research over the past five decades, resulting in over 2000 scientific publications; which have shown antibacterial, anti-fungal, anti-tumor, hypolipidemic, hypoglycemic, antiatherosclerotic, hematinic and hypotensive activity (Chooto, 2004). These effects have been demonstrated in both animals and humans.

Ethno-medicinal use of plants and plant products has been practiced in various cultures for thousands of years (Gill, 1992). Since then it has become part of the inherited wisdom rather than being based on knowledge of plants bioactive chemical constituents that beneficially modulate the physiology of an ailing human being (Saxena, 2003). The World Health Organization estimates that, about 85 per cent of the populations in the Sub-Saharan

Africa rely on traditional medicine for their primary health care needs. World wide, more than 10,000 species of higher plants have been used for medicinal purposes and it is estimated that thousands of kilograms of medicinal plants and their parts are collected and used everyday by traditional healers and mothers at home across Africa (Saxena, 2003; Chooto, 2004).

Today, interest is focused in plants as likely sources of new commercial drugs. Modern analytical methods have not only revealed the enormous variety and complexity of bioactive principals of medicinal plants, but also confirmed their potential value for use as medicines or as models for synthetic drugs.

Botanists believe that garlic *(Allium Sativum L.)* was one of the first plants to be cultivated. The remains of ancient garlic have been found in caves inhabited 10,000 years ago. Sources agree that garlic originated in the high plains of West-Central Asia and from there, it entered the New World, including Africa, with various colonial expeditions. Recently, it was estimated that some 200 varieties of garlic are grown around the world (Ho, 2001).

Much has been written about the healing power of garlic. In writings from early civilizations all around the Mediterranean, we learn that garlic was not only a food, but also a medicine and a preservative (Hsu et al., 1986). The first garlic prescription was inscribed in cuneiform on a Sumerian clay tablet. Clay models of garlic heads were crafted in Egypt more than 5000 years ago and garlic heads (6-12 heads) were placed in tombs, including Tutankhamen.

Microbiologist Louis Pasteur (1858) put a few cloves of garlic into a Petri-dish full of bacteria. When he looked at it a few days later, he found a clear zone of killed bacteria around each clove. Both before and after that discovery, garlic preparations have been used to disinfect wounds and to treat illness from cholera to cancer.

Other studies on garlic have shown inhibition of platelet aggregation (Apitz-Castro et al., 1983) and prevention of fat infiltration of the liver (Sang et al., 1995). Extracts of garlic has also been shown to improve the activation of natural killer cells as well as the level of interleukin-2 (Tang et al; 1997).

The use of plants and plant products in disease control has persisted despite use of modern pharmaceutical products and dominance of synthetic drugs, especially in the third world countries. These has also been so for garlic in West-Central Asia, where as millions of Asians swear to the efficacy of garlic treatments, it's pharmacological effects have seldom been subjected to rigorous trials and control, and these claims remain suspect. The practice of using garlic has thus remained a tradition passed from generation to another and mainly employed when other alternatives are beyond reach (due to cost) or as a last resort in desperate situations.

Since there is lack of sufficient scientific information on the effects of garlic consumption on blood cells, particularly RBC, this study therefore proposes to evaluate its effect on the production of RBCs by the bone marrow.

1.1 STATEMENT OF THE RESEARCH PROBLEM

The "Effect of fresh Aqueous Garlic Extract on the formation of Red Blood Cells in Male Adult Rabbits".

1.2 PURPOSE OF THE STUDY

Over the past decade, advances in herbal medicine have come out strongly developing marginal gaps and heavily agued controversies over which field between herbal medicine and modern medicine is worthy to offer treatment in the present world. Because of the emergence of more complicated and mutating disease-causing pathogens, there is need to merge Herbal and Modern medicine. With modern technology, active ingredients can be extracted from herbs and incorporated as components in modern drugs to come up with more potent drugs that would better treat the present complicated diseases. Garlic bulb being a 'herb', a lot of scientific researches have been conducted. Most of them have explored garlic as an antibacterial, antifungal, antitumor, hypolipidemic, hypoglycemic, antiatherosclerotic and a hypotensive. There is minimal evidence of scientific researches conducted on garlic and blood. The purpose of this study is to evaluate the effect of garlic consumption on blood cells, particularly its effect on the red blood cells.

1.3 RATIONALE/JUSTIFICATION OF THE STUDY

Despite garlic's popularity in Kenya as an indisputable solution to a wide range of health problems including infections, its use still remains subject to rigorous trials and controls, as not much scientific evidence support its efficacy.

According to the present literature review, many scientific researches, projects and clinical studies have shown garlic's antibacterial, antifungal, antitumor, hypolipidemic, hypoglycaemic, antiatherosclerotic, hypotensive and anticancer activity. In view of the above, little has been done on haematology, specifically garlic's effect on the specific blood cellular components. Recent studies in haematology have only shown antithrombotic and fibrinolytic activity.

Red blood cells, being the majority blood cellular components, bring about the important reason for this study.

1) There is lack or little credibility, awareness and laboratory data to support garlic's therapeutic claims.

2) The global scenario is now changing towards the use of non-toxic plant products with traditional medicinal value especially in poor countries where conventional drugs are commercially beyond the reach of most people.

3) This research generates information needed about the effects of garlic products, which are currently untested and hopefully will make it more accepted and appreciated by the society.

4) It brings insight on garlic's diverse curative ability.

Because garlic bulbs are affordable, the poor who can not afford frequent conventional treatment for infections, will now appreciate it more, not forgetting it's proved merit of boosting immune response. This research is therefore warranted!

1.4 RESEARCH OBJECTIVES

1.4.1 General Objective

To investigate the effect of Aqueous Garlic Extract (AGE) on red blood cell formation in the rabbits over a 30 day period.

1.4.2 Specific objectives:

1. To investigate how changes in AGE dosage affect the RBC production.
2. To test what effect AGE administration may have on the PCV.
3. To find out whether the levels of Hb in the blood do actually change when AGE is administered.
4. To establish what effect AGE administration would have on the rate of production of reticulocytes.

5. To determine whether there are any differences among the six groups with regard to the blood parameters in question.

1.5 RESEARCH QUESTIONS

1. Does RBC production depend on the dosage of AGE administered?
2. Does AGE administered have any effect on PCV?
3. Are the changes in Hb levels dependent on AGE administered?
4. Does the concentration/amount of AGE administered have any effect on the rate of production of reticulocytes?
5. Are there any differences among the groups with respect to the blood parameters in question?

1.6 HYPOTHESIS

H_0: Aqueous garlic extract (AGE) does not have any effect on the process of red blood cell formation (erythropoiesis).

1.6.1 Specific Hypothesis

H_1: There is no statistically significant relationship between the doses of AGE administered and red blood cell production.

H_2: AGE has no effect on the packed cell volume.

H_3: The level of Hb in blood is independent of the amount of AGE administered.

H_4: The rate of production of reticulocytes has no relationship with AGE administered.

H_5: There are no differences among the six groups with regard to the blood parameters being studied.

1.7 MEDICAL/SCIENTIFIC SIGNIFICANCE

This study is projected to bring significant benefit to the medical field in providing alternative substitute to the conventional hematinic drugs currently being used. Since garlic bulbs are readily available and affordable in the local markets, financially disadvantaged people would be able to access it and include it in their diet daily since it is relatively inexpensive. Consequently, they will be boosting their immune systems and avoid chances of anemia or blood related diseases like pernicious anemia, that are likely to be caused by an imbalance of certain blood parameters e.g. Red blood cell count, Hemoglobin levels, Packed cell volume, Platelet count etc. In addition, the active ingredients in garlic bulbs could be extracted and used as components in manufacturing modern and more potent hematinic drugs, thereby supporting and sealing the gap between Herbal/Traditional medicine and Modern medicine.

CHAPTER 2

2.0 LITERATURE REVIEW

It has been said that your food should be your medicine and your medicine should be your food (Foster and Duke, 1990). Garlic is perhaps the most qualified medicinal food known at least as long as humans have recorded historical events. Perhaps no other plant has been used for so long and in as many cultures, and for as many medicinal and culinary purposes as **Garlic** *(Allium sativum Longicupis).*

2.1 HISTORICAL PERSPECTIVES OF GARLIC

Comparing Mediterranean and American cultures, Mediterranean populations eat a lot of this plant, while Americans in general eat it only occasionally. However, recent studies suggest it is something worth "befriending" it in the kitchen (Pat, 1998).

Garlic, a member of the onion- lily family, is a hearty bulb that grows prolifically under the right conditions. In the Mediterranean cultures, garlic presence, both as a flavouring agent and medicinal plant, is traceable for thousands of years. It is said that Hippocrates himself used garlic vapors to treat certain cancers. When antibiotics were scarce during World War 11, garlic poultices were placed on wounds to prevent infection (Pat, 1998).

Word Root of garlic

The word "**garlic**" originated from the Anglo-Saxon word "gar-leac" or "Spear-plant" (Pickering, 1879). "**Allium**" the ancient for garlic, derived from the Celtic *all,* signifying hot or burning. The species name, "**Sativum,**" means planted, cultivated or sown (not wild) and the variety is '**L**' means Longicupis. The genus *Allium*, of the lily family (liliaceae) includes onion, chives, shallots and leeks (Manniche, 1989).

From the time of the Egyptian pharaohs and the earliest Chinese dynasties, garlic *(Allium sativum)* has been widely consumed as both food and medicine. Is garlic the secret key to the construction of the pyramids? Herodotus (484-425 B.C.), the Greek historian, known as the "Father of History," said that there is an inscription of Egyptian characters on a pyramid which records the quantities of radishes, onions and garlic consumed by the laborers who constructed it (Manniche, 1989). Manniche notes the discovery of garlic cloves in the tomb of Tutankhamen, and the sacred animal temple precinct at Saqqara. As many as two to three garlic cultivators were known at the time of Pliny the Elder (23-79 A.D.), the Roman naturalist.

Pliny explored the virtues of garlic in his *Natural History*. "Garlic has powerful properties, and is of great benefit against changes of water and residence..." Pliny says that garlic is an antidote for the poisonous bites of the shrew, dog bites, snakebites; it neutralizes the poisonous plants acon and henbane. He recommends its use in the treatment of asthma, as a cough suppressant, and to expel intestinal parasites. It is an anodyne (pain killer) for toothaches- as a decocted rinse, crushed and inserted directly into the hollow tooth, or macerated with vinegar. Pliny reports garlic as an aphrodisiac when pounded with fresh coriander and taken in neat wine.

In China, garlic is known as *Da-Suan*, and medicinal use was first recorded by Tao Hong-jing in his book *'Miscellaneous Records of Famous Physicians'*, published during the era of North and South Dynasties around 510 A.D. Garlic is known to have been cultivated in China as early as the Tang Dynasty (618-907 A.D).

2.1.1 General description and characteristics of garlic

Garlic is the common noun. It is scientifically referred to, as *Allium sativum L.* It is a member of the lily family- liliaceae. It belongs in the same group of plants as the onion. During growth, it has long narrow leaves that are flat like grass. The bulb is used as medicinal spice. The herb consists of numerous "bulb lets" or individual cloves grouped together and enclosed in a thin white skin. It has whitish flowers that are found in groups at the end of the stalk that rises directly from the bulb (Murray, 1996).

Photo of Garlic bulbs on a weighing machine: A- Garlic bulbs, B- Garlic cloves (PLATE 8).

Characteristics

Garlic is a perennial plant that grows up to 1.5m tall. It has flat leaves, 8mm wide. The flowers are usually greenish, whitish or rosy, but not very abundant, that stands out with their long peduncle on a head of little bulbs, surrounded by a very long spate. Bulbs of garlic are formed by a white cover, inside which there are several little bulbs (cloves) (Murray. 1996).

Garlic, commonly known as Allium sativum, belongs to the family liliaceae. The plant has many local names and of different varieties (Gill, 1992).

Common name:	-Garlic.
Kingdom:	-Plantae.
Family:	-Liliaceae.
Genus:	-Allium.
Species:	-Sativum.
Variety:	-Longicupis.
Scientific name:	**-Allium Sativum L.**

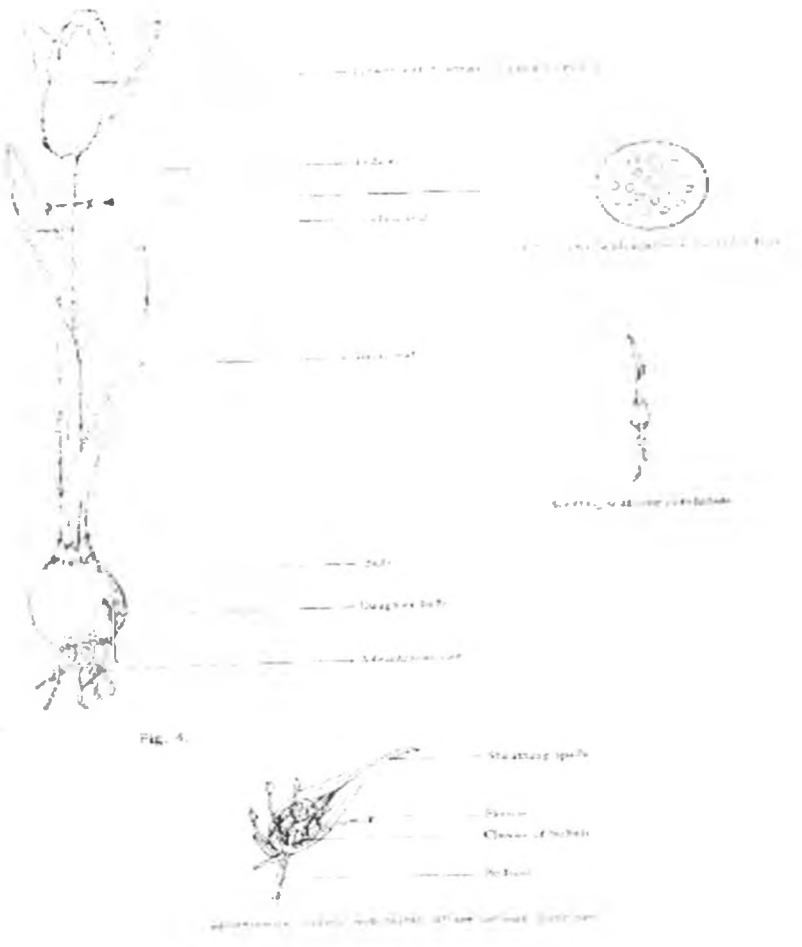


Fig. 2.1 Garlic plant (*Allium Sativum L.*) (Aebi, 1974).

2.1.2 Chemical composition of garlic

1. Sugars: Glucose and fructose.
2. Minerals: Major; manganese, potassium, calcium, phosphorus.

   Minor quantities; magnesium, sodium, zinc, copper, selenium, iron.
3. Amino acids: Valine, aspartic acid, lysine, glutamic acid, leucine and arginine.
4. Vitamins: Mainly: vit B_6, B_{12} and C. In small quantities: niacin, folic acid and pathogenic acid (Agarwal, 1996).

5. Ajoene: Produced by condensation of allicin.
6. Alliin: Which is converted to allicin by enzyme allinase (Aebi, 1974)
7. Essential oils with many sulfur-containing components, -Allyl disulfide and Allyl trisulfide (Ellman, 1959).
8. Quercetin and Saponins.

2.1.3 Functional discoveries of Garlic compounds

-Ajoene is released when garlic is ground, chopped, chewed or cooked. It decreases blood cell clumping- reduces chances of stroke (Tyler, 1993).

-Saponins are believed to lower blood pressure, decreasing chances of stroke (Orekhov, 1995). -Fructants may stimulate the immune system.

-Alliinase is inactivated by heat. Cooked garlic does not have as strong an odor as raw garlic (nor nearly as powerful physiological effects).

-Selenium is an antioxidant contained in high quantities in garlic (Banerjee, 2002). Antioxidants fight oxidation and free radicals inside the body that wear out the body and may lead to cancer.

Recent information has brought forth ample data that support significant antioxidant activity of garlic *(Allium sativum L.)* (Balasenthil, 2000; Grudzinski, 2001; Ide, 1997; Wu, 2001). Among various preparations of garlic supplements, aged garlic extract in particular has been associated with antioxidant activities in sound scientific experiments (Ho, 2001). Chemical composition of garlic for every 100 gm is formed by the following elements (Ryzhenkov et al., 2003):

Table 2.1 Chemical compounds contained in 100g of Garlic.

COMPOSITION.	IN 100 gms of Garlic.
Carbohydrates and vitamins	33.07 g
Lipids	0.5 g
Fiber	2.1 g
Water	59 g
Sodium	17 mg
Manganese	1672 mg
Potassium	401mg
Phosphorus	153 mg
Sulfur	70 mg
Calcium	181mg
Magnesium	25 mg
Iron	0.308 mg
Glutamic acid	0.805 g
Lysine	0.273 g
Arginine	0.634g
Aspartic acid	0.489g
Calories	149 Kcal

Researchers have tried to estimate dozens of compounds contained in garlic. They have identified garlic's volatile oil; including 33 sulfur compounds, 17 amino acids, 12 minerals, vitamins A, B and C (Abdullah et al., 1988). Pizzarno (1996) did a steamed distillation of crushed fresh bulbs yielding 0.1-3.6 percent of a volatile oil. The main components of the volatile oil are sulfur compounds, especially allicin, diallyl disulfide and diallyl trisulfide. These compounds are primarily the active components of garlic (Leung and Foster, 1996).

A study by Al-Naghdy (1988), demonstrated at the University of Qatar provides evidence for the occurrence of prostaglandin from a homogenized garlic extract. Allicin

identified as a by-product produced by crushing or bruising the bulb, in which an odorless sulfur-containing compound called *alliin* comes into contact with the enzyme alliinase, resulting in conversion to allicin. He also said that allicin is strongly antibacterial and is the primary component responsible for the potent characteristic garlic smell. Allicin is unstable and decomposes down to diallyl-disulfide upon steam distillation for essential oil production (Tyler, 1993).

The percentage composition of the bulb as given by E. Solly (1993) is: Water- 84.09%, organic matter- 13.38% and inorganic matter- 1.53%. That of leaves being: Water- 87.14%, organic- 11.27% and inorganic- 1.59%. The bulb has a strong characteristic odor, an acrid taste and yields offensively smelling oil, essence of garlic identical with allyl sulfide ($C_6H_{10}S_2$).

Table 2.2 Nutritional value of garlic. (adapted from Ryzhenkov et al., 2003).
garlic/ half ounce:

Calories	21g
Total fat content (g)	0.1 g
Saturated fat (g)	0
Mono-saturated fat (g)	0
Poly-saturated fat (g)	0
Dietary fibers (g)	0.3 g
Proteins (g) and vitamins (g)	1 g
Carbohydrates (g)	5 g
Cholesterol (mg)	0

2.4 VARIETIES AND CLASSIFICATION OF GARLIC

Some 300 varieties of garlic are grown around the world, but most garlic grows in USA- about 90 percent of it in California. It is of two types; "early" and "late." The early

variety harvested in mid-summer, is white or off-white in colour; the late variety, harvested a few weeks later, has a similarly coloured outer skin, but the sheaths covering the individual cloves are pinkish (De Candolle, 1908).

It is cultivated around the world and is planted between February and March, harvested between August and September (Kamenetsky, et al., 2004). There are approximately 18,000 acres of garlic-cultivated land in the USA. The 1989 garlic harvest in California, the major garlic producing state, yielded about 250 million pounds. The vast bulk of the crop is used to produce dehydrated garlic products. About 50 million pounds of the California crop is sold as fresh garlic worldwide. Americans consume an estimated 80 million pounds of fresh garlic per year, Asians 60 million pounds and only 10 million pounds for the Africans (Foster, 1990). Some varieties of garlic are:

1. Allium Sativum Var. Sativum.
2. Allium Sativum Var. Ophioscordon.
3. Allium Sativum Var. Pekinese.
4. Allium Scorodoprasum Longicupis.

2.2.1 Tribal names

English: -Garli

Kikuyu: -Gitunguru saumu

Luos: -Kitunguu saum.

Luhya: -Estunguu saumu

Giriama: -Thunguu saumu.

Ibo: -Ayo.

Yoruba: -Ayuu.

Hausa: -Tafarnuwa.

Chinese: -Da-Suan.

2.2.2 Available products and other forms of garlic

. Raw garlic.

. Cooked cloves.

. Dehydrated powder.

. Garlic capsules and tablets.

. Garlic oil.

. Aged garlic extracts (Kyolic).

. Garlic syrup.

. Fresh garlic juice.

. Garlic tincture.

2.3 DOSAGES AND TOXICOLOGICAL ASPECT

Garlic has been standardized for its allicin content, determining its "Allicin Potential." At least 4,000mg fresh garlic is equivalent to 1-to-2 garlic cloves, and 10mg allicin or a total allicin potential (Foster, 1990). Standardized preparation of garlic guarantees exact dosing and minimizes the problems of the strong odor of raw garlic.

Though generally regarded as safe, toxic effects have been recorded in domestic and farm animals after over-consumption of garlic or onions due to oxidative damage to erythrocytes and consequent hemolytic anemia in canines (Hu et al., 2002). These effects have been attributed to the aliphatic sulfide contained in garlic.

In human, there are minor side effects as gastrointestinal burning/irritation, disorders of bleeding and blood clotting, indigestion, loss of appetite, diarrhea, and diabetes, chocking thirst due to its pungent odor. Rare side effects include anemia and oxidation of essential body components (Numagami, 1996).

Precaution should be taken when using garlic medicine, especially those used to control sugar content in blood and inflammation. When used externally on the skin, garlic produce dermatitis, hence it must be used cautiously, and certainly never recommended for use on the skin of young children. In any case, it is convenient to consult the doctor, chemist

or dietician to avoid possible contraindications. By no means should one take different preparations of the same product, e.g. capsules, tincture, extracts...etc, simultaneously.

2.3.1 Health benefits and uses of garlic

Garlic is one of the most potent, and from a health perspective, most powerful members of the onion family (Allium). Most of the health benefits derive from the more than 100 sulphur compounds it contains, especially allicin, which is responsible for garlic's characteristic scent and flavour.

Allicin is formed when garlic sulphur compound alliin is converted to allicin by the enzyme allinase that occurs when the cloves are chopped, crushed or chewed. If garlic is cooked immediately after chopping, allicin never forms and the health benefits are lost.

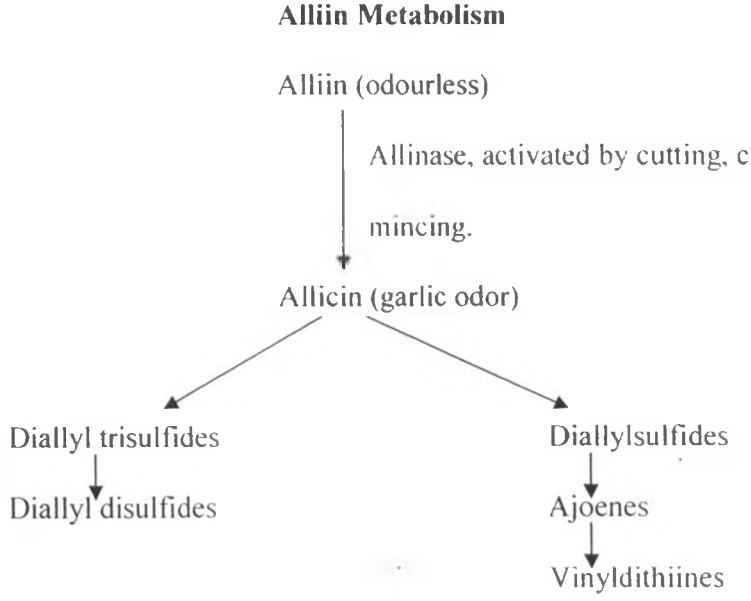


Fig 2.2 A diagram on Garlic's metabolic pathway.

Among the promising health benefits of garlic are:

-It may protect against stomach and colon cancer.

-Slows the build-up of artery-clogging plague.

-Prevents the formation of blood clots.

-Helps lower blood pressure.

-Reduce the chances of infection.

-Improves nasal congestion and sinusitis.

Garlic is a powerful natural antibiotic; it can stimulate cell growth and activity, and decrease blood pressure in hypertensive conditions. A main advantage to using garlic for its antibiotic properties is that it does not destroy the body's natural flora. It is excellent for use in all colds and infection of the body.

2.3.2 A Summary of other uses of Garlic

In summary:

No other herb has served as many culinary and medicinal roles in as many cultures, as garlic. Traditionally, the fresh cloves, garlic tea, syrup, tincture and other preparations have been used as aphrodisiacs, to treat hysteria, dandruff, typhus, epilepsy, antiseptic, colds, fever, flu symptoms, coughs earache, bronchitis, shortness of breath, asthma, sinus congestion, headache, stomach ache, high blood pressure, toothache, atherosclerosis, hypertension, vaginitis, worms, diarrhea, dysentery, gout, rheumatism, whooping cough, pinworms, old ulcers, snakebites and for numerous other ailments, conditions, and applications (Foster, 1990; Gill, 1992; Sumiyoshi, 1997; McMahan, 1993; Sang, 1995).

2.3.3 Recent studies and research

Over 2,000 papers on the various aspects of the chemistry, pharmacological and clinical applications of the genus *Allium* have been published in the past 20 years. Garlic has been studied widely for its role in cardiovascular health. Dioscorides was a well-known first Century physician who wrote that "garlic doeth clear the arteries and opens the mouths of veins." Recent studies have shown contradicting effects of garlic on blood cholesterol

(Michael, 2003). Over a long period of study, a pattern of effective uses emerged which served to guide modern researchers in the 20th Century. Science has now validated these claims by proving that garlic does indeed lower cholesterol and triglyceride as well as helps to reduce clot formation (thrombi) in the blood (Michael, 2003). Numerous studies done recently verify four main activities or modes of action for garlic:

a. Cholesterol-lowering.

b. Anti-thrombotic.

c. Mild but broad-spectrum antibiotic.

d. Anti-cancer potential (specifically, the lowered risks of colon and urinary/bladder cancers).

Schaefer (1998) studied garlic for its immune boosting properties. Numerous studies performed in the recent years indicate that the compound allicin, found in fresh garlic, has antibiotic and antifungal properties (David, 2005). He attributes the potency of garlic to natural plant defense mechanism.

Components of garlic seem to be able to widen blood vessels (Jennifer, 2005). For years, herbal medicine practitioners have touted the health effects of the stinky bulb. David. J (2001) substantiates the idea that garlic and its derivatives are capable of producing vasodilatation (widening of blood vessels), an action which contributes to the purported ability of garlic to reduce hypertension.

Areas that are still being studied in detail are on the potential of garlic for the treatment of high blood pressure, atherosclerosis, digestive ailments, colds, flu, bronchitis, antibacterial and antifungal activities. Other activities that have been the subject of research primarily in the developing countries include the use of garlic to treat amoeba, dysentery, epilepsy, anti-inflammatory, anti-fertility (oxytotic), tuberculosis, and anti-toxic activity for the treatment of poisoning, such as industrial lead poisoning.

Louis Pasteur (1858) was the first to note the antibacterial activity of garlic. Various studies have shown that garlic extracts or various components have a broad-spectrum antibacterial action against Gram-negative and Gram-positive bacteria. Allicin is considered to be the primary antibacterial component. In 1984, Singh and Shukla demonstrated that garlic shows promising activity against eight out of nine clinical strains of bacteria, which are highly resistant to antibiotics.

