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CO-ADMINISTRATION OF CYCLOPHOSPHAMIDE AND ETHANOLIC SEED EXTRACT OF *TELFAIRIA OCCIDENTALIS* (PUMPKIN) PROTECTS TESTICULAR FUNCTIONS IN ADULT MALE ALBINO WISTAR RATS

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ABSTRACT

Cyclophosphamide is used to treat auto-immune and neoplastic diseases. However its use is often limited by a wide range of adverse effects. This research was aimed at determining the effect of co-administration of ethanolic seed extract of *Telfaira occidentalis* (ESETO) and Cyclophosphamide on the testes of adult male wistar rats. Twenty adult male wistar rats weighing between 110-230g were divided into 5 groups (A-E) of 4 rats each. Group A (control) received distilled water and feed *ad libitum* only. Group B received 30mg/kg of Cyclophosphamide. Rats in groups C and D received 30mg/kg of cyclophosphamide each and co-administered with 1000mg/kg and 100mg/kg of ESETO respectively, while group E received 1000mg/kg of ESETO. Both substrates were administered orally once a day for 28days. Twenty-four hours after the last administration, the rats were weighed, anaesthetized, blood samples collected and testes together with the epididymis excised and weighed. Sera samples separated afterwards were assayed for testosterone and Follicle Stimulate hormones (FSH) levels. Epididymal sperm investigations were carried out, and the testes processed for histopathological examinations. Results showed that Cyclophosphamide administration caused losses in both body and testicular weights, reductions in serum levels of testosterone and FSH, as well as degeneration of testicular architecture, loss of sperm bundles and arrest of spermatogenesis. However, co-administration with graded doses of ESETO caused dose

dependent ameliorative effects with improved testicular parameters. Ethanolic seed extract of *Telfaira occidentalis* thus exhibited protective and antioxidant activities against cyclophosphamide induced testicular damage in adult male albino wistar rats.

KEYWORDS: Testis, Cyclophosphamide, *Telfaira occidentalis*, testosterone, Follicle Stimulating hormone.

INTRODUCTION

The testes are the male gonads that primarily produce sperm and androgens.[1] These functions are however affected by environmental factors such as exposure to pesticides, exogenous estrogens, heavy metals and chemotherapy, impacting negatively on male fertility.[2,3]

Cyclophosphamide (N, N-bis (2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine2-oxide), a cytotoxic alkylating agent, is a nitrogenous mustard belonging to the group of cytotoxic or cytostatic drugs and regularly used in the treatment of auto-immune and some neoplastic diseases.[4] Cyclophosphamide incorporates a subgroup of substances called alkylating agents. These agents are relatively inactive until the binding phosphorus-nitrogen is broken via metabolism catalyzed by Cytochrome P450 (a liver enzyme) and transformed into 4-hydroxycyclophosphamide that forms aldophosphamide. Aldophosphamide, is converted in the tissues to mustard phosphoramide (the effectively cytotoxic molecule) and acrolein (which is responsible for the adverse effects).[5, 6]

These adverse effects include hemorrhagic cystitis, pulmonary fibrosis, irreversible azoospermia in man, biochemical and histological alterations in the testis and epididymis, and decreased plasma testosterone levels in humans and experimental animals.[2,7,8] Studies have attributed these effects to the generation of free radicals and reactive oxygen species (ROS) that disrupts tissue antioxidant defense systems and produces highly reactive oxygen free radical, which are mutagenic to mammalian cells.[4,9,10]

Telfairia occidentalis (Pumpkin) a member of the family *cucurbitaceae,* is a vegetable grown and highly reputed in traditional medicine and largely consumed in many African countries. Its seeds are rich natural sources of proteins, polyunsaturated fatty acids, phytochemicals, sterols, antioxidant vitamins such as carotenoids and tocopherol and trace elements such as

zinc and selenium as well as vitamin E.[4,11] It has been documented that pumpkin extracts ameliorates the effect of ROS-induced testicular damage in experimental animals.[12,13,14]

The dearth in literature on the effects of consumption of *Telfairia occidentalis* (pumpkin) seeds on cyclophosphamide induced testicular damage, make this research imperative.

