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Research Article

Intake of Fluted Pumpkin Seeds Rebalances Oxidative Stress Parameters in the Aged Rat's Testes

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The effect of fluted pumpkin seeds (FPS) consumption on the antioxidant status of the testes of aged Wistar rats was evaluated in this study. Sixty (50 aged, 6 months old, and 10 young, 2 months old) rats were divided into six groups of 10 per each group. Testosterone (15 mg kg^{-1} body weight, once weekly for 40 days) was injected intraperitoneally and used as positive control. FPS intake (50, 100, and 200 mg kg^{-1} body weight) or vehicle control (corn oil) were administered orally, twice weekly for 40 days and compared with the untreated aged and young control rats. Changes in antioxidant status in the testis of the aged rats was reflected as increased superoxide dismutase and catalase activities and glutathione and decreased lipid peroxidation levels which were attenuated more efficiently by the lowest dose FPS (50 mg kg^{-1} body weight). Additionally, nitrite concentration that was found to be diminished in the aged rats was raised to the young control values after intake of the FPS (50 mg kg^{-1} body weight). As expected, testosterone injection increased endogenous testosterone concentration and also remained higher in the untreated aged animals than in young control and treated aged rats. In conclusion, compromised antioxidant defense system of the testes that is associated with ageing could be reversed to the status of the young control by the intake of FPS.

1. Introduction

Ageing is a time dependent physiological alterations in an organism that leads to diminish biological functions on the cellular, tissue and system level, and of the organism's ability to adapt to metabolic stress [1–3]. The influence of ageing on male fertility health has not been well studied, this is because men produce sperms throughout their life time starting from puberty, and this has made people believe that advanced patrilineal age does not influence male reproduction, fertility, and progeny outcome [4–6]. It is known that as ageing increases, oxidative stress also increases in the gonads, which results to deoxyribonucleic acid damage, germ cell apoptosis, and impaired spermatogenesis and spermatozoa development [7–9]. Oxidative stress occurs when the imbalance between reactive oxygen species (ROS) production and antioxidant defenses leads to cellular damage occurs because of higher levels of the ROS much more than those of the antioxidants [10, 11]. Reactive oxygen species are generated during multiple endogenous processes and exogenously induced metabolic events, and they play important roles in ageing and age-associated diseases [12]. It happens that the antioxidant defense system gradually becomes weaker to cope with the detoxifying of oxygen radicals as the tissues and cells of ageing males get overwhelmed with the increased level of ROS [10].

Many nonenzymatic (glutathione, ascorbate, vitamin E, and several other antioxidant molecules) as well as enzymatic scavengers of ROS including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) in the testes work cooperatively to prevent increase of oxidative stress [13]. For example, SOD breaks down superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and the H_2O_2 is further inactivated by its conversion to water (H_2O) by CAT. There are other antioxidant mechanisms, e.g., those mediated by GPX and peroxiredoxins that also contribute

to the inactivation of H_2O_2 and peroxynitrites [10, 14]. Furthermore, most studies on changes in testicular morphology have shown that ageing is associated with reduction in testicular weights and morphometric data including seminiferous tubule volume, length of tubules, reduction of spermatogonia, escalated numbers of multinucleated spermatogonia, giant spermatids, and Leydig cells [3, 15, 16].

Adjusting lifestyle and dietary habits, chemical drugs, and herbal remedies have been employed in the restoration to normal testicular functions with advancing age. In most developing countries including Nigeria, where there is relatively no subsidized health care system for the population, accessing health care is very expensive especially for the aged population; therefore, the old population utilizes alternative remedies including herbal remedies for the management and treatment of ageing-induced testicular impairments. Some of these herbs were found useful in the management of ageing and aged-related oxidative stress because of their antioxidant properties which are of benefits therapeutically for the infertile male. Some examples include from green tea [17], white tea [18], resveratrol (grape seed extract) [19, 20], and many other medicinal plants including *ginseng* spp. *Tribulus terrestris*, *Lepidium meyenii*, and *Vernonia amygdalina* [21–23].

