Effect of Calabar Stone (Calabash chalk) on the testes of an adult male Wistar rats

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Maduakolam Justice Desire

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A PROJECT SUBMITTED TO THE DEPARTMENT OF ANATOMY, SCHOOL OF BASIC MEDICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA.

IN PARTIAL FULFILMENT OF REQUIREMENT FPR THE AWARD OF BACHELOR OF SCIENCE (B.Sc)

DEGREE IN ANATOMY.

SUPERVISED BY: Dr. Daniel E. Odiase

January 2023

CERTIFICATION

I, **Maduakolam Justice Desire ,** hereby wish to certify that the research work presented in this dissertation for the award of Bachelor of Science (B.Sc) degree in Anatomy is the result and sole out of an independent research done by me under the supervision of **Dr. Daniel E. Odiase**and any assistance given was duly acknowledged. I also certify that this dissertation has not been submitted anywhere else in part or in full for any other examination or institution. All literatures and other sources of information consulted, cited or used in this research have been duly acknowledged in references.

______________________ ______________________

Dr. Daniel E. Odiase DATE

(SUPERVISOR)

______________________ ______________________

DR INNIH, S.O DATE

(HEAD OF DEPARTMENT)

______________________ ______________________

EXTERNAL EXAMINER DATE

DEDICATION

This work is dedicated to God Almighty for keeping me alive throughout my academic sojourn and especially throughout the duration of this research.

ACKNOWLEDGEMENTS

I ascribe all thanks to the undesigned designer (Jehovah) for allowing me to attain this feat, a special cognizance to my indispensible supervisor DR. Daniel E. Odiase for his untiring support, contributions and his constructive criticism.

A special accolade to Prof. Eze Gerald Ikechi who I regard with high esteem. He has been a father and a friend.

Thanks to the lecturers, Department of Anatomy, University of Benin, and my bosom friend Abdullahi Jemila (Jemiski); Okpadanyeta David, Idele Courage, Anyankoha Kenechi, Christabel Ogunkoro and Abulimen Favour.

This acknowledgement will be incomplete if my indomitable parent (Mr and Mrs. Maduakolam) and siblings are not recognized.

If only gratitude were a personality, if doggedness were a human; my irreplaceable parent would have been the exact replica.

May you all prosper in your life endeavours.

Gracias.

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ABSTRACT

Calabar stone is also known as poto in English is a clay held in a belt-like textile material that is wrapped around the waist and eaten as desired, frequently without water. This study aims to determine the effect of calabar stone on the testis of adult male Wistar rats. Twenty (20) rats were divided into four groups (A-D). Group A served as the control group and the animals were given regular feed and water. Group B served as low dose group and the animals were given 100mg/kg of dissolved calabash chalk along with regular feed and water. Group C served as medium dose group and it animals received 200mg/kg of dissolved calabash chalk along with regular feed. Group D served as the high dose group and the animals were administered with 400mg/kg of dissolved calabash chalk as well as regular food and water. All animals were sacrificed on the??? day. Body weight change and testicular weight index were determined. Testicular tissues were collected for assessment of sperm morphology as well as haematoxylin and eosin staining. Body weight increased in all groups from the initial mean weight of 171.5g, though significantly ($P<0.05$) only in group C. Testicular weight significantly increased ($P<0.05$) in group B. A significant increase ($P<0.05$) was also observed in the testicular index for animals in group B. There was no statistically significant change ($P<0.05$) in the sperm morphology across all groups. H&E staining revealed mild leydig cell hyperplasia across all groups which are a possible evidence of testicular tumour that appear in about 3% of the general population. Increased luminal diameter was seen in group D animals which suggested spermiation. It can be concluded that Calabar Chalk has no significant effect on the testes.

CHAPTER ONE

INTRODUCTION

1.1 Study Background

Calabar stone is also known as poto in English, La craie or argile in French, nzu by the Igbos, ndom by the Efiks/Ibibios of Nigeria, and Mabele by the Lingala of Congo (Ekong *et al*., 2012). Other names for it include Ebumba, Poto, and Ulo (Abrahams *et al*., 2013).

This stone is available in a range of sizes and with varying mineral composition. In a traditional environment, the clay is held in a belt-like textile material that is wrapped around the waist and eaten as desired, frequently without water. The ones typically consumed in Africa include essential elements like as phosphorus, potassium, magnesium, copper, zinc, manganese, and iron, with the intention of extracting these minerals for human consumption (Abraham *et al*., 2006). Geophagia (the practice of eating the earth, including dirt and stone) is a practice related with religious beliefs, medicine, or as part of a normal diet (Abrahams *et al*., 2013; Dean and Ma, 2007). An examination of the literature clearly shows that geophagia is not confined to any age group, race, sex, geophagic area, or period, however nowadays the practice is most plainly frequent among the world's poorer or more tribally oriented people and is therefore especially widespread in the tropics (Abrahams and Persons, 1996).

According to Dean *et al*. (2004), the main component of Calabar stone is aluminium silicate hydroxide- $Al_2Si_2O_5(OH)_4$. This belongs to the Kaolin clay family. At a mean, multi-elemental analysis was able to quantify 22 elements in calabar stone, including lead. Calabar stone is a geophagic material that has traditionally been consumed by people (particularly pregnant women) as a remedy for morning sickness, nausea, and for pleasure. When ground, it may also be used as a face powder and an antiperspirant (Popoola *et al*., 2013). Calabar stone is available in a number of forms, including powders, molded shapes, and blocks. It comes in two varieties: salted and unsalted. Calabar stone is also known as Nzu by the Ibos, Ndom by the Efik/Ibibios, and Ulo in some sections of Bini in Nigeria (Ekong *et al*., 2008).

It is widespread in Nigeria and other Sub-Saharan African nations. When geophagic materials come into touch with digestive fluids, they have the potential to have clinical or sub-clinical harmful effects on the human, according to Abrahams *et al*. (2006).

Calabar stone also contains metalloids and metals such as lead, which accounts for a large portion of its composition, arsenic, iron, aluminum, potassium, zinc, titanium, manganese, barium, chromium, copper, nickel, rubidium, and tin (Campbell, 2002).

Calabar stone research has demonstrated hepatic sinusoidal enlargements, gastrointestinal damage, and anaemic consequences. Calabar stone elements such as lead and even arsenic have been linked to nerve and brain damage, as well as learning and behavioral dysfunctions in both animals and humans. In a recent study, we found that prolonged calabar stone diet intake increased anxiety and pain perception in mice. In addition, alpha lindane, endrin, endosulphan 11, and p,pI- dichlorodiphenyl dichloroethane were found as persistent organic pollutants (DDD). Iron, aluminum, potassium, titanium, chromium, manganese, zinc, nickel, and the metalloid arsenic were also discovered (Dean *et al*., 2004; Ekong *et al*., 2012).

According to Owhorji *et al*. (2019), there are safety issues about the eating of Calabash chalk, which is a prevalent habit in various African and Asian communities. Calabash chalk contains lead (Pb) and arsenic, both of which are thought to be detrimental to the brain and cause cognitive impairment. Consumption of calabash chalk may influence other neural activity in the body, such as movement and social behavior. As a result, the current study looked at the effects of this diet on locomotion and social behavior in mice. 45 Swiss white mice of mixed sex were randomly divided into three groups of 15 animals each. Group 1 was the control group, whereas groups 2 and 3 were given low and high dosages of calabash chalk meals, respectively. After 30 days of feeding, their locomotor and social activities were evaluated. Their locomotor behavior was evaluated using an open field maze, and their social behavior was investigated utilizing a nesting behavior test. The calabash chalk diet-fed mice had a considerably lower (p 0.05) frequency of line crossing compared to the control. When compared to the control, the nesting score of the calabash chalk diet-fed mice was substantially lower (p 0.05). In conclusion, calabash chalk ingestion affects mobility and social behavior in mice.

1.2 Problem statement

Calabar stone contains a lead content of around 40 mg/kg, which is more than the recommended dietary limit (Dean *et al*., 2004; EU,2001). Higher amounts of lead exposure can cause difficulties during pregnancy as well as learning and behavioral issues in young children (Canfield *et al*., 2003; Shannon *et al*., 2003). Previous research has shown that calabar stone causes ovarian follicle atresia, vacuolation, and necrosis Oyewopo *et al*., (2017), fragmentation of the liver parenchymal cells and dilatation of hepatic sinusoids Ekong *et al*., (2013), demineralization of the femur bone and change in growth rate Ekong *et al*., (2013), (2012). Calabar stone was also shown to produce spleen enlargement (Ekong *et al*., 2009), oedema and hemorrhage in the stomach mucosa, and koilocytic and hyperkeratosis alterations in the oesophageal mucosa (Ekong *et al*., 2012). The study was carried out in order to assess the effect of calabash chalk on the uterus, which serves as the conduit for reproduction.

Study justification

Because calabar stone intake contains lead, arsenic, and other neurotoxic compounds that alter neuronal components of the body, it is vital to investigate the potential effects on the testes using adult male Wistar rats.

The Goals and Objectives

The study's goal is to determine the effect of calabar stone on the testis of adult male Wistar rats. The following objectives will be used to attain the study's goal:

Experimental animals' acclimatization

(ii) Preparation, storage, and administration of calabar stone solution (iii)Histopathological examination of the testis (iv)Statistical data analysis with one-way ANOVA and SPSS version 2.0.

CHAPTER TWO

2.1 LITERATURE REVIEW

Calabash chalk is a geophagic substance that is commonly consumed for pleasure in West African countries, as well as by pregnant women as a sickness remedy (Abrahams *et al*., 2013; Akpantah *et al*.,2010; Anon 2012; Dean *et al*., 2004; Ekong *et al* 2012; Moses *et al*., 2012). The practice of eating the ground, particularly dirt and chalk, is known as geophagia. This is not a new or outdated habit that can be related with religious beliefs, medication, or as part of a regular diet (Dean *et al*., 2004; Ekong *et al* 2012; Moses *et al*., 2012). This act may expose the consumer to harmful chemicals and parasites contained in the earth consumed (Ekong *et al*., 2012)

(Obidike 2021)

Calabash chalk is also known as calabar stone (English), la craie or argile (French), mabele (Lingala in Congo), nzu (Igbo of Nigeria), ndom (Efik/Ibibio Nigeria), and eko (Bini/Edo Nigeria). It is also referred to as ebumba, poto, and ulo. Ekong *et al*., 2012; Abrahams *et al*., 2013) It is known as shilè in Ghana and umcako in Zulu (South Africa)

Location

Calabash chalk is mostly found in Nigeria and other West African regions. Although this geophagic material is native to Africa, it may also be found at ethnic stores in the United Kingdom, Canada, and the United States as a result of West African migration (Abrahams *et al*., 2013; Moses *et al*., 2012) Calabash chalk is also consumed by women of African heritage in the United States state of Georgia (Moses *et al*., 2012).

Composition

Calabash chalk is a natural substance made up of fossilized sea shells. It can, however, be created artificially by combining clay, sand, wood ash, and even salt. The calabash chalk is made by shaping and heating this mixture (Ekong *et al*., 2012) It comes as powder, molded shapes, and blocks (Dean at al., 2004; Ekong *et al*., 2012).

Uses

People of African origin of all sexes and ages engage in the practice of eating calabash chalk for recreational purposes. However, it is popular among pregnant women who claim it avoids vomiting, over-salivation, and nausea (Abrahams *et al*., 2013). Calabash chalk is also used in the production of facial masks and soaps.