Ajoene, a compound characterized by Block et al (1984) was found to be the most active component with anti-thrombotic activity (preventing blood platelet aggregation or clotting). Block (1986), citing unpublished studies by Catalfamo of New York State University, states that the antithrombotic activity of ajoene is the result of inhibiting exposure of fibrinogen receptors on platelet membranes. He also notes that while other components of garlic (diallyl disulfide and methyl allyl trisulfide) have been claimed to be the active antithrombotic components, other researchers have found them to be virtually inactive anti-thrombotic components. Attempts are underway to come up with ajoene-containing products that will produce anti-thrombotic activity free from the odiferous antisocial side effects of the fresh garlic bulb (Block, 1986). As an anti-clotting agent, ajoene is at least as potent as aspirin. The platelet antiaggregant activity may be responsible for the potential utility of garlic as a useful protective agent in atherosclerosis, coronary thrombosis and stroke (Tyler, 1993).

Several studies have focused on the ability of garlic to reduce cholesterol, triglyceride and increase high-density lipoproteins in the blood. In 1973, Bordia and Barisal demonstrated that garlic essential oil reduced serum cholesterol three hours after volunteers ate fatty meal with garlic. Studies by Jain (1977) and Bordia (1981) showed that a decrease in serum cholesterol and triglyceride levels in normal and hyperlipidemic patients could be achieved with long-term daily ingestion of garlic. Studies by Bordia (1981) and Nitiyanant (1987) both

demonstrated initial rise in serum cholesterol levels, followed by decline after long-term administration (5 months) of garlic. Nitiyanant studies also showed an increase in high-density lipoprotein in the blood.

Carter et al (1989) did a study in which ten AIDS patients were given garlic extracts for a period of ten weeks. They then analyzed the activity of natural-killer cells and helper/suppressor ratio. The authors reported an improvement of AIDS related conditions including diarrhea, genital herpes, candidiasis and pansinusitis with recurrent fever. Four out of seven evaluable patients had an improved helper/suppressor ratio. There is still need for further study on other effects of garlic on AIDS patients.

The German Government Commission approves the use of garlic as a nonprescription medicine to lower cholesterol and to reduce other cardiovascular risk factors (Blumenthal et al., 1996). Oluwole (2001) did a study on the effect of garlic on hematological and biochemical parameters in rats. He showed that garlic extract increased hematopoiesis in rats by causing tissue hypoxia.

2.3.4 Present pharmacological and clinical applications

The on-going worldwide research on garlic is to unravel the medicinal and pharmacological activities of garlic against many bacteria, viruses, worms (parasites) and fungi.

Some of the broad-spectrum preventive activities of garlic are:

1. *Anti-thrombotic effects:*

   -The presence of sulfurous-containing compounds (allicin and ajoene), makes it very important by giving it anti-thrombotic properties which enhances blood circulation i.e. prevention of blood clot formation, makes blood more fluid and

help to fight against circulatory diseases: arteriosclerosis, cholesterolaemia (Jain, 1993), hypertension, angina pectoris and hemorrhoids.

2. Inhibits platelet aggregation:

-It decreases atherosclerosis, heart diseases and stroke by decreasing plasma viscosity (Tyler, 1993) and decreasing fasting blood glucose (Orekhov, 1995).

3. Fibrinolysis:

-It increases serum fibrinolytic activity- promotes fibrinolysis (Jain, 1993; Block, 1986) and therefore offers benefit in prevention of heart attacks, strokes and various thromboembolic events (Tyler, 1993).

4. Lowering cholesterol:

-Many double-blind placebo controlled studies have been done in relation to cholesterol levels, with preparations of at least 10mg allicin/day or total allicin potential of 4,000mcg/day.

. Decreased total serum cholesterol by about 10-12%.

. Decreased LDL, by 15%.

. Increased HDL, by 15%.

. Decreased triglyceride, by 15%. This assists in prevention of heart diseases and stroke.

5. Prevents LDL-Oxidation:

-There is evidence that LDL oxidation has a role in atherosclerosis development (Orekhov, 1995; Senevirantne, 1999).

6. Hypertension:

-Garlic decreases systolic (about 11mmHg) and diastolic (about 5mmHg) blood pressure (Daniel and Amabel, 2008).

-Decreases risk of stroke by 30-40% (Tyler, 1993).

-Decreases risk of heart attacks by 20-25% (Silagy, 1994).

7. Hypoglycemia:

-Shallot and garlic extracts have a hypoglycemic influence on fructose-induced insulin resistant animals (Razieh et al, 2007).

2.4 BLOOD

The cellular elements of blood are: white blood cells, red blood cells and platelets. They are suspended in the blood plasma. The normal total circulating blood volume is about 8% of the body weight (5600ml in a 70kg man). About 55% of this volume is plasma and 45% the cellular elements.

RED BLOOD CELLS (ERYTHROCYTES).

The major function of red blood cells (erythrocytes) is to transport hemoglobin, which in turn carries oxygen from the lungs to the tissues. In lower kingdom animals, e.g. the helminthes, hemoglobin circulates as free protein in the plasma, not enclosed in the red blood cells. In humans, if it floats freely in the plasma, about 3% of it would leak through the capillary membrane, into the kidneys' glomerular filtrate each time the blood passes through the glomerular for urine formation. Hence, for hemoglobin to remain in the blood circulation, it must exist inside red blood cells.

Other functions of red blood cells other than transport of hemoglobin are: 1) contains *carbonic anhydrase*, which catalyses the reversible reaction between carbon dioxide and water.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

This makes it possible for blood to transport large amounts of carbon dioxide from tissues to the lungs in the form of bicarbonate ions (HCO_3^-). 2) Hemoglobin in red blood cells is an excellent *acid-base buffer* of the whole blood.

Normal red blood cells have a biconcave shape, a diameter of about 7.8 micrometers and a thickness of 2.5 micrometers. The average volume of red blood cells is 90 to 95 cubic micrometers. The cells' deformability allows them to change shape as they squeeze through the thin capillaries in the body tissues. They have a quality cell membrane that allows deformation without rupture of the cells. The normal concentration of red blood cells in normal men per cubic millimeter is 5.2 million (+/-300,000), and in normal women is 4.7 million (+/-300,000). Red blood cells have the ability to concentrate hemoglobin in the cell fluid up to 34g/dl of cells. This concentration cannot be exceeded because this is a metabolic limit of the cells' hemoglobin-forming mechanism. The normal hematocrit (percentage of the cells in blood) is between 40 to 45%, while hemoglobin in men is between 14-18g of hemoglobin per deciliter and of women is between 12-16g/dl of blood.

SITES OF RED BLOOD PRODUCTION

During embryonic life (0-3 months), the primitive, nucleated red blood cells are produced in the *yolk sac* during gestation. The *liver*, *spleen* and *lymph nodes* are also stimulated to produce RBCs between 3 to 5 months. Then during late gestation (5-9 months), and after birth, RBCs are exclusively produced by the *red bone marrow*. Figure 3.1 shows comparative rates of RBC production in the human bone marrow of different bones with age. Bone marrow of nearly all bones produce RBCs until the age of 5 years, after which, marrow of the long bones become infiltrated with fat (yellow marrow). They become inactive after about the age of 20 years, except for the proximal portions of tibiae and humeri. Beyond this age, the vertebrae, sternum, ribs and iliac region of the pelvic bones produce RBCs, but the production decreases with age.[1]

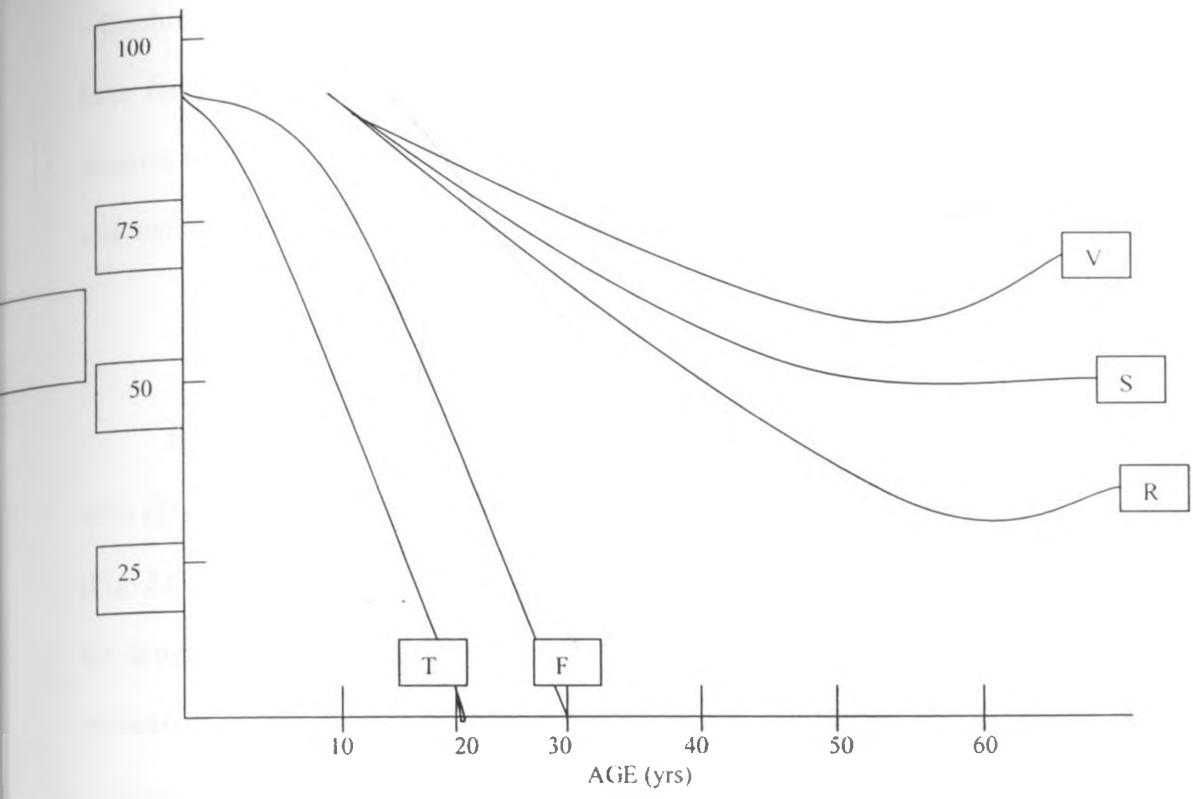


Fig 2.3 Comparative rates of RBC production in the human bone marrow of different bones with age (McDonald and Cruickshank, 1988).

V-Vertebra
S-Sternum
R-Rib
F-Femur (Shaft)
T-Tibia (Shaft)

2.4.1 Formation of blood cells

The bone marrow contains cells called Pluripotential Hematopoietic Stem Cells (PHSC), from which all cells in the circulating blood are formed. Figure 3.2a shows the developmental stages of the stem cells to form different peripheral blood cells. The stem cells differentiate to form the other cells as shown in figure 3.2b. The PHSC differentiate to form Committed Stem Cells (CSC). These grow and produce colonies of specific types of blood cells. Those committed stem cells that produce erythrocytes are called Colony Forming Unit

Erythrocyte (CFU-E). Those committed to form granulocytes, monocytes and lymphocytes, have the designations CFU-G, CFU-M and CFU-L respectively. The formation of RBCs requires the presence of inducers: Interleukin 1, 6, and 3, to promote growth, differentiation and maturation of great numbers of erythrocytes.

2.4.1.1 Developmental stages of erythrocytes

In the adult, erythrocytes are formed in the bone marrow from the pluripotent stem cells (Fig. 2.4). A hormone Erythropoietin (EPO) controls the rate of production of RBCs (Fig. 2.6). Many developmental stages occur as red cells mature. Each time the cells divide the daughter cells are slightly smaller than the parent cells. The synthesis of DNA for the maturation of the nucleus requires the presence of vitamin B_{12} and folic acid. If these vitamins are deficient the erythrocytes formed are larger than normal *(macrocytic)* (McDonald, 1988).

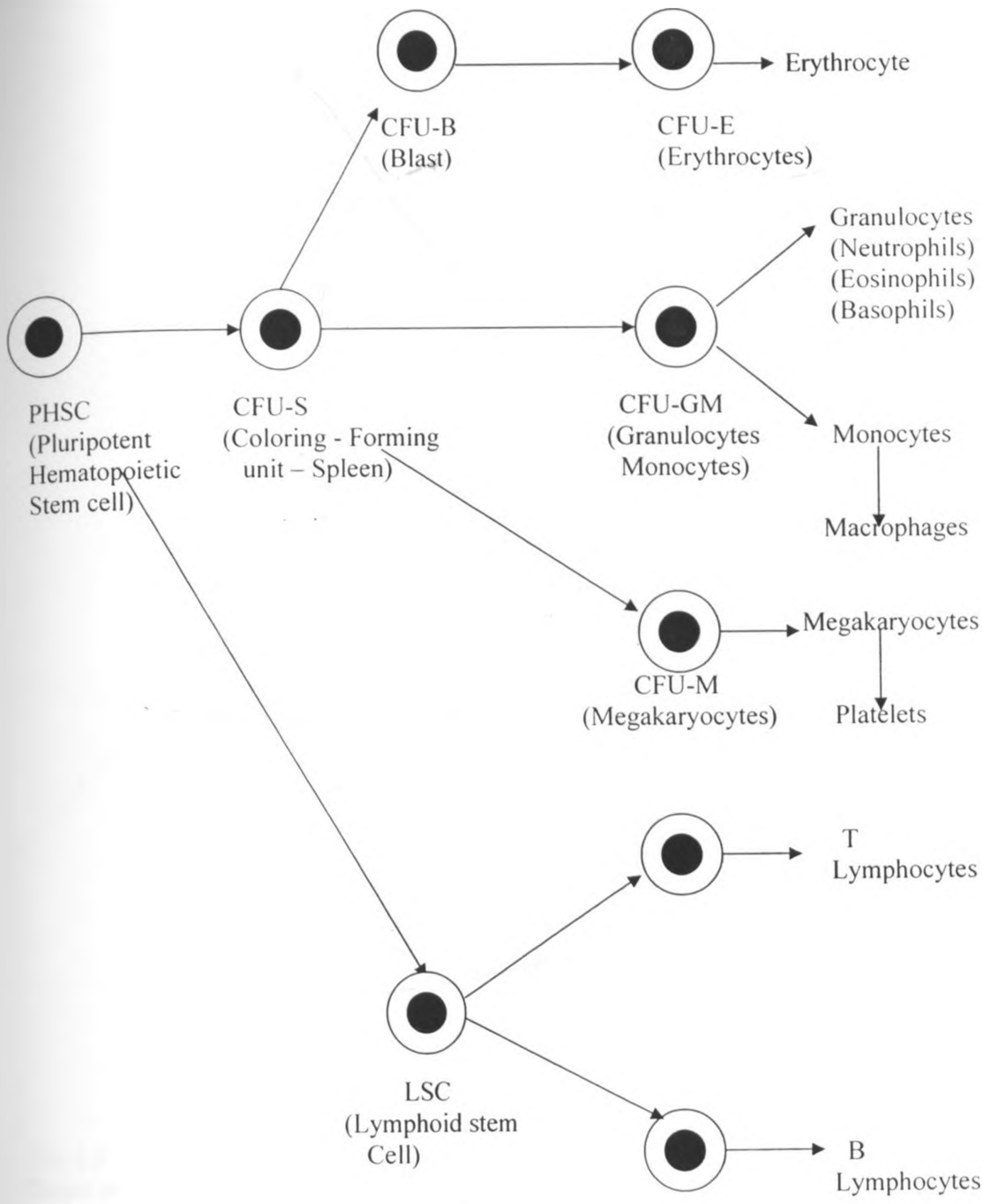


Fig 2.4
Formation of different peripheral blood cells from the original pluripotent hematopoietic stem cells (PHSC) in the human bone marrow (McDonald and Cruickshank, 1988).

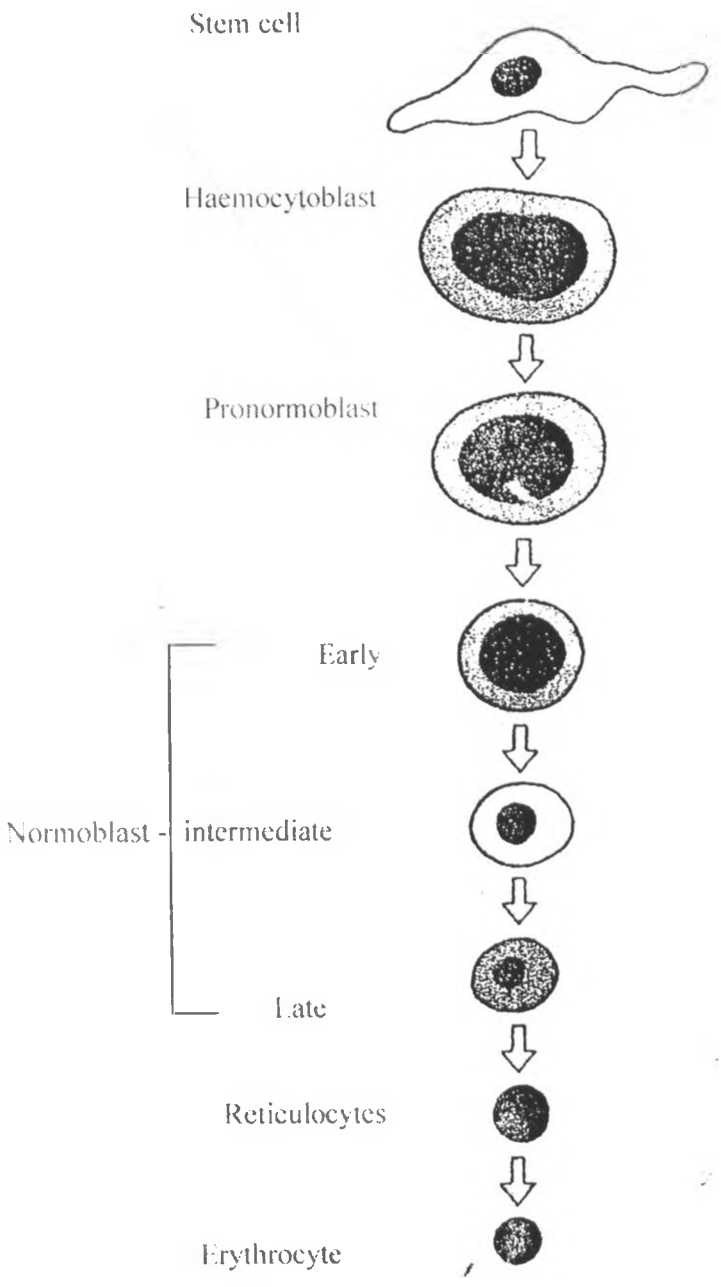
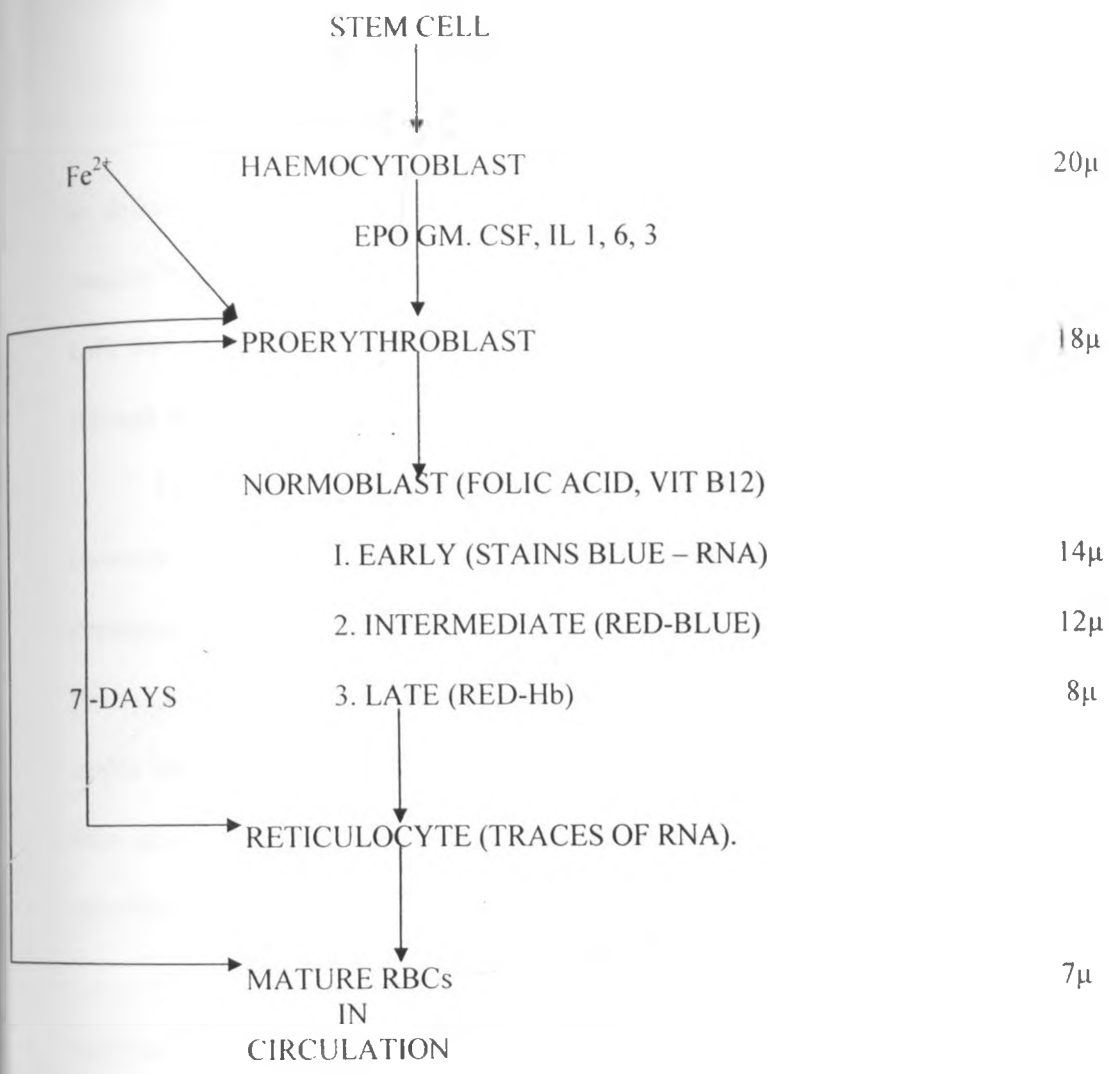


Fig. 2.5
Stages in the development of the Red Cells from stem cells in the bone marrow, to mature erythrocytes in the circulation. Cell division (mitosis) occurs at each stage as far as the late normoblast, so that each pronormoblast gives rise to very many erythrocytes. (Based on McDonald. Paul & Cruickshank 1988.)

Fig: 2.6

ERYTHROPOIESIS:



ERYTHROPOIETIC CHANGES

1. Decrease in size
2. Acquisition of Fe^{2+}
3. Maturation and disappearance of the nucleus.
4. Cytoplasm becomes abundant
5. Loss of organelles.
6. Synthesis of hemoglobin.
7. Mitosis till intermediate stage (McDonald and Cruickshank, 1988).

During development the cells begin to manufacture hemoglobin in very large amounts occupying the cell. Each molecule of hemoglobin consists of four units each of which is made up of: -A polypeptide chain

-A haem moiety

-An iron atom.

In deficiency of iron, cells cannot manufacture as much hemoglobin, and they tend to be smaller than normal in size *(microcytic)*. As hemoglobin makes the erythrocytes red in color, cells that contain less of this protein look paler *(hypochromic)* than normal cells when viewed through a microscope (McDonald, 1988).

From pluripotential Hematopoietic stem cells, cells in the bone marrow become committed to form RBCs, forming **proerythroblast**, as shown in figure 2.6. Under appropriate stimulation, large numbers of these cells are formed from CFU-E stem cells. Proerythroblast then divide multiple times forming **normoblast** that undergoes three distinct stages because they stain differently. The *early normoblast* stains blue with basic dyes; they then accumulate hemoglobin and stain red in *intermediate normoblastic* stage, before transforming in to the *late normoblastic* stage.

In the later stages of development cellular organelles disintegrate and disappear so that the cells emerging from the bone marrow in to the blood contain only residual fragments, which also soon disappear. These fragments stain in a distinctive way giving these first immature erythrocytes the name *reticulocytes* because of the network (reticulum) of residual fragments that are visible microscopically. Reticulocytes make up 1% of the total numbers of red blood cells in the blood (Jelkmann, 1992). The final remnants are extruded forming mature erythrocytes.

2.4.2 Erythropoiesis

Erythropoiesis is the production and release of red blood cells from the bone marrow. This process increases during bleeding or hypoxia due to reduced hemoglobin (Fig. 2.7) and therefore reduced O_2 supply to the tissues (Krantz, 1965). On the other hand, when red blood cell volume is increased i.e. during transfusion, the erythropoietic activity in the bone marrow decreases. This process of erythropoiesis is regulated by secretion of erythropoietin (EPO) (Fig. 2.7 and 2.8) (Grant, 1952).

Erythropoietin (EPO) is an approximately 34-kDa-glycoprotein hormone produced primarily by fibroblast-like type 1 interstitial cells of the peritubular capillary endothelium of the kidney in adults. It contains 165 amino acid residues and 4 oligosaccharide chains (Reissmann, 1950). Other sites are the liver's perivenous hepatocytes in healthy adults. In premature, as well as full term infants, the liver is the primary site of EPO production after birth. The kidney becomes the primary site of EPO synthesis at the age of 2 years and onwards throughout life.

EPO production is also stimulated by reduced oxygen content in the renal arterial circulation, e.g. during high altitude exposure (Fig. 2.6). In this situation, there is reduced O_2 concentration as one ascends up high altitude. This results in an increased hemoglobin concentration in the blood, and consequently an augmented O_2-carrying capacity. The increase in hemoglobin is caused by the release of EPO from the kidneys and possibly from other organs. This hormone accelerates RBC production in the bone marrow to counteract the reduced O_2 content in the general circulation, the vital organs and the rest of the body (Lenfant, 1971).

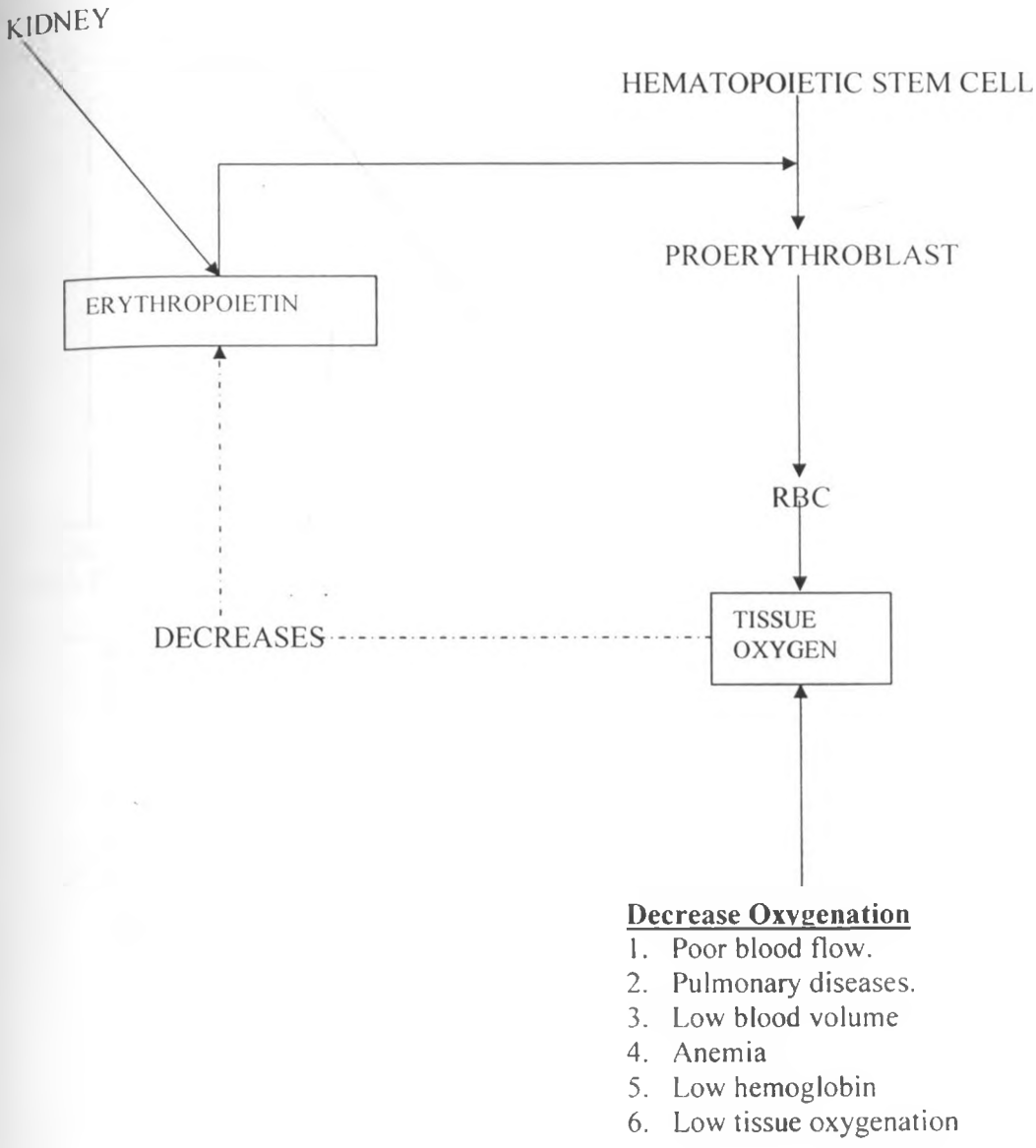


Fig: 2.7
Function of EPO to increase RBC production under various conditions that decrease tissue oxygenation, in humans (McDonald and Cruickshank, 1988).