Fluted pumpkin (*Telfairia occidentalis Hook f*. cucurbitaceae) is a cucurbitaceous leafy green vegetable that is popular in West and Central Africa especially for its edible seeds and leaves [24]. Among peasants in southeastern states of Nigeria, fluted pumpkin seeds (FPS) can also be boiled and consumed as snacks or left to ferment after boiling and use afterwards as a seasoning for soups [25, 26]. In traditional practice, the leaves form part of herbal remedies for the treatment of convulsion, diabetes, chronic fatigue, and malaria [24]. There are also unverifiable claims by herbal practitioners that the fluted pumpkin has aphrodisiac effects in men [27–29]. This seems probably true because several animal model studies have confirmed that extracts of fluted pumpkin inhibits lipid peroxidation, enhances the sperm quality status of rats, and could influence testis function and reproductive health [28, 30]. Fluted pumpkin seeds are rich in some nutrients including vitamins, iron, phosphorus, and potassium and phytochemicals like tannins, flavonoids, alkaloids, erucic acid, and oxalates as well as other phytoconstituents which could induce plethora of biochemical, physiological, and morphological effects in rats [31, 32]. However, none of these phytochemicals have been isolated and applied in a testicular system of a rodent model [28]. Several investigators have validated the pharmacological effects of FPS using animal models. Extractives of the seeds repress blood glucose levels [33], exhibit schizontocidal activity during early, and established infections [34], anticancer, and anti-inflammatory effects [35], stimulatory effects on haematological parameters [36], antithrombopoietic activity [37], antioxidant effects in rats ovaries [38], lactating effects in nursing mothers [39], and antinociceptive effects [40]. Dietary FPS inhibits growth of abnormal prostatic cells in rats [41] and has dual effects on male fertility health [28]. Furthermore, the testes of rats that are injured by noxious chemicals of environmental origins and gonado-toxicants like drugs can be protected and preserved by FPS [24, 29, 31, 42–44]. Therefore, this study was designed to evaluate the effect of intake of FPS on changes in the aged rat's testes due to oxidative stress, and focuses on measuring testicular mass, testosterone, ascorbate, and nitrite concentrations as well as oxidative stress markers and DNA fragmentation.

2. Materials and Methods

2.1. Preparation of the Hexane-Rich Fraction of FPS. Raw and matured pods of fluted pumpkin were bought from Aluu boundary market in Port Harcourt, Nigeria. The pods were cut open and the seeds were collected, sorted (to remove bad ones), and washed. The seeds were air-dried, deshelled, and then grounded into powder. Flours obtained from the seeds and weighing 2 kg were defatted with n-hexane in a Soxhlet apparatus for 8 hours and then air-dried on aluminum trays at room temperature. The hexane-fraction was collected from the FPS marc extract and stored in the refrigerator. The defatted FPS marc extract was then soaked in absolute ethanol for 72 h after which they were sieved using a muslin cloth and thereafter filtered with Whatman paper. The collected filtrate was thickened at low pressure to constant weight in a Soxhlet extractor at 45°C, and the brownish residue was dried in an oven at 40°C. The weight of the extract was 4.22% (w/w). To prepare the hexane-rich fraction, the brownish extract was mixed with the hexane-fraction and the mixture was suspended in equimolar volume of water and hexane (50 ml) for 24 h with constant intermittent shaking. After 24 h, the partitioned hexane-layer was collected with a separating funnel. After three cycles of successive separation with hexane, the resulting hexane-fraction extracts were pooled together and evaporated under a vacuum using a rotary evaporator to yield a brownish-dark residue. The extract was dried in an oven at 40°C and kept in the freezer until it was used. The final yield of the extract was 5.43% (w/w). The extraction yield (%) was calculated as follows:

$$\%\text{extraction yield} = \frac{\text{Weight (g) of the dry extract after solvent extraction}}{\text{Weight (g) of dry plant sample before extraction}} \times 100\%. \quad (1)$$

2.2. Animals and Experimental Designs. Sixty male Wistar rats (*Rattus norvegicus*), 50 aged rats (219 ± 33 g; 6 months old) and 10 young rats (87 ± 18 g; 2 months), were bought from animal breeder and kept in the Department of Biochemistry, University of Port Harcourt animal house. The rats were allowed one week to acclimatize to the environment and had unrestricted access to normal rat chow and water *ad libitum*. They were kept in plastic cages with dry sawdust as bedding material under standard conditions of temperature (23 ± 1°C), humidity (50-60%), and 12-hour light/12-hour dark cycle throughout the 40 days study period. The study received authorization of the Department of Biochemistry Research Ethics committee on 18 January 2022 with an ethical approval reference number of UPH/BCHREC/2022/009; and the methodology for the handling

of the animals was also in accordance with the requirements of the National Institute of Health Guidelines for Animal Care and Use of Laboratory Animals (Publication Number, 85–23). The rats were divided into 6 groups of 10 rats per each group. Testosterone group was administered testosterone (15 mg kg^{-1} body weight) intraperitoneally, once a week for 40 days. FPS group was treated with FPS extract (50, 100, and 200 mg kg^{-1} body weight) orally, twice a week while the aged and young controls was administered with corn oil (2 ml kg^{-1} body weight) orally, twice a week for 40 days [42]. The ages of the rats at 2 and 6 months were selected because the 8 weeks-old rats are young animals and the 24 weeks-old rats are adult animals and they exhibit differences in the capacity of their testes to respond to oxidative stress [45, 46]. At the end of the study, the rats were weighed and killed; blood samples were collected, tissue samples were removed, washed in ice-cold 1.15% potassium chloride and damped gently in-between a Whatman paper before they were weighed and processed for biochemical assays.