MATERIALS AND METHOD

Ethical approval

Ethical approval for this study was sort and obtained from the Ethical Committee of the Faculty of Basic Medical Sciences, College of Health Science, Nnamdi Azikiwe University, Nnewi Campus, Okofia, Anambra State, Nigeria.

Animal procurement, care and treatment

Twenty-one (21) adult male wistar rats weighing between 110-230g were purchased, housed in well ventilated plastic rat cages, under room temperature (27-31°C) and allowed to acclimatize for two weeks in the Animal House of the Department of Anatomy, Faculty of Basic Medical Sciences Nnamdi Azikiwe University, Nnewi Campus. They were fed with grower's mash (Top feeds, Nigeria Ltd) and distilled water *ad libitum* throughout the duration of the experiment.

Procurement and Preparation of Cyclophosphamide

Cyclophosphamide was purchased from Styleon-C pharmacy opposite Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State and a stock solution of 6.42ml was obtained by dissolving ten tablets of about 53.5mg each in 100g of water.

Procurement and preparation of ethanolic seed extract of *Telfairia occidentalis*

One kilogram of *Telfairia occidentalis* (pumpkin) seeds were purchased from Nkwo market in Nnewi, Anambra State, Nigeria, shed dried and milled into powder. 100g of the seed powder was soaked in 80% ethanol for 24 hours with frequent stirring using the modified method of Abd El-Ghany *et al.*[15] The resulting ethanol extracts were filtered and concentrated using a rotary evaporator (Heidolph.VV2000, Germany). The residue was lyophilized using a vacuum freeze dryer (Tilburg, Holland; 145Fm-RB) and the final extract was weighed and kept refrigerated until further use.

Acute toxicity study of Cyclophosphamide and ethanolic seed extract of *Telfairia occidentalis*

The median lethal dose (LD_{50}) of Cyclophosphamide via oral route was carried out in the Department of Physiology, Faculty of Basic Medical Science, Nnamdi Azikiwe University, Nnewi Campus. This was determined using the method of Lorke[15] and was found to be 100mg/kg for Cyclophosphamide, and above 5000mg/kg for *Telfairia occidentalis*.

Experimental design and Protocol

After acclimatization, the rats were weighed and divided randomly into five experimental groups (A-E) of 4 rats each. Group A served as control and received only distilled water and feed *ad libitum*. Groups B rats received 30mg/kg of *C*yclophosphamide only, Group C and D received 30mg/kg of cyclophosphamide each and a co-administration with 1000 and 100mg/kg of ethanolic seed extract of *Telfairia occidentalis* respectively, while group E received 1000mg/kg of ethanolic seed extract of *Telfairia occidentalis*. All administrations were orally, and once a day for 28days.

Termination of the experiment, Blood sample collection and Organ extraction

A. Blood sample collection

At the end of experiment, blood samples were collected by ocular puncture under the influence of chloroform anaesthetisia. 2ml of blood from each rat was centrifuged to separate the serum. The sera were collected in plain bottles, refrigerated at -20°C and assayed for testosterone and Follicle Stimulate hormones (FSH).

B. Organ collection

While still under the influence of chloroform anesthesia, the abdominal cavities were opened up through a midline abdominal incision and the testes together with the epididymis were excised and weighed. Epididymal sperm investigations carried out, and the testes fixed in freshly prepared Bouin's fluid.

Testosterone and FSH assay

Sera samples were assayed for testosterone and FSH levels using the Microwell enzyme linked immunoassay (ELISA) technique; using analytical grade reagents (Syntron Bioresearch Inc., USA).