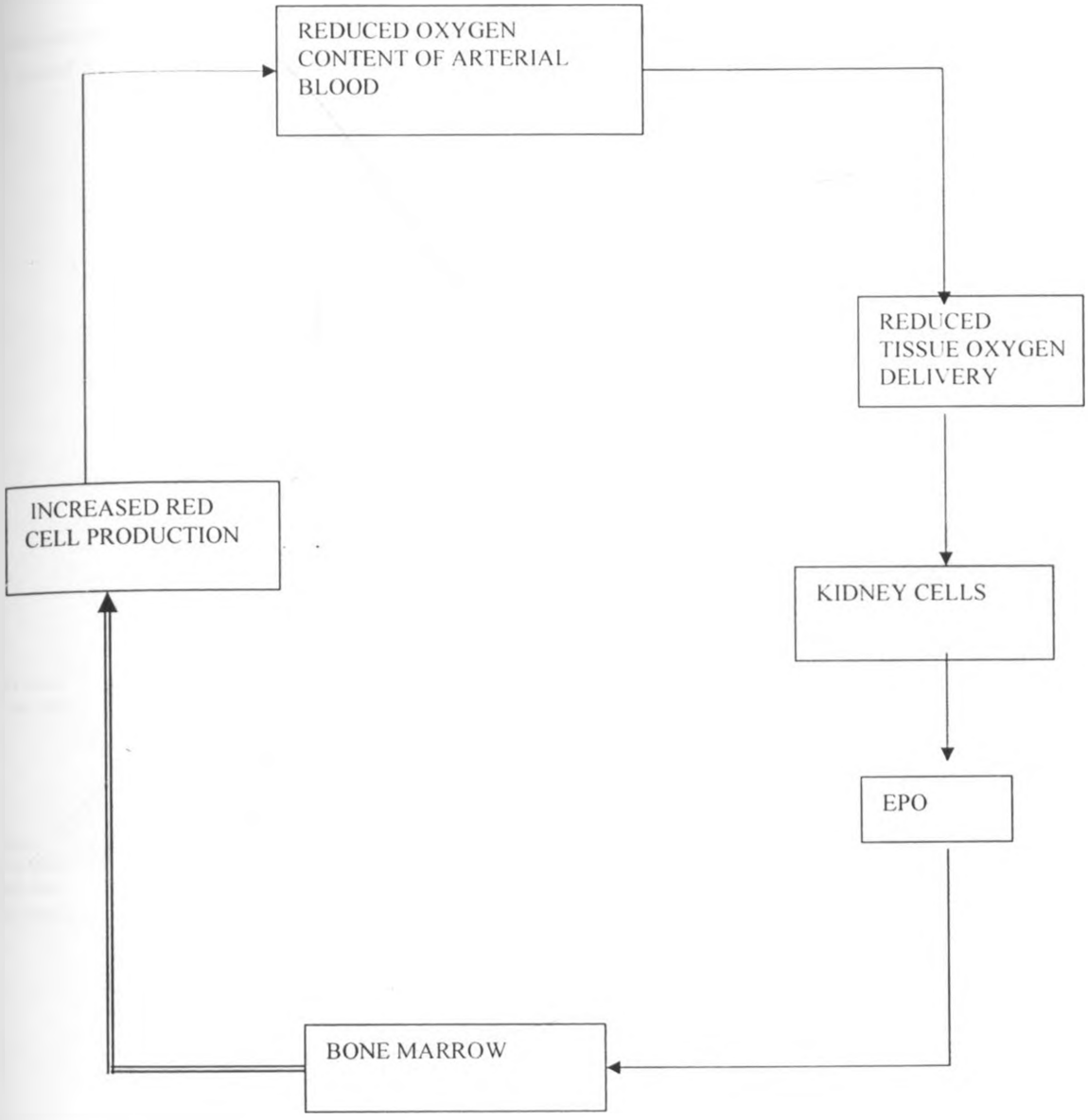


Fig. 2.8
Control of Arterial Oxygen content by regulation of red cell number, in humans (McDonald and Cruickshank, 1988).

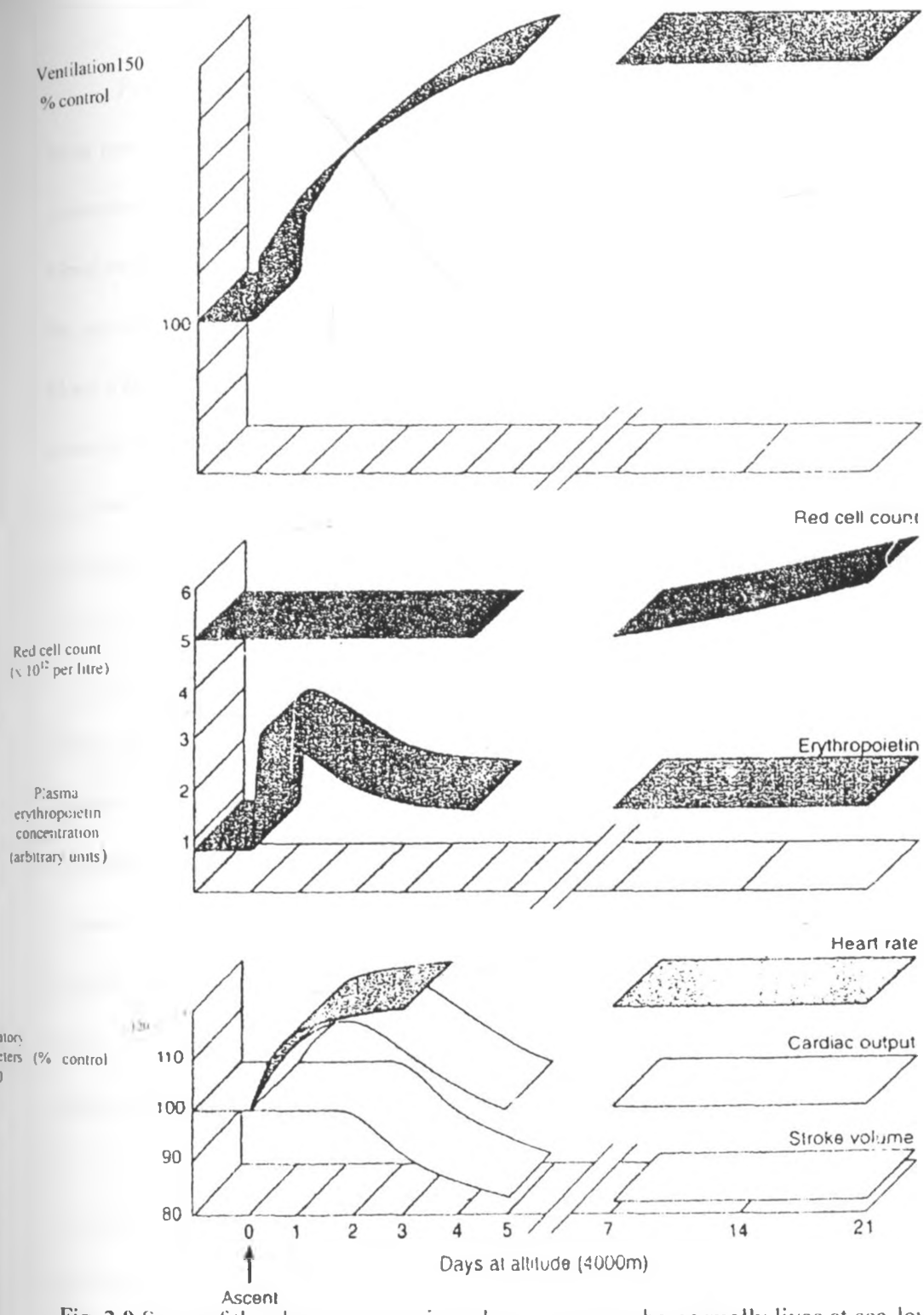


Fig. 2.9 Some of the changes occurring when someone who normally lives at sea-level ascends rapidly to a place in the mountains (4000 meters) and stays there for weeks (Adapted from Case 1985.).

2.4.2.1 Experimental evidence of Erythropoietin production

For many years in the 1900's, the constancy of the circulating red blood cell mass had been noted (Grant, 1952), but the factors involved in maintaining a stable red blood cell concentration by regulating the rate of erythropoiesis were unknown. An increase in red blood cell production at high altitudes with reduced O_2 supply was observed and on this basis the amount of O_2 supplied to the bone marrow was suggested as the direct stimulus to red blood cell production (Schneider, 1921). This was followed by experiments that subjected animals to hypoxia and demonstrated subsequent increase in erythropoiesis (Altland, 1949; Bancroft, 1949). While atmospheric or anemic hypoxia increase erythropoiesis, hyperoxia and plethora created by the transfusion of red cells, were shown to reduce erythropoiesis (Campbell, 1927).

In the 1950s, Carnot and Deflandre hypothesized, that erythropoiesis was controlled not by direct deoxygenation of the marrow, but by a humoral factor; elaborated outside the marrow and secreted into the blood in response to hypoxia (Carnot, 1906). Carnot and Deflandre termed that plasma factor *hemopoietine*, but, as it appeared to be involved exclusively in red blood cell production, **Ervthropoietin** became the adopted name. Reissmann (1950) did an experiment in which he showed that subjecting one parabiotic rat to hypoxia (blood O_2 saturation of 63%), increased erythropoiesis in the partner rat breathing room air and having a blood O_2 saturation of 97%.

In 1989, Fukumoto and Obinata were able to show the mechanism of EPO on erythroid progenitor cell induced from murine erythroleukemia cells. They showed that EPO does not increase cyclic-AMP levels in the cells directly, but as a stimulator of the EPO receptors, and/or as second messenger in combination with other substances in the cascade.

Yamamura (1992) studied the distinct downstream signaling mechanism between EPO receptors and interleukin-2 receptors. Based on his results, he concluded that EPO

receptors and interleukin-2 receptor beta-chains belong to the same cytokine receptor super family.

Arkh (2004) did a study on the characteristics of EPO regulation on population living at high altitude territories in Russia. He examined the serum and erythropoietic-ferritin in women at Tienshan. A specific and long-term adaptation to hypoxia gave rise to an original mechanism of erythropoietin regulation when all iron participates in hemoglobin synthesis.

2.4.2.2 Source and regulation of EPO secretion

In adults, the kidneys secrete 85 % of EPO, and the liver secretes only 15%. The two organs contain mRNA messages for EPO synthesis. The spleen and the salivary glands also secrete some small amounts. The interstitial cells of the peritubular capillary beds in the kidney, the perivenous hepatocytes and kupffer cells in the liver produce EPO. Some brain cells, where it protects against exocitotic damages triggered by hypoxia, also produce EPO. In the uterus and the oviduct, its production is stimulated by prostaglandin E_2. Circulatory EPO binds to EPO receptors on the surface of erythroid progenitor cells resulting in replication and maturation of functional erythrocytes.

Ersler (1989) produced four lines of evidence indicating that decrease in local oxygen triggers an increase in EPO synthesis: 1) Synthesis of EPO increase with anemia, 2) EPO production increase with lowered renal blood flow, 3) EPO production is increased in central hypoxia (lowered arterial O_2, pulmonary diseases, people living in high altitudes), and 4) EPO production increases where hemoglobin has high oxygen affinity; i.e. mutant hemoglobin with high O_2 affinity and alkalosis. In all of these cases, local PO_2 falls as tissues respond to a fall in O_2 delivery by extracting more O_2 from each volume of blood that passes through the kidneys (Fig. 3.3a).

Maxwell and Nichols (1997) showed that there are other non-renal sensors and hormones that stimulate EPO production. Prostaglandin E_2, adenosine, androgen, TSH, ACTH, growth hormone, thyroid hormone, epinephrine, nor epinephrine and angiotensin stimulate erythropoietin secretion by increasing intracellular levels of cyclic Adenosine Monophosphate (cAMP), while pharmacological amounts of estrogen, depress EPO secretion. Poor blood flow, cobalt salts, low hemoglobin levels, low red blood cell count and anemia stimulate EPO production (Adamson, 1996). People with severe kidney diseases or have had their kidneys removed and placed on hemodialysis, suffer severe anemia as a result of decreased EPO production because 15% of EPO from the liver is insufficient to cause enough red blood cell formation needed by the body. Recent studies have shown that, oxygen sensors regulate EPO secretion in the kidney and liver by stimulating or inhibiting transcription of the EPO gene to form EPO mRNA (Walter, 2003).

Tissue oxygenation is the most essential regulator of red blood cell production. Any conditions that cause reduced oxygen supply to the tissues ordinarily increase the rate of EPO secretion, and hence, the rates of red blood cell production. For example, anemia due to hemorrhage, X-ray therapy causes hyperplasia (increased cell size and number) of the bone marrows to meet the demand for red blood cell in the body. Diseases of the circulation that cause reduced blood flow to peripheral vessels, i.e. those causing failure of oxygen absorption in the lungs, also increase the rate of red blood cell production. Cardiac failure and lung diseases due to hypoxia stimulate EPO release resulting in increased rate of red blood cell production, with a resultant increase in the hematocrit and hence the total blood volume (Adamson, 1996).

2.4.2.3 Mechanism of action of EPO

EPO is a growth factor related to other cytokines and works by recruiting more EPO-sensitive committed stem cells in the bone marrow, which are converted to red blood cell

precursors that form erythrocytes. The EPO receptors are members of the cytokine receptor super family. The receptors work via tyrosine-kinase activity and activates serine and threonine pathway to stimulate the production of proerythroblasts in the bone marrow as well as the growth and development of red blood cells from progenitor cells (Ersler, 1989). EPO in the circulation has a half-life of about 5 hours and is released into the circulation within 24 hours of kidney stimulation (Fisher, 1997).

In oxygen deficiency situations, erythropoiesis is triggered, but there will be no new red blood cells in the circulation, until 3-5 days later. When EPO stimulates the production of proerythroblasts from hemopoietic stem cells in the bone marrow, different erythroblast stages occur before red blood cells mature (Fig. 2.6) (McDonald et al., 1988). The rapid production of cells continues as long as the person remains in the low oxygen state, or until enough red blood cells have been produced to supply adequate amounts of oxygen to the tissues. The rate of EPO production decreases to a level that is required to maintain the required number of red blood cells in the circulation, but not an excess (Ratcliff, 1997).

2.5 RABBITS

Rabbits *(Oryctolagus cuniculus)* are classified with hares, pikas and American cottontail rabbits in the Phylum ***Chordata***, Class ***Mammalia***, Order ***Lagomorpha*** and Family ***Leporidae.*** The domesticated rabbit is derived from the European wild rabbit, apparently native to continental Europe and perhaps North Africa. The species is most common in Southern and Mediterranean Europe, particularly Spain, and has been domesticated at least since Roman times. Wild rabbits had been introduced to many countries including Britain, since the Middle Ages, and Australia and New Zealand in the 19th Century (National Research Council, 1977).

Domesticated rabbits are raised for meat, fur and pellets. Rabbits are important livestock in some parts of the world, including Europe and China. As meat producers, they are highly efficient converters of low-grade plant materials (e.g. hay, forage, crops and cereal by-products) to animal protein, with best conversion ratios of around 2.5:1. As laboratory animals, rabbits have been particularly used for anti-serum production, pyrogen testing, cardiovascular studies including atherosclerosis, teratology, and ocular studies. Their advantages as laboratory animals include easy access to blood vessels, size, reproductive rate, and suitability for cage housing. Laboratory rabbits used include the following breeds:

1. New Zealand White: These are large-bodied albinos with erect ears, developed as meat rabbits. Mature body size, 4-5kg.
2. Lop-eared rabbits: These are large coloured rabbits with broad pendulous ears, used originally for ear chamber implantation studies. Mature size, 4kg.
3. Dutch Belted and English Multicoloured rabbits: These are smaller coloured rabbits with erect ears. Mature size, about 3kg.

Rabbits are listed and protected in the Animals (Scientific Procedures) Act 1986 and must therefore be obtained from accredited breeding and/or supplying establishments. Rabbits with coloured coats may be identified readily by their appearance. Other suitable methods of marking include microchip implants, wool dyes or marker pen. Tattoos or leg rings are less suitable, since tattooing may cause discomfort and leg rings applied when the animal is young may become too tight as the animal grows.

2.5.1 Behaviour and Environment

Wild rabbits are crepuscular or nocturnal, becoming active and emerging from their burrows to feed at dusk or during the night. In the laboratory, periods of activity are seen throughout the day and night. Rabbits are social animals that are able to utilize a complex,

three-dimensional environment. If given sufficient space, domestic and laboratory rabbits will exhibit the full range of behaviours seen in their wild ancestors, including climbing up to a good vantage point, exploratory and tunnelling behavior, social activity and aggression. Aggressive behavior is seen most in breeding and pubertal animals, and adult males. Males are most aggressive when competing for food, territory or females. It is usual for them to be separated from 10 weeks of age to prevent fighting (National Research Council, 1996).

Rabbits require temperatures between 15 and 25^0C. Neonates cannot maintain their body temperature until they are 7 days old, so they must be kept in a warm environment. Humidity should be kept between 40 and 65%. Females require 14-16 hours of light daily, and males 8-10, with or without periods of twilight. Shortened light cycles may results in reduced sexual activity in the autumn. Low intensity light should be provided for albino animals.

Rabbits can hear in the ultrasound range, from 2 to 16 kHz and possibly up to 42 kHz, so care should be taken not to expose them to excessive ultrasound. Background noise can help to prevent the animals from being startled by sudden noises. Ventilation is particularly important for rabbits. Since they are susceptible to respiratory diseases, and poor ventilation allows a build-up of ammonia, which predisposes them to these ailments. Draughtless ventilation and efficient tray liners reduce the ammonia level. At least 12-15 air changes per hour should be provided.

2.5.2 Feeding and Water

Rabbits are Coprophagic (they naturally eat their own special faecal matter; mostly due to lack of vitamin B), and this is an important part of their digestion (Blount, 1957). They require a diet with high fibre content, and although much fibre remains undigested, it is required for bulk and reduces incidence of hairballs and diarrhoea. A diet with 12-22% fiber

and 12% protein for maintenance or 15-17% for growth is recommended (National Research Council, 1996). *Ad lib* feeding for rabbits in cages may sometimes result in obesity. Overeating may be a stereotypic behavior, and is seldom seen in floor-housed rabbits. Providing environmental enrichment and a variety of foodstuffs can reduce this problem. Hay may be given as a supplement to provide fiber and as a plaything.

High-energy diets are required for rabbits that are reproducing and for the dwarf breeds, with 10500kJ per kg of feed. For maintenance, 8800kJ per kg is sufficient. ***Rabbits need 6-8g of high-energy food per 100g-body weight daily***. As the gut flora plays an important part in digestion, changes in diet should be done gradually, over a 4-to-5-day period, to allow the flora to adapt. Failure to do this will result in diarrhea or anorexia (Cheeke, 1987). Plate 7 depicts weighing of food (Standard Rabbit Pellet) and amount of water.

Water should be supplied ad lid, and be fresh and clean. Automatic systems are often used. ***Rabbits normally consume 10ml water per 100g-body weight. Lactating does may drink up to 90ml per 100g-body weight***. Rabbits have a tendency to play with water bottles, so they should be checked frequently to ensure they are not empty and that the floor has not become wet (National Research Council, 1977).

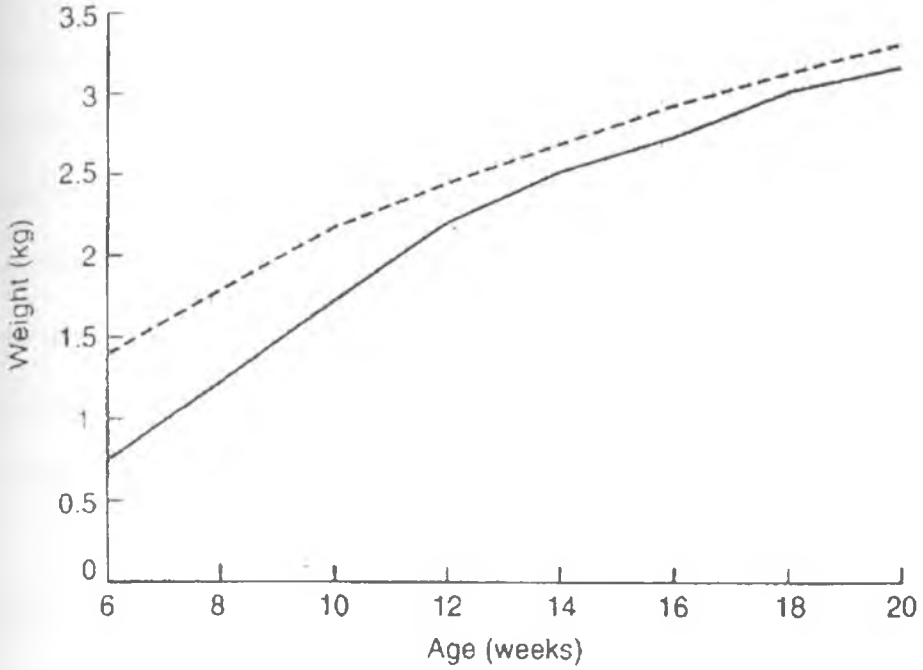


Fig. 2.10 Typical New Zealand White rabbit growth chart. (-) Minimum weight; (- - -) Maximum weight (McDonald and Cruickshank, 1988).

2.5.3 Housing and Handling

In the laboratory, it is necessary to confine rabbits for practical reasons, but it is essential to provide sufficient room for the animals to perform majority of their natural locomotory behaviours. This will prevent skeletal problems, such as hypoplasia of bone tissue and osteoporosis, with the increased risk of fractures and nerve damage leading to cage paralysis. Prolonged periods of inactivity of the animals will lead to increased stereotypic behavior. Space is always limited, but careful design of housing will optimize the use of the available space. Single caging should be used only where group housing is inappropriate, such as for adult males or if an animal needs to be isolated for measurement of food and water intake. Pair housing in large cages may be an acceptable alternative in many circumstances.

Cages should allow the rabbits to stretch out in at least one direction and to be high enough for the animal to stand. Giving hay, chew-sticks or cardboard boxes to play with can provide environmental enrichment, or they can be taken for exercise for short periods. Rabbits should always be housed where they can see other rabbits. Grid floors are generally provided in cages, and should be carefully designed to allow urine and faeces to drain without predisposing to sore hocks (Pododermatitis). Trays beneath the grid should be lined with an absorbent pad to lock in the ammonia. Otherwise, high ammonia levels predispose the animals to the development of respiratory diseases.

Rabbits will thrive particularly well if group housed in floor pens. The incidence of aggressive behavior depends on many factors, including strain (Dutch rabbits are more aggressive than New Zealand white), sex, age and weight, as well as the pen size and construction, the relatedness of the individuals, and to the proximity of other rabbits of the opposite sex. Ample dividers, cardboard boxes or large tubes within the pen should be provided so nervous or frightened animals have bolt holes where they can hide from aggressive co-species. Groups of animals that can be successfully housed together include breeding females, if they are siblings or have been put together before or soon after weaning; a doe and litter; single-sex groups of newly weaned animals; and stable groups of animals on procedure.

Rabbits communicate by the sense of smell and this has to be considered when designing rabbit housing. They will feel secure and confident if surrounded by their own smell, and are disturbed by strong smelling disinfectants. Floor pens should be lined with bedding materials such as straw, shredded paper, non-resinated saw dust, cotton-wool, meadow hay, cellulose wadding, peat moss litter and softwood shaving or woodchip. Floor pens should measure 4.5-6 sq ft, and 4ft x 18in x 18in for cage housing. The ideal bedding and nesting materials should be:

1. Harmless to the animal, i.e. non-toxic and non-staining, non-edible and free from pathogenic organisms and parasites.
2. Absorbent.
3. Disposable.
4. Readily available and easily stored.
5. Relatively cheap.

A Photograph of Caged rabbits vs. Floor-housed rabbits (PLATE 7).

Group housing in pens has many advantages over traditional single cages. The improved welfare of group-housed animals manifests in improved physical and psychological well-being. Floor pens are more economic to purchase and maintain than conventional cages. Identification and treatment of sick individuals is easier.

Rabbits will urinate and defecate in latrine areas, which can be cleaned frequently, i.e. twice a week. They produce copious, turbid urine, which may be yellow to dark red due to the presence of a varying quantity of porphyrins. The urine tends to leave scales on the litter trays due to the calcium content, so they may need to be cleaned with acidic agents. Rabbits are generally docile and amenable animals, can be readily trained, and rarely bite if handled correctly. The animal will become accustomed to handling, which will facilitate the performance of procedures. The animal should **never** be lifted by the ears; and the back must be supported at all times to avoid injuries to the spine. Rabbits have powerful hind legs, and the handler must be careful to avoid being kicked or scratched.

Hold the animal by the loose scruff behind the neck together with the ears, supporting the hindquarters with the other hand through the abdomen (Biological Council Animal Research and Welfare Panel, ₦992) (PLATE 9).

2.6 HAEMATOLOGY

2.6.1 Morphology of peripheral blood cells

The reported means of erythrocyte diameter fall generally in the 6.5 to 7.5micrometers range, with the values for adult rabbits near the lower end of the scale (Albritton, 1952; Schemer, 1967). The thickness is reported as a consistent 2.4micrometers. Erythrocytes of the newborn exceed 9micrometers in diameter and show adult values at 20 to 120 days of age (Laird et al., 1970). Polychromasia results of 2 to 4% reticulocytes were consistent findings related to the relatively short life span and consequent rapid turnover. Numerous crenated forms characterize smears of rabbit blood. Normal erythrocyte values appear in Table XVIII, Appendix I.

2.6.2 Erythrocyte life span

The erythrocyte life span as determined by techniques employing ^{55}Fe, ^{59}Fe, or ^{15}N averages around 50 days, with values reported as low as 45 and high as 68 days (Burwell, 1953). This relatively short life span is associated, as it is in other small species of laboratory animals, with a consistently significant number of reticulocytes. The polychromasia and anisocytosis observed in rabbit erythrocytes have a similar basis (Schalm, 1965). A summary of normal hematological values on the New Zealand white rabbit is presented in Appendix F.