2.3. Tissue Processing and Biochemical Assays. The testis (0.25 g) was homogenized using mortar and pestle in a 2 ml ice-cold phosphate buffer solution (pH 7.4, 0.1 M). The homogenate samples were centrifuged at 8950 × g per minute at 4°C for 30 min. The collected supernatants were immediately used for biochemical assays.

2.4. Oxidative Stress Markers Evaluation. Ohkawa et al. [47] protocol was adopted for the lipid peroxidation assay. In this method, thiobarbituric acid was used as substrate for measuring the absorbance of malondialdehyde (MDA) level in the sample at 532 nanometer wavelength. Moron et al. [48] method was used for glutathione (GSH) evaluation at 412 nanometer wavelength using Ellman's reagent for colour development. The activity of catalase was monitored in a spectrophotometer at 240 nanometer wavelength as described previously by Chance and Maehly [49]. Kakkar et al. [50] procedure was adopted for measuring the enzymatic activity of superoxide dismutase (SOD). In this method, nicotinamide adenine dinucleotide-reduced (NADH) was used as the substrate for the formation of the bluish-coloured methosulphate-nitroblue tetrazolium formazon at 560 nanometer wavelength. Nitrite level in tissue sample was determined at 540 nanometer wavelength using the Griess reaction as described by Green et al. [51]. Total ascorbate concentration in tissue sample was determined at 520 nanometer wavelength according to the protocol of Omaye et al. [52] using dinitrophenylhydrazine for colour development. Lowry et al. [53] was employed for the measurement of protein concentration.

2.5. Quantification of DNA Fragmentation by Diphenylamine Reaction. Tissues were homogenized in the DNA lysis buffer (10 mM Tris-hydrochloric acid, 1 mM ethylenediaminetetraacetic acid, pH 8) containing 0.2% Triton X-100. After centrifugation (3000 × g, 20°C) for 10 min, the supernatant was discarded and pellet was resuspended in the lysis buffer and shaken vigorously. The pellet was resuspended in the lysis buffer and after centrifugation (5000 × g, 20°C) for 30 min, the supernatant was collected in new test tubes. After addition of trichloroacetic acid (25%) to the supernatant and pellet samples, the mixture was incubated overnight at 4°C and thereafter assayed for DNA content by diphenylamine reaction at 600 nm as described by Sandau et al. [54].

2.6. Quantification of the Testosterone Concentration by Enzyme Immunoassay. Blood samples were put in plain bottles and after 30 min, they were centrifuged (4000 revolutions per minute) for 20 min for the extraction of serum. Enzyme immunoassay was performed with the Accu-BindTM testosterone kit (CAT NO. 3725-300) according to the manufacturer's instructions (Monobind Inc. CA, USA). The absorbance of sample was recorded at 450 nm and testosterone concentration was extrapolated from a testosterone calibration curve (0–12 ng/ml). The assay sensitivity and cross-reactivity with other tested steroids are 0.0576 ng/ml and <0.0001%, respectively.

2.7. Statistical Analysis. Statistical analyses of all data were performed with GraphPad Prism software (San Diego, CA, USA). All data are presented as mean ± standard deviation of the mean. Comparisons of multiple groups were performed using One-way analysis of variance (one-way ANOVA) and *post-hoc* test was conducted using Tukey's multiple comparison test. Student's *t*-test was used when only two pairs of data were compared. Statistical difference was considered significant when $P < 0.05$.

3. Results

3.1. Final Body, % Body Weight Gain, Absolute Paired Testis Weight, and Relative Testis Weights of the Ageing Rats after Treatment with FPS for 40 Days. The percentage body weight gain of young animal was significantly increased compared to aged control. FPS at 200 mg kg^{-1} b.w increased the percentage body weight gain compared to 50 mg kg^{-1} b.w ($P = 0.00442$). It was observed that testosterone administration significantly decreased the percentage body weight gain compared to the aged control. As expected, the final body weight of all aged animal groups was increased compared to the young animals ($P = 0.00062$). Absolute testicular weight of animals administered 200 mg kg^{-1} b.w FPS was significantly increased compared to the young control ($P = 0.0185$). FPS extracts at 50 mg kg^{-1} b.w ($P = 0.001813$) and 100 mg kg^{-1} b.w ($P < 0.0001$) decreased testicular weight compared to the 200 mg FPS group. It was also observed that the young rats had significantly increased relative testicular weights compared to aged animals (Figures 1(a)–1(d)).