Sperm Analysis

i) Sperm count assessment

Assessment of epididymal sperm count was carried out using a modified method of Yokoi and Mayi.[17] The epididymis was minced with anatomic scissors in 5mL physiologic saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5g sodium bicarbonate and 1mL formalin (35%). Total sperm number was determined by using the new improved Neubauer's counting chamber (haemocytometer). Approximately 10µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and allowed to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-called squares, and multiplied by 10^5 to get the number of sperm/ml homogenate.

ii) Assessment of sperm motility

Sperm motility was evaluated both subjectively and using a computer-assisted semen analyzer (CASA) (Sperm Vision Minitube™ of America, Inc., 2002). For the analysis, a 300-µl aliquot of a thoroughly but gently mixed semen sample was placed into an open 3-ml tube. The tube was kept in a 35°C water bath (Grants Instruments Ltd., Cambridge, UK) for 5 min before semen analyses. A 5-µl aliquot was placed on a pre-warmed 38°C microscope slide, covered with a coverslip (24 × 24 × 1.5 mm) and the proportions of total motile spermatozoa were recorded in percentage.

iii) Assessment of sperm morphology

Sperm morphology was evaluated using the method as described by Zemjanis,[18] with the aid of light microscope at a magnification of x400. Caudal sperm were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin. One hundred sperm cells from the sample were scored for morphological abnormalities. In this study, a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head and was expressed in percentage.

Tissue processing and photomicrography

The fixed testes were processed at the histology laboratory of the International Center for Research and Cancer Diagnosis, Abakiliki, Ebonyi State. After fixation the tissues went

through several processes of fixation, dehydration, clearing, infiltration, embedding, sectioning and staining. Dehydration was carried out using different ascending grades of alcohol ranging from 50% - absolute Alcohol for 30mins each. Clearing was by immersing the tissues through 3 changes of xylene for 30mins. The cleared tissues were impregnated and infiltrated to remove the clearing agent (xylene) by passing tissues through three changes of molten paraffin wax for 6 to 8 hours. Infiltrated tissues were embedded with molten paraffin wax and allowed to solidify. Sections of 5micron were obtained using rotary microtome. These sections were dried on a hot plate and stained using Haematoxylin/Eosin (H/E). Stained tissues were viewed under light microscopy.

Data analysis

Differences between the mean sperm count, sperm morphology and serum testosterone levels of the control and treatment groups were compared for statistical significance by one-way analysis of variance (ANOVA) and post hoc Scheffe's test using SAS 9.2 Enterprise Guide 4.3 software (SAS Institute Inc., Cary, North Carolina, USA). Data was expressed as Mean ± Standard Deviation (SD) and were considered statistically significant when $P < 0.05$.

RESULT

Physical and Behavioral Observations

During the course of acclimatization, their stool was normal and they adapted well to their environment. During the 28 days of substrate administration, increased breathing rate immediately after administration of cyclophosphamide as well as loss of appetite were observed in groups administered with cyclophosphamide (groups B,C and D). Rats in group B showed the least mobility, when compared to other groups.

Body weight changes

Table 1: Mean initial, final and weight differences of experimental animals.

Groups	Mean Initial body weight(g) ±SD	Meal Final body weight(g) ±SD	Mean Weight difference(g) ±SD	P-value
A	147.00±4.00	220.00±3.00	73.00±1.00	
B	220.00±2.00	190.00±1.00	-30.00±2.00	0.00*
C	148.00±3.00	203.00±3.00	55.00±3.00	0.00*
D	150.00±1.00	200.00±5.00	50.00±5.00	0.00*
E	128.00±3.00	215.00±2.00	87.00±3.00	0.07

*= P <0.05 when compared to control.

Testicular weight observations

Table 2: Mean Relative testicular weights of experimental animals.

Groups	Mean Relative testicular weight(g) ±SD	P-value
A	0.015±0.04	
B	0.010±0.01	0.00*
C	0.021±0.03	0.00*
D	0.018±0.04	0.00*
E	0.019±0.02	0.00*

*= P <0.05 when compared to control.

Observations on sperm motility

Table 3: Sperm motility levels (in percentage) of the experimental animals.

Groups	Mean% ±SD	P-value
A	70.00±0.00	
B	45.00±7.07	0.00*
C	70.00±0.00	1.00
D	65.00±7.07	0.31
E	70.00±0.00	1.00

*= P <0.05 when compared to control.