Health Risk

Geophagical materials have the potential to cause clinical or sub-clinical harmful effects when consumed and come in touch with digestive fluids (Abrahams *et al*., 2013). There have been various studies on the health dangers of taking calabash chalk, including changes in hemoglobin concentrations, red blood cell counts, and erythrocyte sedimentation rate. Another potential negative effect of consuming this geophagia is a change in growth rate and demineralization of the femur bone (Ekong *et al*., 2011) According to some studies, calabash chalk causes a variety of gastrointestinal ailments such as nausea, ulcers, and gastritis. This is due to histomorphological alterations in the stomach and esophagus caused by calabash chalk (Moses *et al*., 2012).

Calabash chalk on the uterus

Calabash chalk caused histological abnormalities in the rats' uterus. This study found that calabash chalk had a deleterious effect on the uterus of adult female Wistar rats, whose tissues are similar to those of humans. According to the findings of this study, calabash chalk may affect growth rate and create histological alterations in the uterus, which could be harmful to the reproductive system and health. This chemical should be avoided by women, especially those of reproductive age (Opara and Nwagbaraocha 2018).

Calabash chalk induced histological abnormalities in the stomach and esophagus, which could lead to various pathophysiological disorders (Ekong *et al*., 2011).

Obidike identified ten (10) reasons why calabar chalk should be avoided in 2021. Among these causes are:

1. **Empty nutrition:** Calabar chalk has no nutritional value for the body because it lacks carbohydrate, protein, dietary fiber, fats, and vitamins.
2. **Calabar chalk is addictive:** The salty and earthly smell that reminds people of the first fall of rain, similar to nicotine or drugs, can lead people to consume more calabar chalk.
3. **Ingestion of toxic chemicals:** Calabar chalk is primarily made up of aluminium silicate hydroxide, which is a form of kaolin. It also has a lead content of 10-50mg/kg. An amount of lead in food that exceeds the acceptable value of 1mg/kg. Furthermore, it contains arsenic, chromium, aluminum, silicon, alpha lindane, endosulfan 11, and other organic pollutants that are known to harm the body (Dean *et al*., 2004).

4. **Risk of infertility:** A 2021 study discovered that regular and sustained ingestion of calabar chalk in animal models may affect growth rate and create changes to the uterus- which could be harmful to the reproductive system. It was recommended that women of reproductive age avoid consuming calabar chalk (Opara and Nwagbaraocha 2018).
5. **Toxicity in pregnancy:** Consuming calabar chalk during pregnancy was discovered to have a harmful effect due to its high lead and arsenic concentration. One study in pregnant rats given calabar chalk discovered that calabar chalk consumption induced pregnancy loss, stillbirth, and decreased mother weight gain and foetal growth (Aprioku and Ogwo-Ude 2018).
6. **May cause developmental issue in Children:** Toxic minerals, such as lead, can cause slower growth and developmental difficulties in infants and developing children because lead quickly crosses the placenta to the foetus during pregnancy. One study discovered high levels of lead in the blood of the umbilical cord of pregnant women who drank calaber chalk, implying that lead might readily be passed from mother to newborn during pregnancy (Brice *at al*., 2017).
7. **May affect bone health:** Pregnant mothers who consume calabar chalk may unwittingly transfer lead to their unborn children, resulting in delayed bone formation. In one animal investigation, rats ingested calabar chalk, which induced demineralization of the femur bone and affected their growth rate (Ekong *et al*., 2012).
8. **Increase risk if digestive issues:** Calabar chalk can cause constipation, stomach distress, nausea, and vomiting due to the excessive ingestion of hazardous compounds. Edema and bleeding were discovered in the stomachs of animal models fed 40mg/ml calabar chalk for 14, 21, and 28 days, respectively (Moses *et al*., 2012).

9. **Increase risk in anaemia:** Calabar chalk ingestion on a regular and extended basis might result in anaemia, particularly in women of childbearing age. According to one study, this chalk alters the concentrations of haemoglobin, red blood cell count, platelet count, and erythrocyte sedimentation rate in rats (Akpantah *et al*., 2010).
10. **Risk of parasitic infestation:** Most calabar chalk producers are simply concerned with monetary gain and will go to any length to maximize profits, including mining the chalk from contaminated soil surfaces with faeces and organic debris. This method's chalk may include parasitic species' eggs, such as tapeworm and other nematodes, resulting in infestation (Akpantah *et al*., 2010).

ORGAN OF STUDY: TESTES

TESTES EMBRYOLOGY

The gonads are formed from three sources: mesothelium (the epithelium that lines the posterior abdominal wall), underlying mesenchyme (embryonic connective tissue), and primordial germ cells (the first undifferentiated germ cells) (Moore *et al* 2016).

The genital or gonadal ridges arise first as a pair of longitudinal ridges. They are generated by epithelial proliferation and condensation of underlying mesenchyme. It is not until the sixth week of development that germ cells arise in the vaginal ridges. Primordial germ cells form in the epiblast, travel via the primitive streak, and by the third week are found in the yolk sac wall near the allantois, amid endoderm cells. They travel through the dorsal mesentery of the hindgut as ameboid movement during the fourth week, arriving to the primitive gonads at the beginning of the fifth week and entering the genital ridges in the sixth week (Saddler, 2015).

2.2 TESTES ANATOMY IN GENERAL

The testes are male sex glands that have both endocrine and exocrine (release of sperm) functions. The testes are two oval-shaped reproductive organs located in the scrotum and separated by the scrotal septum. The testes are bean-shaped and range in size from three to five centimeters in length and two to three centimeters in breadth. When palpated via the scrotum, the testes are smooth and velvety. The spermatic cord suspends the superior portion of the testes. The scrotal ligament, a gubernaculum remnant, binds the testes to the scrotum at the inferior end. In general, the left Testes is connected somewhat lower than the right Testes (Tsili *et al*., 2019; Yang, Workman and Wilson, 2018).

THE TESTES HISTOLOGY

The tunica albuginea is a thick fibrous membrane that covers each testes. The tunica albuginea connective tissue grows into a thick mass in the posterior region of the testes, projecting into the testes substance to produce the mediastinum testes. Septa connect the mediastinum testes to the tunica albuginea, which divides the testes material into many lobules. Each lobule has one or more highly convoluted seminiferous tubules and is generally conical in shape. These tubules are bordered with cells involved in the generation of spermatozoa (Singh, 2011).

LEYDIG CELLS OR INTERSTITIAL CELLS

The crevices between the seminiferous tubules within a lobule are filled with connective tissue including mast cells, macrophages, neurons, lymphatics, and blood arteries, including fenestrated capillaries. Interstitial cells, also known as Leydig cells, grow into giant round or polygonal cells with central nuclei and eosinophilic cytoplasm rich in tiny lipid droplets throughout puberty. These cells generate testosterone, which aids in the development of secondary male sex characteristics.

TUBULES SEMINIFEROUS

Each seminiferous tubule is lined by germinal or spermatogenic epithelium, a complex, specialized stratified epithelium. This epithelium's basement membrane is covered by fibrous connective tissue, with an innermost layer containing flattened, smooth muscle-like myoid cells. The germinal epithelium is made up of big non-dividing Sertoli cells and dividing spermatogenic lineage cells (Mescher., 2016).

In the young adult, sperm is generated at a rate of around 2 108 per day in the seminiferous tubules. Each testes has between 250 and 1000 tubules in its lobules, each measuring 150-250m in diameter and 30-70cm in length. The overall length of one testes' tubules is approximately 250m (Mescher., 2016).

CELLS FROM SERTOLI

Sertoli cells are tall columnar epithelial cells that feed spermatogenic cells and split the seminiferous tubules into two compartments (basal and adluminal). All spermatogenic lineage cells are intimately linked to the expanded surfaces of Sertoli cells and rely on them for metabolic and physical support. Sertoli cells sustain around 30-50 developing germ cells. Sertoli cells are found to have a lot of smooth endoplasmic reticulum, some rough endoplasmic reticulum, golgi complexes, mitochondria, and lysosomes. Their nuclei are ovoid or triangular in shape, euchromatic, and feature a conspicuous nucleolus. These characteristics distinguish Sertoli cells from neighboring germ cells (Mescher., 2016). Sertoli cells perform a variety of activities which includes:

Developmental support, protection, and nourishment of spermatogenic cells (Mescher., 2016).

Sertoli cells expel water containing fresh sperm from the Testes into the seminiferous tubules (Mescher., 2016).

During spermiogenesis, Sertoli cell lysosomes phagocytize and digest excess cytoplasm discharged as residual bodies (Mescher., 2016).

SPERMATOGENESIS

Spermatogenesis is the process through which male gametes (spermatozoa) are generated from primitive spermatogenic cells (spermatogonia) in the Testes. The spermatogenic cells have cytoplasmic attachments with sertoli cells, which supply all of the required ingredients for spermatogenesis via the cytoplasmic connection (Sembulingam., 2012). Spermatogenesis is separated into four phases namely: Stages of proliferation, growth, maturation and transformation

• **Proliferation Stage**: Each spermatogonium has diploid chromosomes (23 pairs). Each pair has one member of maternal and one of paternal ancestry (Sembulingam., 2012). There are 22 autosomal chromosomal pairs and one sex chromosome pair among the 23 pairings (X or Y chromosome). Spermatogonia divide by mitosis throughout the proliferative stage, with no change in chromosomal number. The last generation of spermatogonia begins the stage of development as a primary spermatocyte. Spermatogonia, like Sertoli cells, move to the lumen of the seminiferous tubule (Sembulingam., 2012).

• **Growth Stage**: At this stage, the initial spermatocyte develops into a big cell. Apart from growth, no further changes occur in spermatocytes at this stage (Sembulingam., 2012).

• **Maturation Stage**: Once mature, each primary spermatocyte swiftly undergoes meiotic or maturation division, which happens in two stages: Each main spermatocyte differentiates into two secondary spermatocytes during the first phase and gets only a haploid number of chromosomes (22 autosomes and an X or a Y chromosome). In the second phase, each secondary spermatocyte divides for the second time, producing two haploid cells known as spermatids (Sembulingam., 2012).

• Transformation Stage: There is no more separation. Spermatids are converted into developed spermatozoa (sperms) and discharged during spermination (Sembulingam., 2012).

TESTOSTERONE SECRETION REGULATION

The Leydig cells are stimulated by luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH), and the amount of testosterone released is directly proportionate to the amount of LH available (Sembulingam., 2012). LH secretion from the anterior pituitary gland is triggered by hypothalamic luteinizing hormone releasing hormone (LHRH). The negative feedback mechanism of testosterone controls its own secretion (Sembulingam., 2012). It operates on the hypothalamus, inhibiting LHRH secretion. When LHRH production is blocked, LH is not produced from the anterior pituitary, causing testosterone secretion from the testes to cease (Sembulingam., 2012). When testosterone synthesis is low, a lack of inhibition of the hypothalamus leads to testosterone release via LHRH and LH (Sembulingam., 2012).

SPERMIOGENESIS

Spermeogenesis is the process through which spermatozoa develop from spermatids (Sembulingam., 2012). Changes that occur during spermeogenesis include: Nuclear material condensation, the formation of acrosome, mitochondrial spiral filament, and tail structures and extraction of superfluous (non-essential) cytoplasm (Sembulingam., 2012).

GROSS TESTES BIOLOGY

The testes are the male gonads that seem to be identical to the female ovaries. It is a double ovoid/ellipsoidal generative organ responsible for spermatozoa generation and testosterone discharge (Singh V., 2014). It weighs approximately 10-15g and spans about 4cm long, 2.5cm wide, and 3cm anteroposteriorly. The overall architecture of the male reproductive systems in rats mimics that of men.