2.6.3 Morphology and differentiation of bone marrow cells

Methods are available for the quantitative determination of marrow cells (Hulse, 1964). However, the most widely used procedure is the differential count of aspirated marrow smears. To the degree the smear is representative of the entire marrow; meaningful information can be secured relative to the myeloid to erythroid ratio (M:E), the presence of abnormal cells, and assessment of the normality of maturation. The problem in morphological

identification is concerned primarily with the earlier states of the erythroid cells. However, there are fewer problems in rabbits than in rats. When more specific answers are required, additional histochemical techniques should be employed. Published colored plates can also be of benefit in morphological identification (Schemer, 1967).

Published myeloid to erythroid ratios of normal rabbits are generally around 1:1 (Schemer, 1967). The results of Sabin et al (1936) reveal a significant departure in one-week-old rabbits with an M: E ratio of 0.19:1, which was back to 1.09:1 by four weeks of age. Table XXI, Appendix J is considered a representative normal of the differential marrow cellularity of the normal rabbit.

2.6.4 Blood volume

Blood volume of the domestic rabbits as measured by the T-1824 (Evans Blue) dye method was reported by various authors as 5.7 +/- 0.48 and 6.98 +/- 0.91 ml/100g body weight (Aikawa, 1950; Armin et al., 1952). These differences are possibly related to the age of the animals. Measured red cell volume, plasma volume, and blood volume all decrease significantly from birth to four months of age (young adult), with the greatest decreases noted in the plasma and blood volume.

2.6.5 Sources of variation in hematological values

Some of the sources of variation in hematological values of a rabbit as reported by Weisbroth et al (1974) are:

a) Age.

b) Sex.

c) Breed and Strain.

d) Seasonal and Diurnal Changes. i.e. high in the morning and low in the afternoon and evening; high values present in spring.

e) Nutritional aspects.

f) Trauma, i.e. cold stress was reported to result in increase in platelet and erythrocyte counts, PCV, clotting time, total plasma proteins, and beta-globulin. Decreases were observed in serum albumin and prothrombin time. (Sutherland, 1958).

2.7 REMOVAL OF BLOOD

The removal of blood from an animal is a procedure with three potential stressful components.

1) Handling and restraining the animal. To minimize the distress, humane methods of handling and restraining the animal should be considered, particularly use of appropriate sedative or anesthetics.
2) Venepuncture causes some pain and discomfort whatever the site.
3) The removal of blood causes physiological responses, the magnitude of which depends on the volume of blood removed (as a percentage of the total) and the speed of withdrawal. The rapid removal of large quantities of blood will cause

the animal to go into hypovolemic shock, and may even cause death. The percentage of blood loss required to cause hypovolemic shock varies with the speed of withdrawal, whether or not fluid is replaced concurrently, and the psycho-physiological state of the animal at the time. Chronic slow hemorrhage is tolerated well than acute blood loss, and placid animals tolerate greater losses than nervous ones, again indicating the need for competent animal handlers (Weisbroth, 1974).

The experimental techniques should be refined such that the quantity of blood removed is minimized. This is particularly important in small mammals, such as mice, where the blood volume is small and sample volume is critical.

To achieve meaningful results, any sample taken must be of good quality and must be preserved in the best possible manner. If the sampling technique is poor, blood may clot or hemolyse, rendering results invalid. Blood may be collected using syringes and hypodermic or butterfly needles, through indwelling cannula and evacuated tubes (e.g. anti-coagulant treated vacutainers).

Different anticlotting agents are suitable for different purposes:

a) No anticoagulant: blood clots and serum can be removed after centrifugation.
b) Lithium heparin: anticoagulant for most biochemical assays. Sodium heparin is sometimes used if preservation of WBC is required.
c) Potassium EDTA: used for hematological analysis.
d) Oxalate/fluoride: used for blood glucose determination.

After collection into anticoagulant, the blood should be mixed thoroughly by ***rolling***, not by shaking as this can damage the cells and lead to hemolysis.

2.7.1 Preparation of site and collection of blood

Before piercing the skin, it is important to be certain of the location of the vein either by visualizing it or palpating its course, and to have it immobilized. Blood should be collected using an aseptic technique. The area should be clipped to remove hair, and then cleaned. The use of warm water with or without disinfectant will help dilate superficial veins as well as cleansing the skin. The skin can be swabbed with 70% ethanol or disinfectant. In some species, it may be advantageous to apply local anesthetic cream to the site 30-60

minutes before venepuncture to prevent any discomfort in such animals as the cat, dog, rabbit, or pig. **Plate** 7 shows bleeding and removal of blood from a rabbit

Blood can be collected relatively easily from the lateral or marginal ear vein using an over-the-needle cannula, hypodermic or butterfly needle. A peripheral vasodilator may be applied to the clean shaved skin over the vein (e.g. vasolate, xylene), 5-10 minutes before blood collection. Once the vein is engorged, a 23-gauge hypodermic needle is inserted, attached to a 2 or 5ml syringe, and blood collected by suction. After collection, the vasodilator is wiped off and pressure is applied until the bleeding ceases. Bleeding from the central ear artery is possible, and causes damage to the ear if direct and prolonged pressure is not applied over the vessel.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 ANIMAL MODEL AND SAMPLE SIZE

A. Setting: Animal house in the Department of Medical Physiology, Chiromo Campus, and Kenyatta National Hospital (KNH) Haematology laboratory, Nairobi.

B. Study design: A Simple Controlled Experimental study.

C. **Sample size**: 30 Adult male New Zealand White rabbits. (30/50 sample size is Statistically adequate {>50%} and documented by Banerjee, 2002; Oluwole, 2001; Iranloye, 2001; Razeih et al, 2007 and Daniel, 2008 in their research).

D. Eligibility criteria for the animal model: -Adult male New Zealand White rabbits. -8 to 12 weeks old. -Weigh between 2.0 to 3.0kg. -Healthy.

E. Exclusion:

-Female rabbits (Oestrous hormones may interfere with the results).

-Sick male rabbits.

F. Why Rabbit as the Experimental animal model?

-The rabbit has larger blood volume (Appendix F) that can withstand the frequent bleeding without affecting the body's physiology.

-The rabbit has largely accessible marginal ear veins from which easy venepuncture can be performed.

-It has adequate quantities of bone marrow which is easily accessible. This is due to the relatively large bones from which marrow aspiration was done.

-The sample size used was large enough, hence not much volume of blood was obtained from each animal (only 1ml was removed).

3.1.1 Animal preparations and their welfare

Male adult New Zealand White rabbits were used for this study. They were obtained from a local farmer who was licensed to breed and supply the rabbits to different institutions for different purposes, i.e. to international hotels for meat, science-based institutes for experiments e.t.c. The rabbits used were inbred and thus had very little, or no genetic variations that could have interfered with the results. The physiological variations would therefore not have significantly affected the results, because production of inbred strains is currently successful in rabbit genetics.

The rabbit's total blood volume is about 57 to 65ml/kg body weight; therefore, withdrawing 1ml of blood every 7 days was expected to cause only minimal physiological imbalance (Appendix F). Diurnal effects on haematology have been reported (Sutherland et al., 1958), with records indicating higher levels of blood parameters in the morning and low levels in the evening. Therefore, to obtain consistent mean values on the haematological parameters being investigated, bleeding and blood sampling was done between 9.00am and 11.00am.

All the animals were weighed twice a week (Monday and Thursday) to ensure that the experiment utilized animals that weighed between 2.0 to 3.0kg. They were housed in individual spacious cages (4ft x 18in x 18in) in the animal house. They were kept under standard laboratory conditions; room temperature monitored between 15-25^{o}C. relative humidity 45-65% and a normal photoperiod (12hrs dark/12hrs light). The rabbits were fed on standard rabbit pellets obtained from and manufactured by UNGA Farm and Care Limited, Nairobi, and vegetables twice a week (Wednesday and Sunday), to provide dietary fibers and boost blood iron content, and water *ad libitum*.

The animals were handled humanly, in accordance to the Animal Care Committee Guidelines (Appendix E). The animals were left to habituate and acclimatize for 3 weeks

before starting the experiment. The faecal/urine collection trays were emptied and cleaned daily using a diluted antiseptic (savlon 1ml/liter of water). The entire room was cleaned and disinfected before starting the experiment. The floor was cleaned twice a week.

3.2 EXPERIMENTAL PROTOCOL

3.2.1 Fresh Aqueous Garlic Extract (AGE) preparation

Fresh garlic bulbs were purchased from a Farmer (Fresh from the farm). Since there are several species of garlic fruit, the species of interest for this experiment was *Allium Sativum L*, which has about 6 to 12 cloves covered within a white coat. Five garlic bulbs were peeled, sliced and ground using a blender machine. A Fresh aqueous homogenate of garlic (semisolid garlic fibre and its active ingredients) was made and used within 30 minutes of preparation. Five doses of different concentrations from the homogenate were separately weighed as follows; 26, 52, 104, 208 and 416mg/kg body weight of the animal, which were extrapolated using Paget and Barnes Conversion Table and orally delivered as the following volumes 0.5, 1.0, 2.1, 4.78 and 8.4ml of absolute AGE respectively. 2ml distilled water was used as a **vehicle** to ensure that the animals swallowed the whole content in solution (Appendix G).

3.2.2 Test system

30 male adult rabbits were randomly divided into 6 groups, i.e. control group, experimental group 1, 2, 3, 4 and 5. Each group contained 5 rabbits. The five freshly prepared doses of AGE (0.5, 1.0, 2.1, 4.78 and 8.4ml) were given orally to 5 groups, 1, 2, 3, 4, and 5 respectively. Animals in the control group were each given 2ml of distilled water as a placebo. Each morning between 8-9am, fresh AGE was prepared and the above process was repeated at a fixed time (9.00am), daily for 30 days.

Every 7th day, all the animals were bled through the marginal/lateral ear-vein. Using a 23-gauge needle, 1ml of blood from each animal was collected in mini-collecting Ethylene diamine tetra-acetic acid (k_3 EDTA) treated tubes and taken to Kenyatta National Hospital's Haematology and Blood Transfusion laboratory for analysis of Red Blood Cell count (RBC), Packed Cell Volume (PCV), Reticulocyte count (Retics) and Hemoglobin levels (Hb).

Phase 1. RBC, PCV and Hb level were determined by an automated haematological Cell-Counter, MS-4 series, from a full haemogram (Appendix H).

Phase 2. Reticulocyte count was done manually. Three laboratory assistants were provided and supervised by Dr. W. O. Mwanda, The Chairman, Department of Haematology and Blood Transfusion, KNH.

Phase 3. At the end, 2 animals from each group were randomly selected, non-reversibly sedated using diethyl ether and Ketamine (50mg/kg i.m.), that produced sedation and immobilization lasting about 30 minutes (National Research Council, 1996). Using the procedure in subsection 4.9, Bone Marrow (BM) aspirates were collected from the sternum, ulna, humerus, tibia and femur for histological analysis of the marrow's erythropoietic activity.

Throughout the experiment, changes in body weight, food and water intake patterns of rabbits in all groups were monitored so as to detect any behavioural changes, disease infection or parasitic infestation.

3.3 PILOT STUDY

Before setting out the main experiment, a pilot study was conducted for one month in the same department. Six adult male New Zealand White rabbits were used. They were randomly selected to form a control, group A, B, C, D and E. The procedure under subsection 4.3.1 and 4.3.2 was conducted.

The purpose for the pilot study was to assess the feasibility, challenges and other limitations expected before carrying out the main experiment. The results of the pilot study revealed that the study was feasible and the tools designed were appropriate, except for a few modifications. The researcher found that rabbits, as animal models for the study, were very sensitive and highly prone to infectious respiratory and digestive tract infections, hence high rate of mortality. They therefore required very close observation, care and high standards of hygiene.

The positive results were encouraging, and produced the determination to perform the rest of the study.

3.4 TECHNIQUE FOR CALCULATING DOSES

The doses of each drug tested in an experimental study should be chosen according to equivalent effective therapeutic doses given in a clinical study. The equivalent dose is not normally equal per kg body weight for different animal species, since they differ widely according to their surface areas (Paget and Barnes, 1964). These investigators stated that the dose of any drug cannot be transferred from an animal species to another, purely on the basis of relative weights. The drug's effects were best transferred between species on the basis of relative surface areas. They concluded that this approach should be used when extrapolating therapeutic or toxic doses of any drug from one species to another or to man, and vice versa, as illustrated in Table 4.5.

Table 3.1 Illustration of the surface area ratios of different animal species and of man (Paget and Barnes, 1964).

Animal species	200g. Rat	1.5kg. Rabbit	12kg. Dog	70kg. Man
200g. Rat	1.0	3.9	17.8	56.0
1.5kg Rabbit	0.25	1.0	4.5	14.2
12.0kg. Dog	0.06	0.22	1.0	3.1
70kg. Man	0.018	0.076	0.32	1.0

The absolute for a species in a column is determined by the product of the given dose, and the factor given at the intersection of relevant row and column. Thus an effect produced in a 70kg man by a dose of 2mg/kg; the absolute dosage to man is 140mg.

Extrapolated to a dog, -according to surface area – the effect might be expected at an absolute of 140 x 0.32 = 44.8mg for a dog of 12kg weight, i.e. 3.73mg/kg instead of 2mg/kg in human subject. For this reason, much bigger doses were administered to experimental animals than to human subjects in order to get the equivalent effect of each dose tried. Even in other species, the doses used to produce an equivalent effect were different.

3.5 DATA COLLECTION, ANALYSIS AND PRESENTATION

At the end of every 7 days, RBC, PCV, Hb and Reticulocyte analysis were done to check the effects of AGE and the progressive dose-response by the automated haematological cell-counter machine. Counts of RBC, PCV and Retics were compared within and between groups by a one-way ANOVA at 95% confidence interval. Control graphs for each of those parameters were used for determining their upper and lower limits. Line graphs were used to plot the results before analysis and interpretation.

Comparisons of Hb levels were done by looking at the percentage proportions in the various groups of rabbits. A post-hoc test (Turkey's HSD) pair-wise multiple comparisons

was used to analyze structural significance difference on the baseline between garlic fed and the controls. All values were expressed as Mean +/- SEM. P-value of less than 0.05 was considered statistically significant. Data was tabulated and graphs drawn to depict variations of the measured haematological parameters. All the data was analyzed statistically by computer using the Statistical Programme for Social Sciences (SPSS) version 11.5.

3.6 CHEMICALS

All chemicals that were used in this study were of Analytical grade obtained from Dialab Supplies Limited, Nairobi, distributors of Laboratory equipments, Medical supplies and Health Care Products. Solutions were prepared with glass-distilled water.

3.7 BLOOD FILM PREPARATION

A fresh drop of blood was put onto one end of a clean grease-free glass slide. Using a bevelled piece of glass a little narrower than the slide, the drop of blood was spread along it until the whole drop had been smeared. Care was taken not have too large a drop, or incline the spreader at too great an angle, as the film will be too thick for satisfactory microscopic examination.

The thickness and the even distribution of the cells play an important part in obtaining accurate results. The smear should be slightly thicker at the origin than at the tail end. An ideal area for microscopic examination was related towards the tail end of the smear.

3.7.1 Staining thin blood smears

Giemsa stain was used to stain the thin blood smears. This is an alcohol-based Romanowsky stain that requires dilution in pH 7.1-7.2 buffered water before use. It gives the best staining of thin films. It also stains thick films well provided they are completely dry

(overnight drying is recommended), as the concentration of stain is low and the staining time is sufficiently long. Preparation of Giemsa stain required:

-Giemsa powder..... (Appendix D).

-Glycerol (glycerine)..... (Appendix D).

-Methanol (methyl alcohol).

-Buffered water..... (Appendix C).

3.7.2 Giemsa staining technique for RBC morphology

Required: -Giemsa stain.

-Buffered water, pH 7.0-7.2.

Immediately before use, dilute the Giemsa stain as required:

3% solution for 30 minute staining.

-Measure 50ml of buffered water (pH 7.0-7.2).

10% solution for 10 minute staining.

-Measure 45ml of buffered water, pH 7.0-7.2 in a 50ml cylinder. Add 5ml of Giemsa Stain (to 50ml mark) and mix gently.

Thin blood films (smears) were prepared as described in subsection 4.7.1. The dried films were fixed by covering them with methanol (methyl alcohol) for 2-3 minutes. They were then allowed to air-dry at room temperature. The slides were placed *downwards*, in a staining jar and supported on each side. The diluted stain was poured in the staining jar and covered with a lid. *Note:* The inverted method of staining prevents stain particles from being deposited on the smear. The smears were left to stain for 30-45 minutes. The stain was washed off and the smears rinsed with buffered water. The backs of the slides were wiped and placed in a draining rack for the smears to air-dry at room temperature. The smears were microscopically examined, first with the 40X objective to see the distribution of material

(RBC`s) and to select a suitable portion of the smear to examine with the oil immersion objective lens. Results: RBC`s- mauve-blue, nucleus of WBC`s- dark purple, and cytoplasm of WBC`s- pale blue or greyish-blue.

3.7.3 New Methylene blue for reticulocyte staining

The New Methylene blue technique is a rapid method, which can be used to show the basic morphology of Reticulocytes in a blood smear. The New Methylene blue stain preparation is describe in Appendix A.

Procedure:

Using a plastic Pasteur pipette, fresh blood was pipetted from the EDTA treated tubes. Three drops of blood were put in small test tubes. With prior filtering, 3 drops of New Methylene blue stain were added to each test tube and mixed gently. The test tubes containing the blood and the stain were put in a water bath for 20-30 minutes at 35-37^{0}C (best stain is obtained when the tubes are kept at room temperature overnight). The tubes were gently shaken to resuspend the RBC`s. On the slides, thin smears of incubated media were made and allowed to air-dry at room temperature. The smears were microscopically examined, first with the 40X objective lens to see the distribution of the material (RBC`s and Retics), and then with the oil immersion objective 100X to make the reticulocyte count. Result: RBC`s were pale green-blue, and Retics- violet-blue stained granules of ribosomal RNA they contain. The count was done systematically (i.e. consecutive fields) in 1000 RBCs, noting the numbers that are Reticulocytes. The percentage of Reticulocytes was calculated as follows.

$$\% \text{ Retics} = \frac{\text{No. of Retics}}{\text{1000 RBCs}} \times 100\%$$

The Reticulocyte count was reported as the average of two counts performed from two separate preparations.

Reticulocyte Index (RI): When an RBC count is not available, it is recommended that the reticulocyte count be expressed as a Reticulocyte Index (RI).

$$RI = \frac{\text{Reticulocyte \%} \times \text{PCV}}{45}$$

3.8 BONE MARROW ASPIRATION

Two animals from each group were non-reversibly anaesthetized using diethyl ether (plus Ketamine, 50mg/kg i.m.) that produced sedation and immobilization lasting about 30 minutes. The skin of the area to be aspirated was cleaned with 70% alcohol (e.g. ethanol). A disposable bone marrow needle (Islam's bone marrow aspiration needle) was used. With a boring movement, the needle was pushed past the skin, subcutaneous tissue and periosteum overlying the selected site into the bone. When the bone had been penetrated, the stiletto was removed, a 2ml syringe was attached, and the marrow content was sucked up into the syringe (Waynforth, 1992). Only a small amount (less than 1ml) of the marrow content was aspirated. This procedure was done on the ulna, the sternum, tibia, femur and the humerus of the rabbits. Bone marrow clots faster than peripheral blood, so films were made from the aspirated material without delay.

3.8.1 Preparing films from bone marrow aspirates

Immediately after aspiration, using a plastic Pasteur pipette, a drop of aspirated material was put on one end of clean slides. At least 2 thinly spread smears of the aspirated material, 3-5cm in length, were made using a smooth-edged glass spreader of not more than 2cm width. Only a small quantity of aspirate was required and dilution with blood was

avoided. The smears were rapidly air-dried for 3-5 minutes at room temperature. Fixation was done by covering the films of bone marrow with few drops of absolute methanol (methyl alcohol). However, a large fixation time (20 minutes) is essential for rich quality staining. They were air-dried at room temperature and stained with Giemsa stain (subsection 4.7.2.1). After drying, the smears were microscopically examined under oil immersion. The 40X objective was used to scan the smear for an ideal area and the 100X objective to identify the cellular components

3.9 QUALITY CONTROL/ANALYSIS

These are processes done to ensure precision, reliability, consistency and accuracy of the results. One needle and syringe was used per animal during blood collection. Blood was collected in K_3-EDTA treated vacutainers to avoid clotting. Counts for RBC's, PCV and Hb levels were produced in duplicates and their means were used as accurate results.

Quality control of Reticulocyte counts included filtering the stain before use and checking the best staining time to use when a new batch of stain is made. Duplicate counts were always performed (using a different person for each count) and the two counts should agree within 20% range of each other. The same procedure was applied for use of Methylene blue for morphological examination of RBC's.

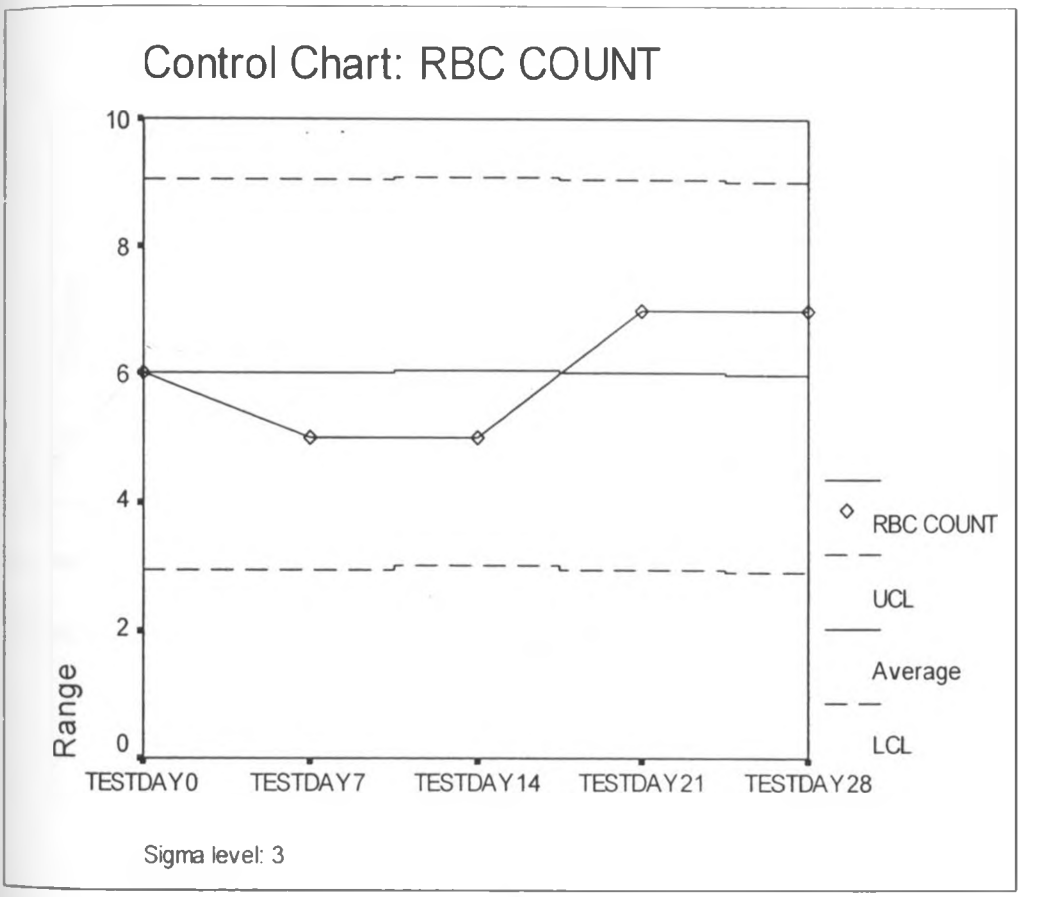
During morphological analysis of RBC's and Reticulocytes in both peripheral blood and bone marrow, duplicate slide smears were made for accurate microscopic examination via X40 objective lens for general distribution of cells, and oil emersion lens for distinctive morphological identification.

CHAPTER 4

4.0 RESULTS

Control charts were used to investigate whether the treatment processes as captured in the slides as shown concerning blood parameters were in statistical control over the four – week study period.

Fig. 4.1 Control Chart for RBC counts.



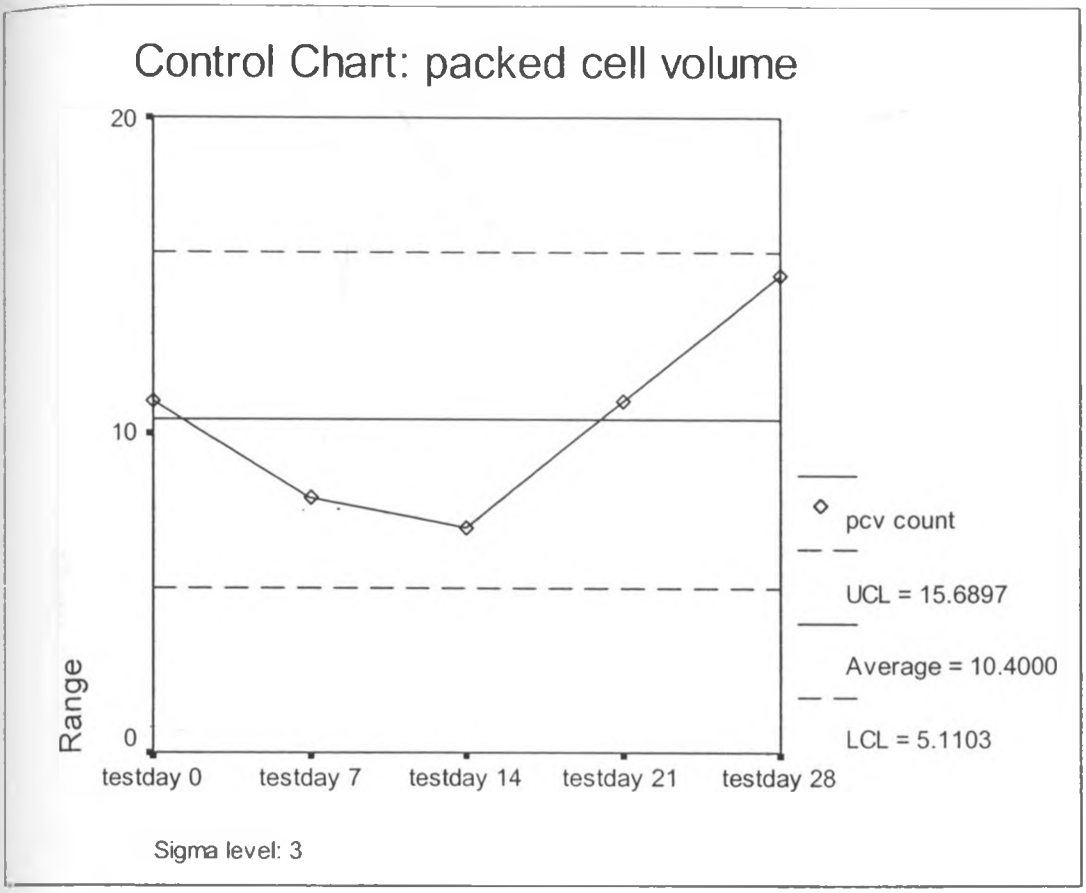
From the control chart above, it was clear that;

(i) Most points were near the average,

(ii) No points were beyond the control units.

This pattern therefore indicated a stable treatment process for the RBC over the four-week period.