Observations on sperm count

Table 4: Sperm count ($x10^5$) of the experimented animals.

Groups	Mean Sperm Count ($x10^5$) ±SD	P-value
A	876.00±0.00	
B	126.50±17.67	< 0.00*
C	689.50±91.21	< 0.04*
D	329.50±127.98	< 0.00*
E	770.00±8.48	< 0.19

*= P <0.05 when compared to control.

Observations on sperm morphology

Table 5: Semen morphology of experimental animals.

Groups	Normal(%) ±SD	Abnormal(%) ±SD	P-value
A	85.00±0.00	15.00±0.00	
B	70.00±0.00	30.00±0.00	1.00
C	85.00±0.00	15.00±0.00	1.00
D	85.00±0.00	15.00±0.00	0.17
E	75.00±0.00	25.00±0.00	1.00

Observations on Serum Testosterone and FSH levels in the experimental animals

Table 6: Serum levels of FSH and Testosterone of the experimented animals.

Groups	FSH ±SD (µIU/mL)	P-value	Testosterone ±SD (ng/mL)	P-value
A	4.53±0.03		5.70±0.01	
B	2.06±0.05	< 0.001*	2.89±0.01	0.001*
C	3.44±0.18	< 0.001*	3.43±0.09	0.001*
D	3.01±0.04	< 0.001*	2.95±0.13	0.001*
E	4.50±0.12	0.862	5.80±0.04	0.001*

*= P <0.05 when compared to control.

Histopathological Findings

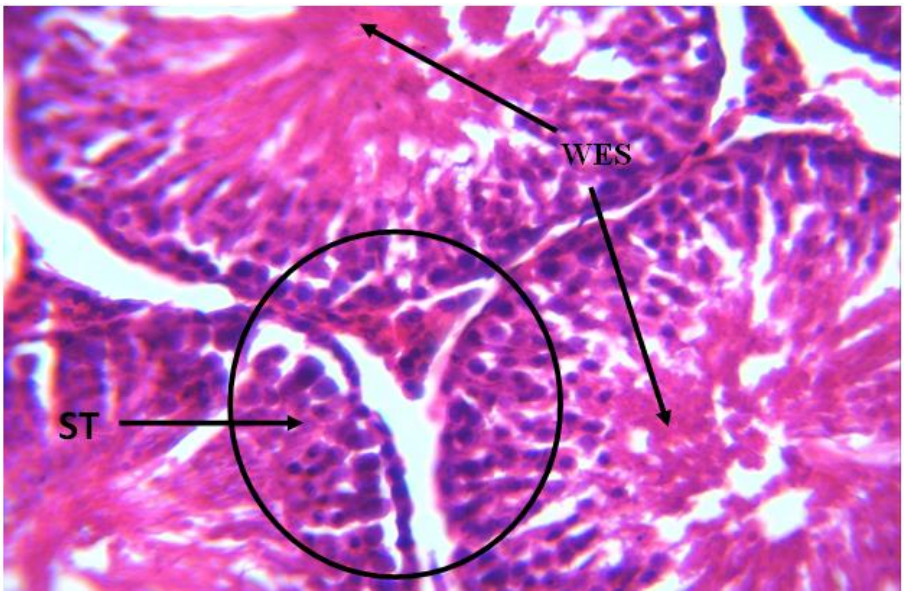

Fig.1 Photomicrograph of testis of Group A (control): showing normal testicular architecture with seminiferous tubules (ST) with well enhanced Spermatogenesis (WES).