EXTERNAL FEATURES OF THE TESTES

The testes have two poles (upper and lower), two borders (anterior and posterior), and two surfaces (medial and lateral) (Singh, 2014). Both poles are convex and smooth, with the upper pole connecting to the spermatic cord. The testicular appendix is a tiny oval structure that is frequently observed connected to the top pole of the Testes (Singh, 2014). The anterior border is spherical and entirely covered by tunica vaginalis, whereas the posterior border is straight, partially covered by tunica vaginalis, and serves as an attachment point for the epididymis (Singh, 2014). The epididymis sinus is an outgrowth of the tunica vaginalis cavity that separates the epididymis from the Testes on the lateral side (Singh, 2014). Both surfaces are smooth with a modest convexity. Singh V. (2014)

COVERINGS OF THE TESTES

TUNICA VAGINALIS

The tunica vaginalis is the testes's most superficial covering. The persisting lower section of the processus vaginalis is represented by a serous sac. It is invaginated from behind by the Testes, resulting in outer parietal and inner visceral layers with a possible void between them (Singh, 2014). On the front and lateral sides of the testes, the outer parietal layer lines the scrotum while the inner visceral layer covers the tunica albuginea (Mescher., 2016).

TUNICA ALBUGINEA

It is a dense layer of fibrous tissue that surrounds the testes. Except where it comes into direct contact with the epididymis, it is covered with tunica vaginalis (Singh, 2014). The tunica albuginea thickens on the testes's posterior side to form the mediastinum Testes (Mescher, 2016).

TUNICA VASCULOSA

It is the testes's deepest vascular layer, lining the testes's lobules (Singh, 2014).

ARTERIAL SUPPLY

The testicular artery starts from the abdominal aorta and descends into the scrotum via the inguinal canal to nourish the testes and epididymis (Drake *et al*., 2015). It separates into several little branches and two major branches (medial and lateral) at the posterior border of the Testes. The tunica vasculosa is formed by the medial and lateral branches piercing the tunica albuginea and ramifying on the surface of the testes lobules (Singh, 2014).

VENOUS DRAINAGE

Venous blood from the testis is drained through the pampiniform plexus of veins, which is made up of veins issuing from the testis. This plexus ascends and condenses at the superficial inguinal ring to generate four veins that pass through the inguinal canal within the spermatic cord. They connect at the level of the deep inguinal ring to generate two testicular veins that follow the testicular artery. The testicular vein empties into the inferior vena cava at an oblique angle on the right side, whereas it drains into the left renal vein at a straight angle on the left (Singh V., 2014).

LYMPHATIC SUPPLY

The lymph veins from the testis drain into the pre-aortic and para-aortic group of lymph nodes at the second lumbar vertebral level (Singh V., 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1Collection and identification of Calabash chalk.

Calabash chalk was obtained at Uselu market in Egor Local Government area of Edo state, Nigeria.

The substance was identified at the department of anatomy, University of Benin, Edo State, Nigeria.

3.1.2 Experimental animals

Twenty Wistar rats weighing between 150g and 193g were procured and housed in the Department of Anatomy Animal House unit for a period of two weeks for acclimatization. They were kept in clean, well-ventilated aluminium cage at room temperature with a natural light schedule of 13 hours daylight and 11 hours of darkness, and they were fed a conventional laboratory feed of water and feed at regular basis.

3.1.3 Equipments

Automatic tissue processor (Manufactured by Thermo SCIENTIFIC), centrifuge (Manufactured by LMG international Inc, microwave oven (Lot No 910381507E), water bath (Manufactured by MEDIFIED EQUIPMENT AND SCIENTIFIC LTD with No DK-420), rotary microtome (Manufactured by Leica Microsystem Inc with Serial No. 5461752.2013), weighing scale (manufactured by KERRO Laboratory with Model No. BL-P3/6002).

Laboratory mortar and pestle, separating funnel, filter paper, , evaporating dish, spatula, , plastic tubes, syringes, canula, timer, chloroform chamber, scalpels, scissors, forceps, blades, icebox, ice, , Pasteur pipettes, Apetman Micropipettes, test tubes, curvettes, spectrophotometer, beakers, measuring cylinders, eppendorf microcentrifuge tubes, plastic homogenizer, , gel electrophoresis machine, gel documenting machine, couplin jars, funnel, , glass slides, cover slips, drying racks, light microscope, Armscope MD900

3.2 Methods

3.2.1 Preparation of extract

Calabash chalk, which is a solid mast, was hand-ground to powder and then placed in a basin of water until entirely dissolved. It was then carefully poured into a storage container before being administered to the animals.

3.2.2 Determination of dose

LD50 of calabash chalk was determined to be greater than 5000mg/kg (Ekong *et al*., 2015) and this was used to set the dose for administration.

3.2.3 Experimental Design

The twenty (20) rats were divided into four groups, each with five animals with an average weight of 170g, using the block method.

Group A served as the control group and the animals were given regular feed and water.

Group B served as low dose group and the animals were given 100mg/kg of dissolved calabash chalk along with regular feed and water.

Group C served as medium dose group and it animals received 200mg/kg of dissolved calabash chalk along with regular feed.

Group D served as the high dose group and the animals were administered with 400mg/kg of dissolved calabash chalk as well as regular food and water.

3.2.4 Totoxicity induction

A modified version of Carson and Pruett's binge-drinking model was used to cause testicular damage.

3.2.5 Sacrifice of animals

At the end of the trial, all twenty rats were sacrificed under moderate anaesthesia with chloroform. In preparation for regular histology procedures, the testes and epididymis were removed, weighed, and preserved in Bouin's solution.

Sperm cells were extracted from the vas deferens and analyzed for viability, morphology, and count using a counting chamber and a bi-ocular microscope.

3.2.6 SPERM ANALYSIS

During the rat sacrifice, sperm cells were retrieved from the epididymis by litigating the extremities. To let the sperm to disperse, the litigated extremities of the vas deferens were teased.

Under the microscope, the segment was examined for sperm motility. The outcomes were assessed using two factors. The following parameters were analysed:

1. A mean progressive motility score is used to indicate progressive (or qualitative) motility.

Progression of motility (PM), Non-progressive motility (NPM), Immovable (IM) and Total sperm count.

2. Percentage of motile spermatozoa usually presented with 5% intervals since the precision is not higher, and spermatozoa can show good motility and viability in seminal plasma 24hours after ejaculation but in some semen samples, the motility declines much faster.

Spermatozoa are picked from the petri dish using a micro Pasteur pipette and dispenses on a clean slide. It is then covered with a coverslip and viewed under the microscope (×10 and ×40) objective lenses. The percentage motility score was evaluated according to their nature as progressively motile, non-progressively motile and immotile (Itani, 2013).

Vitality test was carried out to evaluate the living and dead spermatozoa. This was done with a supra-vital stain (eosin Y) (Itani, 2013).

One volume of semen is milted with two volumes of eosin Y solution (1% in distilled water). After 30seconds three volumes of 10% nigrosine solution (in distilled water) was added and the sample is mixed. It is air dried using a thin smear. The smear can be viewed under normal oil immersion microscopy (×100 objectives). Live spermatozoa are unstained while dead spermatozoa are stained red (Itani, 2013).

Sperm morphology was evaluated making a smear of sperm cells on a grease-free slide and allowed to air dry. The sample was stained using the Bryan-Leishman technique (Besley *et al*., 1980) for 30mins. The smear was rinsed with distilled water, bloated and finally air dried. It was then viewed with oil immersion objective. The scores of results were recorded as a percentage of normal and abnormal sperm cells. Normal sperm shows normal characteristics of the head, axoneme, middle piece and a tail. Abnormal sperm cells were characterized by large heads, headless, tailless, bulgy mid-piece, curved tail and joined heads.

The sperm count is determined by first making a 1:20 dilution of the spermatozoa with 10% formal saline in a test tube. Then counting chamber is charged with a few drops of mixed solution into the chamber. It is viewed with the microscope with the ×10 objective lens. The sperm cells are counted and are scored in $10^6/mm^3$.

It is important to note that during these procedures the dilution must be precise and subsequent mixing should be carefully carried out. A suitable diluent is composed of 50g $NaHCO_3$, 10ml of 35^oC formalin, 5ml saturated aqueous gentian violet and distilled water up to 1000ml.

3.2.7 HISTOLOGICAL ANALYSIS

The tissues were dehydrated in ascending grades of alcohol (ethanol), cleared in xylene and embedded in paraffin wax. The deparaffinised sections were stained routinely with Haematoxylin and Eosin.

3.2.7.1 PARRAFIN TISSUE PROCESSING

Following fixation in 10% formal saline, the tissues were processed as shown below:

The tissue was dehydrated through 50% to absolute alcohol for one hour each; it was then cleared in xylene for one hour each; for three changes. Infiltrated was done in three changes of paraffin at 60C for one hour each. It was then embedded in molten paraffin wax. Sectioning was done at 5 microns thick using a Rotary microtome.

3.2.7.2 HAEMATOXYLIN AND EOSIN STAINING METHOD

Sections were brought to water through descending grades of alcohol. They were stained in iron Haematoxylin for 10-15 minutes. Excess stain was removed by washing under tap water. Differentiated in 1% acid alcohol for 10 seconds, it was blued in running tap water for 5 minutes. The sections were counter-stained with 1% Eosin for 5-10 minutes and rinsed in water. It was then dehydrated rapidly through 50% graded alcohol to absolute alcohol. It was cleared in xylene and mounted in DPX (Distrene Plasticizer and Xylene).

Digital photomicrographs of the tissue sections were taken at various magnifications. Photomicrographs were snapped with digital Armscope MD900

3.8 STATISTICAL ANALYSIS

The data will be analyzed using descriptive and inferential statistics. All values will be presented as the standard error of mean (SEM) for five rats each of the four groups. The significance of the difference in the means of all parameters will be determined using one-way analysis of variance (ANOVA; 95% confidence interval). All statistical analysis will be carried out using Statistical Package for Social Sciences (SPSS).

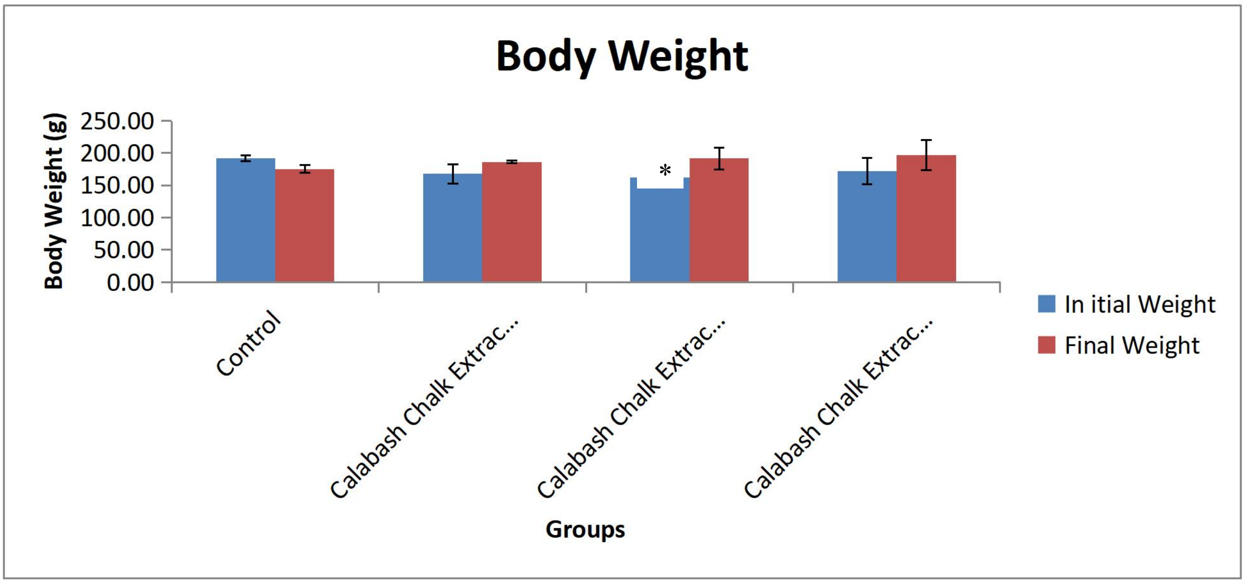
CHAPTER FOUR

Result

Results from the statistical analysis showed a significant increase (P<0.05) in the body weight of animals that received medium dose (200mg/kg) of calabar stone extract when compared with that of the control, a significant increase (P<0.05) was also observed in the testicular index of animals that received low dose (100mg/kg) of calabar chalk when compared to the control. In other parameters which include: Testicular weight, Progressive Motility, Non-Progressive Motility, and Immotile sperm cells, no statistically significant difference (P<0.05) was observed across the groups.