Fig. 4.2 Control Chart for Packed Cell Volume

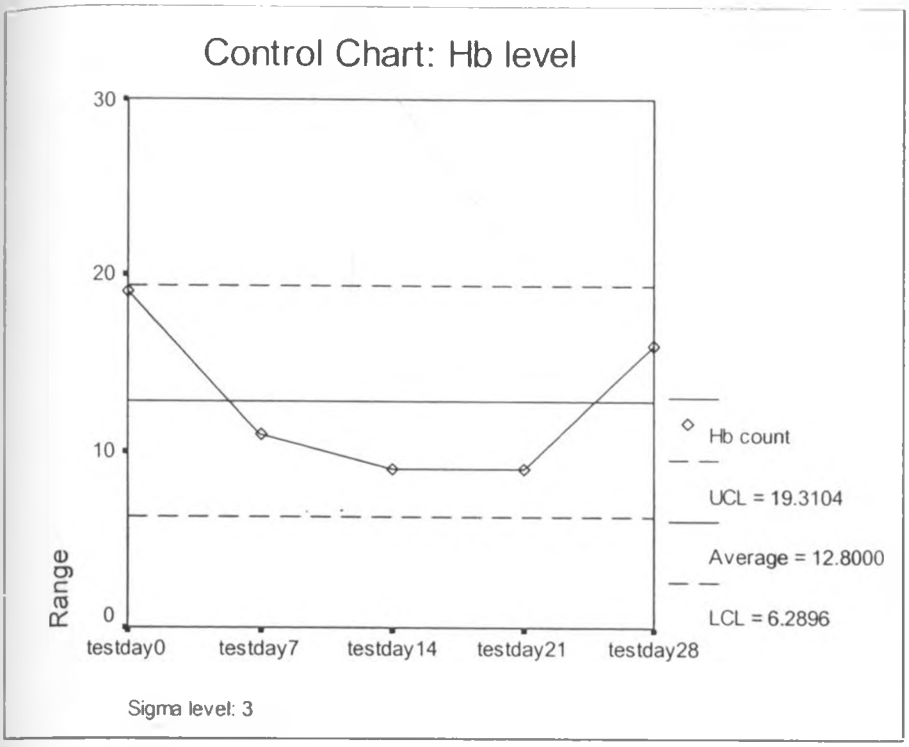


As was the case with the RBC, the control chart for packed cell volume also exhibited the characteristics of a process that was in control:

- Most points were near the average.
- Only one point was near the upper control limit.
- No points were beyond the lower control limit.

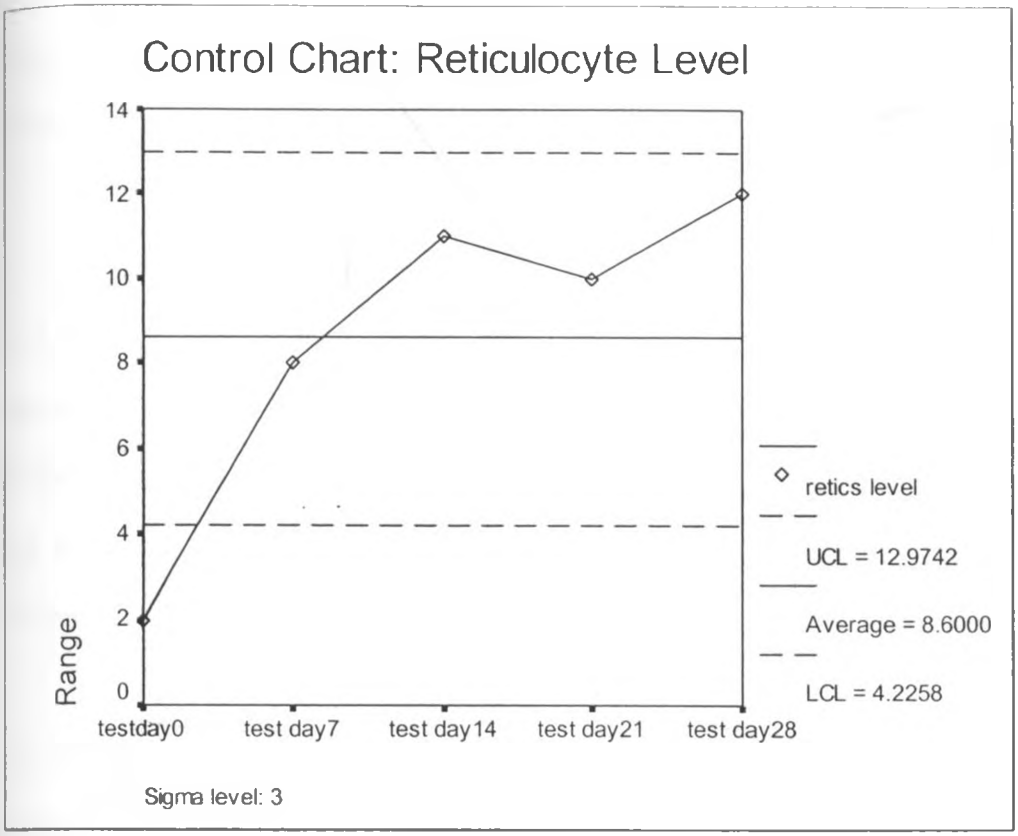
Thus the treatment process for packed cell volume was in statistical control over the four – weeks of the study.

Fig. 4.3 Control Chart for Hb Level



The treatment process for the Hb level was equally within statistical range over the four-week period as shown in Fig. 5.1c. It clearly showed that, most of the points were near the average; only one point was near the control limit; and no point was beyond the control limit.

Fig. 4.4 Control Chart for the Reticulocyte count.



The control chart for the Reticulocyte count seem to indicate the presence of a special cause such as acclimatization to the new environment for the test day 0 since the point was below the lower control limit. The process however attained statistical control over the next three-week study period.

4.1 Data Analysis and Results

In order to investigate the parameter dose response for the various groups, the respective parameter means were compared for the six experimental groups. Dose response curves were then drawn for each parameter.

In order to investigate the hypothesis that Aqueous Garlic Extract (AGE) does not have any effect on red blood cell formation, the researcher was keen on the effect AGE administration had on (i) RBC production, (ii) Packed cell volume, (iii) The Hb level and (iv) the rate of production of Reticulocyte.

The researcher therefore used a one-way analysis of variance (ANOVA) to test for the difference in the means of the respective parameters verses the different doses administered.

To investigate structural differences (real difference between the 6 groups and not just by chance) among the six groups with regard to the parameters, Turkey's (Honesty Significant Difference) pair-wise comparison and range test was used.

Table 4.1 The effect of Aqueous Garlic Extract on RBC Production

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CONTROL	25	5.1200	.83267	.16653	4.7763	5.4637	3.00	7.00
EXP.GROUP 1	25	5.1200	1.39403	.27881	4.5446	5.6954	1.00	7.00
EXP.GROUP 2	25	6.0800	1.63095	.32619	5.4068	6.7532	3.00	11.00
EXP.GROUP 3	25	6.3600	1.84572	.36914	5.5981	7.1219	1.00	9.00
EXP.GROUP 4	25	7.2400	1.09087	.21817	6.7897	7.6903	5.00	9.00
EXP.GROUP 5	25	7.3200	1.57374	.31475	6.6704	7.9696	5.00	11.00
Total	150	6.2067	1.66808	.13620	5.9375	6.4758	1.00	11.00

From the descriptive statistics shown in Table 5.2a, it is clear that the mean RBC count gradually increased from the control group to the experimental group 5. The control group and the experimental group 1 with the least dosage of AGE had the least RBC mean count of 5.1200 while the experimental group 5 with the highest AGE dosage had the largest RBC mean count of 7.3200. The dose response curve in Figure 5.2 further showed that the RBC count stepwise increased with increase in AGE dosage.

Fig. 4.5 Shows a dose-response curve for mean RBC count

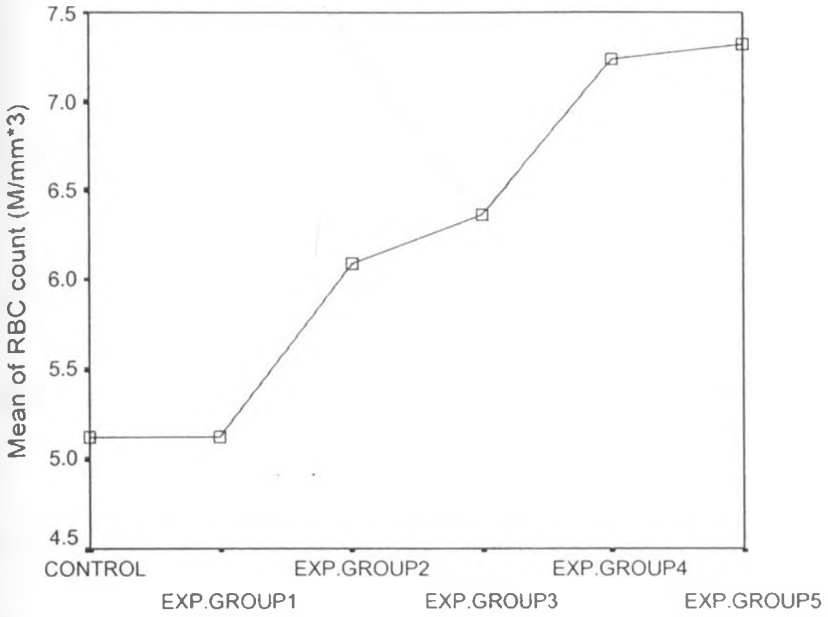


Figure 5.2: Dose response curve for mean RBC count

To further investigate whether the differences in the RBC mean counts between the groups were statistically significant, the following hypotheses were used:

H_0: $M_1 = M_2 = M_3 = M_4 = M_5 = M_6$ (There is no difference between the mean RBC counts between the groups).

H_1: $M_1 \neq M_2 \neq M_3 \neq M_4 \neq M_5 \neq M_6$ (The RBC mean difference between the groups differs significantly).

Table 4.2 Shows the ANOVA results on mean RBC differences between and within groups.

ANOVA

RBC COUNT

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	117.713	5	23.543	11.419	.000
Within Groups	296.880	144	2.062		
Total	414.593	149			

The ANOVA output in Table 5.2b indicated that the observed difference in the means is highly significant at the 0.01 level. $F(5) = 11.419$, $p = 0.000$. We rejected H_0 in favour of H_1, i.e. the RBC mean difference between the groups differed significantly, and was not by chance. The researcher concludes that dose of AGE does make a difference in RBC production. A highly significant increase in the RBC count occurred in the experimental groups compared to the control group.

Figure 5.2.1 further shows a decreasing rate of increase in the mean RBC counts within the 4-week study period. There was a steady sharp rise in the mean RBC count between days 0 and 7, then a slight rise to day 14, and a levelling off to almost a plateau at day 28. This was analyzed from rabbits in group 5.

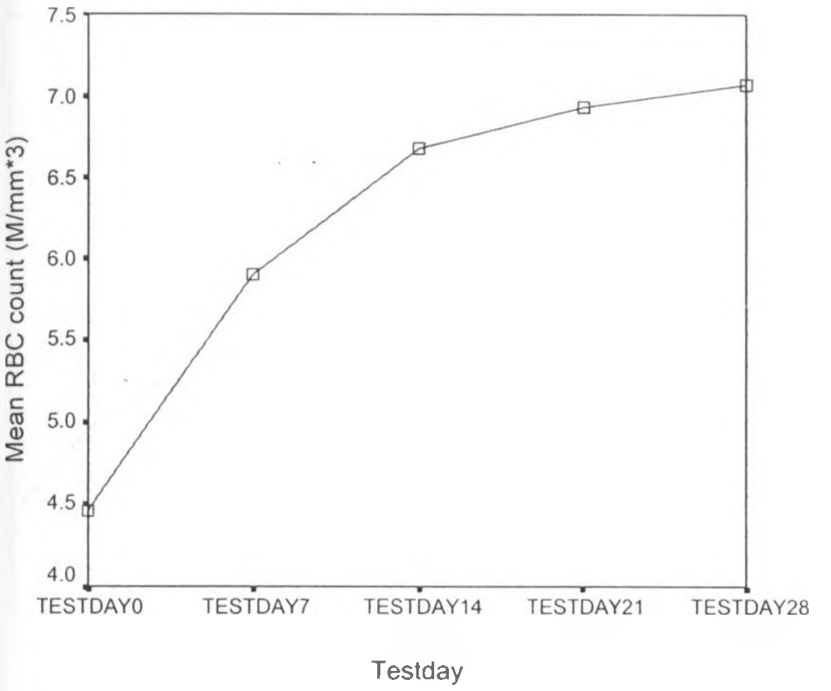


Fig. 4.6 Mean RBC counts between days.

TABLE 4.3 The effect of aqueous garlic extract on packed cell volume

PCV count

Descriptives

Group	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	25	7.4000	2.27303	.45461	6.4617	8.3383	3.00	12.00
exp Group1	25	8.2800	2.17025	.43405	7.3842	9.1758	4.00	13.00
exp Group2	25	8.9600	4.36348	.87270	7.1588	10.7612	1.00	22.00
exp Group3	25	8.8000	2.73861	.54772	7.6696	9.9304	3.00	14.00
exp Group4	25	9.5200	3.09731	.61946	8.2415	10.7985	4.00	15.00
exp Group5	25	10.9600	2.60576	.52115	9.8844	12.0356	6.00	16.00
Total	150	8.9867	3.11736	.25453	8.4837	9.4896	1.00	22.00

Means and standard deviations, as well as the 95% confidence interval of the mean, for the six groups are shown in Table 5.3a. From the table, the control group without any dose of AGE had the least mean volume of 7.4000, while the experimental group 5 receiving 8.4ml of AGE had the highest mean volume of 10.9600. The table also shows that there is a 95% chance that the fine control mean volume is contained in the interval 6.4617 to 8.3383, where as the time mean value for experimental group 5 is contained within the internal 9.8844 to 12.0356.

Fig. 4.7 Shows a dose-response curve for PCV between the groups.

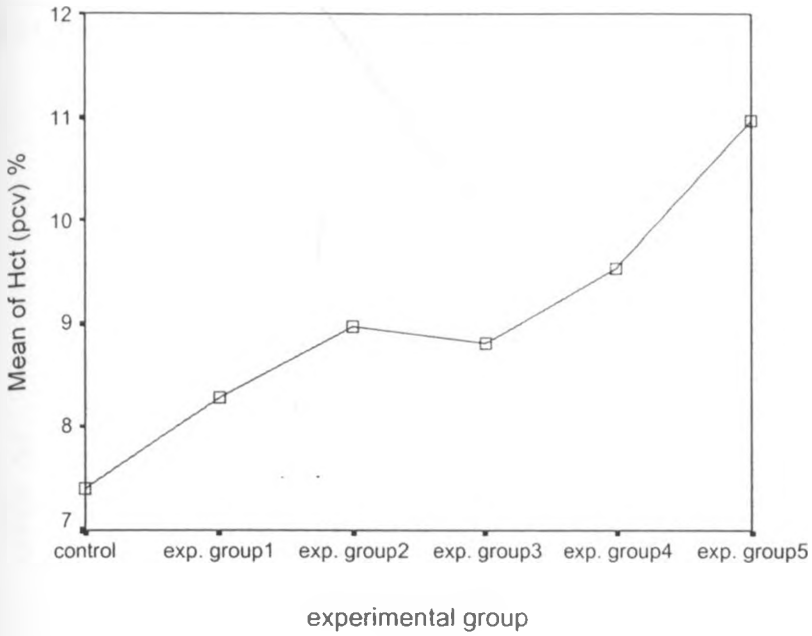


Figure 5.3: Dose response curve for PCV

The dose response curve in Figure 5.3a further indicated an almost linear relation between the control group and experimental group 2. A slight drop in volume was observed between experimental group 2 and group 3, followed with a steady increase in volume observed between experimental group 3 and group 5.

To investigate whether the observed mean differences in the packed cell volume were statistically significant, the following hypotheses were used.

H_0: $M_1 = M_2 = M_3 = M_4 = M_5 = M_6$ (There is no difference between the mean packed cell volume between the groups).

H_1: $M_1 \neq M_2 \neq M_3 \neq M_4 \neq M_5 \neq M_6$ (The mean packed cell volume differs significantly between the groups).

Figure 4.8 below, further showed that the mean PCV increased over the 4-week study period as measured in rabbits of group 5. There was a steady increase in PCV between day 0 and 14, then a gradual fall in the magnitude of increase between day 14 and 28.

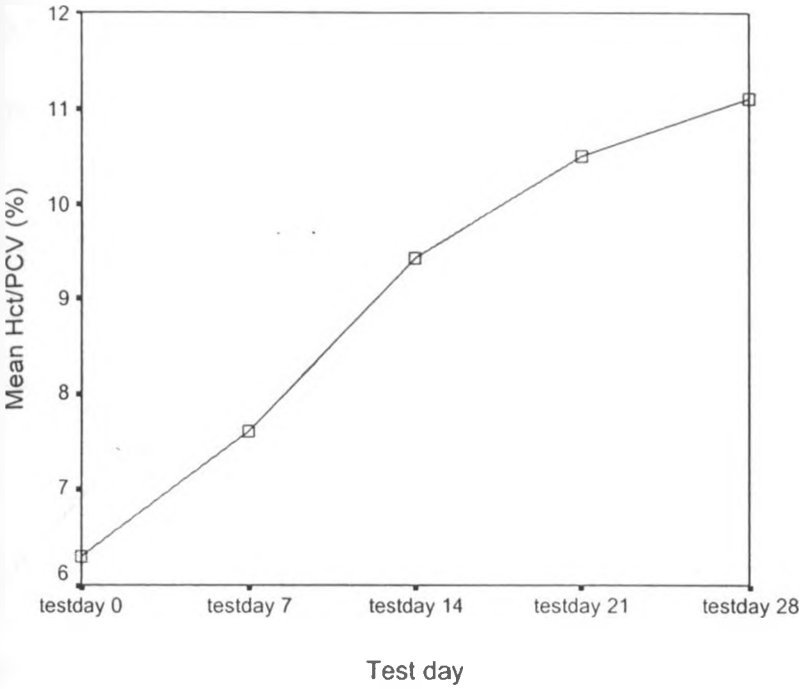


Fig. 4.8 Mean PCV between days.

Table 4.4 ANOVA results on mean PCV between and within groups.

ANOVA

PCV count

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	180.773	5	36.155	4.108	.002
Within Groups	1267.200	144	8.800		
Total	1447.973	149			

The ANOVA output in Table 4.4 above indicated that the observed difference in the mean packed cell volume was highly significant at the 0.01 level. $F(1,5) = 4.108$, $p = 0.002$. We therefore rejected H_0 in favour of H_1. The researcher concluded that the dose of AGE does make a difference in the packed cell volume. A highly statistically significant increase in the PCV occurred between and within the experimental groups as compared to the control group.

Table 4.5 The effect of Aqueous Garlic Extract on the Hb Level

Descriptives

Hb count

Group	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	25	7.2800	2.49199	.49840	6.2514	8.3086	3.00	13.00
Experimental group1	25	7.7600	2.38537	.47707	6.7754	8.7446	2.00	11.00
Experimental group2	25	8.2400	3.96106	.79221	6.6050	9.8750	1.00	21.00
Experimental group3	25	9.2000	3.53553	.70711	7.7406	10.6594	1.00	17.00
Experimental group4	25	10.8400	3.38723	.67745	9.4418	12.2382	3.00	20.00
Experimental group6	25	11.0000	2.98608	.59722	9.7674	12.2326	6.00	17.00
Total	150	9.0533	3.44036	.28090	8.4983	9.6084	1.00	21.00

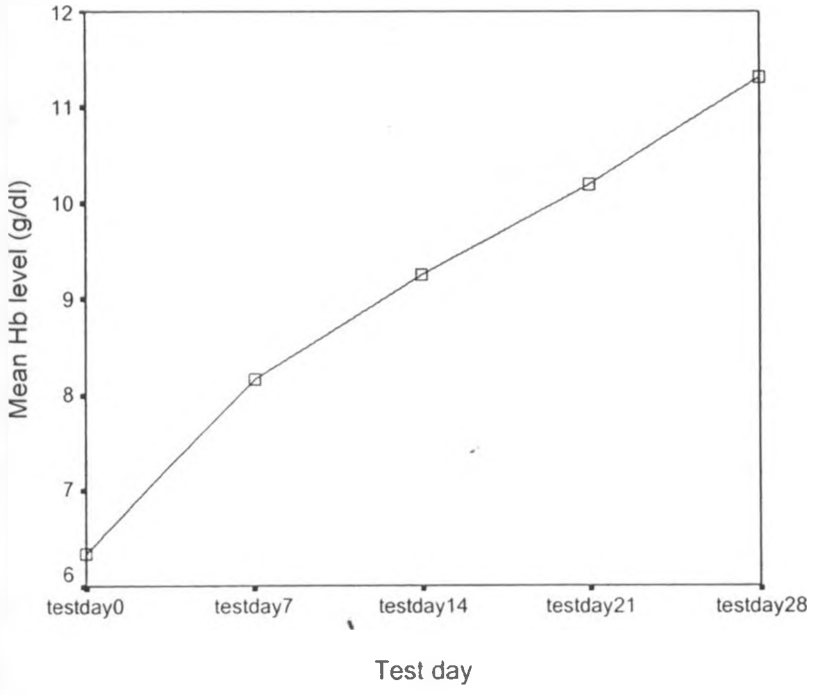


Fig. 4.9 Mean Hb levels between days.

Fig. 4.10 Shows a dose-response curve on Hb level between groups

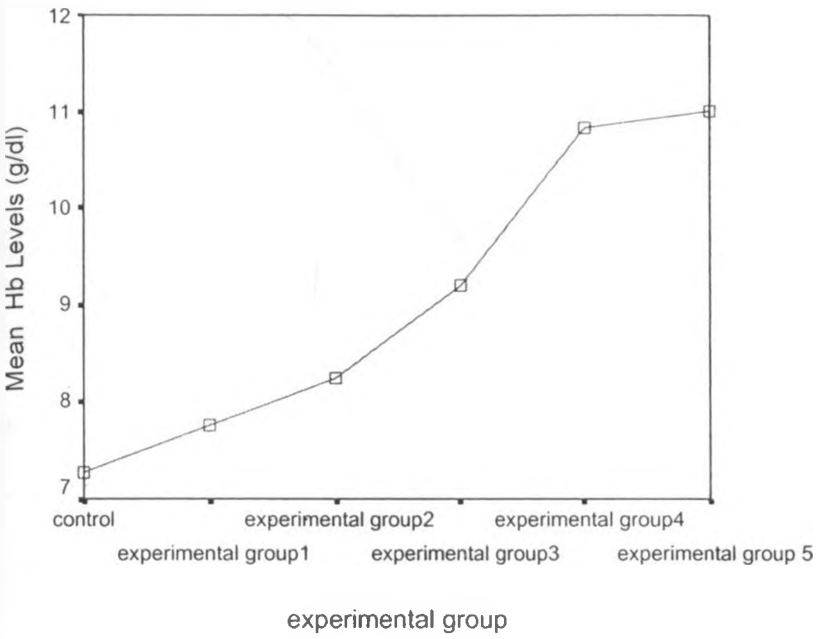


Figure 5.4: Dose response curve for Hb Levels

The means, standard deviations and the 95% confidence intervals of the mean, reflected an increase in Hb levels for all the treatment groups (Table 4.5). The dose response curve in Figure 4.10 further shows an exponential increase in Hb level between the control group and experimental group 4, and a very minimal linear increase between experimental group 4 and group 5. Results of the ANOVA for Hb level as seen in Table 4.6 revealed a highly statistically significant effect for dosage, $F(1, 5) = 6.192$, $p = 0.000$. The researcher therefore concluded that dosage of AGE made a difference in the Hb level. A highly significant increase in the Hb level occurred in the experimental groups compared to the control group.

Figure 4.9 further shows the relationship between Hb levels and the study period taken from rabbits in group 5. There was a sharp increase in Hb level between days 0 and 7, leading to an almost linear increase to day 28.

Table 4.6 ANOVA results for the mean Hb levels between and within groups

ANOVA

Hb count

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	312.053	5	62.411	6.192	.000
Within Groups	1451.520	144	10.080		
Total	1763.573	149			

Fig. 4.11 Shows the dose-response curve on the rate of production of Reticulocytes.

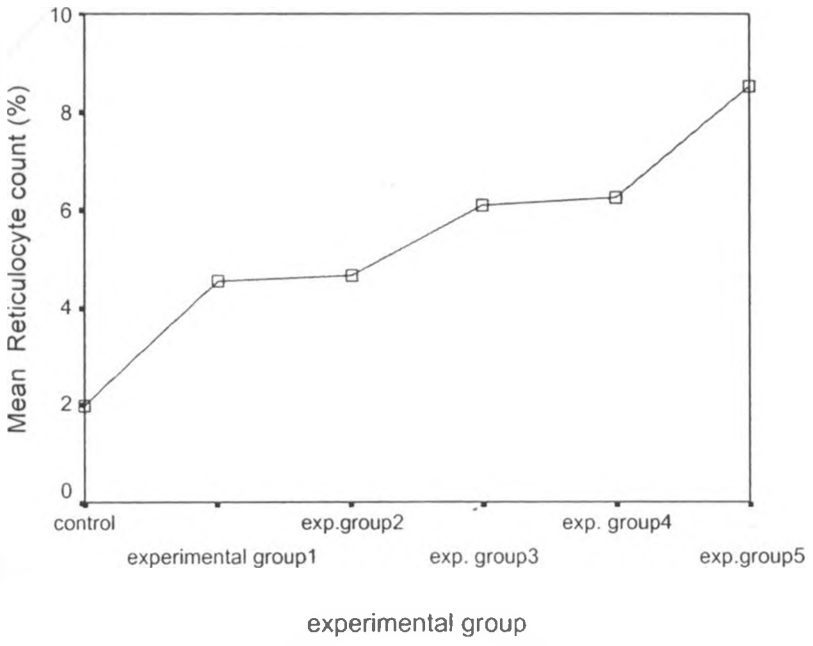


Figure 5.5: Dose response curve for Reticulocyte production

Table 4.7 Shows the effect of AGE on the rate of production of Reticulocytes.

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval of Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	25	2.0000	.57735	.11547	1.7617	2.2383	1.00	3.00
Exp. Group1	25	4.5600	1.87261	.37452	3.7870	5.3330	1.00	8.00
Exp. Group2	25	4.6800	2.03552	.40710	3.8398	5.5202	1.00	9.00
Exp. Group3	25	6.1200	2.31517	.46303	5.1643	7.0757	1.00	9.00
Exp. Group4	25	6.2800	2.99333	.59867	5.0444	7.5156	2.00	14.00
Exp. Group5	25	8.5200	3.77624	.75525	6.9612	10.0788	1.00	13.00
Total	150	5.3600	3.14372	.25668	4.8528	5.8672	1.00	14.00

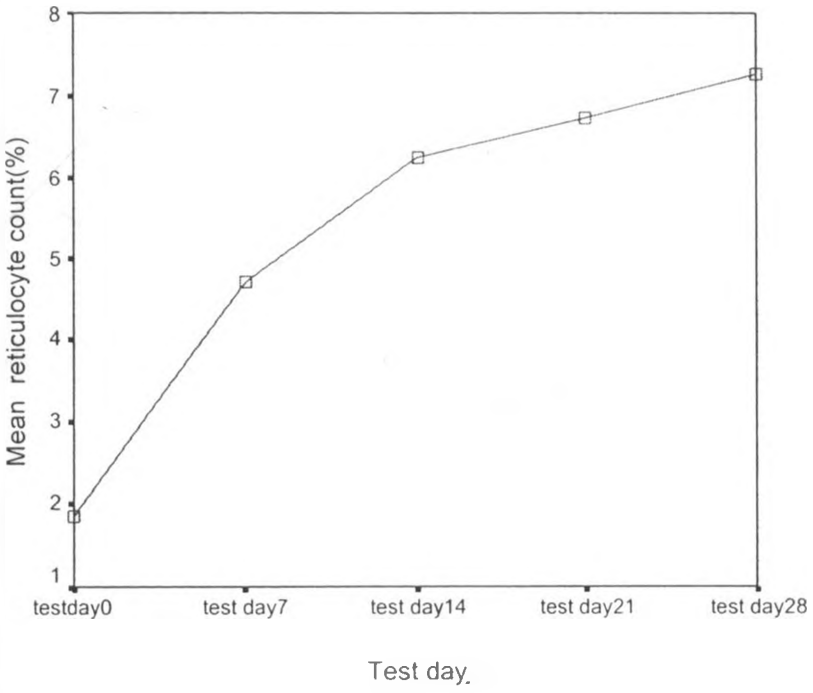


Fig. 4.12 Mean Reticulocyte counts between days.