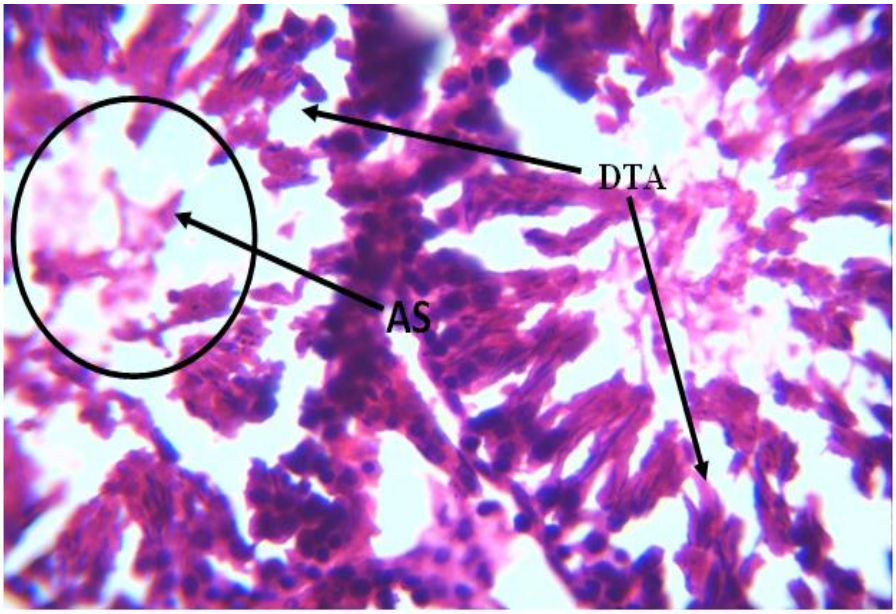

Fig.2 Photomicrograph of testis of Group B (administered with 30mg/kg of Cyclophosphamide only): showing degeneration of testicular architecture (DTA), and arrest of spermatogenesis (AS).

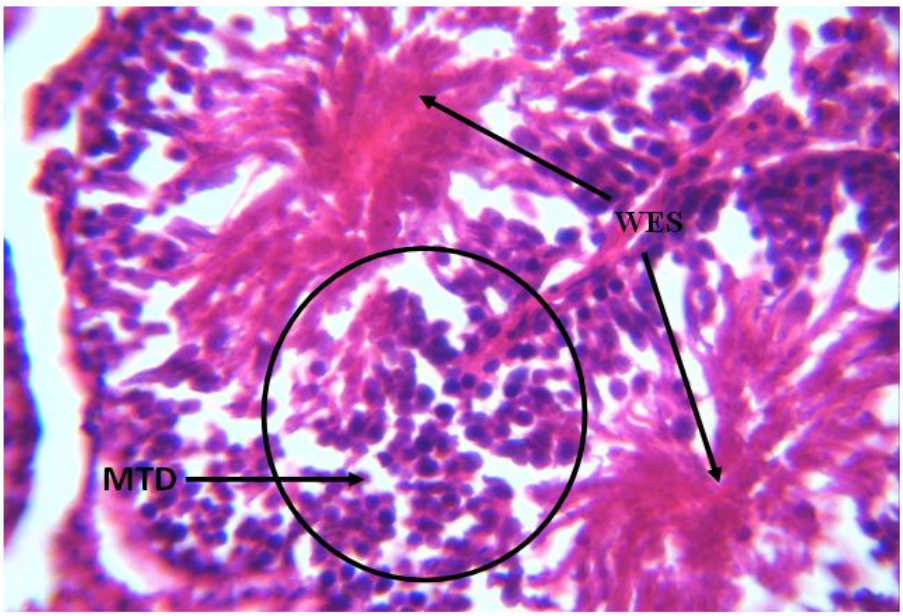

Fig. 3 Photomicrograph of testis of Group C (x400) (H/E) (received a co-administration of 30mg/kg of cyclophosphamide and 1000mg/kg of *Telfairia occidentalis* extract): showing mild testicular degeneration (MTD) with well enhanced spermatogenesis (WES).