Result from the photomicrograph showed increased luminal diameter in the group that received high dose (400mg/kg) of calabar stone when compared to the animals in the control group.

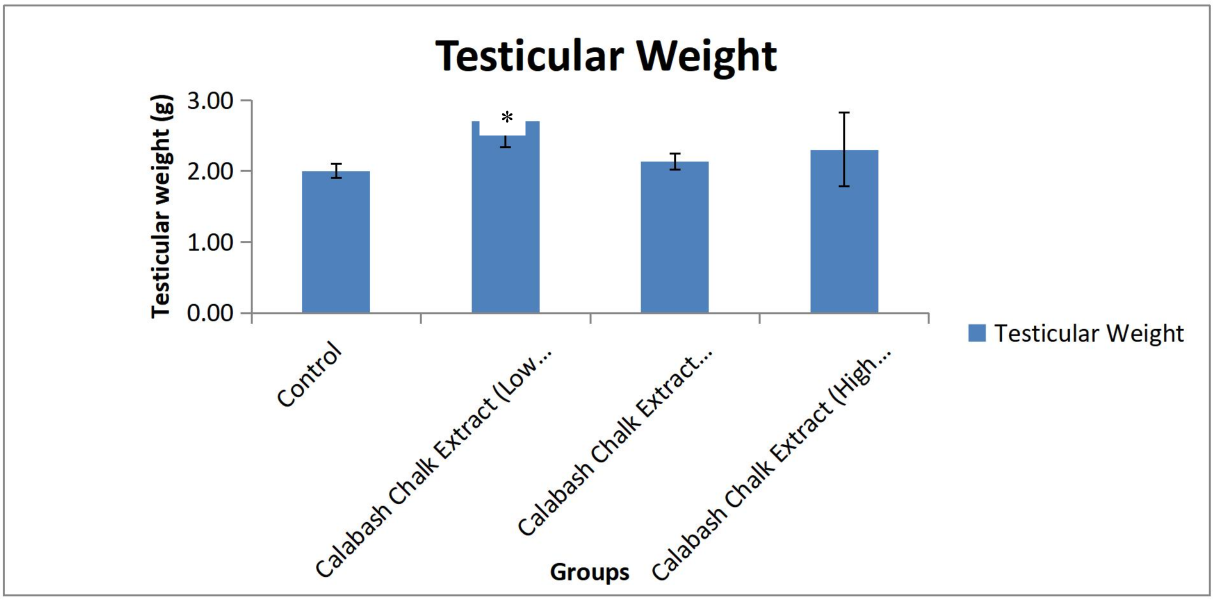
Statistical Analysis



* Significant difference from the control group.

Chart 1 Showing the Difference between initial and final Body weight after the period of experimentation across all groups.

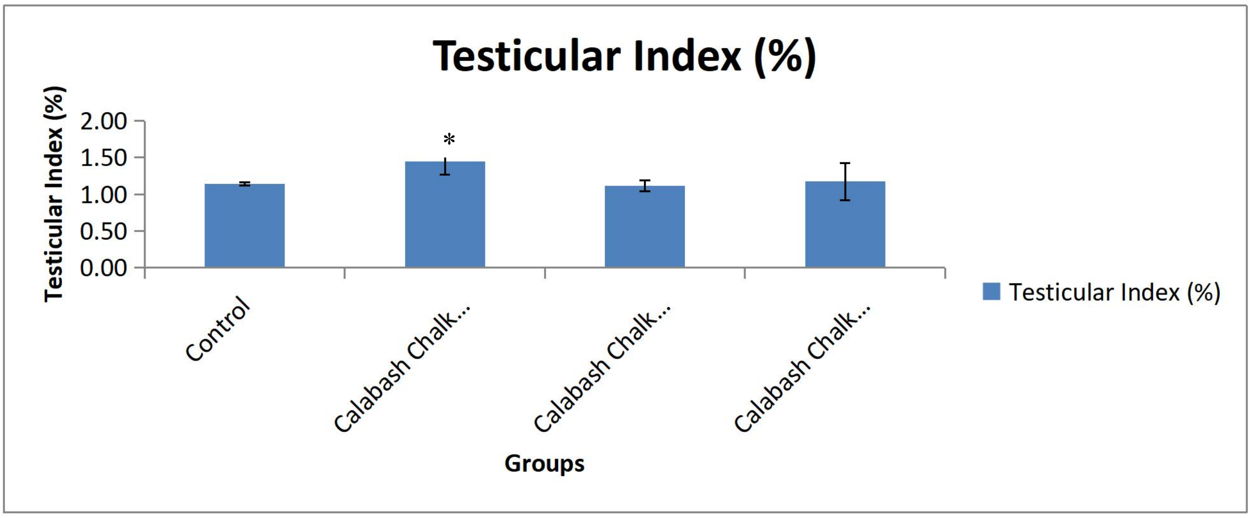
There was a statistically significant increase (P<0.05) in the body weight of animals in the group that received medium dose of extract as compared to the control group.



* Significant difference from the control group.

Chart 3 showing the testicular weight after the period of experimentation across all groups.

There was a statistically significant increase (P>0.05) in the testicular weight of animals in the group that received low dose of extract as compared to the control group.



* Significant difference from the control group.

Chart 4 Showing the testicular index after the period of experimentation across all groups.

There was a statistically significant increase (P>0.05) in the testicular index of animals in the group that received low dose of extract as compared to the control group.

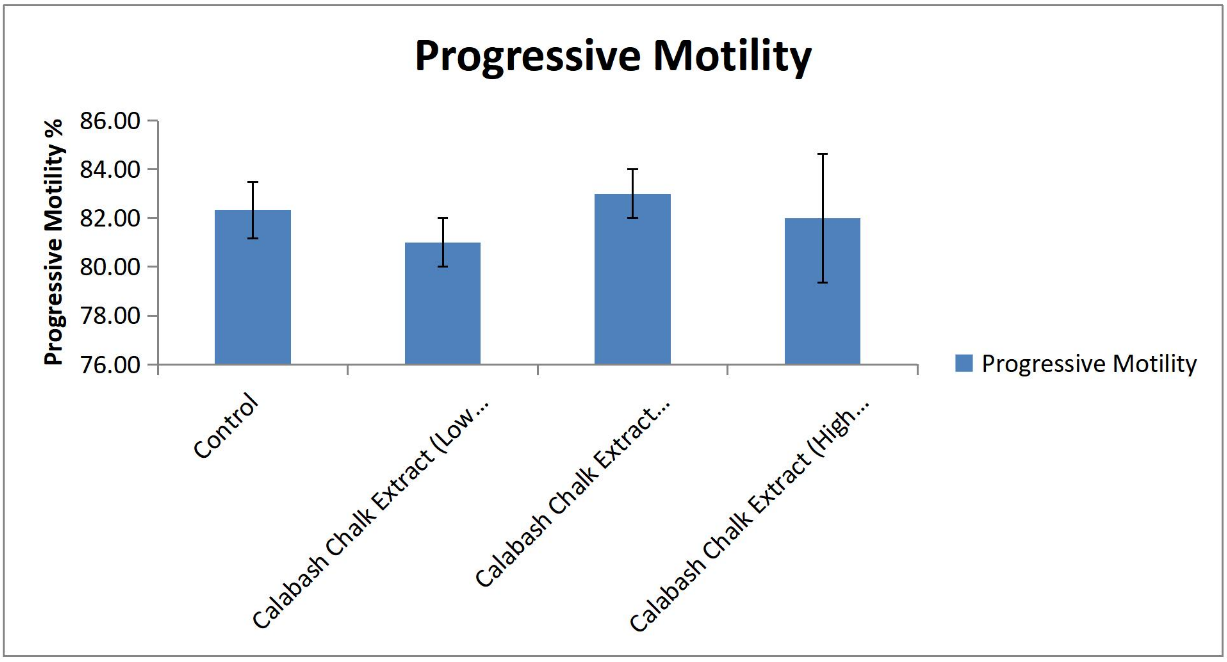


Chart 5 Showing Progressive motility weight after the period of experimentation across all groups.

There was no statistically significance difference across all groups

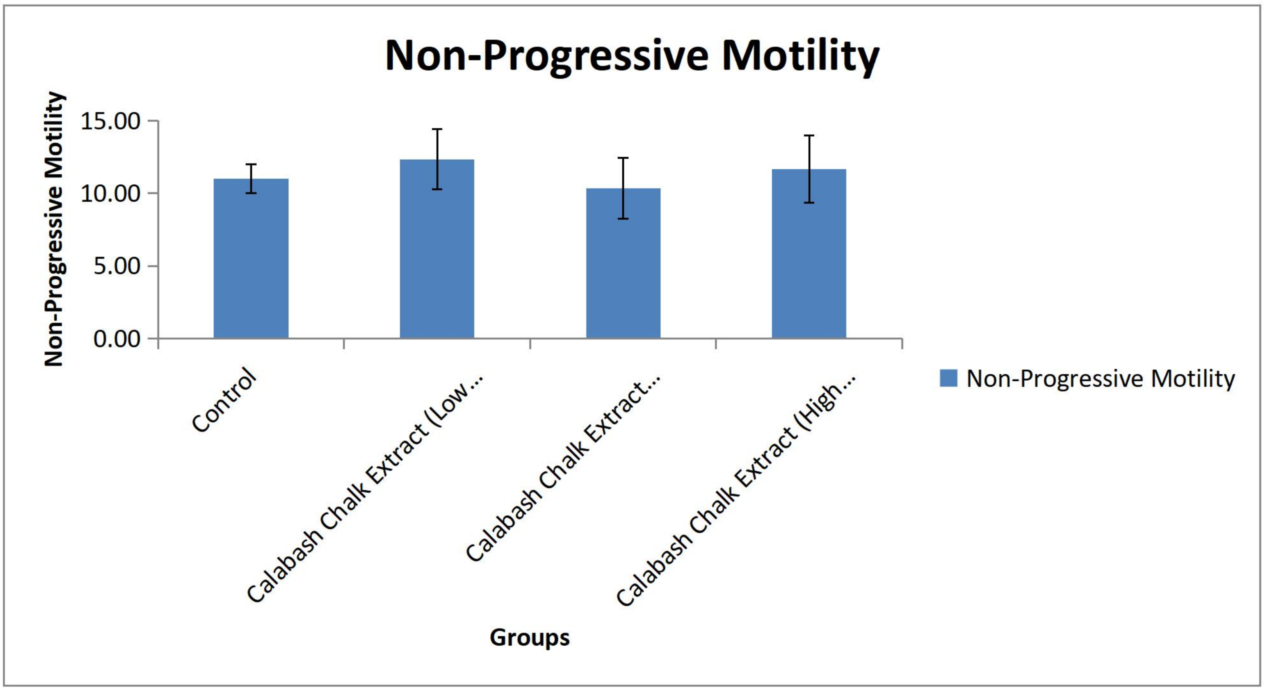


Chart 6 showing Non-Progressive motility after the period of experimentation across all groups.