Figure 4.12 above showed a similar trend as that of mean RBC (Fig 4.6), with a gradual reduction in the rate of increase between days 14 and 28. The dose response curve in Figure 4.11 indicated an overall stepwise increase in the rate of production of reticulocyte between the control group and the experimental groups. The descriptive statistics in Table

4.7 further indicate that the mean rate of reticulocyte production was 2.00 for the control group. This rose to 4.56 for experimental group 1, 4.68 for group 2, 6.12 for group 3, 6.28 for group 4 and finally to a high of 8.52 for group 5.

To further examine the particular effect AGE had on the rate of production of reticulocyte, the following hypotheses were used.

H_0: $M_1 = M_2 = M_3 = M_4 = M_5 = M_6$ (Aqueous Garlic Extract has no effect on the mean rate of production of Reticulocytes).

H_1: $M_1 \neq M_2 \neq M_3 \neq M_4 \neq M_5 \neq M_6$ (Aqueous Garlic Extract increases the mean rate of production of Reticulocytes).

Table 4.8 Shows ANOVA results of the mean rate of Reticulocyte production between and within groups.

ANOVA

Retics level

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	595.040	5	119.008	19.529	.000
Within Groups	877.520	144	6.094		
Total	1472.560	149			

From the ANOVA output in Table 4.8 above, $F(1,5) = 19.529$, $p = 0.000$. The mean difference was highly significant at the 0.01 level. The researcher therefore rejected H_0 in

favour of H_1, and concluded that there was a highly significant increase in the mean rate of production of reticulocytes in the experimental groups, compared to the control group. Thus, an increase in the AGE dose led to an increase in the mean rate of production of reticulocyte.

The F test conducted showed that the doses of AGE were significantly related to the increase of blood parameters tested, proved by differences in the mean and variance of the six groups. However, the test did not tell us about the magnitude of the differences among the six groups. This was revealed by Turkey's (Honestly Significant Difference) pair wise multiple comparisons for each tested blood parameter, for all the 6 groups.

Table 4.9 Structural differences among the groups with regard to the RBC count.

Multiple Comparisons

Dependent Variable: RBC COUNT
Turkey's HSD

(I) EXPERIMENTAL GROUP	(J) EXPERIMENTAL GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CONTROL	EXP.GROUP1	.0000	.40612	1.000	-1.1731	1.1731
	EXP.GROUP2	-.9600	.40612	.176	-2.1331	.2131
	EXP.GROUP3	-1.2400(*)	.40612	.032	-2.4131	-.0669
	EXP.GROUP4	-2.1200(*)	.40612	.000	-3.2931	-.9469
	EXP.GROUP5	-2.2000(*)	.40612	.000	-3.3731	-1.0269
EXP.GROUP1	CONTROL	.0000	.40612	1.000	-1.1731	1.1731
	EXP.GROUP2	-.9600	.40612	.176	-2.1331	.2131
	EXP.GROUP3	-1.2400(*)	.40612	.032	-2.4131	-.0669
	EXP.GROUP4	-2.1200(*)	.40612	.000	-3.2931	-.9469
	EXP.GROUP5	-2.2000(*)	.40612	.000	-3.3731	-1.0269
EXP.GROUP2	CONTROL	.9600	.40612	.176	-.2131	2.1331
	EXP.GROUP1	.9600	.40612	.176	-.2131	2.1331
	EXP.GROUP3	-.2800	.40612	.983	-1.4531	.8931
	EXP.GROUP4	-1.1600	.40612	.054	-2.3331	.0131
	EXP.GROUP5	-1.2400(*)	.40612	.032	-2.4131	-.0669
EXP.GROUP3	CONTROL	1.2400(*)	.40612	.032	.0669	2.4131
	EXP.GROUP1	1.2400(*)	.40612	.032	.0669	2.4131
	EXP.GROUP2	.2800	.40612	.983	-.8931	1.4531
	EXP.GROUP4	-.8800	.40612	.260	-2.0531	.2931
	EXP.GROUP5	-.9600	.40612	.176	-2.1331	.2131
EXP.GROUP4	CONTROL	2.1200(*)	.40612	.000	.9469	3.2931
	EXP.GROUP1	2.1200(*)	.40612	.000	.9469	3.2931
	EXP.GROUP2	1.1600	.40612	.054	-.0131	2.3331
	EXP.GROUP3	.8800	.40612	.260	-.2931	2.0531
	EXP.GROUP5	-.0800	.40612	1.000	-1.2531	1.0931
EXP.GROUP5	CONTROL	2.2000(*)	.40612	.000	1.0269	3.3731
	EXP.GROUP1	2.2000(*)	.40612	.000	1.0269	3.3731
	EXP.GROUP2	1.2400(*)	.40612	.032	.0669	2.4131
	EXP.GROUP3	.9600	.40612	.176	-.2131	2.1331
	EXP.GROUP4	.0800	.40612	1.000	-1.0931	1.2531

* The mean difference is significant at the 0.05 level.

From the Turkey's HSD Table 4.9 above, some of the mean differences have asterisks, indicating that the differences were statistically significant at the 0.05 level. Thus -1.2400 indicated that the mean difference between the control group and experimental group 3 is -1.2400 and was significant at the 0.05 level with the flip coefficient, 1.2400 appearing later for the difference of experimental group 2 from the control group. Looking down the list, it was clear that most of the significant differences in the RBC count concern the fact that the AGE dosage was higher in experimental groups 3, 4 & 5. Though the ANOVA test showed that RBC count was related to AGE dosage, this was not consistent for every experimental group.

Table 4.10 Structural differences among the groups with regard to the packed cells volume (PCV)

Multiple Comparisons

experimental oup	(J) experimental group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
ontrol	exp. group1	-.8800	.83905	.900	-3.3036	1.5436
	exp. group2	-1.5600	.83905	.432	-3.9836	.8636
	exp. group3	-1.4000	.83905	.555	-3.8236	1.0236
	exp. group4	-2.1200	.83905	.123	-4.5436	.3036
	exp. group5	-3.5600(*)	.83905	.001	-5.9836	-1.1364
xp. group1	Control	.8800	.83905	.900	-1.5436	3.3036
	exp. group2	-.6800	.83905	.965	-3.1036	1.7436
	exp. group3	-.5200	.83905	.989	-2.9436	1.9036
	exp. group4	-1.2400	.83905	.679	-3.6636	1.1836
	exp. group5	-2.6800(*)	.83905	.021	-5.1036	-.2564
xp. group2	Control	1.5600	.83905	.432	-.8636	3.9836
	exp. group1	.6800	.83905	.965	-1.7436	3.1036
	exp. group3	.1600	.83905	1.000	-2.2636	2.5836
	exp. group4	-.5600	.83905	.985	-2.9836	1.8636
	exp. group5	-2.0000	.83905	.169	-4.4236	.4236
xp. group3	Control	1.4000	.83905	.555	-1.0236	3.8236
	exp. group1	.5200	.83905	.989	-1.9036	2.9436
	exp. group2	-.1600	.83905	1.000	-2.5836	2.2636
	exp. group4	-.7200	.83905	.956	-3.1436	1.7036
	exp. group5	-2.1600	.83905	.110	-4.5836	.2636
xp. group4	Control	2.1200	.83905	.123	-.3036	4.5436
	exp. group1	1.2400	.83905	.679	-1.1836	3.6636
	exp. group2	.5600	.83905	.985	-1.8636	2.9836
	exp. group3	.7200	.83905	.956	-1.7036	3.1436
	exp. group5	-1.4400	.83905	.523	-3.8636	.9836
xp. group5	Control	3.5600(*)	.83905	.001	1.1364	5.9836
	exp. group1	2.6800(*)	.83905	.021	.2564	5.1036
	exp. group2	2.0000	.83905	.169	-.4236	4.4236
	exp. group3	2.1600	.83905	.110	-.2636	4.5836
	exp. group4	1.4400	.83905	.523	-.9836	3.8636

* The mean difference is significant at the 0.05 level.

The Turkey's HSD Table 4.10 indicated that the differences in the mean packed cell volume were not very significant. Thus the only significant mean differences concern the coefficient -3.5600 showing that the mean par was significantly different between the control

group and experimental group 5. -2.6800 indicated that the mean PCV was significantly different between the experimental group 1 and group 5. Thus though the ANOVA F test concluded that the packed cell volume was related to the variation in AGE dose, but the mean difference was not very significant among the groups.

Table 4.11 Structural differences among the groups with regard to the Hb level.

Multiple Comparisons

Dependent Variable: Hb count

(I) Experimental group	(J) Experimental group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Experimental group1	-.4800	.89800	.995	-3.0738	2.1138
	Experimental group2	-.9600	.89800	.893	-3.5538	1.6338
	Experimental group3	-1.9200	.89800	.274	-4.5138	.6738
	Experimental group4	-3.5600(*)	.89800	.002	-6.1538	-.9662
	Experimental group5	-3.7200(*)	.89800	.001	-6.3138	-1.1262
Experimental group1	Control	.4800	.89800	.995	-2.1138	3.0738
	Experimental group2	-.4800	.89800	.995	-3.0738	2.1138
	Experimental group3	-1.4400	.89800	.598	-4.0338	1.1538
	Experimental group4	-3.0800(*)	.89800	.010	-5.6738	-.4862
	Experimental group5	-3.2400(*)	.89800	.006	-5.8338	-.6462
Experimental group2	Control	.9600	.89800	.893	-1.6338	3.5538
	Experimental group1	.4800	.89800	.995	-2.1138	3.0738
	Experimental group3	-.9600	.89800	.893	-3.5538	1.6338
	Experimental group4	-2.6000(*)	.89800	.049	-5.1938	-.0062
	Experimental group5	-2.7600(*)	.89800	.030	-5.3538	-.1662
Experimental group3	Control	1.9200	.89800	.274	-.6738	4.5138
	Experimental group1	1.4400	.89800	.598	-1.1538	4.0338
	Experimental group2	.9600	.89800	.893	-1.6338	3.5538
	Experimental group4	-1.6400	.89800	.452	-4.2338	.9538
	Experimental group5	-1.8000	.89800	.345	-4.3938	.7938
Experimental group4	Control	3.5600(*)	.89800	.002	.9662	6.1538
	Experimental group1	3.0800(*)	.89800	.010	.4862	5.6738
	Experimental group2	2.6000(*)	.89800	.049	.0062	5.1938
	Experimental group3	1.6400	.89800	.452	-.9538	4.2338
	Experimental group5	-.1600	.89800	1.000	-2.7538	2.4338
Experimental group5	Control	3.7200(*)	.89800	.001	1.1262	6.3138
	Experimental group1	3.2400(*)	.89800	.006	.6462	5.8338
	Experimental group2	2.7600(*)	.89800	.030	.1662	5.3538
	Experimental group3	1.8000	.89800	.345	-.7938	4.3938
	Experimental group4	.1600	.89800	1.000	-2.4338	2.7538

Turkey's HSD * The mean difference is significant at the 0.05 level.

From the Turkey's HSD Table 4.11, it was clear that a high dose of AGE, led to inter group change in the mean difference in Hb level. Thus the mean differences between experimental groups 4 and 5 and the control group, experimental group 1 and experimental group 2 were significant at the 0.05 level.

Table 4.12 Structural differences among the groups in relation to production of Reticulocyte.

Multiple Comparisons

(I) Experimental group	(J) Experimental group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Experimental group1	-2.5600(*)	.69822	.005	-4.5768	-.5432
	Exp. Group2	-2.6800(*)	.69822	.003	-4.6968	-.6632
	Exp. Group3	-4.1200(*)	.69822	.000	-6.1368	-2.1032
	Exp. Group4	-4.2800(*)	.69822	.000	-6.2968	-2.2632
	Exp. Group5	-6.5200(*)	.69822	.000	-8.5368	-4.5032
Experimental group1	Control	2.5600(*)	.69822	.005	.5432	4.5768
	Exp.group2	-.1200	.69822	1.000	-2.1368	1.8968
	Exp. Group3	-1.5600	.69822	.229	-3.5768	.4568
	Exp. Group4	-1.7200	.69822	.142	-3.7368	.2968
	Exp. Group5	-3.9600(*)	.69822	.000	-5.9768	-1.9432
Exp.group2	Control	2.6800(*)	.69822	.003	.6632	4.6968
	Experimental group1	.1200	.69822	1.000	-1.8968	2.1368
	Exp. Group3	-1.4400	.69822	.313	-3.4568	.5768
	Exp. Group4	-1.6000	.69822	.204	-3.6168	.4168
	Exp. Group5	-3.8400(*)	.69822	.000	-5.8568	-1.8232
Exp. Group3	Control	4.1200(*)	.69822	.000	2.1032	6.1368
	Experimental group1	1.5600	.69822	.229	-.4568	3.5768
	Exp. Group2	1.4400	.69822	.313	-.5768	3.4568
	Exp. Group4	-.1600	.69822	1.000	-2.1768	1.8568
	Exp. Group5	-2.4000(*)	.69822	.010	-4.4168	-.3832
Exp. Group4	Control	4.2800(*)	.69822	.000	2.2632	6.2968
	Experimental group1	1.7200	.69822	.142	-.2968	3.7368
	Exp. Group2	1.6000	.69822	.204	-.4168	3.6168
	Exp. Group3	.1600	.69822	1.000	-1.8568	2.1768
	Exp. Group5	-2.2400(*)	.69822	.020	-4.2568	-.2232
Exp.group5	Control	6.5200(*)	.69822	.000	4.5032	8.5368
	Experimental group1	3.9600(*)	.69822	.000	1.9432	5.9768
	Exp. Group2	3.8400(*)	.69822	.000	1.8232	5.8568
	Exp. Group3	2.4000(*)	.69822	.010	.3832	4.4168
	Exp. Group4	2.2400(*)	.69822	.020	.2232	4.2568

* The mean difference is significant at the 0.05 level.

Table 4.12 indicated that a change in the AGE dose resulted in a positive change in the mean differences in Reticulocyte production among the groups. Thus the mean difference in Reticulocyte production was statistically significant across the experimental groups 1, 2, 3, 4, 5, compared to the control group. Similarly, it was also observed that the mean

Reticulocyte production differed significantly between one experimental group and the next. Thus though, the ANOVA F test indicated that Reticulocyte production was related to a change in AGE dosage, the difference in the mean Reticulocyte production was however not the same for every experimental group.

CHAPTER 5

5.0 DISCUSSION

5.1 PERIPHERAL BLOOD ANALYSIS

Although garlic bulb has been used to conduct research in various scientific fields, it still remains an interesting herb to study. The present study investigated the effects of freshly prepared Aqueous Garlic Extract (AGE) on the process of Erythropoiesis in rabbits. From the results, the study showed that daily feeding of different doses of AGE significantly increased the Red blood cell count (RBC), Haemoglobin levels (Hb), Packed cell volume (PCV/hematocrit) and the Reticulocyte count in the rabbit's peripheral blood. However, a contrasting report was given by Oluwole (2001), when he reported a reduction in hematocrit from his own study. Takasu et al (2002) also showed that aged garlic extract had no significant effect on RBC, Hb and PCV in his study on the effect of garlic on sickle Red blood cells.

The rabbits were subjected to the same environmental condition. No significant changes were observed in animals in the control group. The blood parameters under investigation for the animals in the control group were maintained within physiological ranges as indicated by the control charts. The charts classify products or services as either acceptable or unacceptable based on normal scales of measurement. The relationship allows limits to be set up around the sample means to show how much variation can be expected for a given sample size between the upper and the lower control limit.

5.1.1 RBC Count

Figure 4.5 showed that the mean RBC counts for the control group rose slightly because of the animals' physiological demands and adaptation, but this was within the normal physiological ranges. On the contrary, much significant change was observed between and

within experimental groups after the administration of AGE during the study period. This study was consistent with previous results observed by Oluwole (2001), for which he showed that garlic extract had significant effect on haematological and biochemical parameters in mice. Other studies have previously showed that low doses of aged garlic extract only cause minimal effect on the mean number of erythrocytes in the peripheral blood. The present study utilized 5 distinct freshly prepared absolute doses of AGE. The control group and experimental group 1 with the least dosage of AGE (0.5ml) had the least mean RBC count, as compared to the rest of the experimental groups. Experimental group 5 had the largest mean RBC count after these rabbits were fed on the highest dose of AGE as shown by the dose-response curve for mean RBC count (Fig. 4.5). An increase in the mean RBC count with increasing dosage was observed within the groups.

Figure 4.6 further showed a steady increase of the mean RBC count, followed by a plateau to the last day of the study in group 5 animals. Similar observations were made earlier by Senevirante (1999) using similar animal models. The plateau probably arose from the fact that, as the stimulatory effect of AGE acts on the erythroid precursor cells in the bone marrow to produce more RBCs, the body's physiological response acts back on the process of Erythropoiesis to maintain the required number of RBCs in the peripheral blood within certain ranges of normality, as required by the body. From the graph, that point seemed to have been attained between days 21 and 28. From the Turkey's HSD table 4.9, some of the mean differences for RBC have asterisks, indicating that there were significant structural differences between the groups at $p<0.05$. This categorically showed that significant differences in the mean RBC count concerns the fact that the production of erythrocytes has significant relationship with the dosage of AGE administered. This indicated that at least in male rabbits, there was an increase in the number of erythrocytes circulating in the peripheral blood after multiple oral administrations of the various doses of AGE.

5.1.2 Packed Cell Volume

The second blood parameter under investigation was Packed cell volume (PCV/Hematocrit). The study results showed a slight change in the mean PCV, though not statistically significant. With reference to Figure 4.7, at some point, there was a drop in volume in experimental groups 2 and 3, while groups 4 and 5, receiving larger doses had higher means of PCV. The Turkey's HSD table 4.10 indicated not much significant difference in the mean PCV, because not much change was observed between the control and experimental groups 1, 2, 3, and 4. However, there was significant difference between the control group and experimental group 5. Figure 4.8 illustrates the behaviour of mean PCV as depicted within the study period. There was generally a slight increase in the mean PCV between weeks, with a steady increase between days 0 and 14, followed by a gradual fall in magnitude of increase between days 21 and 28.

Comparisons done between and within groups (Table 4.4), showed marked difference in the mean PCV which was statistically significant at 0.01 level, with $F(1, 5) = 4.108$, $p = 0.002$. H_0 was therefore rejected in favour of H_1 This was contrary to the study conducted by Takasu et al (2002) on aged garlic extract therapy on sickle cell anaemia, where garlic had no effect completely, on the packed cell volume.

5.1.3 Haemoglobin Levels

Haemoglobin levels (Hb) were also observed. It is interesting to note that data reflects a mean increase in Hb levels for all the treatment groups, despite the insignificant changes in the PCV. There was an exponential increase in Hb levels between the control and the first 4 experimental groups, followed by a minimal linear increase in group 5 (Fig. 4.10). From the Turkey's HSD table 4.11, it was evident that high dose of AGE led to inter-group mean differences in Hb levels. The behaviour of Hb levels in the peripheral blood as observed in

Figure 4.9, produced almost a linear growth curve, with a slight change noted a week after starting the project. The highest mean Hb level was observed on day 28. This was not in line with Takasu et al (2002) study on aged garlic extract therapy on sickle cell, in which he showed that aged garlic extract had no significant effect on RBC, Hb, Hematocrit and Reticulocytes, in addition to other biochemical parameters.

5.1.4 Reticulocytes

The rate of production of Reticulocytes was also analysed. The dose-response curve in figure 4.11 confirmed an overall increase in the rate of production of Reticulocytes between the control and the experimental groups. Like the mean RBC dose-response curve, the Reticulocyte curve also showed a reduction in the rate of increase from day 0 to day 28. The ANOVA results concluded a highly statistically significant increase in the mean rate of Reticulocyte production in the experimental groups compared to the control. The Turkey's HSD table 4.12 indicated that a change in the AGE dose resulted in the change of the mean differences in Reticulocyte production among the groups.

5.2 ERYTHROCYTE MORPHOLOGY

5.2.1 RBC Size and Shape

Reticulocytes are juvenile red cells; they contain remnants of the ribosomal ribonucleic acid, which was present in large amounts in the cytoplasm of the nucleated precursors from which they were derived. Ribosomes have the property of reacting with certain basic dyes such as azure B, brilliant cresyl blue or New Methylene blue to form a blue or purplish precipitate of granules or filaments. Hence, in this study, Reticulocyte count was a very major part in determining the effect of AGE on the process of red cell formation. The reaction above takes place only in vitally stained unfixed preparations. Stages of maturation

were identified by morphological features (Plates 3-6). The most immature Reticulocytes had the largest amount of precipitable material. In the least immature, only a few dots or short strands of ribonucleic granulophilic materials were seen. Reticulocytes were classified into four groups ranging from the most immature Reticulocytes, with large clumps of Reticulin (Proerythrocytes, Basophilic erythrocytes, Polychromatic erythrocyte and Erythroid cells - Plate 4); to the most mature, with a few granules of Reticulin (Reticulum cells -Plate 5).

Complete loss of basophilic material probably occurs in the bloodstream, and particularly in the spleen, after the cells have left the bone marrow. The ripening process is thought to take 2 to 4 days, of which about 24hours are spent in the circulation. The number of Reticulocytes in the peripheral blood is a fairly accurate reflection of erythropoietic activity; assuming that the Reticulocytes are released normally from the bone marrow, and that they remain in circulation for the normal period of time. On the contrary, these assumptions are not always valid as an increased erythropoietic stimulus leads to premature release of Reticulocytes into the circulation. The maturation time of these so-called 'stress' or stimulated Reticulocytes may be as long as 3 days (Gilmer, 1976). In such cases, it is possible to deduce the Reticulocyte maturation time, and to calculate a 'corrected' Reticulocyte count by using plasma-iron turnover data (Seip, 1953; Hillman, 1969). Nevertheless, adequate information is usually obtained from a simple Reticulocyte count recorded as a percentage of the red cells or preferably, if the red cells count is known, expressed as absolute numbers.

In health, the red blood cells vary relatively little in size and shape (Plate 1 and 2). In well-spread, dried and stained films the greatest majority of cells have round smooth contours and have diameters within the comparatively narrow range (mean+/- 2SD) of 6.0 to 8.5micrometers. In this study, AGE was not shown to have any adverse effects on the shape and size of erythrocytes. This indicates that garlic is safe to consume for dietary purposes and would not affect the body's physiological functions. It will also not hamper the function of

RBCs. These results were consistent with previous haematology studies in rats done by Moriquchi (2001), in which he conducted a study on the effect of aged garlic extract on lipid peroxidative damage and the deformability of erythrocytes. He found that AGE significantly prevented the decrease of erythrocyte deformability induced by lipid peroxidation in a dose-dependent manner. The addition of AGE significantly inhibited an increase in thiobarbituric acid-reactive substances (TBARS) and hemolysis rate, and prevented the loss of intraerythrocytic ATP and 2, 3-diphosphoglycerate (2, 3-DPG) in oxidised erythrocytes. Both results suggested the possibility that AGE improved microcirculation and rheological blood properties, and preserved the structure and function of erythrocytes, not only through an antioxidant process, but also via the glycolytic pathway and membrane stabilization of erythrocytes.

Microscopic observation of the RBC shapes from the smears showed membranes that were intact with normal turgor. The RBC membranes were preserved, and no cells were deformed. This was concurrent with experiments conducted by Morihara (2002) using rats. The addition of AGE to a rat erythrocyte suspension significantly reduced the rate of peroxynitrite-induced hemolysis in a concentration-dependent manner. This suggested that AGE protects erythrocyte from membrane damage induced by peroxynitrite. This was as a result of an increase in Nitric oxide (NO) derived from Constitutive Nitric Oxide Synthase (cNOS) and its protection against peroxynitrite, both of which are important factors in the prevention of cardiovascular diseases associated with oxidative stress or dysfunction of NO production. Peroxynitrite was/is recognized as a powerful oxidant, and results in vascular or tissue damage. Previously, AGE has been shown to significantly improve erythrocyte deformability through stabilization of erythrocyte membrane in non-sickle RBCs. These phenomena were attributed to the antioxidant activities of AGE (Morihara, 2002).

5.2.2 Staining Intensity

The red cells stained quite deeply with the Giemsa component of Romanowsky dyes, particularly at the periphery of the cell as a consequence of the cells' normal biconcavity. As observed in plates 1 and 2, a small but variable proportion of cells in the film (almost 10%), were definitely oval rather than round in shape. A very small percentage was contracted and had irregular contours or appeared to have lost part of their cytoplasm as the result of fragmentation (Schistocytes). According to Marsh (1966), the percentage of 'Pyknocytes' (irregularly contracted cells), and Schistocytes in normal blood does not exceed 0.1%. Sometimes in disease conditions, abnormality in the red cell picture stems from four main causes;

1. Abnormal erythropoiesis, which may be effective or ineffective.
2. Inadequate hemoglobin formation.
3. Damage to, or changes affecting the red cells after leaving the bone marrow, indicating the effects of reduced or absent splenic function.
4. Attempts by the bone marrow to compensate for anaemia by increasing erythropoiesis.

These processes result, respectively, in the following abnormalities of the red cells:

1. Increased variation in size (*Anisocytes* = unequal) and shape (*Poikilocytes* = varied shapes) and *punctate basophilia* (punctured cells).
2. Reduced or unequal hemoglobin content (*Hypochromasia* or *anisochromasia* or *dimorphism*).
3. *Spherocytosis*, irregular contraction, *elliptocytosis* (ellipsoid shape) or fragmentation (*schistocytosis*); the presence of Pappenheimer bodies, Howell-Jolly bodies and a variable number of certain specific Poikilocytes (target cells, acanthocytes and spherocytes).

4. Signs of immaturity (Polychromasia and erythroblastaemia).

As microscopically observed in this study, AGE did not affect the shape and size of erythrocytes; meaning that the presence of cells larger than normal (macrocytosis), or cells smaller than normal (microcytosis), or both, was not observed.

5.3 BONE MARROW ANALYSIS

The final phase was to study the erythropoietic activity in the bone marrow. Generally, biopsy of the bone marrow is an indispensable adjunct to the study of diseases of the blood and may be the only way in which correct diagnosis can be made. Marrow can be obtained by needle aspiration, percutaneous trephine biopsy or surgical biopsy. In the present study, the bone marrow was obtained from the rabbits using the bone marrow aspiration technique. The morphological features of individual cells were studied by making imprints from the material obtained.

Satisfactory samples of bone marrow can usually be aspirated from the sternum, iliac crest or anterior or posterior iliac spines in humans. In this study however, to asses erythropoietic effects of AGE, bone marrow was aspirated from the sternum, the femur, the tibia, the humerous and the ulna of the rabbits. Histologically analysed, the percentage of marrow in the bones that was cellular, rather than fatty, was high and normal. This was consistent with Berman and Axelrod (1950), who reported that cellular content in a normal marrow should be 48-79% to fat.