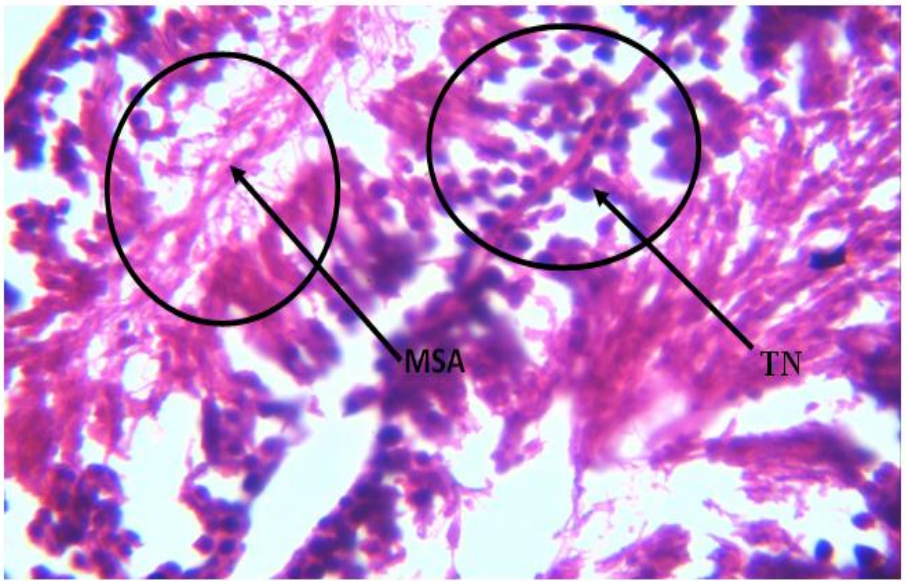

Fig. 4 Photomicrograph of testis of Group D (received a co-administration of 30mg/kg of cyclophosphamide and 100mg/kg of *Telfairia occidentalis* extract): showing moderate spermatogenic arrest (MSA), and testicular necrosis (TN).

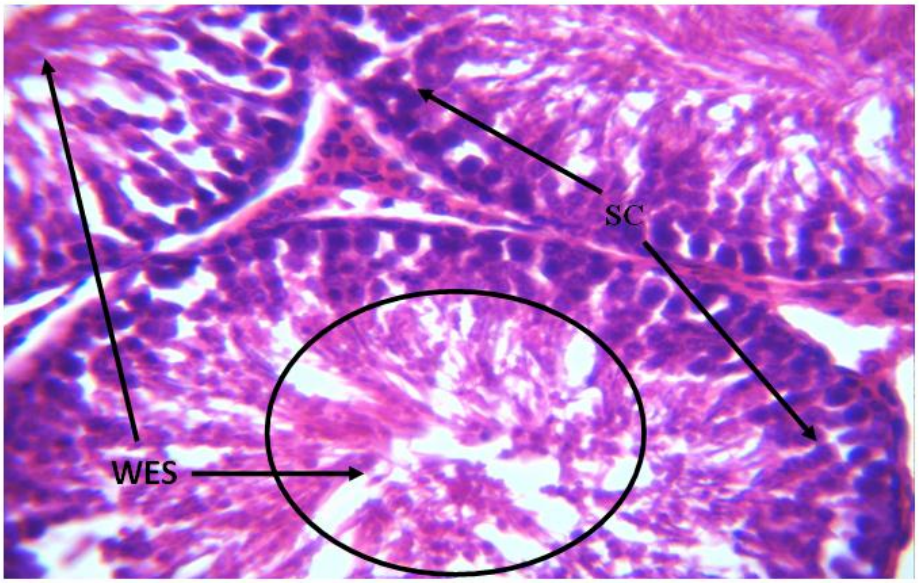

Fig. 5: Photomicrograph of testis of Group E (administered with 1000mg/kg of pumpkin seed extract) showing normal testicular architecture lined with Sertoli cell (SC) and well enhanced spermatogenesis (WES).

DISCUSSION

In this current study, it was observed that oral administration of cyclophosphamide affected the behaviour of the experimental animals. Animals, in the groups that received cyclophosphamide, were more lethargic and less mobile when compared to the control group.