There was no statistically significance difference across all groups

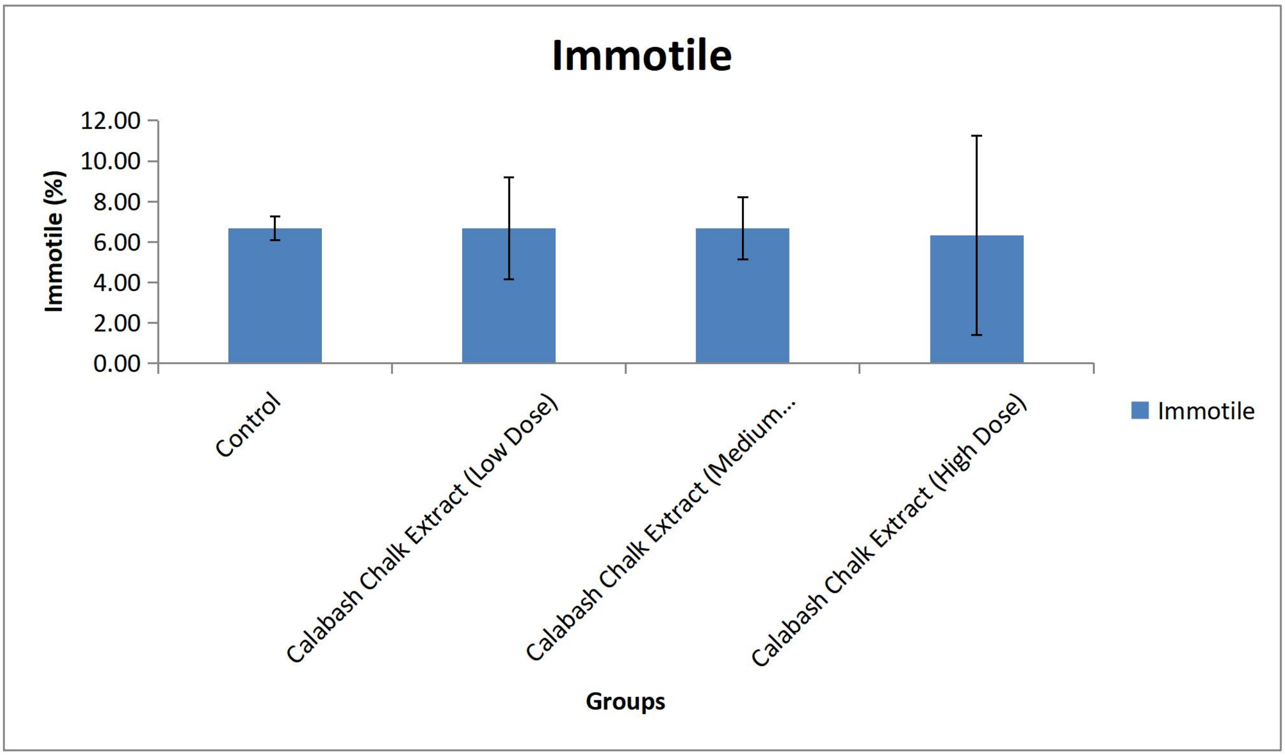


Chart7 Showing immotile sperm after the period of experimentation across all groups.

There was no statistically significance difference across all groups.

Tables

	Control	Calabash chalk extract (Low dose) (100mg/kg)	Calabash chalk extract (Medium dose) (200mg/kg)	Calabash chalk extract (High dose) (400mg/kg)	P- value
Progressive motility (%)	82.33±0.67	81.00±0.58	83.00±0.58	82.00±1.53	0.525
Non-progressive motility (%)	11.00±0.58	12.33±1.20	10.33±1.20	11.67±1.33	0.637
Immotile sperm cells	6.67±0.33	6.67±1.45	6.67±0.88	6.33±2.85	0.999

No statistical differences

	Control	Calabash chalk extract (Low dose) (100mg/kg)	Calabash chalk extract (Medium dose) (200mg/kg)	Calabash chalk extract (High dose) (400mg/kg)	P- value
Testicular weight (g)	1.02±0.04	1.26±0.09	1.08±0.04	1.08±0.13	0.287
Testiculosomatic index (%)	0.58±0.01	0.69±0.04	0.63±0.04	0.59±0.06	0.327

There was a statistically significant increase (P>0.05) in the testicular weight of animals in the group that received low dose of extract as compared to the control group.

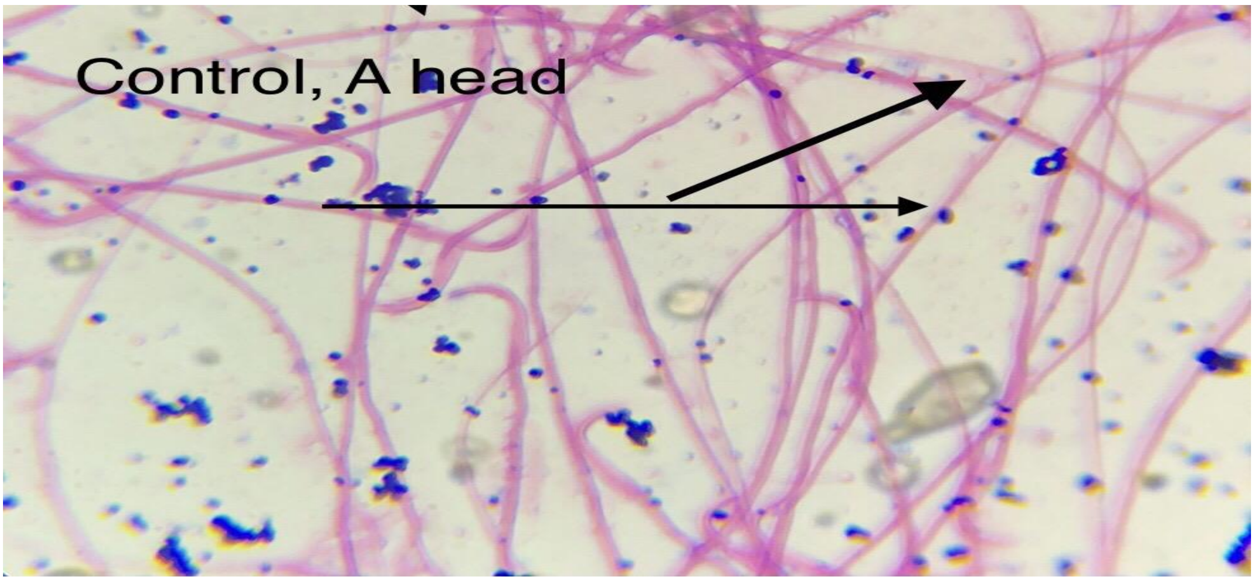
There was a statistically significant increase (P>0.05) in the testicular index of animals in the group that received low dose of extract as compared to the control group.

Body weight

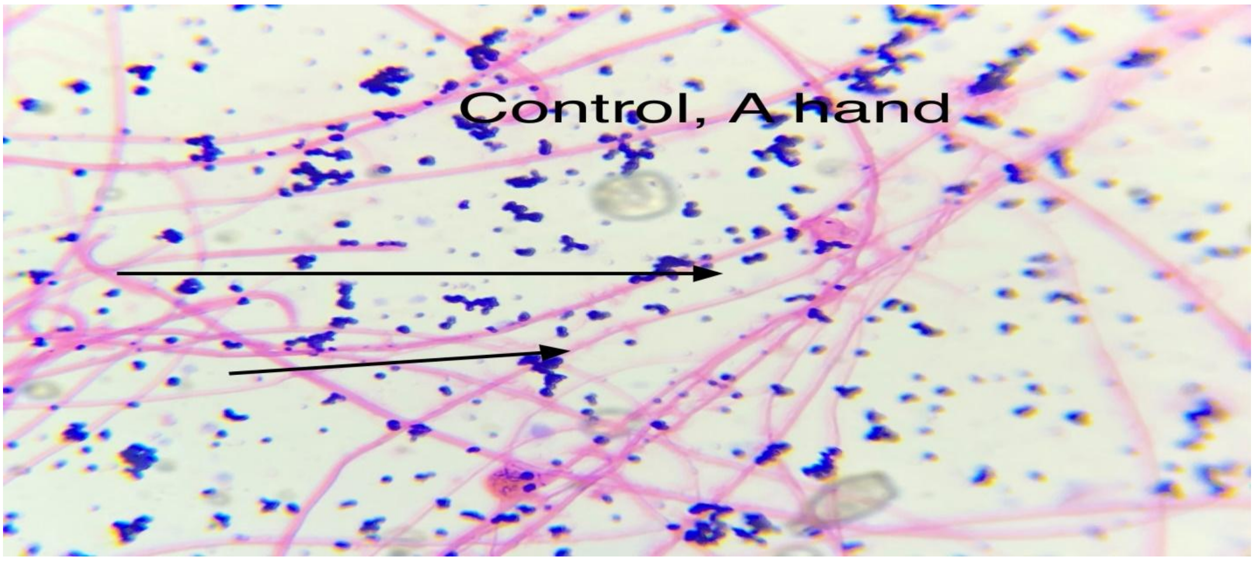
GROUPS	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	P-VALUE
Control	189.00±2.00	175.33±3.53	0.120
Calabash chalk extract (Low dose) (100mg/kg)	175.60±7.01	182.80±3.77	0.513
Calabash chalk extract (Medium dose) (200mg/kg)	170.00±12.50	175.00±18.45	0.863
Calabash chalk extract (High dose) (400mg/kg)	173.00±8.44	182.75±17.20	0.432

There was a statistically significant decrease (P>0.05) in the initial body weight of animals in the group that received medium dose of extract as compared to the control group.