Quantitative cell counts in aspirated marrow is usually difficult, but for practical purposes in studying the effect of AGE on erythropoietic activity in the marrow, the degree of activity and marrow cellularity was assessed within broad limits as *increased*, *normal* or *reduced* by inspecting the stained films containing marrow particles. Marrow from animals in group 5 had high cellularity (75-80%). High cell percentage was observed in plate 4

(Hypercellularity). It implied that there was increased erythropoietic activity in bone marrow of animals in group 5 that were fed on the highest dose of AGE. On the contrary, animals in the control and group 1 had almost an equal percentage of cellularity (25-30%). This, as showed in plate 5, was hypocellular, because the haematopoietic cells were few, indicating that there was normal erythropoiesis without any external stimulation. Experimental groups 2, 3 and 4 showed a slight increase in cellular activity (40-60%), which was interpreted as normocellular erythropoietic activity. The idea of rating bone marrow cellular activity in percentage was first determined by Kerndry et al (1980).

The maturation stages in all the bone marrow aspirate films, microscopically observed were normal, starting with mature red cells and working backwards to the most immature erythroid precursor cells. The Proerythroblasts were normal, no evidence of dysfunctional maturation, no changes in proportion with regard to primitive and mature erythrocytes, and there were no morphological abnormalities.

Laboratory signs of accelerated erythropoiesis were as follows:

1. Peripheral blood:
    (a) Reticulocytosis: There were evidences of increased number of Reticulocytes. This is the most readily and widely used index of accelerated erythropoiesis, even though the statistical errors in enumerating them are common. The Reticulocyte count consistently increased to levels that correlate fairly well with AGE doses administered.
    (b) Increased RBC counts above the normal physiological range, supported by increase in Hb levels and PCV.
2. Bone marrow:
    (a) Erythroid hyperplasia – an increase in erythrocyte precursors.
    (b) Increased erythrocyte turnover, analysed every 7^{th} day of the study period.

It is interesting to note that different plants and plant extracts can also stimulate differentiation and proliferation of cells in the bone marrow. Indeed, many pluripotent stem cells differentiated into normoblasts, to many Reticulocytes and thereafter, in to mature RBCs, increasing the number of Red blood cells in the peripheral circulation.

The interindividual variations in erythropoiesis observed among the population of caged male adult rabbits may be due to the differences in the utilization of garlic components and the concentration of the active ingredients. Those that are stimulatory cause an up-regulation of the Erythropoietin (EPO)-receptors on the proliferative bone marrow cells. According to Song et al (1987), garlic extract is an active oxygen scavenger. It is thus possible that garlic components compete with Hb in the RBC for oxygen resulting in hypoxia, which then stimulates Hb synthesis and RBC production. It is also possible that the end products of garlic metabolism in the body stimulates the kidney directly to cause formation and secretion of EPO, a potent stimulator of the bone marrow's pluripotent stem cells.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

In conclusion, the results show that garlic has a positive effect on the process of Red blood cell formation (Erythropoiesis) and the closely related blood parameters. Together with data accumulated during the past decade on research using rats and mice, these results reinforce the assumption that garlic is medicinal and its ingredients can therefore be incorporated into conventional hematinic drugs to make them more beneficial. However, differences among our cultures, both in health and eating patterns, have encouraged scientists to look closely at the many properties of garlic. These results so far suggest that we may do well to include more garlic in our diet! We will therefore have a more active bone marrow, increased erythrocyte turnover, more circulating red blood cells, increased plasma iron turnover, and improved oxygen delivery to the body tissues

6.1 SUGGESTIONS FOR FURTHER RESEARCH

- More research is needed to determine the specific effects of garlic on the other specific blood cellular components to fully provide knowledge about its effects on blood.
- There is also need to conduct an assay of the hormones closely related to the production of blood cellular components, especially Erythropoietin, to clarify whether AGE acts on the kidney to enhance its secretion.
- Because garlic bulbs exist in several varieties, there is also need to conduct a comparative study to identify which variety of garlic has the most effect in the body.

- Onions, Leeks, Shallots and Chives are species closely related to garlic. Do these four species have similar impact on haematopoiesis, or even in the body, as garlic? That is a research worth exploration.
- Marked differences in experimental conditions are cited as difficulties in making direct comparisons of studies with garlic. Use of various formulations of garlic including; fresh garlic, dried garlic, powdered garlic, freeze-dried garlic, essential oils of garlic, should be investigated on their effects on blood parameters and other cellular components.
- Using garlic, authors have reported an improvement of AIDS related conditions including diarrhoea, genital herpes, candidiasis and pansinusitis with recurrent fever. There is still need for further study on other effects of garlic on AIDS patients, especially on the improvement of helper/suppressor ratio.

6.2 STUDY LIMITATIONS

1. Funds were not enough; hence the researcher was unable to carry out deeper research that would have provided additional information to this project, e.g. Erythropoietin Assay.
2. Some of the equipment were unavailable locally, expensive, and the protocol that would have been used for their importation was tedious and demanding!

But despite the above limitations, the project was within the required time and budget. The researcher was able to conduct and collect valid and reliable data as shown in the findings.

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APPENDIX A

New Methylene Blue Stain.

To make 1 liter:

Methylene blue chloride dye.....0.15g.
Distilled water.....1 liter.

1. Weigh the chemical and transfer to a brown bottle.
2. Add the water and mix until the dye is completely dissolved.
3. Store in the dark at room temperature. Renew if it becomes contaminated.
4. Filter before use.

APPENDIX B

Field's Stain A.

To make 500ml:

Field's stain powder A .....10g.
Distilled water (hot) 500ml

1. Weigh the powder on a piece of clean paper (pre-weighed), and transfer it to a large Pyrex beaker or high-density polyethylene reagent bottle.
2. Measure the water and heat to boiling.
3. Add the hot water to the stain and mix to dissolve the powder.
4. When cool, filter the stain into a storage bottle.
5. Label the bottle and store it at room temperature. The stain is stable indefinitely.

Field's Stain B.

To make 500ml:

Field's stain powder B .....10g.
Distilled water (hot) .....500ml.

Prepare as described for Field's stain A. Label the bottle and store at room temperature. The stain is stable indefinitely.

APPENDIX C

Buffered Water, pH 7.0-7.2.

This is best prepared from stock phosphate buffer solutions A and B as follows:

Stock Phosphate solution A.

Sodium dihydrogen phosphate-1-hydrate .....27.6g.
($NaH_2PO_4.H_2O$).
Distilled water .....1 liter.

1. Accurately weigh the chemical and transfer it to a 1-liter volumetric flask.
2. Half fill the flask with water, and mix to dissolve the chemical. Make up to the 1 liter mark with distilled water, and mix well. Transfer to a leak-proof bottle.
3. Label the bottle stock phosphate solution A. Store in a cool place or preferably at 2-8^0C. The solution is stable for several months.

Stock Phosphate solution B.

Di-Sodium hydrogen phosphate anhydrous .....28.39g.
(Na_2HPO_4)
Distilled water .....1 liter.

Prepare as described above for solution A. Label the bottle Stock Phosphate Solution B. The solution is stable for several months.

To make 1 liter buffered water, pH 7.2.

Stock Phosphate Solution A .....140ml.
Stock Phosphate Solution B .....360ml.
Distilled water .....500ml.

1. Accurately measure the stock phosphate solutions and water, transfer to a clean leak-proof bottle, and mix well. Check the pH using narrow range pH papers or a pH meter.
2. Label the bottle and store it at room temperature. The buffer is stable for several months.

APPENDIX D

Giemsa Stain.

Purchase ready-made or prepare using the following formula:

To make 500ml:

Giemsa powder .....3.8g.
Glycerol (glycerine) .....250ml.
Methanol (methyl alcohol) .....250ml.

1. Weigh the Giemsa powder on a piece of clean paper (pre-weighed), and transfer to a *dry* brown bottle of 500ml capacity which contains a few glass beads.
   Note: Giemsa stain will be spoilt if water enters the stock solution during its preparation or storage.
2. Using a *dry* cylinder, measure the methanol, and add to the stain. Mix well.
   Caution: Methanol is toxic and highly flammable; therefore handle it with care and use well away from an open flame.
3. Using the same cylinder, measure the glycerol, and add to the stain. Mix well.
4. Place the bottle of stain in water bath at 50-60^{0}C, or if not available at 37^{0}C, for up to 2 hours to help the stain to dissolve. Mix well at intervals.
5. Label the bottle, and mark it *Flammable and Toxic*. Store at room temperature in the dark. If kept well stoppered, the stain is stable for several months.

For use: Filter a small amount of the stain into a *dry* stain-dispensing container.

Glycerol Jelly.

To make about 310ml:

Gelatin .....15g.
Glycerol (glycerine) .....50ml.
Distilled water .....250ml.

1. Measure the water and heat to boiling.
2. Weigh the gelatin and add to the hot water. Stir until the gelatin is completely dissolved.
3. Measure the glycerol and mix with the gelatin water.
4. Dispense in 10-20ml amounts in screw-cap bottles, and allow to gel. Label each bottle and store at 2-8^{0}C.

For use: Liquefy a container of glycerol jelly by placing it in hot water (about 50^{0}C).

APPENDIX E

ANIMAL COMMITTEE GUIDELINES ON RABBIT HANDLING.

Stock Rabbits.

It is recommended that group pen housing of stock rabbits could be considered appropriate in the following circumstances:

- Use with compatible adult female rabbits, young rabbits of either sex up to 10-12 weeks of age, or castrated adult males.
- Use particularly for long term holding purposes (over 6 weeks).
- Place no more than 6-8 rabbits in a group.
- Preferably form the group from weaning or as soon after as possible.
- Use with animals free from infection with *Pasteurella multocida*, ear mites, and coccidiosis.
- Provide microenvironments including retreating and hiding places.

Where rabbits are held in cages, it is recommended that:

- Provide sufficient space for adequate exercise, cages should provide room for hopping or jumping (a horizontal dimension of at least 0.8-1m and vertical space for stretching upright 45-50cm (Lehmann, 1987).
- Rabbits should be caged in compatible groups of two or three animals where possible in sufficiently large cages (Whary et al., 1993).
- Where rabbits are held in smaller cages that do not provide room for adequate exercise, access to exercise pens for several hours should be provided at least several times weekly. Such access can also be used for singly caged rabbits for the opportunities for social interaction with other compatible rabbits.
- Environmental enrichment such as toys, sticks, climbing surfaces, and retreating and hiding places within the cages should be provided Stauffacher, 1992).
- Cage floor of stamped out sheet metal or plastic are preferable to wire floors. However, more frequent cleaning of cage floor may be required.
- After weaning, young stock rabbits should be held in sufficiently large cages in litter mate groupings for as long as practicable and while the animals are compatible. Intact males must be separated by 12 weeks of age.
- Singly caged rabbits must always be able to see other rabbits in adjacent or facing cages.
- Whenever possible, experimental rabbits should be held in single cages for short-term purposes only (6-12 weeks or less).

Breeding Rabbits:

More work needs to be done to assess the usefulness of a large enriched group breeding cage holding two or three female rabbits with or without the permanent presence of a male rabbit, as suggested by Stauffacher (1992). The concept of an enriched group mating pen as described by Stauffacher containing one male and five to six females also has much merit but will not hold many rabbits per unit of room floor area, rendering it impractical for most situations, and it may cause higher pre-weaning mortality rates.

For breeding rabbits held singly in cages, it is recommended that:

- Cages should provide room for hopping or jumping (Lehmann, 1987).

  Breeder rabbits held in smaller cages should be given access to exercise pens for several hours at least several times weekly.

- Cages should provide some secluded area for nesting purposes for does with young litters.
- Litters should be weaned by four to five weeks, or does should have retreating areas where they can escape attention from their offspring.

  Does should be kept breeding (i.e. remated by the time of weaning the previous litter) or else housed with one or two other compatible females in sufficiently large cages in between litters. Aggression between breeding does is quite likely. Some large facilities in Australia use a single doe floor-penned breeding system successfully. Such system ensure that the does have full environmental and behavioral enrichment without the unattractive aesthetics of caging and without the problems of group floor pens.

Grasping and lifting a rabbit:

Grasp by the scruff of the neck, together with the ears, and support the body with the other hand through the abdomen.

APPENDIX F

Useful Data of a Normal Rabbit.

Biological data.

Adult weight (kg)	2-5
Birth weight (g)	30-100 (average 50-70)
Growth rate	15-20gm/day to 8 weeks; 100-150gm/week to 26 weeks
Diploid number	44
Food intake	5g/100g bodyweight
Water intake	10ml/100g bodyweight
Life span (years)	6-12
Rectal temp (^{0}C)	38.5-40
Heart rate/min	130-325 (average 200-240)
Blood pressure systole (mmHg)	90-130 (average 110)
Blood pressure diastole (mmHg)	60-90 (average 80)
Blood volume (ml/kg)	57-65 (average 60)
Respiratory rate/min	30-65 (average 35-56)
Tidal volume (ml/kg)	4-6
Oxygen consumption	0.42-0.48ml/gm/hr

Hematological data.

RBC (x 10^6/mm^3)	4-6.5 (average 4.8-6.3)
PCV (%)	30-48 (average 36-44)
Hb (g/dl)	9-15.5 (average 10-14)
WBC (x 10^3/mm^3)	9-12.5 (average 9-11)
Neutrophils (%)	20-75
Lymphocytes (%)	30-85
Eosinophils (%)	0-4

Monocytes (%)	1-4
Basophils (%)	2-7
Platelets (x 10^3/mm^3)	250-656
Reticulocytes (%)	2-4
Clotting time (min)	5 (at 37^0C)

Breeding data.

Puberty (days)	90-120
Age to breed male	6-10 months
Age to breed female	4-9 months
Gestation (days)	30-32
Litter size	4-10
Weaning age (weeks)	4-8
Estrous cycle	Induced ovulator
Post-partum estrous	None (not used)

Biochemical data.

Serum protein (g/dl)	5.4-7.5
Albumin (g/dl)	2.7-4.6
Globulin (g/dl)	1.5-2.8
Glucose (mg/dl)	75-150
Blood urea nitrogen	17-23.5mg/dl
Creatine (mg/dl)	0.8-1.8
Total bilirubin (mg/dl)	0.25-0.74
Cholesterol (mg/dl)	35-53

APPENDIX G

Calculation of Doses from Garlic Bulbs.

Average weight of Whole garlic bulb:

Garlic weight (g).

1	2	3	4	Average
50.13	42.22	45.75	39.16	42.41

From Paget and Barnes' conversion table, translating a dose from a 200g rat to a 1.5kg rabbit, the conversion factor to give absolute dose is 3.9.

Weight of crushed garlic (homogenate) = 42.41g (approx. 40g)

40 x 3.9 = 156.0mg (absolute dose for a 1.5kg rabbit).

If 1.5kg rabbit = 156mg homogenate
2kg rabbit = 2 x 156 / 1.5
= 208mg (absolute for a 2kg rabbit)

Therefore, 1kg gets 208 / 2 = **104mg/kg** body weight.

This is the **Standard dose** that can elicit a similar effect in a 2kg rabbit.

To calculate the concentration (ml) given:

10g of homogenate was completely dissolved in 100ml of distilled water (vehicle).

Therefore 10g/100ml = 1g/10ml distilled water.
= 0.1g/ml or **100mg/ml**.

If 100mg = 1ml
104mg (2kg rabbit) = 104mg x 1ml / 100mg
= **1.04ml** (approx. 1ml)

To calculate doses for a Dose-Response curve, multiples of 2 (up and down), were calculated from the standard dose (104mg/kg b.wt), and converted in terms of concentration/ml as shown in the table below:

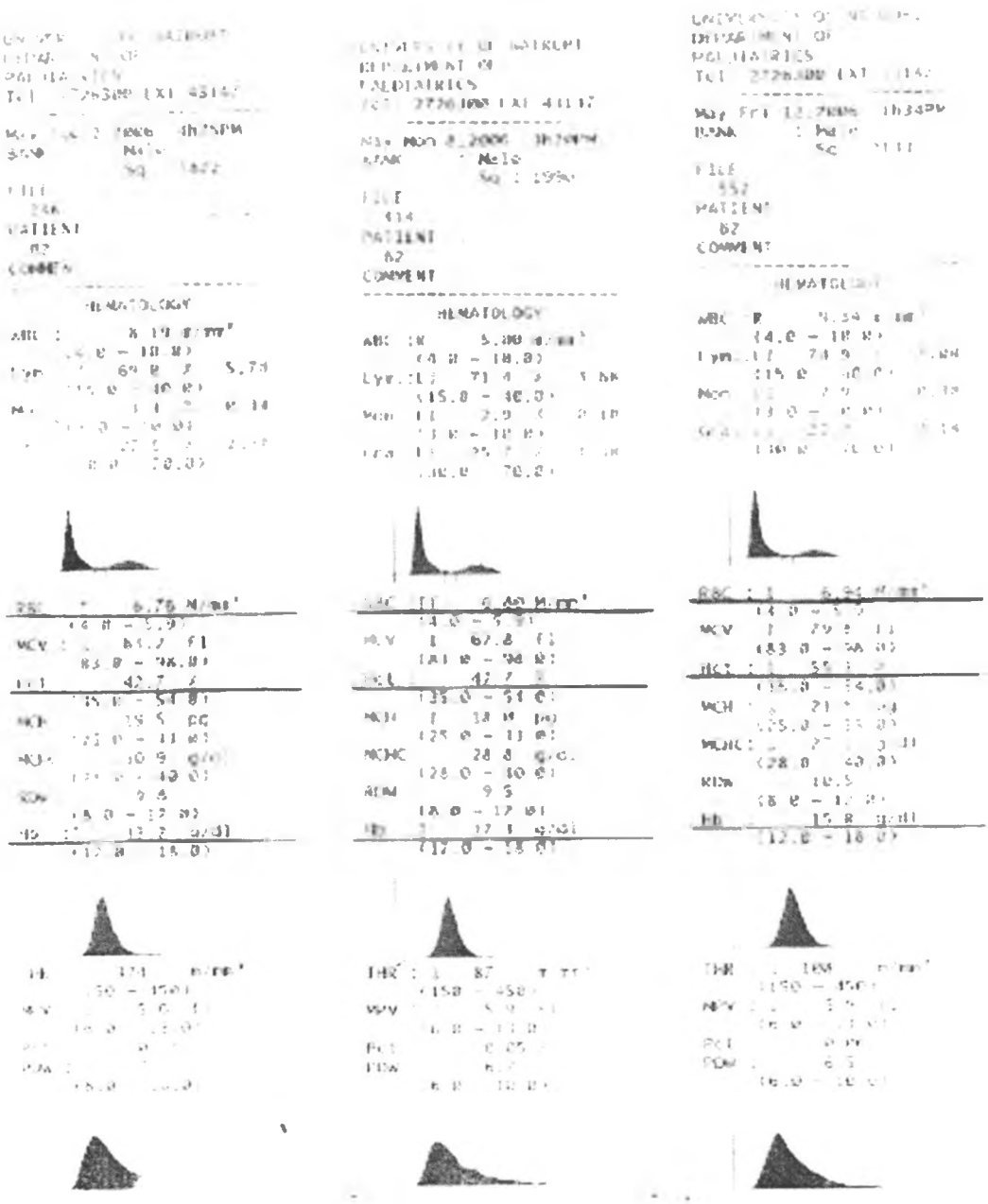
Group	Mg/kg b.wt.	Absolute conversion (ml)	Absolute amount given (in 2, 5 or 10ml syringe (ml).
A	26	0.52	0.5
B	52	1.04	1
C	**104** (standard)	**2.08**	**2.1**
D	208	4.16	4.2
E	416	8.32	8.4

The control group (x) was given 2ml of distilled water. All these doses were given orally to the rabbits with respect to their groups.

APPENDIX H.

Copies of how RBC, PCV and Hb Data were collected.

Full Haemogram from an automated Hematological Cell-Counter Machine, Series MS-4 (Department of Hematology and Blood Transfusion Laboratory, Kenyatta National Hospital (KNH).



APPENDIX I

TABLE XVIII
ERYTHROCYTE AND RELATED PARAMETERS IN THE RABBIT[a]

RBC × 10^6	Hb (%)	PVC (%)	Reticulocytes (%)	RBC diameter (μm)	Ref	Note
4.5–7.0				5–8 (6.7)	94	
5.98 ± 0.78					18	
5.4 ± 0.55	11.8 ± 1.06		3.64 ± 1.22		87	
		36–48				
(5.61)	11	(41)		(6.9)	42	
3.0–8.0						
(5.2 ± 0.63)	8 – 15.5				84	
4–6.4			1–7			
(5.25)	(12.4)		(5)		96	
4.2 – 7.1	10.8–16.0	35–50				
(5.31)	(13.1)	(40.0)			16	
5.36	13.35				91	
5.67 ± 0.77	12.0 ± 1.38				35	
5–6	12–12.5	36–38	2–4		95	
6.27	13.1	39.7		6.7–6.9	109	
4.5–7.0	8–15	33–50	2–3			
(5.7)	(11.9)	(45.5)	(2.2)	6.5–7.5	2	
6.33–7.21	13.95–15.56	40.28–44.99			38	*c*
4.33–6.82	9.37–13.94	31.59–50.0		6.4–9.3	69	*d*
	11.91–16.3	36.67–43.53			69	
5.30 ± .14	14.21 ± .35	41.79 ± .91	34.0		16	*b,e,f*
5.45 ± .13	13.98 ± .25	41.1 ± .71	33.9			*b,e,g*

4.33-6.82 x 10^6 M/mm | 9.37-13.94% | 31.59-50.0 % | 2-4% | 6.4-9.3micrometers

APPENDIX J

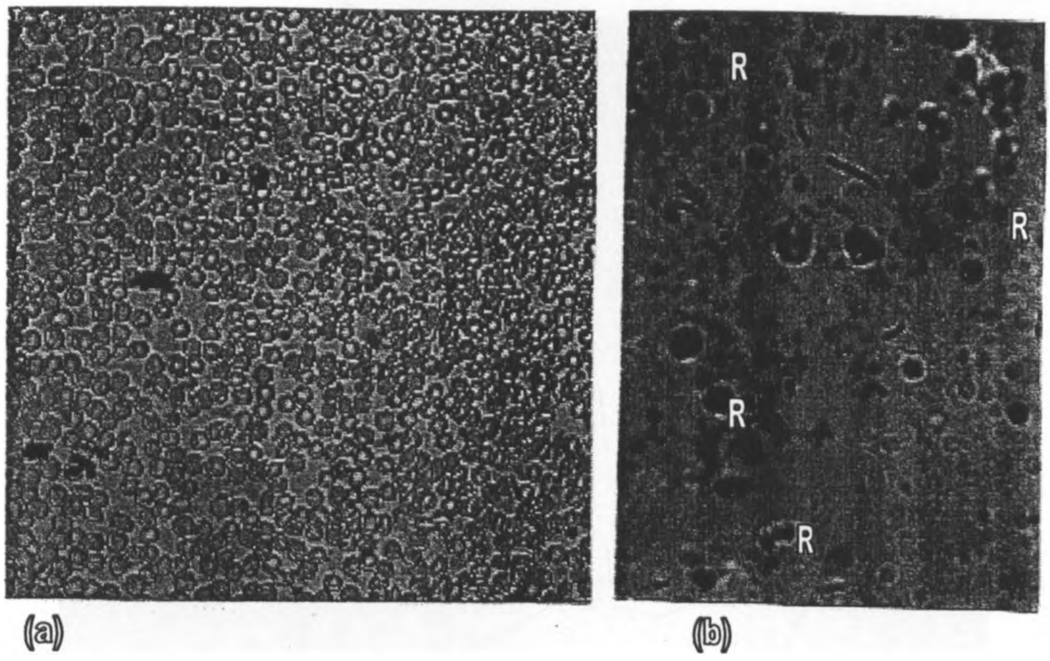
TABLE XXI

DIFFERENTIAL CELL DISTRIBUTION IN THE MARROW OF NORMAL RABBITS[a]

Cell type	Minimum (%)	Maximun (%)	Average (%)
Erythroid series			
Rubriblasts	0.2	0.8	0.2
Prorubricytes	0.2	2.0	0.6
Basophilic rubricytes	0.4	10.8	5.5
Polychromatophilic rubricytes	10.9	26.6	18.9
Metarubricytes	6.6	24.3	16.7
Total erythrocytic			41.9
Granulocytic series			
Myeloblasts	0.2	1.6	0.7
Progranulocytes	0.1	1.6	0.6
Myelocytes	1.1	1.6	0.6
Metamyelocytes	2.8	10.0	7.4
Band pseudoeosinophils	10.8	33.6	23.2
Segmenter pseudoeosinophils	2.0	9.0	5.3
Basophils	0.1	2.4	0.7
Eosinophils	0.2	2.4	1.4
Total granulocytic[b]			42.4
Other cells in the marrow			
Megakaryocytes	0.1	0.3	0.1
Lymphocytes	4.1	21.3	12.6
Monocytes	0.4	3.6	1.6
Plasma cells	0.1	1.2	0.2
RE nuclei	0.2	1.7	1.0
Hemocytoblasts	0.1	0.8	0.2

[b] Myeloid to Erythroid ratio equals 1.01: 1.0.

PLATE 1



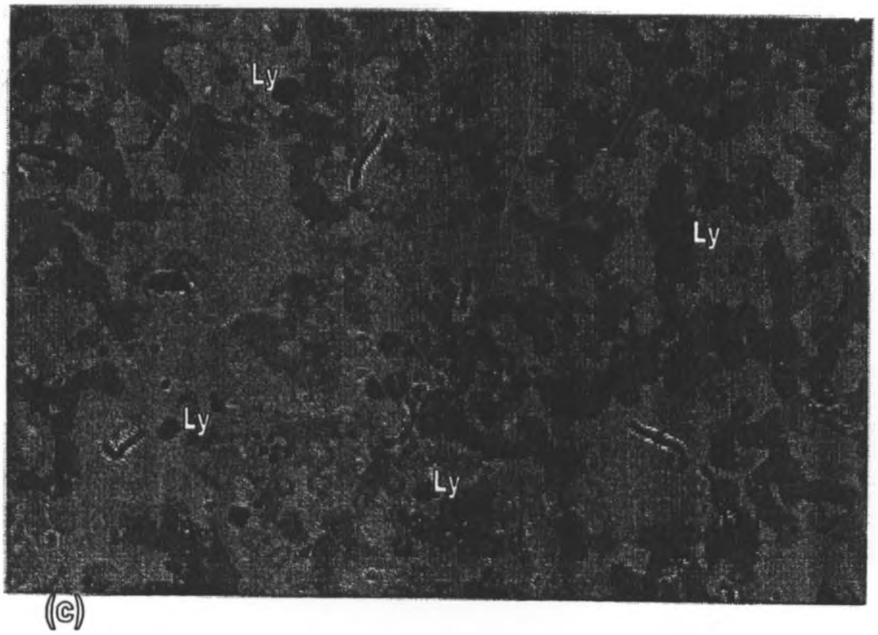


PLATE 1. **(a)** Photomicrograph of peripheral blood smear from a rabbit given an over-dose of AGE. Some cells are irregularly shaped (Giemsa stained). **(b)** Reticulocytes seen in the blood smear of the same rabbit in (a) (New Methylene blue stained). **(c)** Isolated lymphocytes from the same blood smear (Giemsa stained). All slides magnified X400.

PLATE 2.