3.2. SOD and CAT Enzymatic Activities As Well as GSH and MDA Concentrations in the Ageing Rat Testis after Treatment with FPS for 40 Days. There was an increase in SOD activity in the testes of aged control compared to young control ($P = 0.000452$). The SOD activity in all the other test groups was lower but the decrease in SOD activity in the 50 mg FPS group reached statistically significant level to the aged control while in the testosterone group there was an increase in SOD activity compared to 50 mg FPS (Figure 2(a)) and decrease when compared to the aged

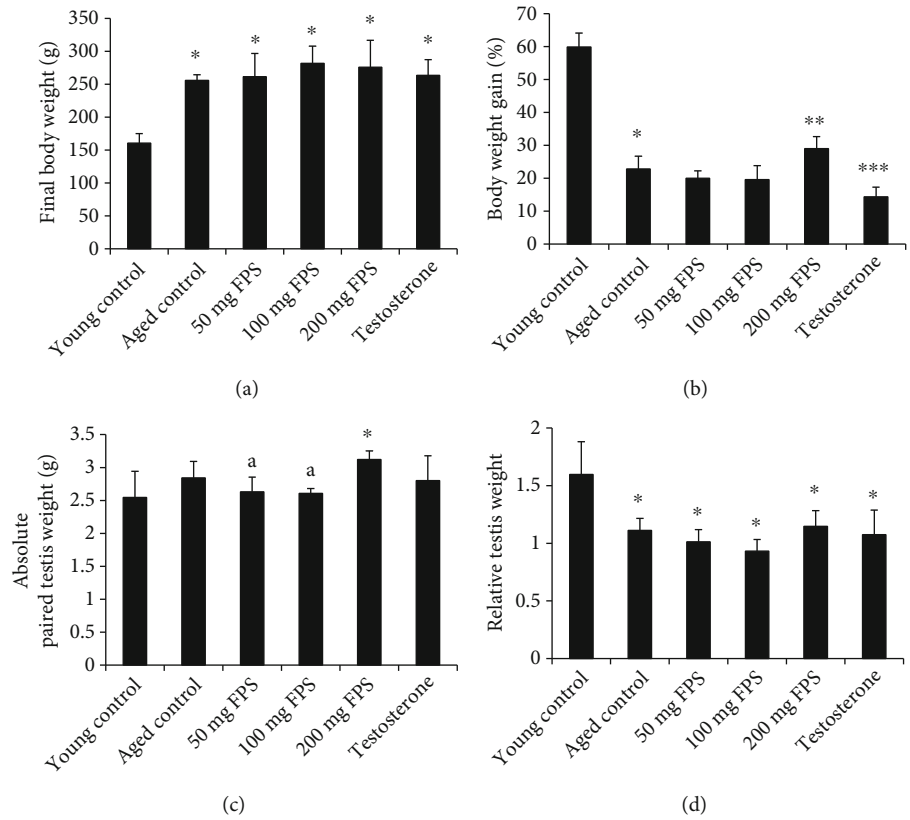

FIGURE 1: Effect of fluted pumpkin seeds (FPS) on the final body weight (a), % body weight gain (b), absolute paired testis weight (c), and relative testis weight (d) of aged rats after 40 days of treatment. Data are mean ± SD ($n = 10$). n = number of rats in each group *versus young control ($P < 0.0001$); **versus 50 mg FPS ($P = 0.0021$); ***versus aged control ($P = 0.004$); a 200 mg FPS versus 50 mg FPS ($P = 0.002$); a 200 mg FPS versus 100 mg FPS ($P < 0.0001$).Relative testis weight = testis weight (g)/body weight (g) × 100%.

control although it was not statistically different. The catalase activity in aged control was also significantly higher than the young control ($P = 0.0058$). Treatment with 50 mg kg^{-1} FPS significantly decreased CAT activity compared to the aged control whereas the testosterone group increased CAT activity compared to 50 mg FPS group and did not change its activity relative to the aged control (Figure 2(b)). Higher doses of FPS did not alter CAT activity relative to the aged control ($P > 0.05$). It was also observed that GSH concentration was elevated in the untreated aged control compared to the young untreated control rat ($P = 0.00736$). Treatment with testosterone ($P = 0.0105$) or 50 mg FPS kg^{-1} b.w ($P = 0.00395$) decreased glutathione level relative to the aged control. The GSH level after treatment with higher doses FPS (100 and 200 mg FPS) was higher than those of the 50 mg FPS animals ($P = 0.00987$) (Figure 2(c)). There was a significant decrease in lipid peroxidation level in the testis of the aged control as compared to young control animals ($P = 0.000289$). The concentration of testicular MDA increased in the FPS groups when compared to the aged control but the increase was lowest in the 200 mg FPS group ($P = 0.000319$). The level of MDA was found to be elevated in the testosterone group relative to the aged control, although it was not statistically different from the young and aged controls (Figure 2(d)).