Sperm Morphology

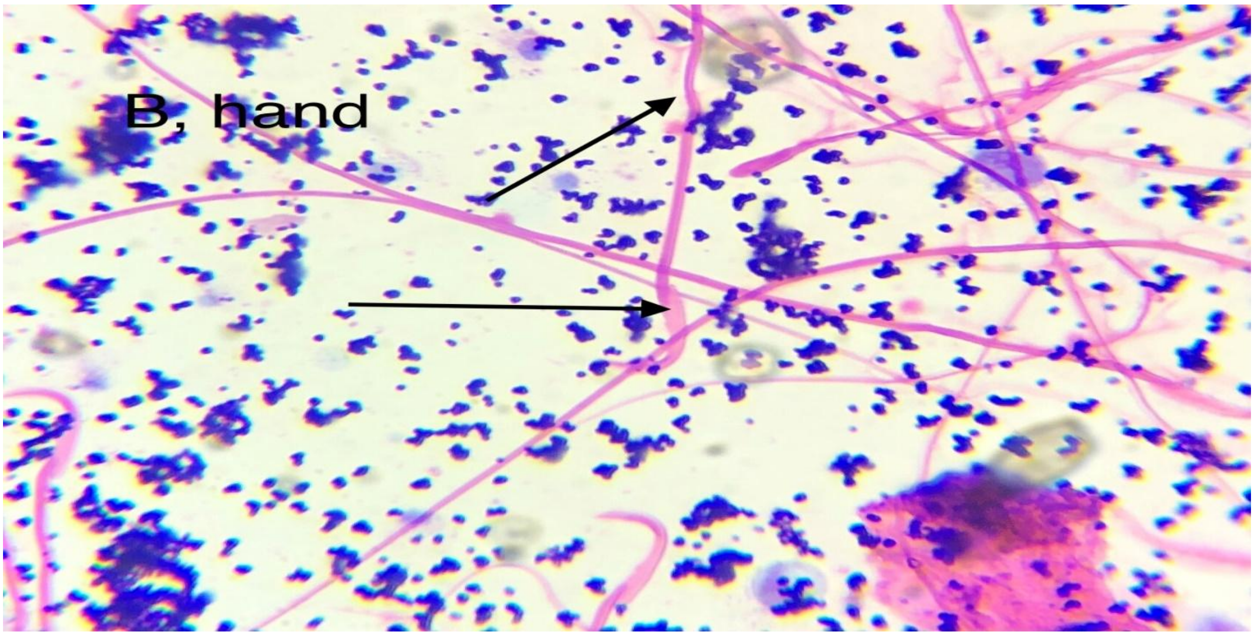


Slide 1 (control): Normal form sperm morphology shown with dark arrows

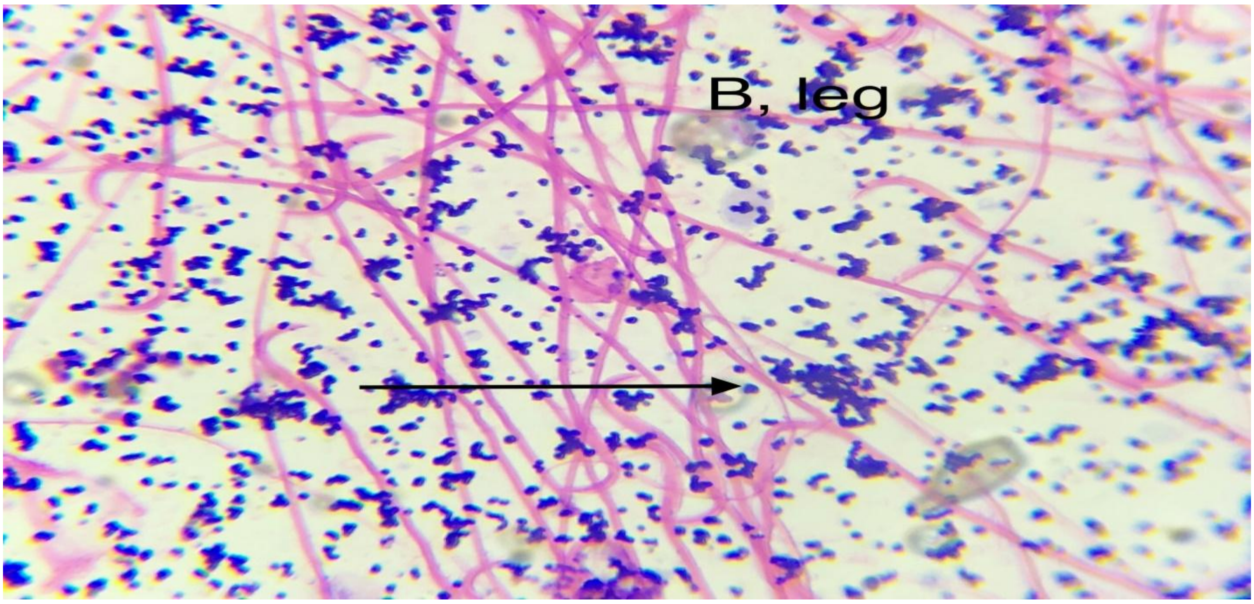


Slide 2 (control): Normal form sperm morphology shown with dark arrows

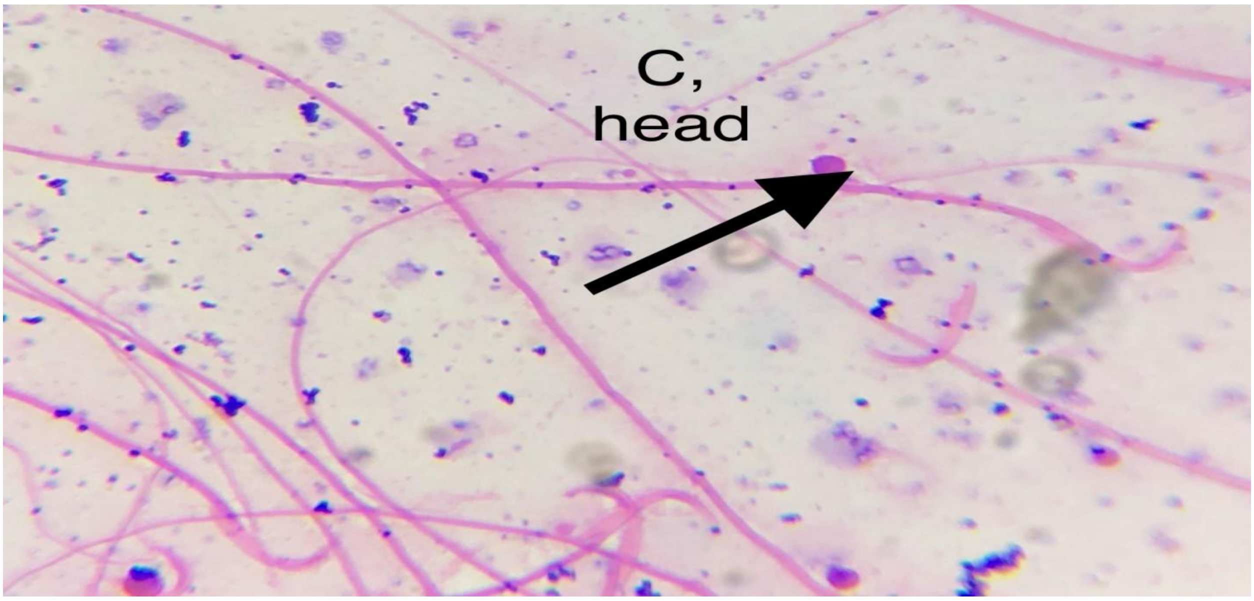
Slide 4 (control): Normal form sperm morphology shown with dark arrows



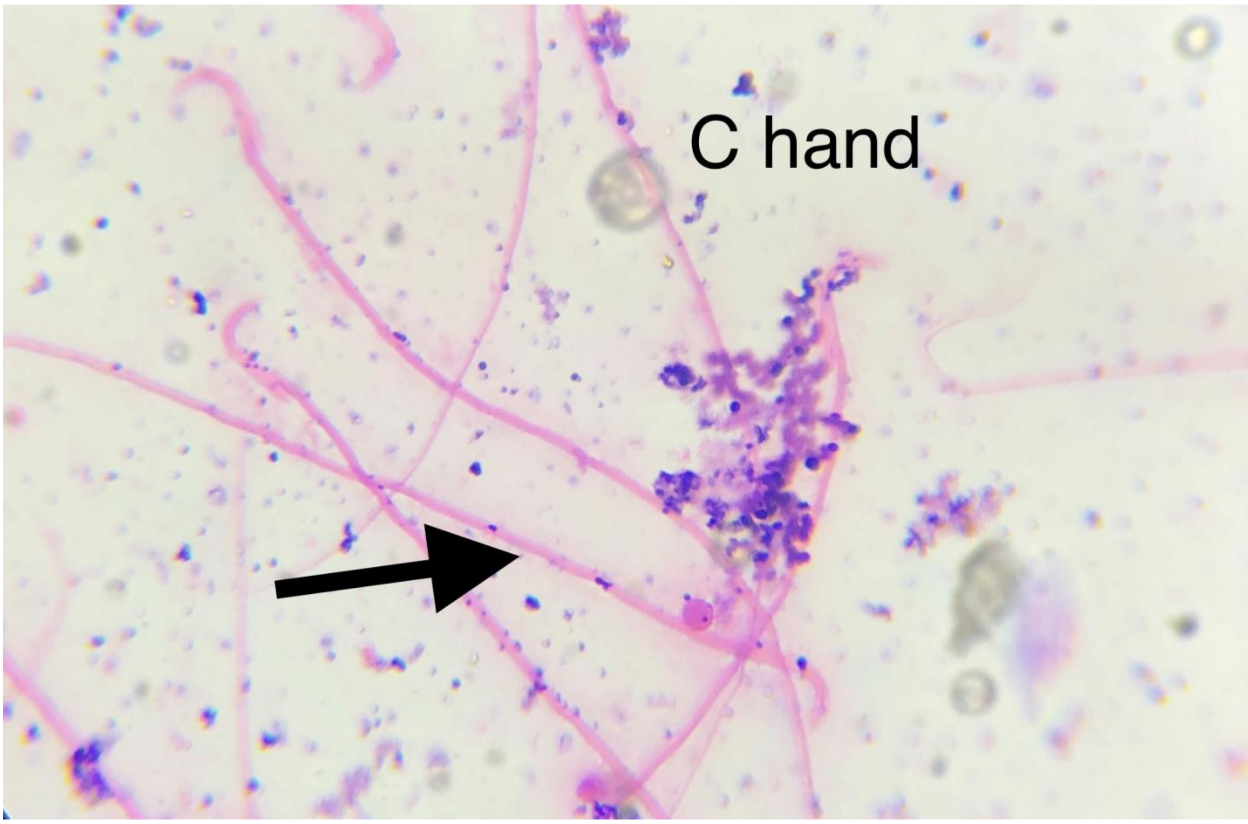
Slide 4 (GROUP B): Normal form sperm morphology shown with dark arrows



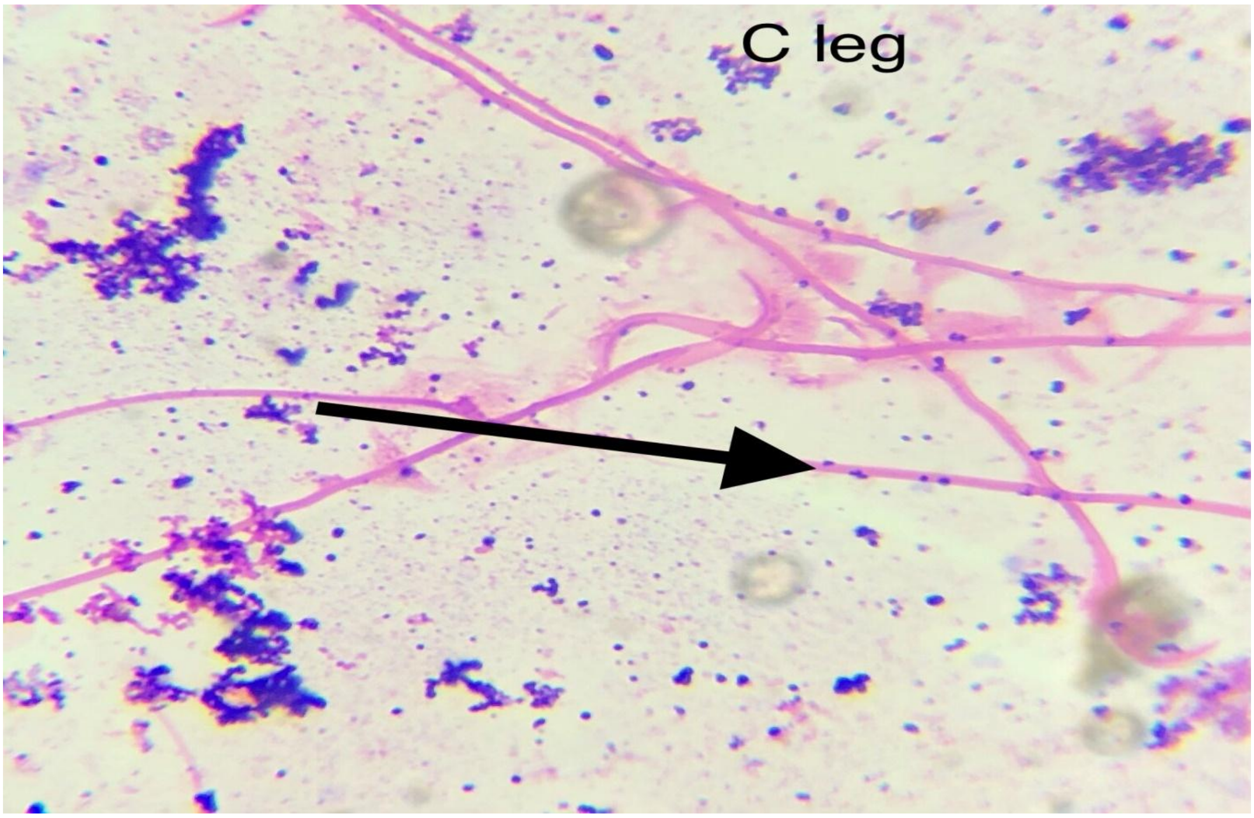
Slide 5 (group B): Normal form sperm morphology shown with dark arrows