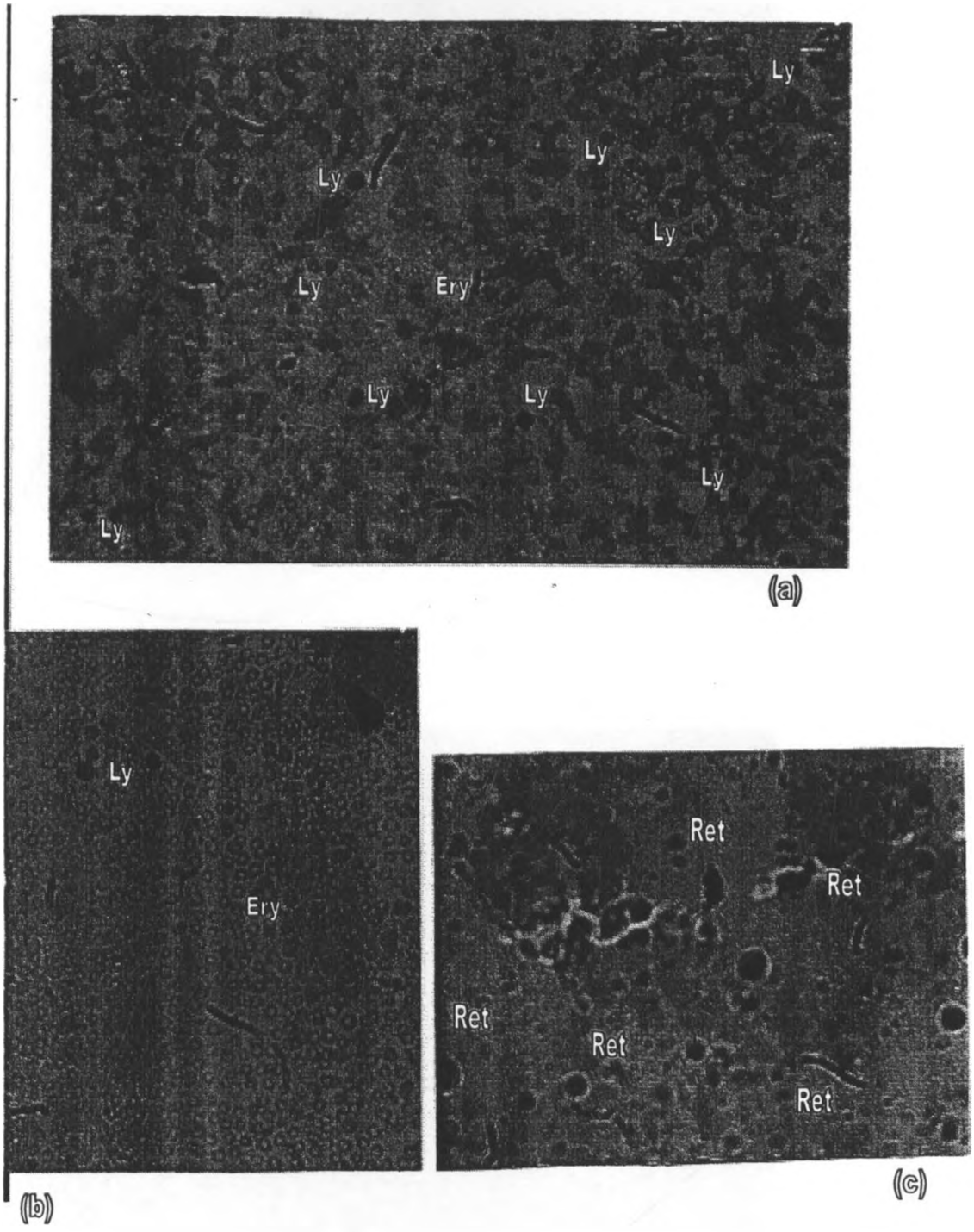
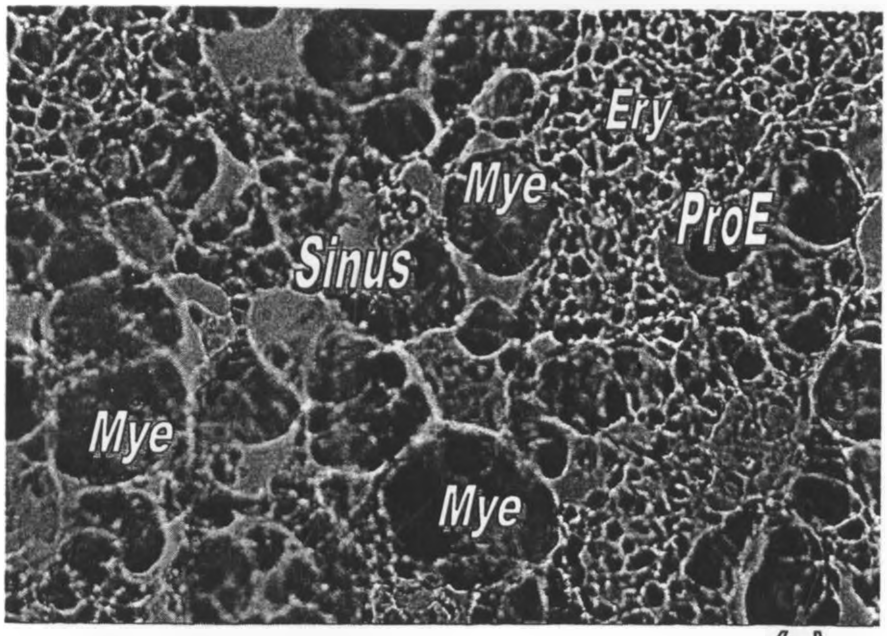


PLATE 2. Photomicrographs showing increased peripheral Erythropoiesis from a rabbit in-group 5, scattered lymphocytes (Ly) with numerous mature Erythrocytes (Ery) in **(a)** and **(b)** Giemsa stained. **(c)** Reticulocytes as seen from the same blood under a light microscope (New Methylene blue stained) X250.

PLATE 3



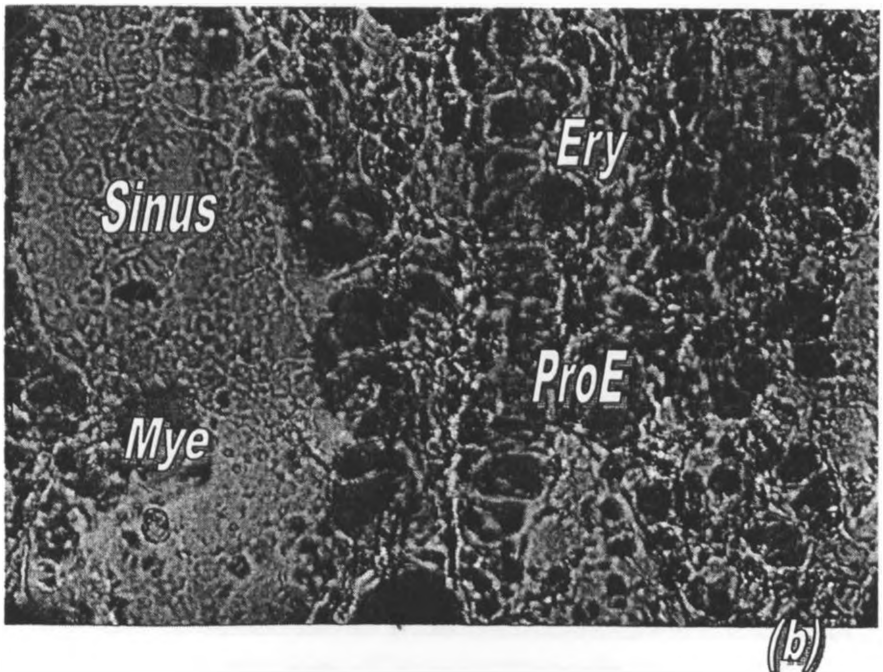


PLATE 3. (**a**) Rabbit bone marrow cells. These photomicrographs contain Myelocytes (Mye), Proerythrocytes (ProE) together with Erythrocytes (Ery) from the Ulna-X400 and the Sternum (**b**) X250. All Giemsa stained

PLATE 4

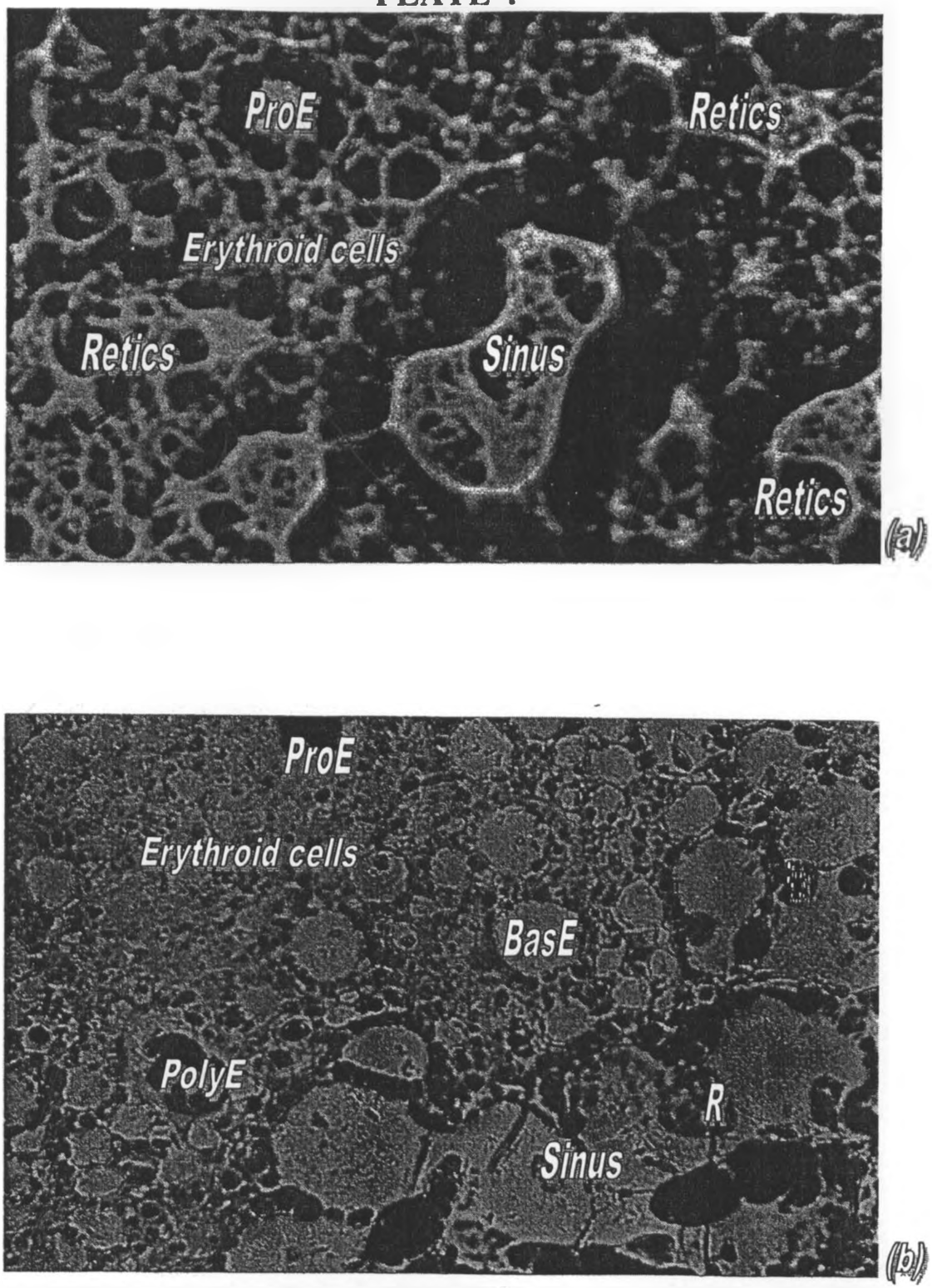
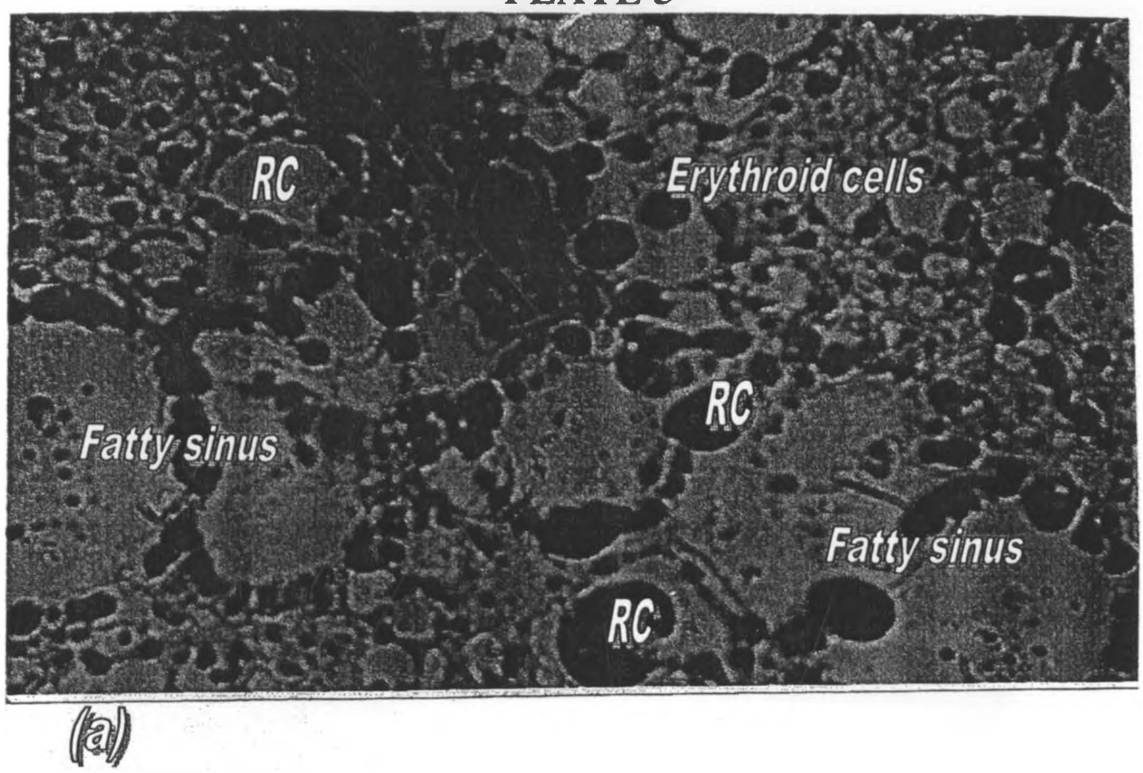


PLATE 4. (a) Morphology of bone marrow cells from the Tibia of a rabbit. The film shows high cellularity in Giemsa stain X250. (**b**) A film of normal Erythropoiesis from a rabbit's Humerus with Proerythroblast (ProE), Basophilic erythroblast (BasE) with Polychromatic erythroblasts (PolyE) predominating the field in Giemsa stain,X400.

PLATE 5



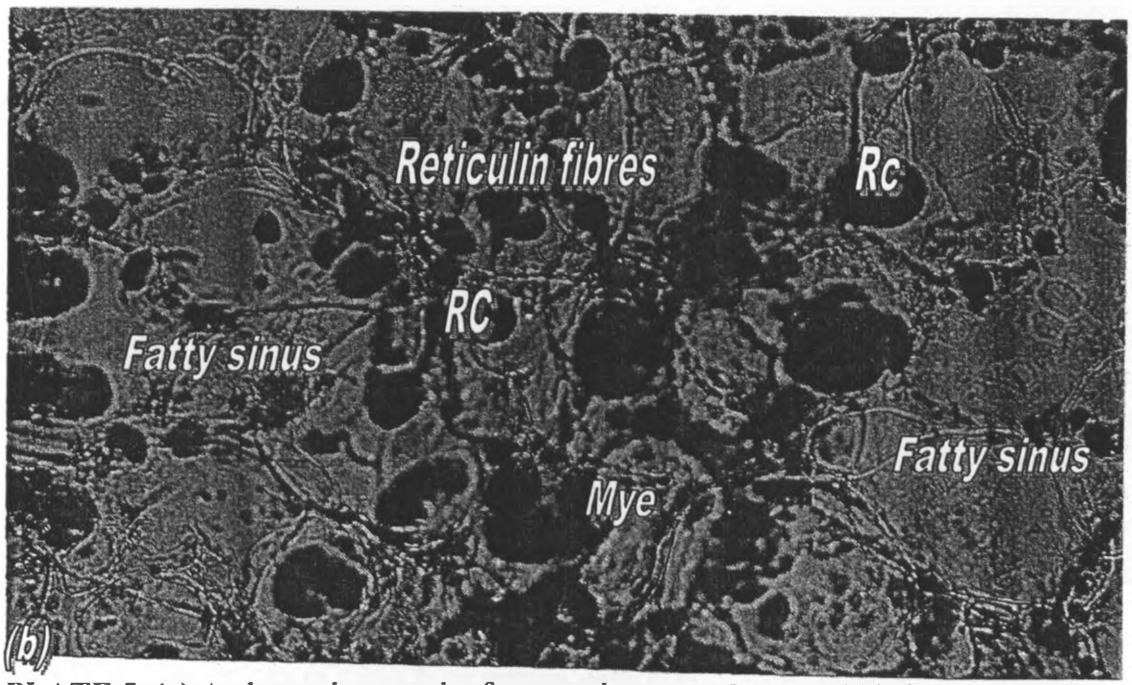


PLATE 5. (a) A photomigrograph of a normal marrow from a rabbit femur in group 1. It shows low cellularity with lots of fatty sinuses and Reticulm cells (RC). **(b)** Section of the same marrow showing Reticulin fibres from an Erythroblastic island-Giemsa stained X400.

PLATE 6

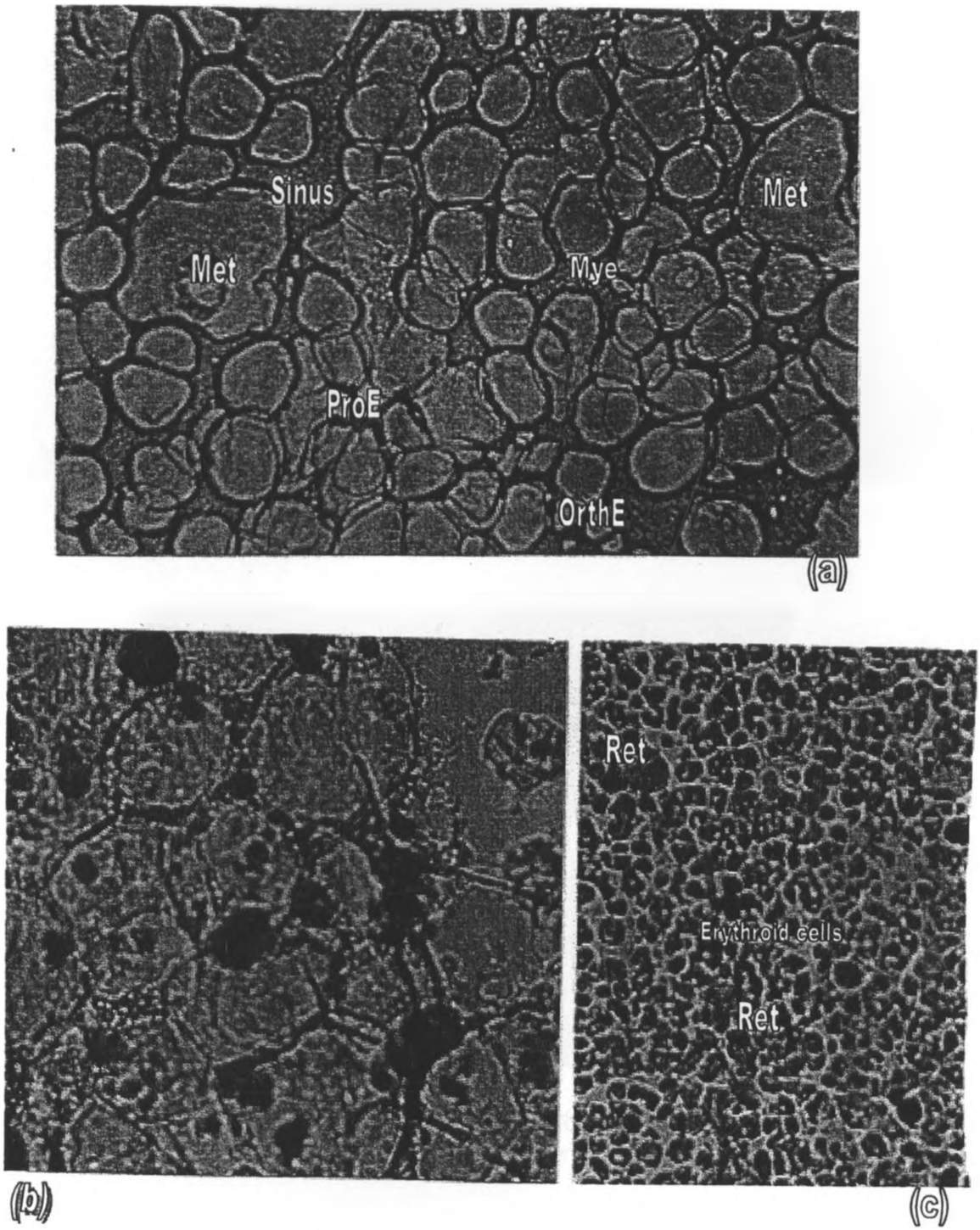
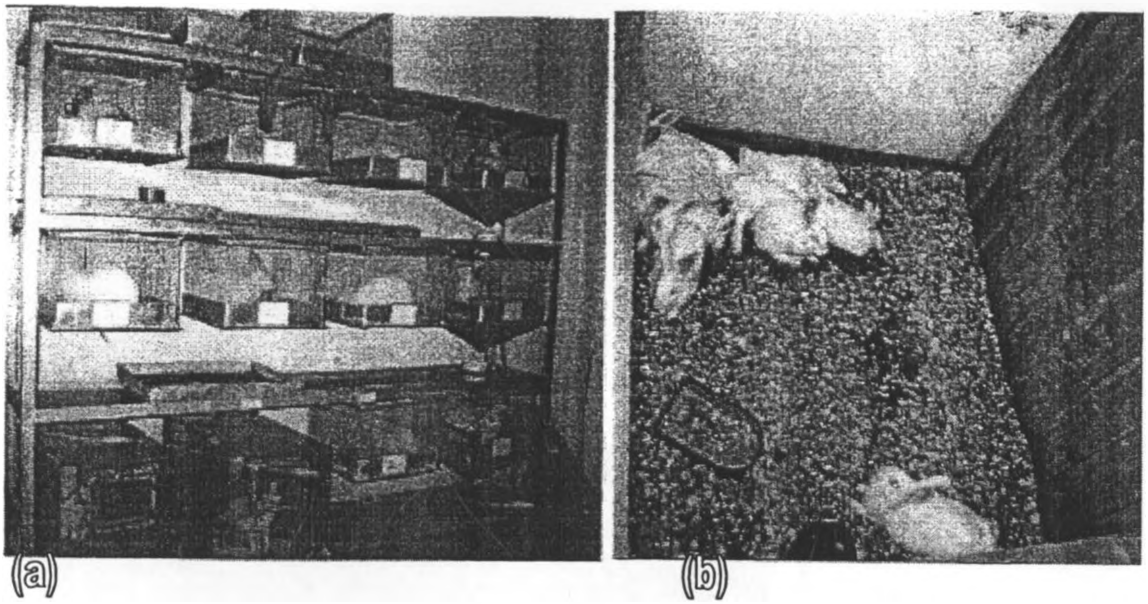


PLATE 6. Morphology of rabbit bone marrow cells at their different stages of maturation in Giemsa stain. **(a)** Bone marrow from the Femur of a rabbit in the control group showing Proerythroblast (ProE), Orthochromatic erythroblast (OrthoE), Metamyelocytes (Met) and Myelocytes (Mye) X400. **(b)** Low cellularity from the Femur of a rabbit in group 3, X400. **(c)** High Erythropoietic activity with two isolated Reticulocytes X250.

PLATE 7



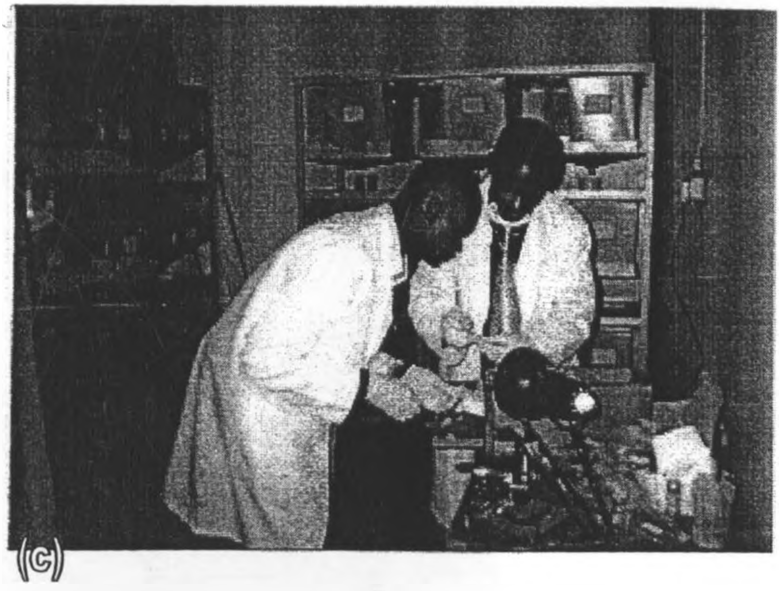
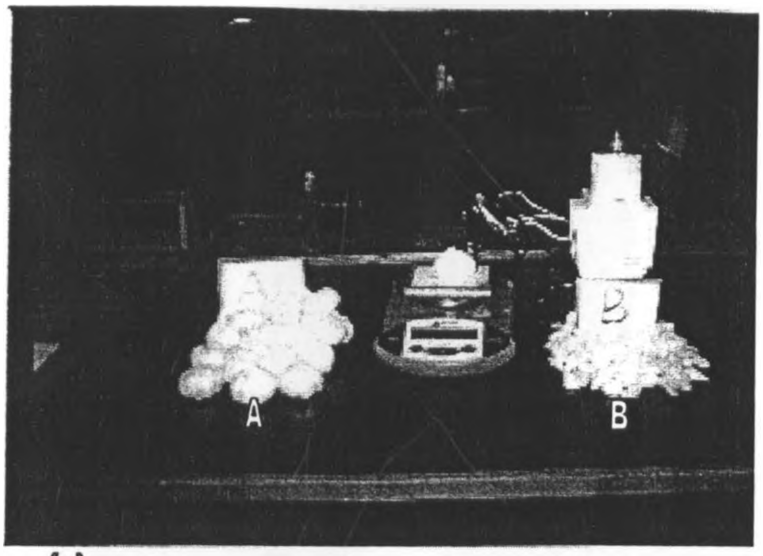
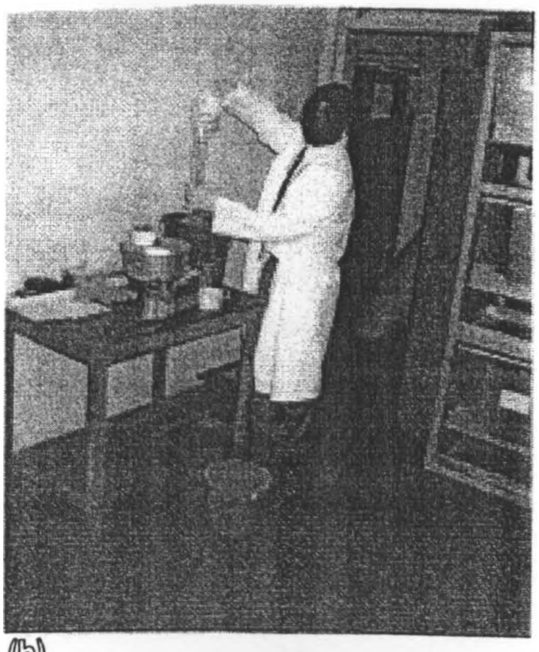


PLATE 7. Above: A comparison of Floor-housed rabbit **(a)** and Single-caged rabbits **(b)**. **Below:** Researchers collect 1ml of blood from the lateral ear vein of a rabbit **(c)**.

PLATE 8



(a)



(b)

PLATE 8. Above (A) Garlic bulbs and **(B)** Garlic cloves.
Below: A Researcher weighs food (Standard rabbit pellet) and required water quantity during animal feeding.

PLATE 9

PLATE 9. How to handle the rabbit.