Loss of appetite was also observed in these groups following the administration of cyclophosphamide. This appetite loss was seen mostly in Group B (administered with 30mg/kg of Cyclophosphamide only) when compared to Group A (Control) that received water and feed and Group E that received 1000mg/kg of seed extract of *Telfairia occidentalis* throughout the duration of the experiments. It was also observed that administration of ethanolic seed extract of *Telfairia occidentalis* was able to ameliorate these physiological problems caused by the administration of cyclophosphamide in the experimental groups C and D that received 30mg/kg of cyclophosphamide and 1000mg/kg and 100mg/kg of seed extract of *Telfairia occidentalis* respectively as animals in these groups, became energised following the administration of the seed extract of *Telfairia occidentals* as the experiment progressed. The increased activity and positive mobility of the animals in groups that received the seed extract of *Telfairia occidentalis* could be as a result of high nutritive contents such as carbohydrates, proteins, fats, fibres and minerals in *Telfairia occidentals* seeds which can boost energy levels.[19,20,21] Whereas the lethargies and less mobility of the animals in the group that received cyclophosphamide alone could be as a result of the impact of this drug on muscle mass and quality as cyclophosphamide has been documented to cause muscle wasting which in turn can cause alterations in energy balance.[22]

Results obtained also showed that there was an increase in the body weights of animals in all groups when their initial weights were compared to their final body weights, except in Group B that received 30mg/kg of cyclophosphamide only throughout the duration of the experiment where loss of weight was recorded. This result agrees with the findings of Kanno *et al*.[23] The loss in weights of the animals observed in Group B could be as a result of loss of appetite following treatment with cyclophosphamide as was observed in their feeding habits. However, increased body weights observed in control group (A) could be physiological as they were fed with only water and animal feed throughout the duration of the experiment. Animals that received only 1000mg/kg of ethanolic seed extract of *Telfairia occidentalis* (Group E) as well as the groups that received co-administration of cyclophosphamide and ethanolic seed extract of *Telfairia occidentalis* also showed weight gains. These findings agree with the findings of Kuku *et al*.[21] This significant weight gain in all groups that received seed extract of *Telfairia occidentalis* either alone or after the administration of cyclophosphamide shows that *Telfairia occidentalis* is associated with weight gain because of its high protein and vitamin levels.[20]

Mean relative testicular weights obtained from this study showed that there was a lower relative testicular weights in group B rats (that received 30mg/kg of cyclophosphamide only throughout the duration of the experiment) when compared to control ($p<0.05$). There were however higher relative testicular weights in other treated groups (C, D and E) when compared to control ($p<0.05$). This result agrees with the work of Oremosu et al.[24] These increases in the weights of the testes obtained in this current study could be attributed to the presence of antioxidants and beneficial nutritional components such as essential fatty acids and the high concentration of zinc and iodine present in pumpkin seeds.[20,25,26] This probably led to enhanced spermatogenesis, improved testicular architecture, with large volumes of sperm cells produced as observed in their various histological slides. It has been reported that essential fatty acids as well as diets rich in zinc does positively influence the normal development of seminiferous epithelial cells which in turn helps improve sperm quality and quantity and also lead to enhanced sperm production[4,27] and by so doing, could be behind the increases the weight of the testes of all groups that received seed extract of *Telfairia occidentalis* either alone or co-administered with cyclophosphamide. Iodine is a trace mineral found in *Telfairia occidentals* seeds, and is of great importance and essential to the function of the thyroid gland, which manufactures the hormones thyroxine and triiodothyronine. These two hormones regulate several enzymes and organic processes necessary for life, including cell division, a necessity for spermatogenesis.[28]

Findings from this study also showed that cyclophosphamide negatively affects the testicular function in adult male rats. This negative effect of cyclophosphamide was manifested in Group B that was administered with 30mg/kg of cyclophosphamide only throughout the duration of the experiment. This caused reduced sperm count, reduced sperm motility, altered sperm quality (Morphology), decreased sera levels of testosterone and FSH when compared to control, as well as alterations in testicular histology with degenerative changes such as necrosis of testicular architecture, marked depletion of the spermatogenic cell populations, and severe loss of germ cells as manifested in the histology slide. These results correlate with those reported by Kanno *et al*,[23] Watson *et al*,[29] and Kenney *et al*.[30] The decrease in testicular function observed in this current study could be as a result of the sensitivity of testicular cells to cyclophosphamide. Cyclophosphamide has been reported to cause the generation of reactive oxidation species (ROS). ROS generated in turn causes alterations in the sperm chromatin structure, mitochondrial DNA damage in the middle piece of spermatozoa, alterations in the epididymal environment, as well as alterations in the