Slide 6 (GROUP C): Normal form sperm morphology shown with dark arrows

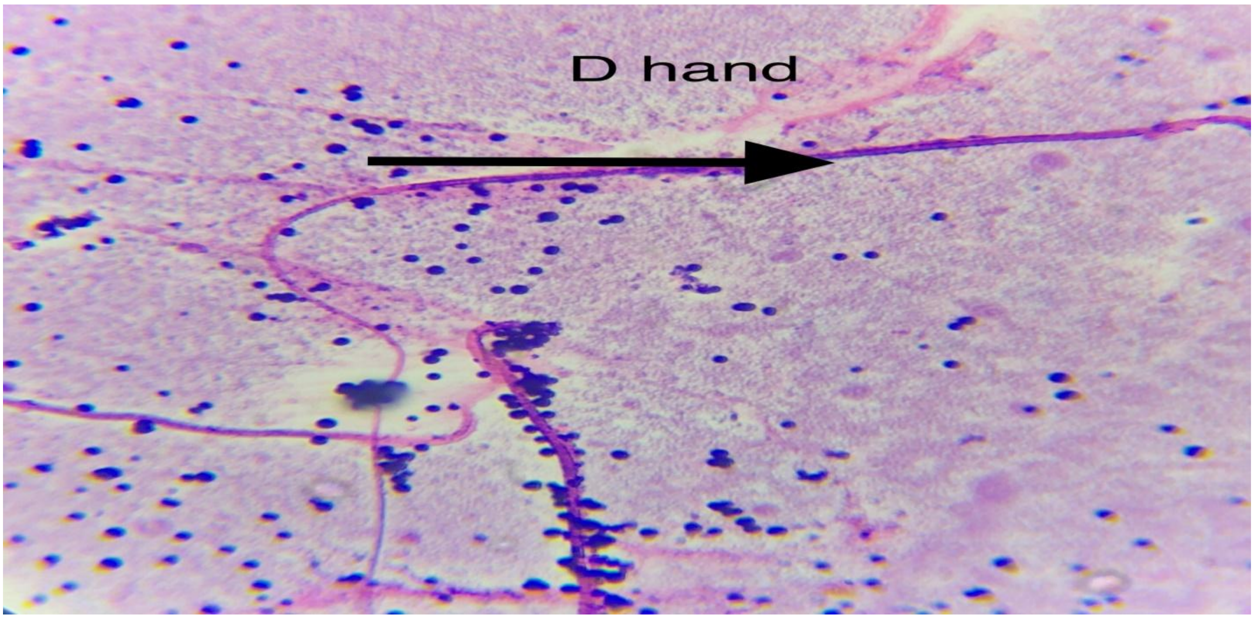


Slide 7 (GROUP C): Normal form sperm morphology shown with dark arrows



Slide 8 (GROUP C): Normal form sperm morphology shown with dark arrows

Slide 9 (GROUP D): Normal form sperm morphology shown with dark arrows



Slide 10 (GROUP): Normal form sperm morphology shown with dark arrows

Slide 11 (GROUP D): Normal form sperm morphology shown with dark arrows

Photomicrography

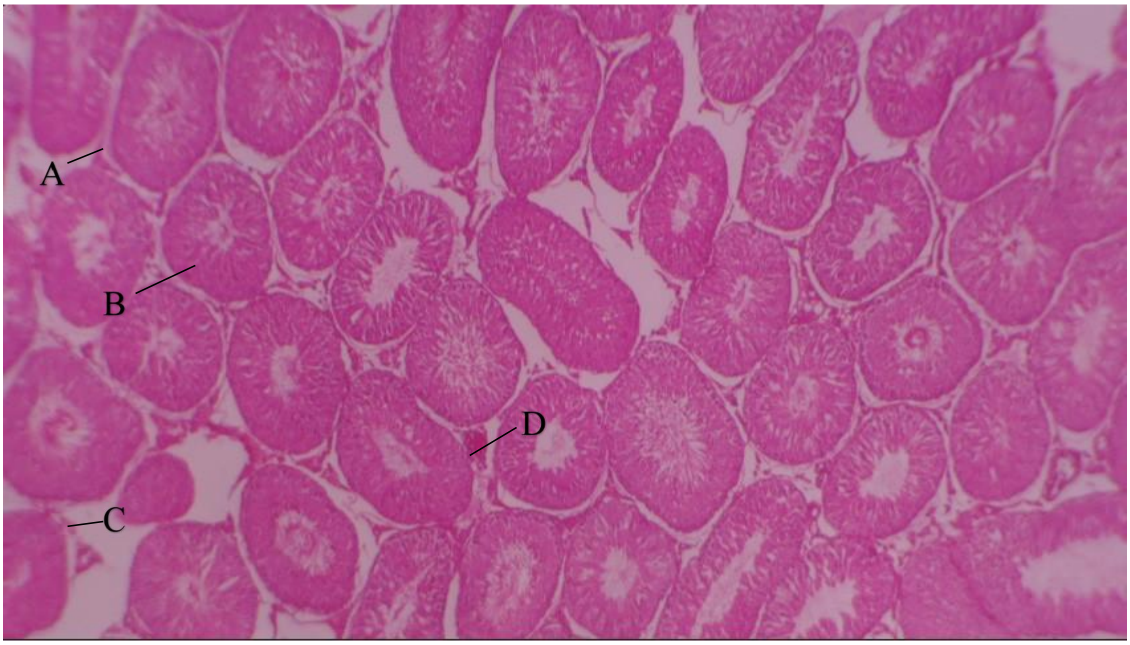


Plate 1. Rat testis. Control. Composed of normal testicular architecture: A. seminiferous tubules lined by spermatogenic series, B. Sertoli cells, C. Leydig cells, D. testicular artery (H&E x 40)

Plate 2. Rat testis. Control. Composed of normal testicular architecture: A. seminiferous tubules lined by spermatogenic series, B. Sertoli cells, C. Leydig cells D. testicular artery (H&E x 100)

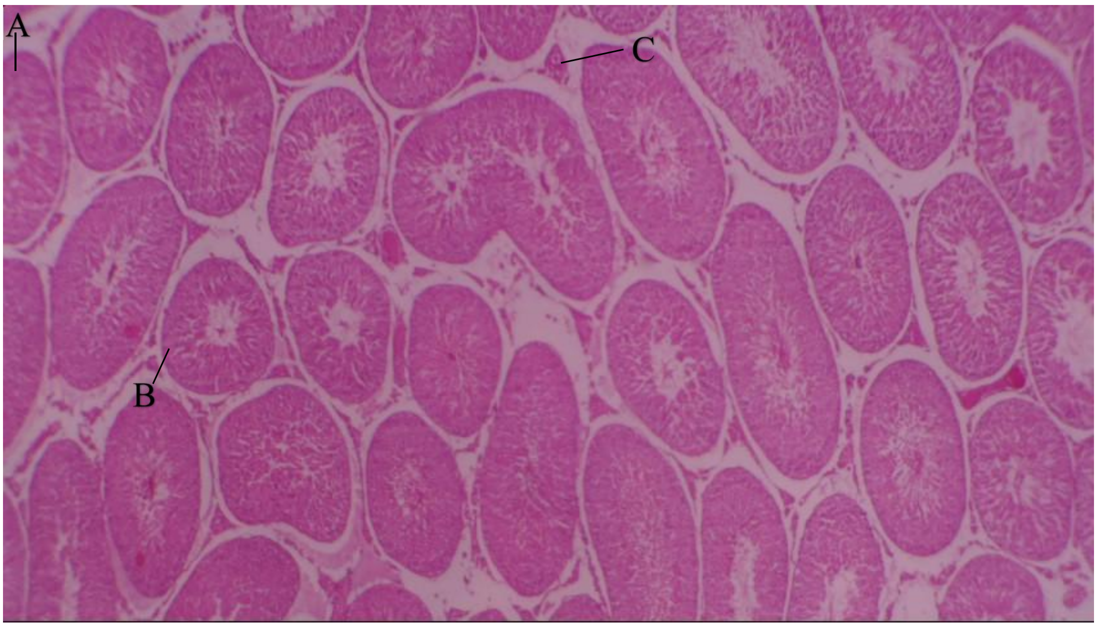


Plate 3. Rat testis given low dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, B. Sertoli cells, C. Leydig cell (H&E x 40)

A

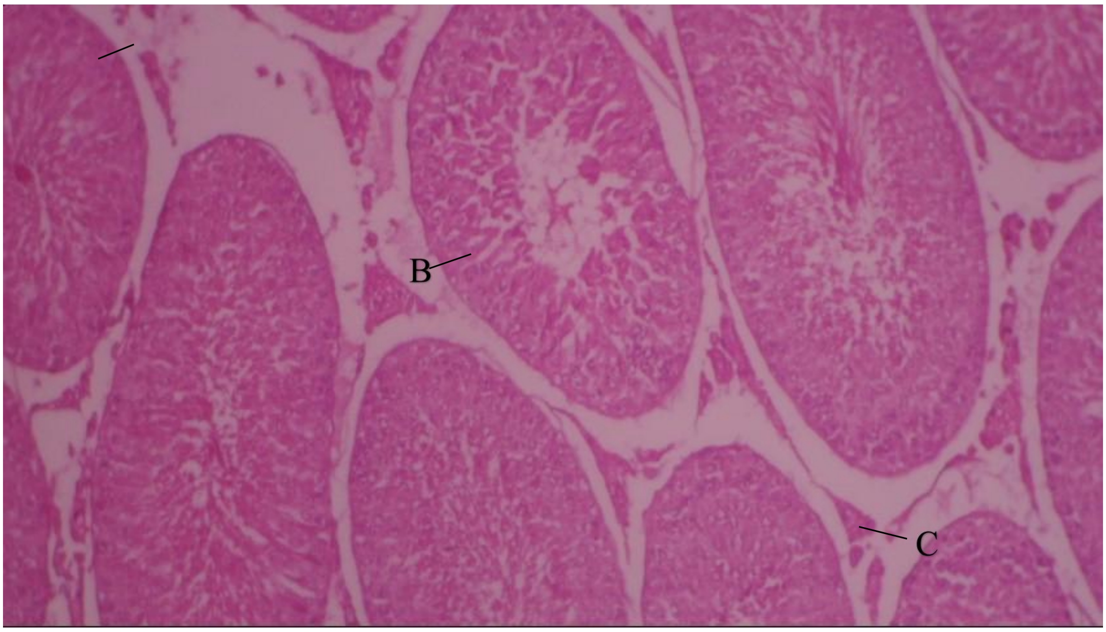


Plate 4. Rat testis given low dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, B. Sertoli cells, C. mild Leydig cell (H&E x 100)