3.3. Nitrite and Ascorbate Concentrations in the Ageing Rat Testis after Treatment with FPS for 40 Days. It was also observed that nitrite concentration was reduced in the testis of the aged control rats ($P = 0.0026$) and testosterone group ($P = 0.00112$) compared to the young control. Treatment with 50 mg kg^{-1} FPS could significantly increase nitrite level when compared to aged control ($P = 0.027$). Nitrite concentration was not significantly altered in the higher dose FPS groups compared to the aged control ($P = 0.477$) (Figure 3(a)). There was an increase ascorbate level in the testes of the aged control animals relative to the young control ($P = 0.00027$). The testes of the young control rats had the least ascorbate concentration across all groups while the aged control had the highest level. Ascorbate concentration was not significantly affected in the higher dose FPS groups compared to the aged control ($P > 0.05$). There was a decrease in ascorbate concentration in the 50 mg kg^{-1} FPS ($P = 0.0279$) and testosterone group ($P = 0.0027$) when compared with aged control (Figure 3(b)).

3.4. Serum Testosterone Level in the Aged Rat after Treatment with FPS for 40 Days. Testosterone concentration changed significantly in the aged rats (6 months) compared to the young control (2 months) ($P = 0.00039$). As FPS increases in dose, the testosterone concentration also

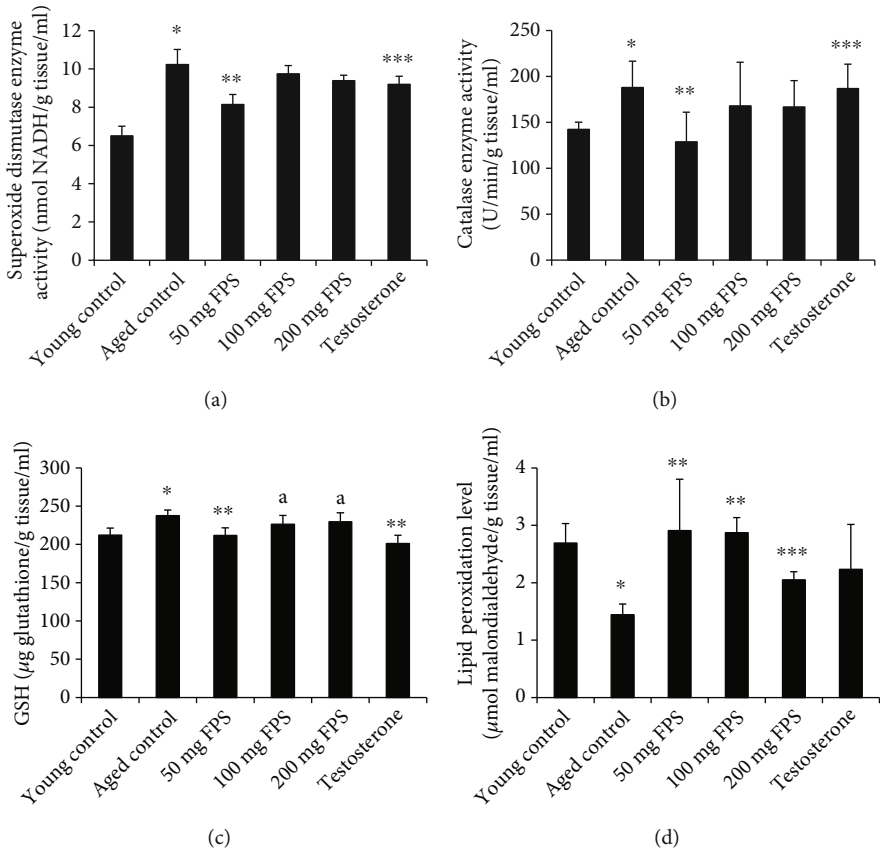

Figure 2: Effect of fluted pumpkin seeds (FPS) on oxidative stress parameters. Superoxide dismutase enzyme activity (a), catalase enzyme activity (b), glutathione (GSH) (c), and lipid peroxidation level (d) in the ageing rat's testis after 40 days of treatment. Data are mean ± SD ($n = 10$). n = number of rats in each group; superoxide dismutase: *versus young control ($P = 0.0005$); **versus aged control ($P = 0.0075$), ***versus 50 mg FPS ($P = 0.0041$); catalase: *versus young control ($P = 0.006$); **versus aged control ($P < 0.0001$), ***versus 50 mg FPS ($P = 0.027$); GSH: *versus young control ($P = 0.0078$); **50 mg FPS versus aged control ($P = 0.0062$), **aged control versus testosterone ($P = 0.0001$), a testosterone versus 100 mg FPS ($P = 0.0079$), a testosterone versus 200 mg FPS ($P = 0.0025$); lipid peroxidation: *versus young control ($P = 0.001$); **50 mg FPS versus aged control ($P = 0.001$), **100 mg FPS versus aged control ($P = 0.0002$), ***versus 50 mg FPS ($P = 0.041$).