composition of the basic proteins in the sperm head probably leading to the death of testicular cells resulting in depletion of germ cells thus causing oligozoospermia (a reduction in the sperm count) or azoospermia, as well as reduction in serum testosterone and FSH levels if usage is prolonged.[4,23,29,31] However, testicular function (sperm count, motility, morphology and testosterone and FSH levels) of rats in Groups C and D that each received 30mg/kg of cyclophosphamide and afterwards treated with 1000mg/kg and 100mg/kg ethanolic seed extract of *Telfairia occidentalis* respectively showed a significant ameliorative effects, with marked improvements in all parameters when compared to those of Group B. This result agrees with previous studies by Aghaei *et al*,[4] Oremosu *et al*,[24] and Akang *et al*.[32] This dose dependent ameliorative effect observed in this current study following treatment with *Telfairia occidentalis* (pumpkin) seed extract could be as a result of high content of unsaturated fatty acids such as omega-6, -9 and -3, L-tryptophan, protein and very high concentration of vitamin E, and zinc contained in pumpkin seeds.[11,33] These constituents have been documented to produce dehydrogenase activity which is essential in testosterone synthesis pathway.[3] This enhanced testosterone production stimulates the spermatogenic cells which undergo successful spermatogenesis, sperm maturation in the epididymis and the secretory activity of the accessory sex glands.[34] In addition, Pumpkin seed oil is rich in many powerful antioxidants and useful nutritional supplements such as essential fatty acids and polyunsaturated fatty acids including linoleic acid, oleic acid, palmitic acid, omega 3, 6 and 9, carotenes, lutein, gamma and P-tocopherols, phytosterols, chlorophyll, selenium and zinc.[35] The presence of these nutrients reduces the susceptibility of the testis and epididymis to lipid Peroxidation.[36]

Histopathological findings in this current study revealed that experimental animals in Group B that received 30mg/kg of cyclophosphamide showed testicular degeneration, with arrest of spermatogenesis and testicular cell necrosis of both Sertoli cells and interstitial cells of Leydig when compared to Control (group A) that received water and feed only throughout the duration of the experiments. The damage observed following cyclophosphamide administration in this current study could be attributed to the ability of cyclophosphamide to induce oxidative stress, and in the process alters DNA structure thus affecting testicular microarchitecture and functions. This is in agreement with the works of Selvakumar *et al*,[37] Pryzant *et al*,[38] and Drumond *et al*.[39] On other hand, histopathological investigation of group E that received 1000mg/kg of ethanolic seed extract of *Telfairia occidentalis* throughout the duration of the experiment showed production of sperm cells with normal

testicular cells and seminiferous tubules lined with interstitial cells of Leydig and Sertoli cell, as well as enhanced spermatogenesis. Ameliorative changes in testicular tissue were seen in the testicular histology of animals in group C and group D, with marked improvements in testicular architecture and spermatogenesis. These improvements in testicular tissues observed in this current study could be as a result of antioxidants present in pumpkin seed extract which was in agreement with that previously reported by Nwangwa *et al*,[13] Akang *et al*,[32] and Saalu *et al*.[40] These antioxidants such as Vitamin E, Vitamin A, and Carotenoids present in pumpkin seeds can break down the oxidative chain reaction and play a very significant role in increasing the body's capacity to fight free radical-induced oxidative stress,[41] and therefore improve the process of spermatogenesis.[24,42]

5.2 CONCLUSION

Findings from this study shows that ethanolic seed extract of *Telfairia occidentalis* (Pumpkin seed) not only exhibited protective and antioxidant activities against cyclophosphamide induced testicular damage in adult male albino wistar rats but also provided ameliorative properties and displayed pro-fertility activity.

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