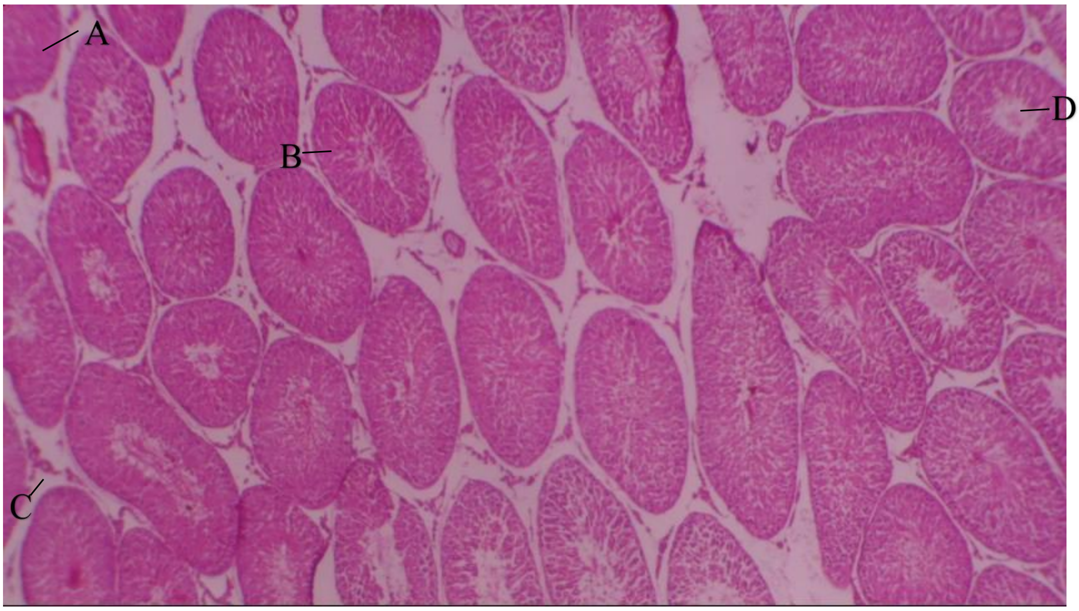


Plate 5. Rat testis given medium dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, B. Sertoli cells, C. mild Leydig cell D. Lumen (H&E x 40).

A

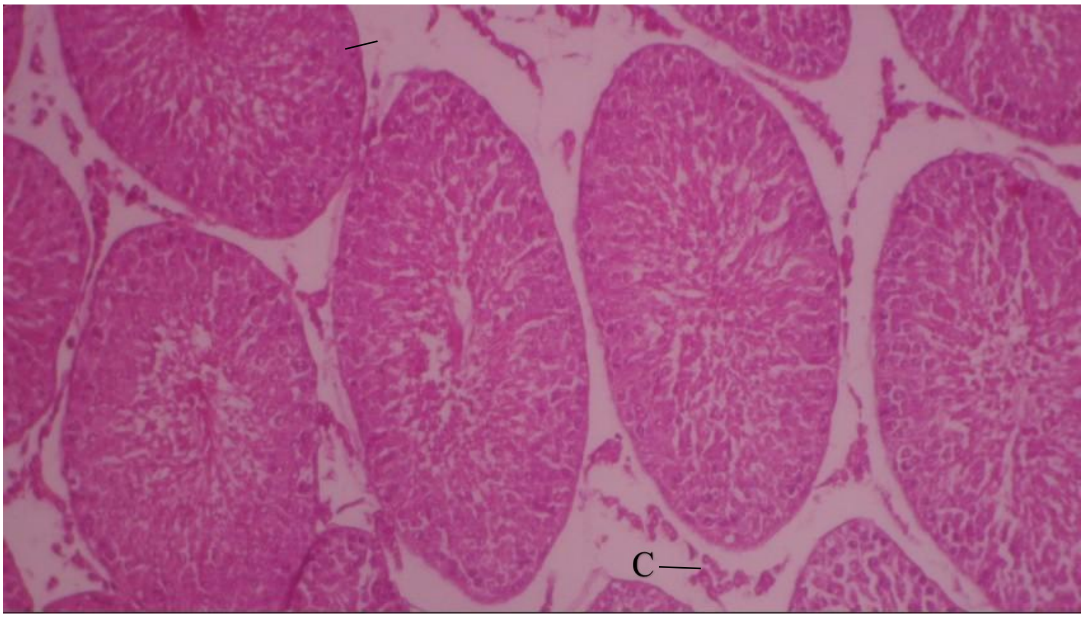


Plate 6. Rat testis given medium dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, C. mild Leydig cell (H&E x 100)

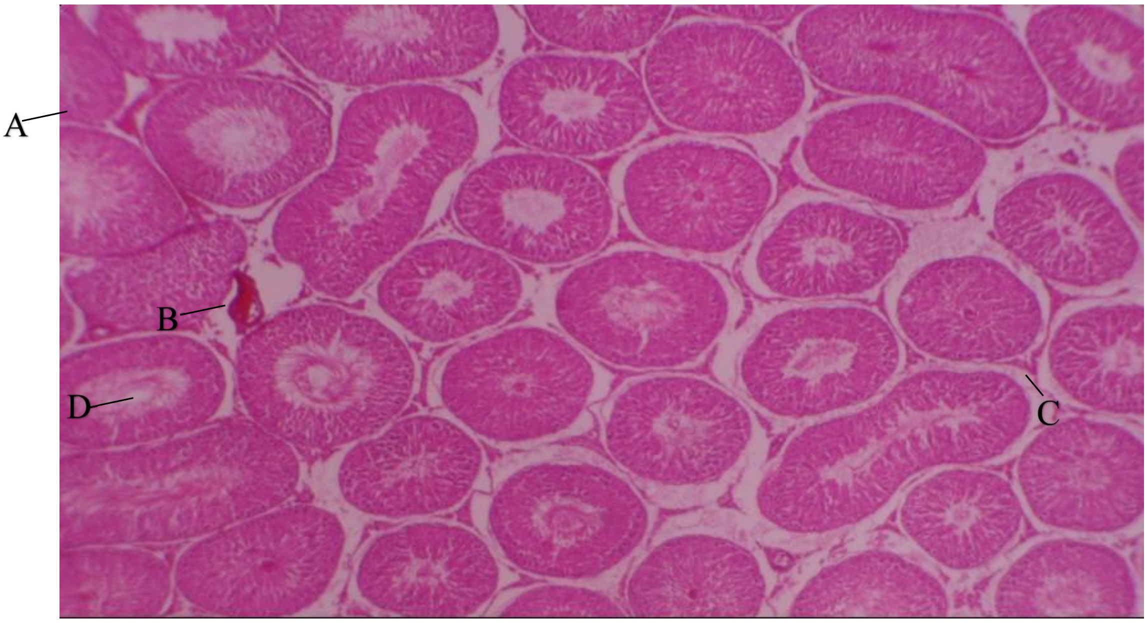


Plate 7. Rat testis given high dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, B. Blood Vessel, C. Leydig cell, D. Increased luminal diameter (H&E x 40)

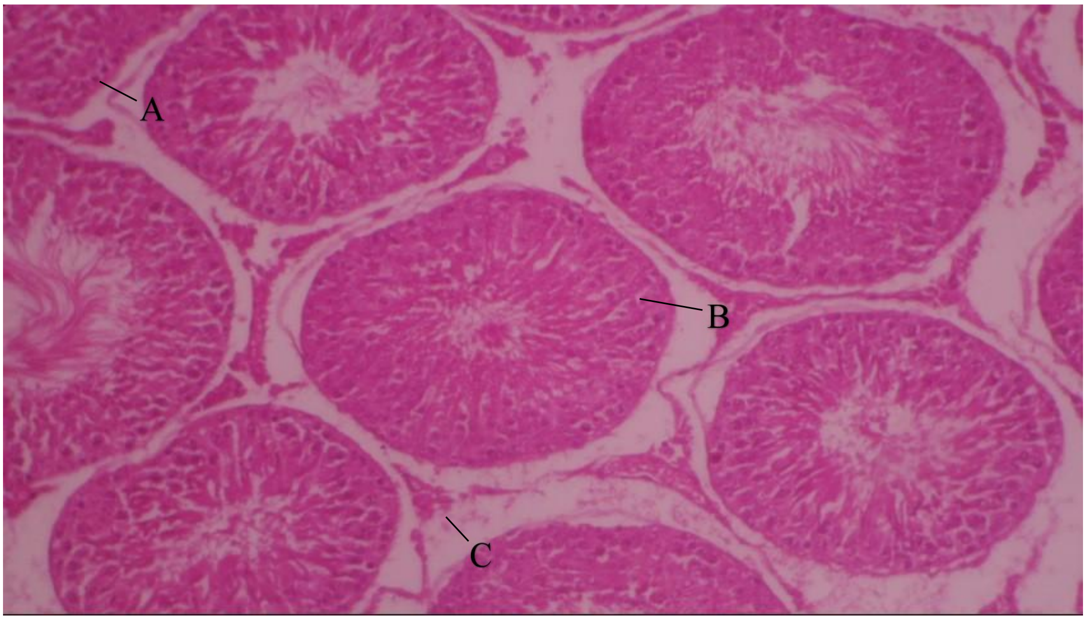


Plate 8. Rat testis given high dose Calabar Stone showing normal testicular architecture: A. spermatogenic series, B. Sertoli cells, C. mild Leydig cell (H&E x 100).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

Discussion

An increase in the final body weight was observed in all the groups when compared to the initial body weight. Although most of this increase was not significant, but in the group that received medium dose of calabash chalk where there was a statistically significant decrease in the initial body weight and a statistically significant increase in the final body weight. It has been argued that increase or decrease in either absolute or relative weight of an animal after administering a chemical or drug is an indication of the toxic effect of that chemical or drug (Orisakwe *et al*., 2003).

In this study, it was observed that the testicular index significantly increase in the group that received low dose of Calabash chalk when compared to control.

In the histological slide of this study, mild Leydig cell hyperplasia was observed in the testes across all dosage of animals administered with calabash stone when compared with control. It has been established by Olumi *et al* (1996) and Naughton *et al* (1998) that Leydig cell hyperplasia is a rare benign condition that is characterised by an increased number of testicular Leydig cells with increased nucleoli, decreased lipofuscins and decreased endoplasmic reticulum. These cells are responsible for the production of testosterone in human males (Mohammad *et al*., 2022). The histology also shows increased luminal diameter of the seminiferous tubule in animals that received high dose of Calabar Chalk when compared with the control group animal. The significance of this change has received little attention, although it is presumed to be related to Spermiation. If so, it may be that the spermatids about to be released are involved in the control of the increase in luminal size (Sharpe, 1989).

CONCLUSION

Findings from this study indicates the aqueous extract of Calabar chalk has no effect on the testes of an adult male Wistar rat.

RECOMMENDATION

It can be recommended based on this study that further research be done on calabar stone in male fertility.

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