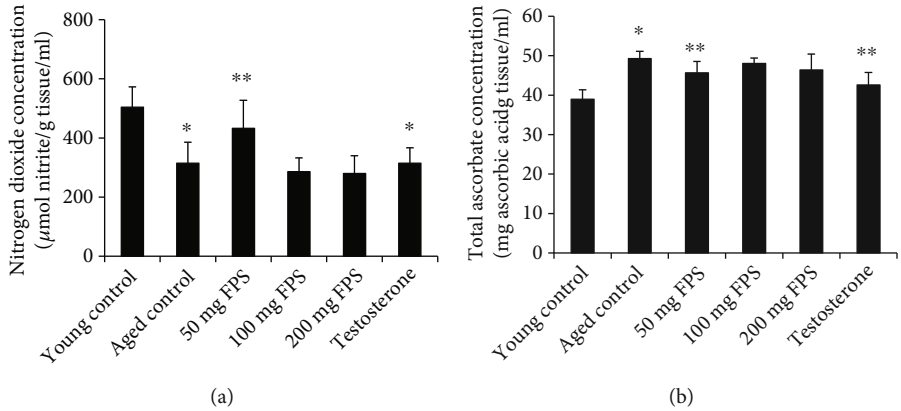

Figure 3: Effect fluted pumpkin seeds (FPS) on nitrite (a) and ascorbate (b) concentrations in the ageing rat's testis after 40 days of treatment. Data are mean ± SD ($n = 10$). n = number of rats in each group; *young control versus aged control ($P = 0.0026$), *young control versus testosterone ($P = 0.0011$) **versus aged control ($P = 0.027$); ascorbate: *young control versus aged control ($P = 0.00027$), **aged control versus 50 mg FPS ($P = 0.0279$) **aged control versus testosterone ($P = 0.0027$).

appears to be decreased compared to the aged group and could reach statistical significant level in the highest FPS dose cotreated groups ($P < 0.0065$). However, testosterone concentration was not significantly changed across the FPS groups ($P > 0.9999$). As expected, testosterone administration elevated testosterone concentration compared to the young control group ($P = 0.0002$) as well as the 50 mg kg^{-1} FPS ($P = 0.0003$), 100 mg kg^{-1}, and 200 mg kg^{-1} b.w ($P = 0.0002$) treated groups (Figure 4). The 27% increase in testosterone concentration in the aged control could not reach statistically significant level when compared to the testosterone group ($P = 0.168$).

3.5. % DNA Fragmentation in the Ageing Rat Testis after Treatment with FPS for 40 Days. Percent fragmented DNA in testis of the untreated aged rats did not change relative to the untreated young control ($P = 0.943$). There was no significant effect on % DNA fragmentation in the 50 mg FPS, 100 mg FPS, and 200 mg FPS groups when compared with the aged control ($P = 0.9963$). Testosterone injection did not also change % fragmented DNA compared to the aged control and young control rats ($P = 0.9989$) (Figure 5).

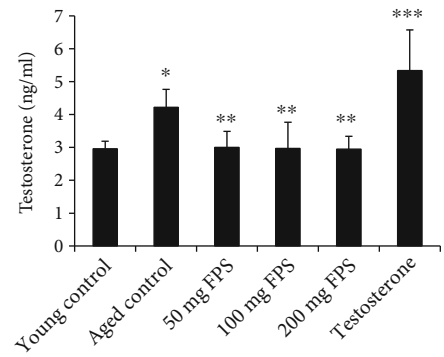

Figure 4: Effect of fluted pumpkin seeds (FPS) on serum testosterone concentrations in aged rats after 40 days of treatment. Data are mean ± SD ($n = 10$). n = number of rats in each group; *versus young control ($P = 0.00039$), **versus aged control ($P = 0.0065$), ***versus young control ($P = 0.0002$), and FPS groups ($P = 0.0003$).

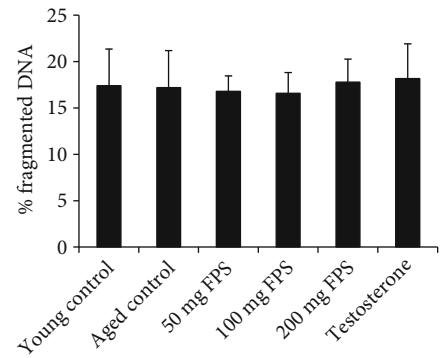

Figure 5: Effect of fluted pumpkin seeds (FPS) on % fragmented DNA in the aged rat's testis after 40 days of treatment. Data are mean ± SD ($n = 10$). n = number of rats in each group. No statistical significant differences across groups ($P > 0.05$).

4. Discussion

The increase in relative testicular weight observed in the young control compared to aged animals in this study might be due to the fact that testis weight diminishes as the animal advances in age [45, 55]. Interestingly, the epididymis of aged rats has also been found to be smaller in size than those of young rats [56] confirming the fact that gonado-somatic index reduces as the animal advances in age [45]. The body weight gain of young animal was increased compared to aged control, this might be that the animals are still growing and as such, the body weights are expected to increase. It is therefore expected that final body weights of the aged animals to be increased compared to the young animals. Furthermore, absolute testicular weight and body weight gain were higher in the 200 mg FPS group suggesting that the higher testis weight is corresponded to the increased body weight. Thus, long-term intake of FPS might be associated with body weight gain, which may be a health risk when clinically administered to humans [57].

Oxidative stress markers evaluated in this study such as SOD and CAT activities and lipid peroxidation and GSH concentration showed an increase in the 6 months old control animals compared to the 2 months young control animals, confirming that the antioxidant defenses are impaired in the aged rats testes [2, 58]. This is similar to the report of Salomon et al. [45], which found that 6 months old rats with or without sexual experience had increased activities of antioxidant enzymes including those of SOD and glutathione *S*-transferase. Superoxide dismutase neutralizes the superoxide anion converting it to hydrogen peroxide whereas CAT removes the hydrogen peroxide produced by the SOD converting it to H_2O. It is well established that SOD and CAT enzymatic antioxidant effects are significant in the reactive oxygen species neutralization pathway whereas reduced glutathione forms the most abundant –SH agent in most mammalian tissues and buffers gonadal tissues against the effect of oxidizing noxious moieties. It is therefore plausible to assume that the elevated level of GSH and antioxidant enzymes, CAT and SOD in the aged rats, are indicative of an adaptive response to the oxidative stress status in the testes of the aged rats. The decrease in lipid peroxidation in testes of the aged animals is therefore expected because the higher antioxidant profiles in the testes of the aged rats is assumed to be inversely related to tissue MDA level, and thus the lipid peroxidation was halted [59]. It must be noted that increases in activity of antioxidant enzymes in rat's testes is progressive from 6 months until the rat reaches 12 months and above when the antioxidant enzymes begin to drop [45, 59, 60]. This supports the conjecture of the free radical theory of ageing [59], thereby supporting our present results. Because the activity of the antioxidant enzyme in the testes of the aged rats is higher than those of the young rats, they are expected to have different capacity to respond to oxidative stress treatment [45, 46]; and therefore, the intake of a suitable antioxidant-rich agent

could shift the antioxidant status in the aged rat's testis to those of the young rats, on the assumption that the altered antioxidant profile in the 6 months old rats is an ageing-initiated process that could be modulated by an antioxidant agent before it manifest as oxidative damage later on when the rats reaches 12-30 months old [45, 46, 61, 62]. Interestingly, the different tested doses of FPS used in this study were able to recover the profiles of the antioxidant enzymes and markers to the level similar to the young control rat's and with the 50 mg kg^{-1} b.w having better effect than higher doses, suggesting that FPS possess regulatory antioxidant effect on the ageing rat testis. Our laboratories have also consistently reported that extracts of FPS have better pharmacological properties at lower than at higher doses including their antioxidant effects in the testes of rats [28, 42–44]. Other antioxidant-rich extractives from natural sources and antioxidants phytochemicals have also been found to modulate the antioxidant status of rats during ageing, and could minimize the onset of age-associated disorders linked with impaired antioxidant activities [61–63]. It is therefore tenable to deduce that FPS rebalances oxidative stress parameters in the aged rat's testis to the antioxidant profiles similar to those of the testes of the young control Wistar rats. This supports the antioxidant-regulatory effects of FPS in the testicular system of an aged rodent model [28, 44].

In tissues, nitrate is reduced to nitrite, and then nitrite is further reduced to nitric oxide via the guanylate cyclase-cGMP signaling pathway [45]. In the gonads, nitric oxide stimulates Leydig cell steroidogenesis pathways responsible for the biosynthesis of testosterone [64]. In this study, nitrite concentrations were observed to be decreased in the testes of the aged rats compared to the young control animals suggesting that nitric oxide is consumed more in the aged rats for important processes that are associated with testicular functions including spermatogenesis and steroidogenesis [45, 65]. It is also plausible to assume that higher enzymatic activities of SOD and CAT could make nitric oxide production insufficient in attempt to protect the aged rat's testis against induced oxidative damage. This seems rationale since nitric oxide could combine with superoxide radicals generated during oxidative stress to form peroxynitrite, an endogenous signaling molecule that induces oxidant stress in tissues [66, 67]. It is therefore likely that the elevated testosterone level in the aged animals is in direct response to the increased consumption of nitric oxide for testosterone production resulting in the diminished level of nitric oxide. This assumption has also been confirmed in the testis of aged Wistar rats in other laboratories [45, 46] thereby supporting our hypothesis that 6 months old Wistar rats could maintain higher levels of antioxidant profiles to support steroidogenesis. Interestingly, 50 mg kg^{-1} b.w FPS better than at higher doses was able to increase nitric oxide level compared to the aged control and to level comparable to the young control rats, further affirming the antioxidant effect of FPS. As expected, nitrite concentration in the testis was also found to be maintained at levels comparable to that of the aged control and lower than those of the young control after injection of testosterone. The higher level of testosterone in these animals, due to increased steroidogenesis, is therefore responsible for the decreased nitrite concentration. Thus, high-testosterone level blunts the nitric oxide effect on the steroidogenesis cascade including its inhibitory effects on testosterone biosynthesis by Leydig cells [68].

Furthermore, ascorbate concentration was least in the testes of the untreated young control animals whereas the aged control had the highest level that was reduced to the level of the young control values in the 50 mg FPS group. This decrease in ascorbate concentration in the young animal and their increase in the aged animals are expected because there is increased requirement of ascorbate for several testicular functions including steroidogenesis and spermatogenesis in the gonads of young animals that are attaining reproductive maturation and gonadal spermatogenesis [13]. Since, ascorbate is itself sustained in a reduced form by a GSH-dependent dehydroascorbate reductase, which is rich in the testis, the higher level of GSH in the testes of the aged animals could also be responsible for the higher ascorbate concentration [13]. Furthermore, the low requirement of ascorbate for downregulated spermatogenesis could result in ascorbic acid been accumulated in the testes of older animals than in young animals [45]. The lower dose FPS (50 mg kg^{-1} b.w) and testosterone injection were found better than the higher doses FPS to lower ascorbate level toward the level seen in the young control animals, confirming the antioxidant regulatory effects and spermatogenesis-inducing properties of FPS.

DNA fragmentation was found not to be affected in the testes of both the young and aged control rats and after the intake of FPS, suggesting that the integrity of testicular DNA contents were not altered in Wistar rats that are 6 months old and it is also not affected by the intake of FPS. Previous studies have found DNA damage in the testes of Wistar rats that are from 9 to 24 months old [46], suggesting that DNA damage may not accumulate in the testes of Wistar rats that are younger than 9 months old thereby supporting our present data on % fragmented DNA in the testes of both the young and aged control adult rats.

The phytochemicals inherent in FPS including tannins, phenolic acids, phytosterols, terpenoids, alkaloids, flavonoids, and saponins have interesting therapeutic properties that may be contributing to their antioxidant-regulatory effects in the gonads of rats [28, 35]. For instance, tannins, phenolic acids, and flavonoids at low dose has antioxidant effect necessary for minimizing oxidative stress changes during ageing; and at high doses, they could become prooxidants [28, 30]; saponins, alkaloids, and phytosterols have regulatory androgenic effects on the gonads of ageing rats [42, 69]; and terpenoids could interfere with the pathophysiology of inflammation associated with ageing [35, 70]. Other components of the FPS extract identified by GC-MS that might have contributed to the observed pharmacological effects on the testis includes 2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene, (E)-9-octadecenoic acid ethyl ester, ethyl 14-methyl-hexadecanoate, 17-octadecynoic acid, 9,12,15-octadecatrienoic acid, 2H-3,9a-methano-1-benzoxepin, 13-oxabicyclo [9.3.1] pentadecane, 2-myristynoyl-glycinamide, and heptadecafluorononanoic acid [24]. This suggests that components in the hexane-rich fraction of

FPS act in harmony to achieve their biochemical and physiological effects in the testis of aged rats. In conclusion, the outcomes of this study begin to provide the evidence that intake of FPS rebalances the antioxidant profiles of aged rat's testes to levels similar to those of the young animals in order to slow down changes due to testosterone concentrations and oxidative stress in the testes. However, it remained to be demonstrated whether a similar biochemical effect can be seen in humans during the process of ageing.

Data Availability

Data is available on request.

Disclosure

The study is a part of the masters (M.Sc) and postgraduate diploma (PGD) degrees dissertations of MAC and CKW, respectively, in the Department of Biochemistry, University of Port Harcourt.

Conflicts of Interest

The authors declare that they have no competing personal relationships or financial interests that could have appeared to influence the publication of this research work.

Authors' Contributions

OEE was tasked for the conceptualization, investigation, and preparation of draft manuscript. SOA was assigned in the conceptualization, review, investigation, supervision, editing, data curation, and approval of manuscript for submission. MAC and CKW were responsible for the investigation. DCB and BMO were charged for the supervision.

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